New additives and ingredients in the formulation of aquafeeds

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Preface

Aquaculture is a young sector which has been showing in recent years an unstoppable, exponential and irreversible growth. This trend can only continue as long as the supply of certain raw materials like fish oils and meals, which are essential for the manufacturing of feed, may grow in line with it. This is the only bottleneck for the future of aquaculture: fish oil production is at a standstill. The massive and traditional sources of fish oil have been based and in fact, they are still supported by the exploitation of great biomass of marine species. These and many other fisheries are nowadays technically declining and socially under question.

Fish meals and oils go through the same processing; both depend highly on wild fisheries as Peruvian anchovy (*Engraulis ringens)* which are under a high fishing pressure. This situation together with other new phenomenon like climatic change could explain its stagnation or even recession. This would be completely negative for the aquaculture sector whose needs of fish oil for year 2030 are estimated at 40 million tonnes.

To consolidate the desired and rapid growth, aquaculture should base its expansion in a greater diversification of the sources of raw material regularly used. This is not a new statement and it has been assumed a long time ago by the whole industry in this activity despite of some remarkable problems. Thus there is a clear research line, not yet fruitful, focused on identifying renewable (vegetal) sources of n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA) as a partial alternative to fish oils. Likewise there are many efforts addressed to obtain protein concentrates of vegetal origin with the same purpose.

Apart from identifying and introducing alternative ingredients the evolution of feed formulation to obtain the best nutritional profiles requires unavoidably new additives. Additional problems such as high densities and the subsequent risk of disease transmission; limited and even forbidden use of antibiotics; together with feed with a high vegetal content, lead to a reduction in survival and growth as well as a lower efficiency in feed use. All these disturbances make aquaculture activities not very profitable. In order to promote growth and an adequate health condition there are several useful tools to be used. Some of them are: the inclusion of natural growth promoters in feed, the use of lactic bacteria to enrich cultures of *Artemia* and rotifers in hatcheries, as well as the combination of mineral vitamin markers with immune stimulant activity. Many of these substances have shown to affect certain species causing benefits derived from their antimicrobial effects, effects on the development of the intestinal epithelium, anti-inflammatory or digestibility improver.

Economically speaking the great price variation in feed ingredients and the low market prices for products from aquaculture, challenge the benefit of many aquaculture activities around the world. As production costs increase, nutritionists look for new ways to reduce them, maximizing digestive and metabolic activities as a base to turn nutrients in growth. Additives to increase digestibility have the ability to improve the use of nutrients from economical ingredients and encourage their transformation in food/meat. They also cause a reduction of fat accumulation in muscle and guts.

The *Centro Tecnológico del Mar-Fundación CETMAR* organized on the 29th September 2011 a seminar titled "Use of New Additives in Fish Feeds" about the relevant role of technological additives and vegetal ingredients in feed formulation (seminar organized within ZIMOFEED project PGIDIT08MMA003CT, financed by Tecnoloxía Sectorial MMA Program, Consellería de Economía e Industria). The seminar aroused a great interest and had a remarkable reception. All along the event the audience showed a great interest about the subject. Thus, the idea of publishing this book was born to collect all the information released during that seminar in a way that all the different issues involved nowadays in this subject were represented. Obviously, the intention is to serve as an orientation to those who want to study in depth these applied aspects of aquaculture research and nutrition.

This book would not have come true if we had not counted on the selfless help of a number of professionals and companies. We could not have reached our goal without their generous contribution. Especially we must mention the following companies DIBAQ SA, NUTRIAD and NOREL SA whose firm commitment with aquaculture has make this book possible. We are in debt with all scientists and company representatives who have given shape to the book as well as to the seminar. To all of them we thank from these pages for their altruistic effort and compromise and confirm our gratitude and recognition.

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Natural growth promoters in aquaculture practices

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Summary The gastrointestinal tract of the aquatic animal is sensitive and responsive to a number of stressing factors, being those common and the cause of disease and poor performance. In order to minimize these negative outcomes and achieve sustainable, safe, and cost-efficient aquaculture practices, nutritionists must focus not only on nutritional specifications but also on promoting gastrointestinal health. In such task, natural growth promoters, as probiotics or organic acids, stand out as a tool to reach an optimal gut flora balance and to stimulate development and integrity of intestinal epithelium.

Introduction

Aquaculture is the world's fastest food production system. Data from World Bank's Global Program on Fisheries and Aquaculture reveals that aquaculture should grow 75 to 85 percent by 2030 to supply the world's growing demand for seafood (Anderson, 2011). In order to achieve such growth, risks and solutions related to disease, nutrition, pollution, genetics, and consequently efficiency, must be immediately addressed.

Nowadays, aquaculture is facing a number of challenges. In first place the high stocking densities and the consequent proneness to pathogen transmission, water contamination, and sediment deterioration. In second place, the increasing limited - or even banned - use of antibiotics and disinfectants which can reduce pathogen transmission and water contamination but on the contrary can lead to the natural emergence of bacterial resistance besides impacting negatively the environment. Last but not least, the feeding of less degradable plant-based diets which results in diminished nutrient utilization and increases the amount of undigested feed excreted to the environment. If not properly confronted, this situation leads to consequences such as impaired water and pond bottom quality, reduced survival, lower efficiencies and growth, and subsequently unprofitable aquaculture practices. With the aim of warranting a successful present and future development of aquaculture, measures applied at present must be effective as well as sustainable.

In both industrialized and developing countries, nutrition and feeding play a critical role in the sustained development of aquaculture, thus particular emphasis is currently being placed on nutritional strategies and their effect on animal health, performance, and environmental integrity. While a first nutritional approach is the increasingly accessible information on nutrient requirements for each of the species as well as on the more effective use of alternative and available ingredients, a second approach is supplementation with natural growth promoters (NGPs). Also termed non-antibiotic growth promoters, NGPs are feed additives contemplating different categories as salts of organic acids, probiotics, prebiotics, phytogenics or yeast. In addition to the beneficial effects in terms of health status and performance, NGPs supplementation does not usually bear any risk regarding bacterial resistance or undesired residues in the edible fish product. In terms of manufacture, worldwide aqua feed production is growing at a rate of 11.1% per year and is expected to reach 70.9 million tones by 2020 (Tacon, 2010) thus the demand for NGPs is expected to augment accordingly (142.000 tones of NGPs by 2020 if average inclusion is about 2 kg of NGP per ton of feed). In such market situation, and as a leading company in NGPs manufacture, it is Norel´s objective to develop innovative and competitive products and consequently to provide customers with the most updated information regarding mode of action, use and benefits. This chapter will review the characteristics and benefits of using probiotics and organic acid salts for aquaculture practices, specially focusing on *Bacillus* spp. and sodium butyrate.

Probiotics: *Bacillus* spp.

A probiotic application for aquaculture practices can be defined as a live microbial supplement that administered via feed or directly into the rearing water provides a benefit to the animal by enhancing nutrient utilization, health status, stress response, disease resistance and performance, and this is partly achieved by optimizing the microbial balance within the animals and water environment (Merrifield et al., 2010). A list of characteristics for potential probiotic bacteria has been reported by several authors (Farzanfar, 2006; Vine et al., 2006; Gómez and Balcazar, 2008) and extended by Merrifield et al. (2010). Among them there are essential properties as being a non-pathogenic microorganism and being resistant to bile salts and low pH. Other favourable properties are adequate and rapid growth at host rearing temperature, antagonistic properties against key pathogens, capacity to produce extra cellular enzymes that improve feed utilization, viability under normal storage conditions and acceptable survival under processing conditions.

Among the different strains of probiotics used at present in aquaculture practices, *Bacillus* spp. are well known due to its sporulated form that confers easy handling, long shelf life, and convenience to be mixed with mash when processing temperature is below 90°C. Numerous studies have demonstrated the beneficial effect of supplementing fish and shrimp diets with *Bacillus* spp. By re-establishing a beneficial gastrointestinal micro biota composition, this application will exert a major effect within the host (Table 1), resulting in enhanced break down of digesta, reduced pathogenic

bacteria load, enhanced disease resistance, healthier absorptive surface area, and consequently better nutrient utilization. These host benefits may be explained by different mechanisms of action such as secretion of exo-enzymes, organic acids and other antimicrobial compounds, as well as by competition for nutrient and attachment sites of intestinal cells (Kesarcodi-Watson et al., 2008). Most recent information reveals an immune stimulating effect of some *Bacillus* spp. by increasing the activity of phagocityc cells and lysozyme (Yung-Zhang et al., 2010).

Water and soil quality management is considered one of the most important aspects of pond aquaculture. While water quality parameters such as dissolved oxygen, pH, or ammonia concentrations affect essential body functions, the condition of pond bottoms and the exchange of substances between soil and water strongly affect water quality. Low dissolved oxygen level is the major limiting water quality parameter (Boyd and Tucker, 1998). Waste accumulation will increase oxygen demand by bacteria in order to perform organic matter break down, consequently favoring the development of anaerobic patches. These anoxic sediments will contact the more oxygen enriched water column leading to disruption of the adequate environmental conditions supporting healthy growth. Additionally ammonia may diffuse into the water column not only as a result of fish excretions but also following microbial decomposition of organic material. In conditions of high pH and temperature, ammonia accumulation can cause sub-lethal effects as poor growth or disease proneness (Hargreaves and Tucker, 2004). Therefore, it is of key importance to maintain pond bottom under aerobic conditions and to avoid organic matter and slime. In this sense, *Bacillus* spp. is considered a bioremediation agent, concept referring to the use of microorganisms that help to maintain proper pond environment by the secretion of exo-enzymes that break down organic matter (Moriarty, 1997). In addition, *Bacillus* spp. may use ammonia nitrogen as nitrogen source for their growth (Ghosh et al., 2010). Many of the benefits reported by Table 1, particularly increased survival, are the consequence of improved pond bottom conditions as a result of reduced concentration of organic matter and ammonia. These improvements are generated not only by direct application into water but also when *Bacillus* spp. are included in feed, as a significant portion of the probiotic leaches from the pellet and feces becoming a component of the pond ecology. While water quality is also assessed by pathogen load, it has been reported that Bacillus spp. can improve water quality reducing pathogenic bacteria load and consequently balancing bacterial population in water (Vaseeharan et al., 2004). This is achieved by competitive exclusion based on secretion of antimicrobial compounds as well as competition with other pathogens for nutrients (Merrifield et al., 2010). Likewise, the direct application of probiotics into the water results in reduced improved microbiota balance at the host's intestinal level, indicating the ability of probiotics to reach the animal's gastrointestinal tract (Li et al., 2007).

Norel manufactures and markets probiotic products containing *Bacillus* spp. as *B. amyloliquefaciens*, *B. licheniformis*, and *B. cereus*. Although the combination of these bacteria is also commercially available for aquaculture

Table 1. Effects of using *Bacillus* spp. in aquaculture practices

Potential probiotic	Parameters investigated	Species	Reference
Dietary application			
B. subtilis	Enhanced disease resistance against *Aeromonas*	Trout	Newaj-Fyzul et al. (2007)
B. subtilis + *B. licheniformis*	Disease resistance Immunological response	Trout	Raida et al. (2003)
B. subtilis + *B. licheniformis*	Improved FCR Improved SGR Improved PER	Trout	Baherhi et al. (2008)
Bacillus spp.	Increased survival Increased lysozyme activity Increased respiratory burst	Trout	Brunt et al. (2005)
B. pumilus + *B. clausii*	Improved fagocitic activity Improved lysozyme activity Improved growth	Grouper	Zhang-Sun et al. (2010)
B. circulans + *B. subtilis*	Improved protein digestibility	Carp	Bairagi et al. (2004)
B. subtilis	Increased respiratory burst activity Increased serum bactericidal activity	Carp	Kumar et al. (2008)
B. subtilis	Increased respiratory burst activity Increased lysozyme activity Increased immunoglobulin	Carp	Nayac et al. (2008)
B. circulans	Improved digestibility after diet fermentation with probiotic	Carp	Ghosh et al. (2004)
Bacillus spp.	Increased survival	Shrimp	Meunpol et al. (2003)
Bacillus S11	Increased survival	Shrimp	Rengipat et al. (1998)
B. megaterium + *B. subtilis*	Increased digestive enzymatic activity	Shrimp	Solano and Soto (2006)
B. subtilis + *B. nato* + *B. licheniformis*	Improved digestive enzymatic activity Improved growth	Shrimp	Gómez and Shen (2008)
B. subtilis	Improved growth Improved survival rate	Shrimp	Far et al. (2010)
B. subtilis	Inhibitory activity against *Pseudomonas fluorescens* Improved survival Improved growth	Tilapia	Aly et al. (2008)
B. subtilis	Improved phagocytic activity	Seabream	Salinas et al. (2008)
Water application			
B. subtilis + *B. megaterium* + *B. polymyxa* + *B. licheniformis*	No benefit	Shrimp	McIntosh et al. (2000)
B.subtilis	Reduced *Vibrio* load	Shrimp	Vaseeharan et al. (2003)
Bacillus spp. *P7*	Improved removal of total amoniacal nitrogen	Shrimp	Ghosh et al. (2010)
Bacillus spp. (DSM)	Increased survival	Shrimp	Moriarty (1998)
B. licheniformis	Reduced intestinal *Vibrio* load Improved haemocyte counts Improved superoxide dismutase and phenoloxidase activities	Shrimp	Li et al. (2007)
B. subtilis UTM 126	Increased survival against *Vibrio* spp.	Shrimp	Balcázar and Rojas-Luna (2007)
B. coagulans	Increased respiratory burst activity	Tilapia	Zhou et al. (2010)
B. megaterium + *B. subtilis* + *B. polymyxa* + *B. licheniformis*	Increased survival	Catfish	Queiroz and Boyd (1998)

practices, our major efforts have focused on the use of *B. amyloliquefaciens*. By collaborating with research institutions and feed companies, our present R&D program is oriented towards the more efficient utilization of this species at both feed mill and farm level.

Ecobiol Aqua

B. amyloliquefaciens is marketed by Norel as Ecobiol Aqua. In comparison to other probionts, the sporulated *Bacillus* spp. offers advantages in use due to its heat-stability, allowing the product to be stored at room temperature in a desiccated form without any harmful effect on viability and resistance to acidic pH, allowing probionts to pass the gastric barrier (Cutting, 2011). In addition to the abovementioned criteria described by Merrifield *et al*. (2010) and Cutting (2011) two other properties make Ecobiol Aqua a highly functional NGP. First of all a high survival rate through pelleting (Table 2) and secondly a faster activation and high multiplication rate of the vegetative form when compared to other *Bacillus* spp. (Figure 1).

Depending on processing conditions, farm management or purpose of application Ecobiol Aqua can be added directly to the mash mixed with oil and coated after heat treatment or even administered via the rearing water. Either via feed or via water the host benefit will result from both improved nutrient utilization and improved water environment.

Different ways of action have been investigated for Ecobiol Aqua; it is the combination of all of them what leads to host benefit. *B. amyloliquefaciens* is capable to secrete 0.9 g of lactic acid per g of sugar present in the culture medium. Within the gastrointestinal tract of the animal, this lactic acid will reduce pathogenic bacteria while is used by beneficial bacteria such as *Lactobacillus* as substrate to grow. Additionally the vegetative form of *B. amyloliquefaciens* produces barnase which is a bacteriocine. As a result, the antimicrobial properties of Ecobiol Aqua have been demonstrated

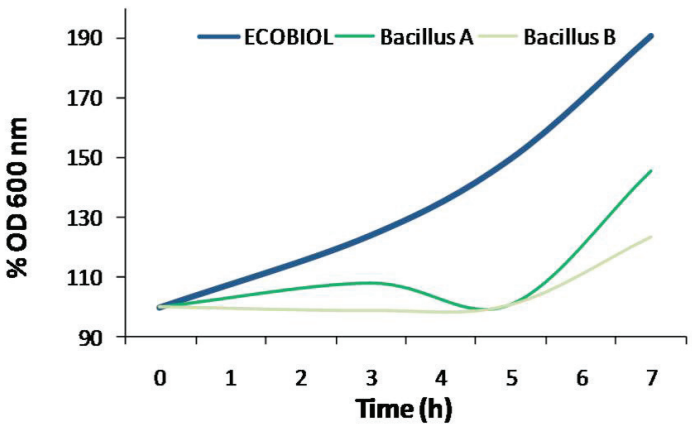


Figure 1. Multiplication rate of Ecobiol Aqua as opposed to other strains of Bacillus *(measured by optical density)*

against pathogens affecting both cold and warm water aquatic species such as *Aeromonas hydrophila*, *Aeromonas salmonicida, Yersinia ruckeri* and *Vibrio parahameolyticus* (Nuez-Ortín, 2011, Unpublished data). At the same time, this inhibitory activity reduces nutrient competition and pathogen colonization of the gut resulting in improved absorptive surface area. It is also known that probiotic bacteria can release extracellular enzymes that help in the digestion process. In a study conducted by the University of Sonora (Mexico) the protease activity in hepatopancreas of shrimp was assessed when Ecobiol Aqua was supplemented at 1 kg/ton of feed (Figure 2). Results showed increased enzymatic activity during the first four hours after feed intake, suggesting enhanced digestibility and consequently better growth performance. Secretion of amylases, cellulases and xylanases has also been reported for *B. amyloliquefaciens* (Cortyl, 2010).

Table 2. *Ecobiol Aqua* survival after processing (90°C during 1 minute)

Mash (UFC/g feed)	Pellet (UFC/g feed)
2.60×10^6	2.25×10^6
1.68×10^6	1.50×10^6
1.27×10^6	1.36×10^6

The efficacy of dietary supplementation of Ecobiol Aqua on performance parameters at a commercial scale has been evaluated in shrimp cultured in Mexico. In a first trial carried out in a semi-intensive farm, diets formulated for *Litopenaeus vannamei* were supplemented with 1 kg Ecobiol Aqua per ton of feed. As shown in Table 3, results showed improvements in survival (+3.7%), average weight gain at 95 days (+4%), and income (+12%) relative to the non-supplemented diet. The same dosage was used in a second trial conducted in semi-intensive culture of *Litopenaeus vannamei* (Table 3), leading to better average weekly gain (+13.5%) and income (+7.2%) with Ecobiol Aqua supplementation.

Therefore, the use of *Bacillus* spp. is common in aquaculture practices, both in feed and in water. There is a number of studies showing that *Bacillus*

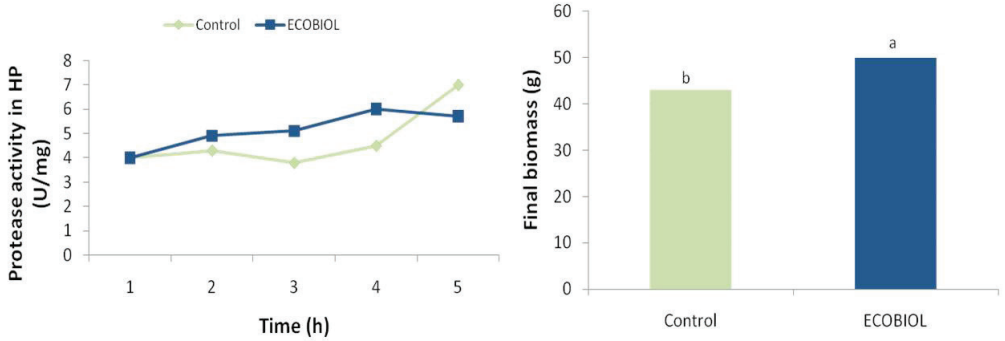


Figure 2. Enzymatic and growing response of Litopenaeus vannamaei *to a diet containing ECOBIOL Aqua*

spp. enhance disease resistance, growth and water quality. Similarly, the use of Ecobiol Aqua (*B. amyloliquefaciens*) is having successful use in shrimp production; not only due to the benefits observed at farm level, but also due to the stability and high survival of the finished product under pelleting temperatures (<95°C).

Table 3. Performance parameters of *Litopenaeus vannamei* supplemented with 1 kg Ecobiol Aqua/ton feed at commercial scale in Mexico

	Trial 1			Trial 2		
	Control	Ecobiol Aqua	Difference (%)	Control	Ecobiol Aqua	Difference (%)
Culture days	101	97	- 4.0	80	80	
Average weekly gain (g)	-	-	-	1.33	1.51	+ 13.5
Average weight at 95 days (g)	13.30	13.83	+ 4.0	-	-	-
Survival (%)	80.90	83.90	+ 3.7	67.5	66.5	- 1.5
Income (US$/ Ha)	3245.2	3618,8	+ 11.4	2841.0	3046.4	+ 7.2

Dosage: 1 kg/ton of feed

Organic acid salts

An organic acid is an organic compound with acidic properties associated to the carboxyl group –COOH. Organic acids, proposed as candidate replacement for antibiotics, have been used in terrestrial animal diets for decades. Although the consistency of the effects on performance achieved by antibiotics is difficult to emulate, organic acids may provide many of the benefits of antibiotics naturally, hence the term NGPs. The antimicrobial and antifungal activity is associated to weak and short-chain organic acids (SCOA) (C1-C7) and highly dependent on concentration and pH. Thus, SCOA such as acetic (C2), propionic (C3), butyric (C4), valeric (C5) and citric (C6) differ in pKa value and consequently in its capability to reduce pH and inhibit pathogens at the different physiological conditions encountered within the gastrointestinal tract (Table 4). However, the effects of SCOA go beyond modification of gut microflora, and other benefits such as improved digestive enzyme activity, increased pancreatic secretion, enhanced development of intestinal epithelium and intestinal barrier integrity, or anti-inflammatory properties, have been described and attributed to likely causes of enhanced performance when supplementing some of these NGPs, particularly butyric acid in its sodium salt form.

Table 4. Organic acids used as additives in animal nutrition

Acid	Formula	Molecular mass (g/mol)	pKa
Formic	HCOOH	46.03	3.75
Acetic	CH_3COOH	60.05	4.76
Propionic	CH_3CH_2COOH	74.08	4.88
Butyric	$CH_3CH_2CH_2COOH$	88.12	4.82
Lactic	$CH_3CH(OH)COOH$	90.08	3.83
Fumaric	COOHCH:CHCOOH	116.07	3.02 4.38
Malic	$COOHCH_2CH(OH)COOH$	134.09	3.40 5.10
Tartaric	COOHCH(OH)CH(OH)COOH	150.09	2.93 4.23
Valeric	$CH_3CH_2CH_2CH_2COOH$	102.13	4.84
Citric	$COOHCH_2C(OH)(COOH)CH_2COOH$	192.14	3.13 4.76

Concerning the use, most of the SCOA are characterized by volatility and corrosiveness, being a problem at the feed mill level where feed is processed under high temperatures. Accordingly, salt forms of SCOA are commercialized, providing convenient storage, stability through processing and consequently high recovery in the pelleted or extruded feed. When it comes to aquaculture a number of studies covering both cold-water and tropical species indicate that a broad range of organic acids, their salts or mixtures of those, can improve growth, feed utilization, disease resistance, and consequently the benefits of the producer (Table 5).

Table 5. Studies using organic acids, either in acid or salt form, in both cold and warm water species

Species	Benefit	Reference
Artic charr	Improved FCR Enhanced health status (diarrhoea)	Ringø (1991) Ringø et al. (1994) Gislason et al. (1996)
Atlantic salmon	Enhanced mineral absorption Improved FCR	Gislason et al. (1994) Gislason et al. (1996) Christiansen and Lückstädt (2008)
Rainbow trout	Enhanced mineral absorption Improved growth rate and FCR	Vielma and Lall (1997) Sugiura et al. (1998a) Sugiura et al. (1998b) Vielma et al. (1999)
Tilapia	Improved growth rate, FCR and PER	Ramli et al. (2005) Zhou et al. (2008) Petcam et al. (2008)
Carp	Reduced intestinal digesta pH Better mineral availability Improved growth rate and FCR	Baruah et al. (2005) Baruah et al. (2007)
Catfish	Improved growth rate and FCR	Owen et al. (2006)

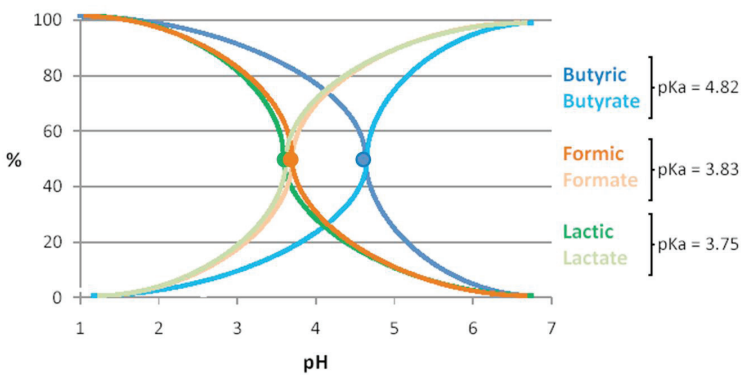
In terms of product development and manufacture Norel is one of top worldwide producers of organic acid salts, being sodium butyrate our primary focus. Our current R&D program concentrates on the investigation of the individual effect of sodium butyrate as well as the synergetic effect when combined with other NGPs on health and performance by elucidating the different genomic and metabolic mechanisms involved.

Gustor Aqua

Gustor Aqua contains sodium butyrate, which is the sodium salt form of butyric acid. Butyric acid is naturally produced and present within the intestine of both carnivorous and herbivorous fish (Holben et al., 2002, Mountfort et al., 2002). As mentioned above, SCOA are commonly known by their specific antimicrobial activity, however, other key benefits such as trophic and immune properties have been attributed to sodium butyrate (Gálfi and Bokori, 1990; Le Gall et al., 2009).

The magnitude of the antimicrobial effects of sodium butyrate is largely dependent on two factors: concentration and gastrointestinal pH. The pKa value of each SCOA represents the pH at which the acid is half dissociated, thus determining the extent of dissociation/undissociation of the acid (Figure 3). The undissociated state of SCOA is strongly lipophilic and capable to diffuse across the membrane of gram negative bacteria (Gálfi and Bokori, 1990). Once inside the bacterial cytoplasm, the more alkaline pH causes dissociation of the acid resulting in two fatal incidents for the bacteria; pH decline -and the consequent disruption of the metabolic processes- and reduced energy availability due to the effort required to transport the proton out of the bacteria (Dibner and Buttin, 2002). Table 6 shows the antimicrobial activity of butyric acid when compared to propionic and valerianic acids. The subsequent reduction in the butyric acid intolerant microorganisms contributes to a diminished risk of subclinical infections and nutrient demand by the gut-associated immune system which leads to enhanced health status and nutrient use. As reported by Page et al.

Figure 3. Dissociation/undissociated state of butyric, formic and lactic acids



(1976) the stomach pH of channel catfish ranges approximately from 1 to 3 proximally and from 3 to 4 distally, while pKa values for butyric, lactic and formic acids are 4.82, 3.83 and 3.77, respectively (Dibner and Buttin, 2002). These values suggest that while the degree of undissociation, and consequently antimicrobial efficacy, in proximal stomach of catfish is similar for the three acids, it is greater for butyric as pH alkalinity increases further down the digestive tract.

Table 6. Minimum inhibitory concentration (in mM) of SCFA against common pathogens affecting fish and shrimp species

Pathogen	Butyric	Propionic	Valerianic
V. anguillarum	22.2	33.3	33.3
V. vulnificus	22.2	33.3	33.3
V. harveyi	22.2	22.2	33.3
V. parahaemolyticus	22.2	22.2	33.3
V. alginolyticus	22.2	22.2	33.3
A. hydrophila	22.2	33.3	33.3
A. caviae	22.2	33.3	33.3
A. salmonicida	22.2	22.2	33.3
F. columnare	22.2	22.2	33.3
Y. ruckeri	33.3	33.3	50

Note that pH reduction can also determine survival of bacteria; however, the similar pKa value among butyric, propionic and valerianic (4.82 vs. 4.88 vs. 4.82) indicates a greater inhibitory activity of butyric by diffusing through the membrane of gram - bacteria

The increasing pH alkalinity as going distally along the intestine leads to a reduced presence of the butyric forms and the subsequent efficacy to diminish pathogenic flora but at the same time favours the presence of butyrate -or dissociated form of the acid-. The stimulatory effect of butyrate on intestinal epithelium development in different animal species has been demonstrated both *in vivo* and *in vitro* conditions, elucidating greater epithelial cell proliferation with the administration of sodium butyrate when compared to other salts as sodium acetate and sodium propionate (Sakata and Tamate 1978, 1979; Sakata, 1987). In a study conducted with pigs, dietary supplementation of 0.17% sodium butyrate increased cell proliferation in ileum by 33%, resulting in 30% increase in villi length (Galfi and Bokori, 1990). This stimulatory effect can be interpreted as a combination of direct and indirect mechanisms along the different sections of the intestine (Sakata and Yajima, 1984). While the caloric content of butyrate is higher than that of propionate and acetate, and it is preferentially oxidized by the epithelial cells when compared to other energy sources (Roediger, 1982; Roediger and Rae, 1982; Ardawi and Newsholme, 1985), butyrate also stimulates insulin secretion, which consecutively exerts a stimulatory effect on intestinal epithelium (Jordan and Phillips, 1978). As

a result of improved villi development, absorptive surface area is enlarged, leading to better nutrient utilization and enhanced animal health status and performance.

Soya is a high protein ingredient with favourable price and available in large quantities on a world-wide basis, thus being an excellent alternative in plant-based diets. The use of whole soya bean as dietary ingredient is not recommended due to its high content of anti-nutrient factors (lecithin, saponins, allergenic proteins, etc.), thus extracted soya is the most commonly used soya product. The alcohol-soluble fraction of extracted soya however, still contains active anti-nutrients being ascribed as the cause of inflammation in distal intestine (enteritis) (Bureau et al., 1998; Francis et al., 2001; Knudsen et al., 2008). Several investigations have reported soya-induced enteritis in salmonids (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2003; Knudsen et al., 2008) and common carp (Urán et al., 2008). This pathological alteration is microscopically characterized by hypertrophic mucosa, infiltration of inflammatory cells, hyperaemia, and shortening of the enterocyte microvilli leading to decreased absorptive capacity and consequently reduced nutrient utilization and performance (van de Ingh et al., 1991; Baeverfjord and Krogdahl, 1996; Urán et al., 2008). Inflammation can either be the cause or the consequence of increased permeability of intestinal epithelium which is in turn structurally limited by the tight junctions (Hamer et al., 2008; Knudsen et al., 2008). These tight junctions are composed of a protein network of sealing strands, and as demonstrated by Knudsen et al., (2008) increased tight junction permeability was observed after feeding soya products to fish. As a result of the increased permeability of intestinal epithelium, susceptibility to bacterial disease augments (Cheeke, 1999). All these observations have led specialists to include not more than 5-15% of soya (weight basis) in salmon diets in order to prevent enteritis, thus limiting higher dietary incorporation levels of this available source of protein. In this situation, sodium butyrate arises as a tool to prevent the development of soya-induced enteritis and allows a more convenient formulation of commercial feeds. As described in mammals, butyrate provides anti-inflammatory properties in the distal intestine by modifying transcription factors (NF-kB), which in turn controls the expression of inflammatory response genes (Hamer et al., 2008; Le Gall et al., 2009). Additionally butyrate at low concentrations (up to 2mM) increases expression of tight junction proteins (Ohata et al., 2005). It is documented that the pathophysiological effects of some parasites as *Enteromyxum* are partly due to the disruption of tight junctions (Woo and Buchman, 2011), thus the reduced epithelial permeability associated to butyrate administration places Gustor Aqua as a new alternative to maintain intestinal barrier integrity and prevent other further pathological processes.

Other mechanisms associated to enhanced intestinal defense barrier have been attributed to butyrate. A first one is production of mucins, which are glycosilated proteins forming a defense barrier that prevents invasion of bacteria and protects against damage from bacterial toxins and

enzymes (Finnie et al., 1995; Hamer et al., 2008). The *in vitro* expression of mucin epithelial genes (MUC) has been shown to increase after butyrate administration (Gaudier et al., 2004). Other components of the intestinal defense barrier being influenced by butyrate are transglutaminase which is actively involved in mucosal healing (D´Argenio et al., 2005), antimicrobial peptides which protect the mucosa against the invasion and adherence of bacteria and thereby prevent infection (Schauber et al., 2006; Sunkara et al., 2011) and heat shock proteins which confer protection against inflammation by suppressing the production of inflammatory modulators (Malago et al., 2005).

Gustor Aqua is manufactured in both free and protected forms. While free sodium butyrate maximizes the antimicrobial activity and confers trophic and immunological properties in the upper-medium gastrointestinal tract of fish, the protected product allows a gradual release of sodium butyrate, therefore promoting antimicrobial, trophic and immunological effects along the entire tract.

The efficacy of dietary supplementation of Gustor Aqua on performance parameters have been evaluated in different aquatic species. In a trial carried out at Nong Lam University (Vietnam) a catfish (*Pangasius hypohthalmus*) diet was supplemented with 0.5 kg Gustor Aqua -free form)-per ton of feed and fed twice daily during 56 days. Results (Table 7) showed significant improvements in weight gain (+35%), specific growth rate (SGR) (+30%), feed conversion ratio (FCR) (-16%) and protein efficiency ratio (PER) (+35%) relative to the non-supplemented diet. In a second trial conducted at the College of Aquaculture and Fisheries at Can Tho University (Vietnam) a shrimp (*Penaeus monodon*) diet was supplemented with 1 kg of Gustor Aqua -protected form- per ton of feed and fed during two months. Coefficients of digestibility were determined by the use of chromic oxide as an inert marker. As shown in Table 8 the supplementation of protected sodium butyrate significantly enhanced digestibility of dry matter (DMd) (+12%), crude protein (CPd) (+5%) and energy (Ed) (+7%) leading to numerical improvements in weight gain, survival and FCR. In both experiments the positive outcomes are attributed to the abovementioned modes of action described for sodium butyrate.

Table 7. Effect of dietary supplementation of GUSTOR Aqua (free form) on performance parameters of catfish (*P. hypohthalmus*)

	Initial weight (g)	Weight gain (g)	SGR (% day)	FCR	PER
Control	6.90[a]	37.56[a]	3.34[a]	1.65[a]	1.34[a]
GUSTOR Aqua	6.75[a]	50.68[b]	4.32[b]	1.39[b]	1.81[b]

Gustor Aqua dosed at 0.5 kg/ton feed
Means with different superscripts in the same column are significantly different (P<0.05)
SGR: Specific growth rate/FCR: Feed conversion ratio/PER: Protein efficiency ratio

Gustor Aqua is a NGP that by providing antimicrobial, trophic and immunological properties effects to the gastrointestinal tract, warrants reduced microbial competition as well as enlarged and healthy intestinal absorptive surface area. In case of pathological conditions such as enteritis due to the feeding of ingredients with high anti-nutrient content or intestinal infection by parasites, the use of Gustor Aqua stands out as a natural tool to enhance intestinal health and nutrient utilization by the animal and consequently productive efficiency.

Table 8. Effect of dietary supplementation of GUSTOR Aqua (protected) on digestibility and performance parameters of shrimp (*P. monodon*)

	DMd (%)	CPd (%)	Ed (%)	Weigh gain (g)	Survival (%)	FCR
Control	64.90[a]	85.20[a]	78.10[a]	13.67	90	3.43
GUSTOR Aqua	72.46[a]	89.11[b]	83.26[b]	4.01	93	3.29

Gustor Aqua dosed at 1 kg/ton feed
Means with different superscripts in the same column are significantly different (P<0.05)
DMd: Dry matter digestibility/CPd: Crude protein digestibility/Ed: energy digestibility/FCR: Feed conversion ratio

General conclusion

In order to meet the world's growing demand for seafood, aquaculture production is increasing significantly. However, current aquaculture practices in many countries are exposed to high risk of disease and reduced performance due to the high stocking densities, declining use of antibiotics and use of plant-based diets. In such situation aquafeed formulators must pay special attention not only to nutritional specifications but also to reduce the potential risk of disease. That is why supplementation with NGPs such as probiotics or organic acid salts has aroused as a natural alternative to develop a successful nutritional program. In this sense, both Ecobiol Aqua and Gustor Aqua have been designed by Norel as tools to promote gastrointestinal health and the resultant maximum nutrient utilization, leading to cost-effective, safe and profitable production when supplemented.

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Innovative approaches to reduce feed cost in aquaculture: optimizing nutrient utilization and gut health

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Summary The strong fluctuations of feed ingredient prices in combination with low market prices for aquaculture products challenge the profitability of many aquaculture operations around the globe. This has accelerated a search for alternative formulations and feed additives to improve the cost efficiency of feeding under various scenarios of ingredient cost and availability. The present paper illustrates a number of new strategies in feed formulation to improve the cost efficiency of feeding fish and shrimp, through the use of feed additives which aim at improving feed utilization and/or gut health. These approaches yield better feed conversion and growth, and reduce the impact of disease on productivity in aquaculture operations.

Introduction

Aquaculture is world's fastest-growing sector in food production reaching an average growth rate since 1970 of almost 9 percent (versus 2.8 percent for land-based animal production). Global feed production for farming fish and shrimp has followed this expansion and is expected to reach 32 million MT by 2012 (Tacon and Metian, 2008). The fast growth of production volumes has resulted for many aquaculture species in a continuous erosion of the sales price at the farm gate and the profitability of the farm operation. In addition, all major aquafeed ingredients -including fishmeal, fish oil, vegetable proteins and fats, wheat flour, feed phosphates, additives, vitamins and minerals- have shown significant price increases over the past years. Despite the increased efforts to reduce the use of ingredients of marine origin, fishmeal remains a major or, in the best case, a significant cost in many aquafeed formulations. The fluctuations of fishmeal prices over the past years are troublesome for feed manufacturers and farmers, which are already under economic pressure due to the low price of the end product (Figure 1).

In the livestock industry, combined research efforts from producers, feed and additive suppliers, and academic institutes have resulted in a vast knowledge basis and a wide range of additives to alleviate increased cost of feed formulations for poultry and pigs. Despite its tremendous growth over the past decades, aquafeed production represented in 2006 only 4%

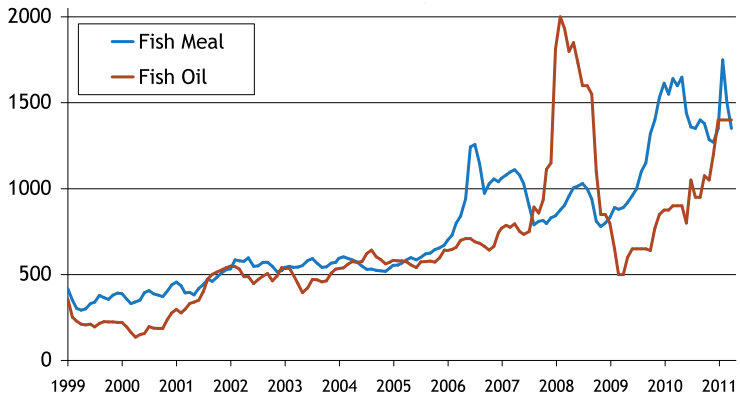


Figure 1: Evolution of fish meal and fish oil prices during 1999-2011 (source: http://hammersmithltd.blogspot.com)

of global animal feed production (Gill, 2007). Furthermore, global aquafeed volume is fragmented over many species produced under different conditions around the globe. This lack of critical mass has limited nutritional research developments, particularly for tropical species of fish and shrimp, to a fraction of what has been done on poultry and pigs. It is obvious that optimizing formulation cost for fish and shrimp requires completing the gaps in the current knowledge of nutritional requirements in function of growth stages and culture conditions (eg. the role of natural feed supplements in semi-intensive farming systems).

Apart from optimizing nutritional inputs and ingredient selection, so far aquaculture nutritionists have spent little attention to the optimal functioning of the digestive system of fish and shrimp. As ingredient prices are rising, nutritionists may find new options for cost reduction in the formulation by maximizing the efficiency of digestive and metabolic processes which are at the basis of converting nutrients into growth. In agriculture, various types of feed additives are being applied to enhance the digestibility and/or utilization efficiency of nutrients, including exogenous enzymes and various types of digestibility enhancers allowing to extract more nutrients out of ingredients, flavours and palatability enhancers to stimulate appetite, and a wide range of products (pre/probiotics, botanical extracts, yeast derived compounds, etc.) to maintain a healthy gut. The feeding biology, digestive physiology and nutritional requirements of warm-blooded land animals differ significantly from those of aquaculture organisms. Therefore, the direct application in aquaculture of nutritional and/or functional feed additives developed for livestock is often not trivial. The present article illustrates the potential to reducing cost of feeding in aquaculture by the application of novel feed additives, targeting mainly to improve the efficiency of digestibility and nutrient utilization. Furthermore, we will illustrate the importance of maintaining gut health and a balanced microflora for maximizing productivity and economics of fish and shrimp farming.

Screening for optimal performance & cost-efficiency of digestibility enhancers

A wide variety of concepts and products is being investigated for improving digestion and feed utilization in pigs and poultry. Potential products for application in fish include botanical extracts and phytobotanical compounds, short and medium chain fatty acids, organic acids, enzymes, and natural emulsifiers (Figure 2). However, it is difficult to predict the functionality of many of these products for a wide range of fish species, covering carnivorous/omnivorous/herbivorous feeding habits, marine/freshwater habitats, cold/tropical climates, and extremely different feed formulations. At the Nutriad Technology Center, a continuous program of empiric screening for different aquaculture species aims at identifying promising compounds for application in aquaculture. Nutriad research has mainly focused on compounds which are heat-stable in order to facilitate application in the mixer under practical processing conditions applied in aquafeed manufacturing.

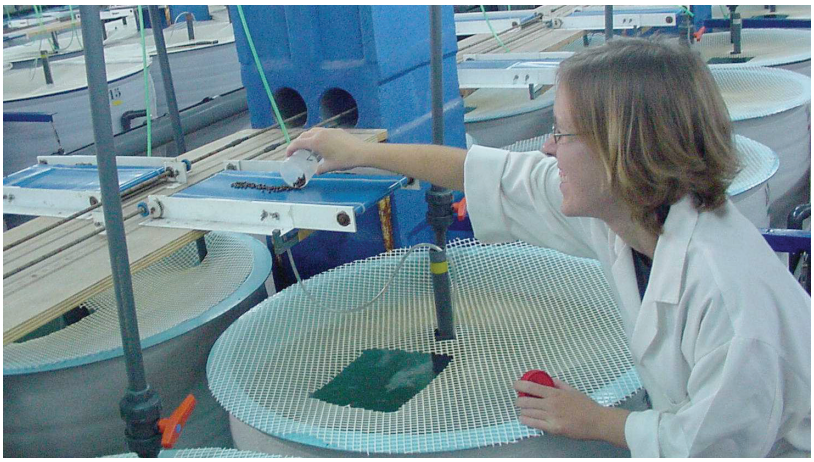


Photo 1: Empiric screening in standardized feeding trials using practical feeds aims at identifying promising feed additives for application in aquaculture

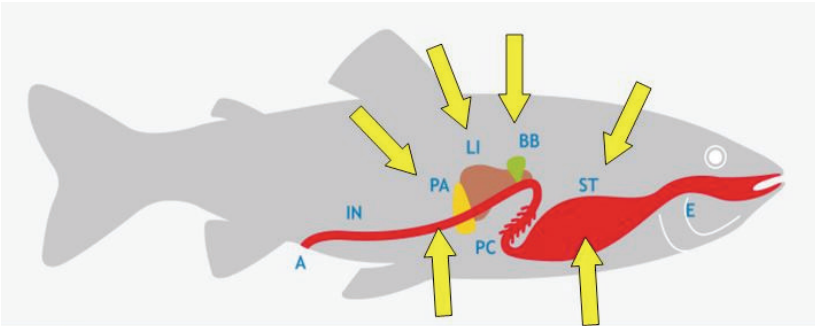


Figure 2. Compounds with potential for improving digestibility and feed utilization in fish (IN=intestine • PA=pancreas • LI=liver • BB=bile bladder •ST=stomach • E=esophagus • PC=pyloric caeca)

Enzymes
Degrade non-starch polysaccharides (NSP)
Make phytate phosphorous available

Short/Medium Chain Fatty Acids
Enhance nutrient absorption

Herbal Extracts & Phytobotanical Compounds
Simulate enzyme secretion & activation
Simulate bile acid production & secretion

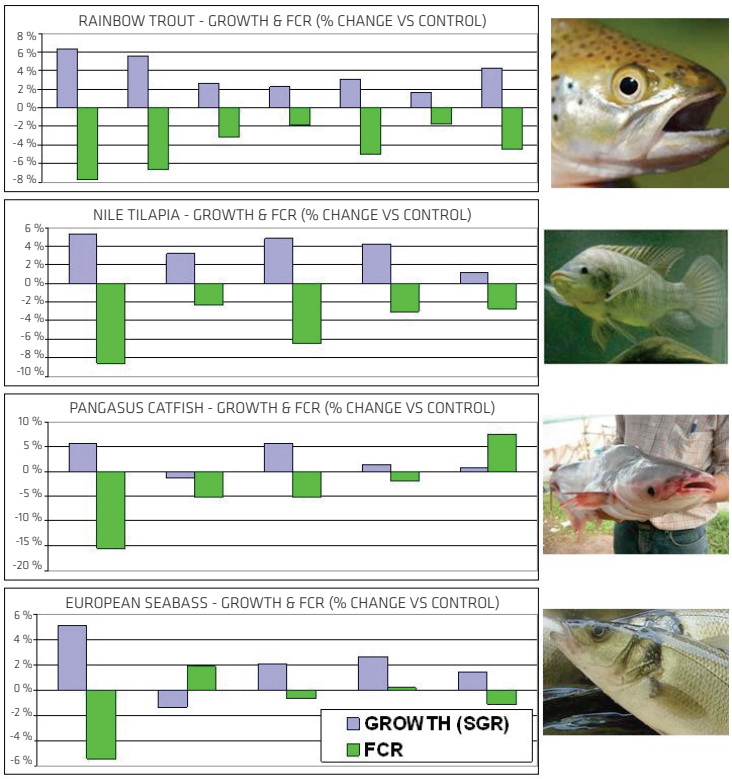
Organic Acids
Enhance acidification of stomach
Improving pepsine digestion

Feed Emulsifiers
Complement bile acid activity

Trace-elements & co-factors
Support enzyme activity in digestion & metabolism

Preselected synergistic blends of digestibility enhancing additives are being screened for their capability to improve growth and food conversion in a number of model species (rainbow trout *Oncorhynchus mykiss*; European seabass *Dicentrarchus labrax*; Nile tilapia *Oreochromus niloticus*; Tra catfish *Pangasius hypophthalmus*). Test feeds are formulated to be representative for average industry standards per species and processed using industrial extrusion technology. Trials executed under controlled lab conditions have revealed enormous differences in efficacy among different additive concepts designed to improve digestion (Figure 3). Some digestibility enhancers seem to be incompatible with the digestive physiology of certain fish species and affected fish performance negatively. The addition of selected blends of digestive aids improved growth/feed conversion ratio in rainbow trout (up to 6% improvement of growth and 8% on FCR versus non-supplemented control group), Nile tilapia (+5/-9% on growth/FCR), pangasius catfish (+6/-15% on growth/FCR), and European seabass (+5/-5% on growth/FCR). These results show the potential of digestibility enhancers to improve feed efficiency in fish farming, resulting in improved economics, more sustainable use of feed ingredients and reduced environmental impact.

Figure 3: Screening of synergistic blends of digestibility enhancers at lab scale comparing product efficacy and cost-efficiency for each fish species



Data show results for growth (SGR, %/day) and feed conversion ratio (FCR) relative to the performance of the non-supplemented control group. Feeding trials were run in triplicate tanks for rainbow trout (trial duration 64 days; starting from 19 g); Nile tilapia (56 days; starting from 9 g); Pangasius catfish (70 days starting from 16 g); and European seabass (84 days; starting from 224 g) (Nutriad, internal reports).

Dose-reponse trial for a digestibility enhancer with Tilapia under laboratory conditions

Dose-response trials under lab conditions provide useful data to explore the most cost-efficient dosage to be recommended for field verification. The supplementation of 1.5 kg per MT of feed of a digestive aid (AQUAGEST® OMF) yielded significantly better FCR (-5%), growth (+4%) and protein efficiency ratio (+4%; Table 1). When increasing the supplementation from 1.5 kg to 3 kg per MT of feed, all these factors were further improved. The hepatosomatic index was reduced by increasing the level of the feed additive, amounting to a reduction of 11% for the 3 kg/MT supplemented diet. The viscerasomatic index was reduced by 8% in the 1.5 kg/MT supplemented diet. The decreased viscerasomatic and hepatosomatic indexes and increased protein efficiency ratio indicate that more dietary energy/protein is made available for growth. This allows the farmer to improve feed conversion, fillet yield and reducing visceral wastes.

Table 1: Dose-response effect for a digestive aid in Nile tilapia (trial duration: 8 weeks; average (n=3) ± SD, different letters in the same row denote significant differences at p≤0.05; Ceulemans et al., 2009)

	Control	AQUAGEST®OMF 1,5 kg/MT feed	% vs control	AQUAGEST®OMF 1,5 kg/MT feed	% of control
Survival (%)	100 ± 0	100 ± 0		100 ± 0	
Initial weight (g)	9.17 ± 0.04	9.15 ± 0.13		8.93 ± 0.21	
Final weight (g)	41.84 ± 0.83	44.32 ± 1.25		44.3 ± 1,99	
SGR (% day)	2.71 ± 0.03^{a}	2.82 ± 0.03^{ab}	*+4.1%*	2.86 ± 0.08^{b}	*+5.5%*
Feed Intake (g/ind)	37.86 ± 0.65	38.83 ± 1.18		38.3 ± 1.15	
Feed Conversion Ratio	1.16 ± 0.01^{a}	1.10 ± 0.01^{b}	*-5.2%*	1.08 ± 0.03^{b}	*-6.9%*
Protein Efficiency Ratio	2.75 ± 0.03^{a}	2.86 ± 0.02^{ab}	*+4.0%*	2.92 ± 0.1^{b}	*+6.2%*
Hepatosomatic Index (%)	1.60 ± 0.15	1.48 ± 0.11	*-7.5%*	1.43 ± 0.01	*-10.6%*
Viscerasomatic Index (%)	8.45 ± 0.09^{a}	7.79 ± 0.11^{b}	*-7.8%*	8.41 ± 0.13^{a}	*-0.5%*

Optimizing the application strategy for a digestibility enhancer with Tilapia under field conditions

Sampaio-Gonçalves et al. (2012) recently evaluated different application dosages of a digestibility enhancer as well as the effect of the basal feed quality on the cost efficiency during a production cycle of Nile tilapia farmed in cages in Brazil. The study evaluated the entire production cycle of tilapia, including the processing of commercial size fish, and was carried out by the Instituto de Pesca in collaboration with a commercial tilapia integration in the Sao Paulo region, Brazil.

Photo 2: Tilapia was grown from 28 g to commercial size in 7m³ cages (pictures provided by Giovani Sampaio Gonçalves)

The trial was performed during two consecutive phases in 7 m³ cages: phase 1 (from 28 g to approx. 170 g) and phase 2 (from 170 g to 750 g, i.e. commercial size). In Phase 1, 880 juveniles of Nile tilapia (GIFT strain, initial weight 28 g) were stocked per cage. 10 replicate cages were fed the control feed, consisting of a commercial feed (36% crude protein). The treatment feed consisted of the control feed supplemented with a digestibility enhancing feed additive (Aquagest® OMF, Nutriad; 3 kg per MT of feed). The treatment group consisted of 15 replicate cages.

In Phase 2, the stocking density was 800 fish per cage and 4 different treatments were run with 5 replicate cages per treatment: (1) Control: fed a commercial feed with 32% crude protein; (2) AG3: control feed supplemented with the feed additive at 3 kg/MT throughout the entire cycle; (3): AG 3/1.5: control feed supplemented with the feed additive at 3 kg/MT until 350g and subsequently at 1.5 kg/MT until the end of the trial; (4) LC-AG2: low cost feed, formulated with protein of lower digestibility and poorer amino acid profile, supplemented with 2 kg/MT of the feed additive (7% reduced formula cost compared to the control feed). During Phase 2, the control group was stocked with fish originating from the control group during phase 1; whereas the other treatments were recruited from fish receiving the additive during phase 1. Feeds were produced in a commercial extrusion line and the additive was included directly in the mixer with all other ingredients prior to extrusion. Feed distribution was based on feeding tables four (Phase 1) and three times per day (Phase 2). At the end of Phase 1, all fish, were weighed, average daily weight gain and feed conversion were determined. At the end of Phase 2, all fish from each experimental cage were weighed and counted. At harvest, 5% of the population per cage were processed for fileting. The evaluated parameters included survival, daily weight gain, feed conversion, fileting yield, viscera weight, liver weight and visceral fat weight.

At the end of Phase 1, fish supplemented with the feed additive showed significant improvements on performance compared to the control group, i.e. 12% better daily weight gain, 5% better survival and 6% better feed conversion (Figure 4).

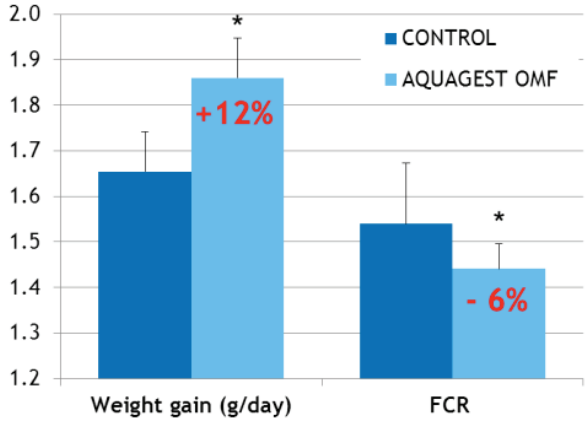


*Figure 4: Daily weight gain and feed conversion of juvenile Nile tilapia during phase 1 (from 28 to approx. 170 g) fed the control diet with or without the supplementation of a digestibility enhancing additive. * indicate significant differences (p<0.05) and deviations from control are shown as percentages in red (Sampaio-Gonçalves et al., 2012)*

During phase 2, the best results were obtained by supplementing the control feed throughout the production cycle with 3 kg/MT of the feed additive (treatment AG3); resulting in a significantly better final weight and reduced amount of feed consumption at the end of the cycle (Table 2). Relatively compared to the control group, treatment AG3 showed improved survival (+2.8%), daily weight gain (+5%), feed conversion (-6.4%), fileting yield (+1.5%), visceral fat deposition (-9.9%), hepatosomatic index (-22%), and viscerosomatic index (-10.7%). Reducing the additive inclusion from 3 to 1.5 kg/MT during phase 2, still resulted in interesting benefits on growth, feed conversion and visceral fat but did not affect fileting yield.

Table 2: Fish performance and processing parameters (fileting yield, visceral fat, hepatosomatic index HSI, viscerosomatic index VSI) of Nile tilapia (from approx. 170 g to commercial size) fed different diets during phase 2

	Control		Control + AQUAGEST OMF 3 kg		Control + AQUAGEST OMF 3-1.5 kg		LC Feed + AQUAGEST OMF 2 kg	
Fish performance								
Survival (%)	92.1	ab	94.7	a	93.0	a	85.6	b
Initial weight (g)	166.9		177.4		166.7		160.8	n.s.
Final weight (g)	714.2	b	751.1	a	730.1	ab	739.2	ab
Growth (g/day)	4.93		5.17		5.08		5.21	n.s.
Feed Intake (kg/cage)	676.6	b	644.5	a	655.25	ab	662	ab
FI as (%ABW/d)	3.09	ab	2.91	a	2.93	a	3.18	b
FCR	1.72	ab	1.61	a	1.63	a	1.77	b
Processing parameters								
Fileting yield (%)	33.4	a	33.9	a	33.3	a	31.5	b
Visceral Fat (%)	4.76		4.29		4.17		5.00	n.s.
HSI (%)	1.07		0.83		0.96		0.84	n.s.
VSI (%)	8.84		7.89		8.18		8.67	n.s.

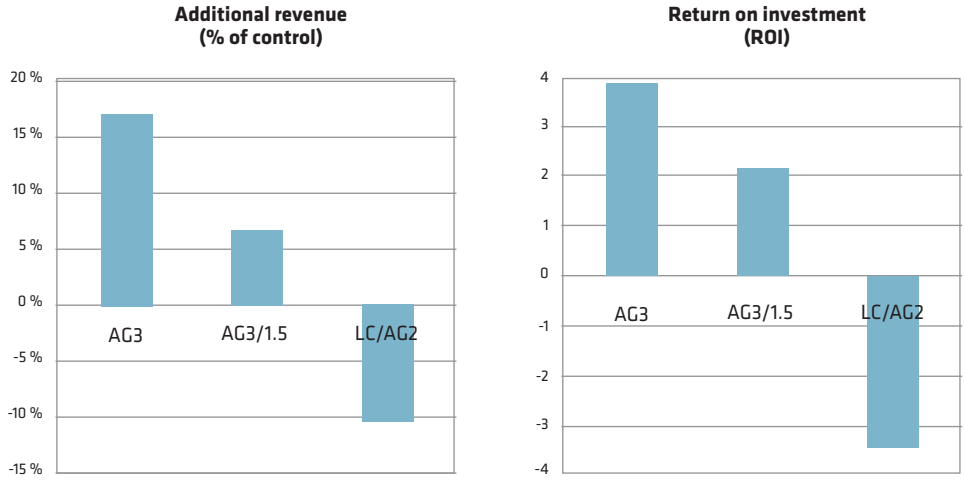
Different letters indicate significant differences (p<0.05, Sampaio-Gonçalves et al., 2012)

The low cost feed performed significantly worse compared to the control feed in most parameters, particularly in terms of survival, feed conversion and fileting yield. The addition of 2 kg/MT of the feed additive was only capable of improving growth but the nutritional impact of reducing the protein digestibility and amino acid balance in this feed heavily affected the overall performance, particularly FCR and survival. The trials results showed that tilapia are highly sensitive to reducing the quality of the dietary protein in the feed. The digestibility enhancer was not capable of rectifying the effect of inferior nutritional specifications, which finally resulted in a less cost-efficient feed for the producer.

Considering the cost efficiency of the different feeds, the optimal additive treatment (3 kg/MT throughout the production cycle) improved farm revenues with 17% compared to the unsupplemented control group and showed a return on investment (ROI) of 3.8:1 (Figure 5). Reducing the additive inclusion to 1.5 kg/MT of feed once fish reach 350 g still resulted in 7% improved revenues compared to the unsupplemented control group and a ROI of 2.1:1. The application of the low cost feed supplemented with 2 kg/MT of the additive resulted in important economic losses (10% reduced revenues compared to control).

This study clearly indicated the potential of improving cost efficiency of tilapia feeds through the use of digestibility enhancing additives. The results showed the importance of maintaining the nutritional balance in the feed in order to maximize the benefits of a digestibility enhancing concept. It is important to note that the economic impacts of performance enhancing feed additives (having a relatively stable cost, independent from standard commodity ingredients) increases dramatically with increasing ingredient cost.

Figure 5: Economic evaluation of different application strategies for a feed additive in tilapia farming. Feed ingredient costs used for this study dated early 2012. Data show change of farm revenues and return of investment, relative to the non-supplemented control group. Treatment groups differ in inclusion of the feed additive and formulation: AG3 (control feed + 3 kg/MT throughout the production cycle), AG3/1.5 (control feed + 3 kg/MT till 350 g; followed by 1.5 kg/MT till harvest), LC/AG2 (low cost feed + 2 kg/MT)



Improving performance of carnivorous marine fish fed low levels of fishmeal

Traditional feed formulations for marine fish are high in marine ingredients, containing 40-50% good quality fish meal and 10-15% fish oil. Animal by-products, including specific qualities of poultry by-products and blood meals, offer interesting alternatives in terms of nutritional profile and digestibility but are not always an alternative to fish meal due to market conditions (eg high demand by the petfeed industry) and/or consumer concerns. Increased replacement of fish meal/fish oil by vegetable protein sources derived from soybean, wheat, rice and corn offers possibilities to stabilize formulation cost in marine fish feeds. However, many nutritionists are hesitant to use high levels of vegetable protein in marine fish feed due to the fear for slower growth and poorer feed conversion compared to traditional feeds containing high levels of fish meal. Carnivorous fish – like any other animal – require a balanced diet which is satisfying nutritional requirements and of acceptable palatability/digestibility to promote ingestion/digestion. Formulating marine fish feed with less marine ingredients requires good knowledge of nutritional requirements and functional/nutritional additives which are capable of solving some of the typical problems encountered in carnivorous fish fed high levels of plant protein, such as reduced palatability and digestibility, anti-nutritional factors and amino acid misbalances.

One of the challenges for replacing fish meal by vegetable protein sources in feeds for carnivorous fish is the effect of vegetable ingredients on lipid digestion, absorption and deposition. This has recently been documented for a number of carnivorous fish species including atlantic salmon (Kroghdal et al. 2003), rainbow trout (Romarhein et al., 2008) and gilthead seabream (Gómez-Requeni et al., 2004). This presents an important challenge for improving low fishmeal diets due to the importance of dietary fat in the energy balance of marine fish.

The addition of a digestibility enhancer with main emphasis on lipid digestion for carnivorous fish (AQUAGEST® CAF) to practical feed formulations containing reduced levels of fishmeal improves significantly growth (with 6.5%), food conversion ratio (with 7%) and protein efficiency ratio (PER, with 7.6%) in a marine, carnivorous fish such as European seabass (*Dicentrarchus labrax;*

Figure 6. Data from a 84-days culture trial with European seabass using triplicate tanks of 600 L per diet. Fish were grown at 20-22°C in a seawater recirculation unit from 224g to ± 318g. The control diet consisted of a practical coated-extruded grow out (crude protein 45%; crude fat 20%), containing 19.6% Peruvian fishmeal (67%CP); 17% fish oil, 5.9% wheat gluten, 6% corn gluten, 5% pea protein concentrate, 32% defatted soybean meal, 8.5% wheat flour, 4% extracted rapeseed meal, 1.15% monocalcium phosphate, methionine 0.1%, lysine 0.2%, premix 0.55% (vitamins, minerals, antioxidant, antimould) (Coutteau et al., 2010)

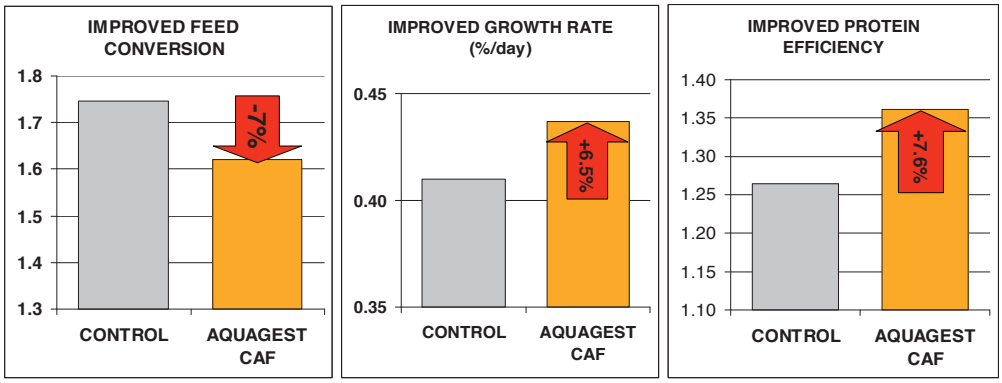


Figure 6) (Coutteau et al., 2010). This clearly demonstrates the nutrients present in feeds containing low levels of fish meal and high levels of vegetable proteins are currently underutilized and require functional additives to maximize their utilization by the fish's digestive and metabolic processes.

Natural emulsifiers to enhance lipid digestion in shrimp

Shrimps do not tolerate high levels of dietary fat very well. A number of studies show reduced growth at levels above 10% of dietary lipid. Nevertheless, shrimp have no or very limited capacity to biosynthesize a number of lipid molecules which are essential for normal growth, including cholesterol, highly unsaturated fatty acids and phospholipids. Cholesterol is a key constituent of cell membranes and precursor for steroid and moulting hormones. It has been found to be most effective in different species of shrimp at dietary levels ranging from 0.20% to 0.5%. Cholesterol levels below 0.10% limit growth in *Litopenaeus vannamei*, even if the other nutrients are formulated to satisfy normal requirements (Duerr and Walsh, 1996). Fishmeal is the major cholesterol source in practical feed formulations for shrimp with some contributions also coming from fish oil, squid and shrimp meal. Shrimp cannot bioconvert highly unsaturated fatty acids (HUFA: 20:5n-3 or EPA; 22:6n-3 or DHA) which need to be provided at 0.8-1% in the diet via the marine ingredients, mainly fish oil and fish meal. Phospholipids are a component of cell membranes, building blocks for lipoproteins (essential for lipid mobilization in the haemolymph), and constitute a highly available source of choline and inositol (Coutteau et al., 1997). Although the optimal level may depend on the formulation and culture conditions, phospholipid requirements are estimated around 1-2% (equivalent to 1.5-3% liquid lecithin). The combination of suboptimal dietary levels of cholesterol and phospholipids may be particularly detrimental to shrimp nutrition due to the interaction between phospholipids and cholesterol requirements (Gong et al., 2000).

Emulsifying agents are a diverse class of compounds which are able to disperse fat in water under the form of small droplets (as in milk). In land animals, emulsifiers are added to the feed to complement the lipid digestive processes by reducing the size of the fat globules released from the diet and increasing the efficiency of the lipase activity. Lipid digestion in shrimp occurs for a big proportion intracellular in the hepatopancreas epithelium

Photo 3: White shrimp (Litopenaeus vannamei; with courtesy of Peter Coutteau)

from where it is transported to the target organs via the haemolymphe under the form of lipoproteins. The formation and absorption of lipid micelles from the lumen of the hepatopancreas tubuli is therefore a limiting step in the lipid digestive process. Digestibility enhancers based on natural emulsifying agents, selected for their compatibility with the shrimp's digestive system, have shown to be capable of complementing the process of emulsification and absorption of dietary fats in the hepatopancreas. This in turn improves the efficiency of shrimp to use fats as essential components and as source of energy for growth.

Coutteau et al. (2011) reported on the use of a digestibility enhancer based on natural emulsifiers to reduce the need for cholesterol supplementation in the diet of tiger shrimp (*Penaeus monodon*). Experimental diets were prepared by lab scale pelletizing. Diets were formulated to satisfy nutritional requirements of P. monodon, except for cholesterol. Three feed formulations were compared:

1) A control diet (CONTROL-) with no cholesterol supplementation and a background concentration of 0.10% cholesterol (provided by the marine ingredients fishmeal and shrimp head meal).
2) A positive control diet (CONTROL+) supplemented with 0.15% cholesterol to a total level of 0.25% cholesterol in the diet.
3) A test diet (AQUAGEST S) supplemented with a digestibility enhancer based on an optimized blend of natural emulsifiers (Aquagest® S, Nutriad International, Belgium).

Overall shrimp growth (up to 1.4 g/week) and survival (close to 90%), was excellent under the conditions of clear water culture. Growth and feed conversion were significantly improved by the supplementation of 0.15% cholesterol. After 70 days of culture, harvested biomass was 33% higher for the shrimp fed the diet supplemented with cholesterol (Table 3). The digestibility enhancer was equally effective as purified cholesterol in improving growth and feed conversion compared to the negative control diet. After 70 days of culture, harvested biomass was 30% higher in the AQUAGEST S treatment compared to the negative control.

Table 3: Survival, growth and food conversion of *P. monodon* fed during 70 days on different experimental feeds

	Control (0.1% cholesterol)	Control (0.25% cholesterol)	Control (0.1% cholesterol+ Aquagest)
Survival rate (%)	88.3±5.8	90.3±5.0	91.7±7.6
Initial Weight (g)	0.65±0.04	0.64±0.02	0.66±0.02
Final Weight (g)	5.9±0.4 a	7.8±0.4 b	7.5±0.2 b
Final Total Biomas (g)	103±2 a	137±9 b	134±8 b
FCR	3.9±0.2 a	3.1±0.2 b	3.4±0.1 b

Average ± SD from triplicate tanks; different letters in the same row denote significant differences $p<0.05$; Coutteau et al., 2011

Photo 4: Feeding trial ponds and harvest of Pangasius catfish in Vietnam (with courtesy of Alexander van Halteren)

Optimizing the application regime to improve feed utilization and fillet yield in Pangasius catfish farming

Pangasius catfish production has grown very rapidly in Vietnam to reach more than a million mt in recent years. This rapid growth has resulted in the industrialization of feed production as well as in culture technology. Whereas traditional catfish farmers produce farm-made feeds by mixing and cooking local raw materials in large kettle cookers, larger integrators have installed state-of-the-art extrusion lines producing compound feed following stringent quality control schemes. Due to declining market prices for Pangasius, farmers and feed producers are continuously searching for ways to improve feed performance and optimize fillet yield to maintain profitability.

The optimal application of novel feed additives requires field evaluations to provide information in terms of farm economics and processing qualities of the fish. The effect of different application regimes for a digestibility enhancer was evaluated for *Pangasius hypophthalmus* ("Tra") in two farm locations of Hung Vuong Mien Tay breeding joint stock company in the Mekong Delta (Van Halteren et al., 2009). The various treatments differed in the inclusion level of the digestibility enhancer (AQUAGEST® OMF, Nutriad, Belgium) in the extruded feed and the period during which it was applied during the production cycle (Table 4). Trials were carried out under standard production protocols in earthen ponds of approximately 5-6,000 m^2 (Trial site Tra Vinh) and 8,500-12,000 m^2 (trial site Sa Dec). The ponds were stocked with juveniles with an average size of 15g at a density of 45-50 fish/m^2 in Tra Vinh, and 30 g fish were stocked at 34-38 fish/m^2 in Sa Dec.

Table 4. Two different application regimes evaluated for supplementing a digestive aid to the feed of *Pangasius* catfish in two farm locations of Hung Vuong Mien Tay breeding joint stock company in the Mekong Delta (van Halteren et al., 2009)

FISH SIZE (g)	Feed specifications (% crude protein)	TREATMENTS		
		Control	AQUAGEST 1	AQUAGEST 2
20-100	28	-	AQUAGEST® OMF 1 kg/MT of feed	AQUAGEST® OMF 2 kg/MT of feed
100-200	28	-	AQUAGEST® OMF 1 kg/MT of feed	AQUAGEST® OMF 2 kg/MT of feed
200-500	26	-	-	AQUAGEST® OMF 1 kg/MT of feed
> 500	22	-	-	-

Overall, the results obtained for FCR and individual daily growth rate were excellent compared to the industry average in Vietnam (FCR: 1.6-1.8; growth: 4-5 g/day). The results from both trial locations showed improved growth and food conversion, and a shortening of the production cycle with the application of the feed additive (Table 5). The improvement on growth performance was modest for the lowest dosage applied only during the initial period (treatment Aquagest 1). However, very significant improvements were obtained for the second treatment (Aquagest 2) compared to the control, including improvements of FCR by 3.3-3.2%, daily weight gain by 10.6-14.8% and a reduction of the culture duration by 5.2-16.4%. All farm results showed better economics due to the application of the digestibility enhancer. This was due to a combination of the improved feed cost per kg of fish produced (0.2-0.7% for the lowest application regime and 1.4-2.4% for the highest application regime) and the significant shortening of the production cycle.

Table 5. Effect of two different application regimes of a digestibility enhancer on duration of the production cycle, growth and FCR of Pangasius catfish in two trial locations in Vietnam (van Halteren et al., 2009)

TRIAL SITE 1: Tra Vinh			
Treatments (see Table 4)	Days of culture to reach 850 g (days)	Individual fish growth rate (g/day)	FCR
Control	210	3.96	1.52
Aquagest 1	207	4.04	1.51
Aquagest 2	199	4.38	1.47
TRIAL SITE 2: Sa Dec			
Treatments (see Table 4)	Days of culture to reach 850 g (days)	Individual fish growth rate (g/day)	FCR
Control	171	4.53	1.55
Aquagest 1	171	4.40	1.53
Aquagest 2	143	5.20	1.50

The fillet ratio is an important criterion for the processor in the Pangasius industry as the main market is sales of fillet. The fillet ratio was determined in the fish processing plant of Hung Vuong Joint stock company after harvesting the ponds.

Pangasius fillets are obtained in three stages, first the total fillet is cut from the fish, secondly the skin is removed from the fillet, and finally the fillet is adjusted to the market demands (less or more fat left depending on the destination country. The first stage "whole fillet" ratio averages 1.9-2.2 in Vietnam and is reported for the trials in Table 6. Overall, the whole fillet yields obtained in the current trials were excellent in comparison to the industrial average in Vietnam (1.9-2.2). Nevertheless, whole fillet yield improved drastically in all trials because of the addition of the digestibility enhancer, i.e. 2.4 to 4.6% for the lowest application and 7.4-7.5% for the highest dosage evaluated.

Table 6. Effect of two different application regimes of a digestive aid on fillet ratio (kg whole fish needed for 1 kg of whole fish fillet) of Pangasius catfish in two trial locations in Vietnam (percentage shows difference compared to non-supplemented control) (van Halteren et al., 2009)

Treatements (see Table 4)	TRIAL SITE 1: Tra VInh	TRIAL SITE 2: Sa Dec
Control	2.14	2.16
Aquagest 1	2.09 (-2.4%)	2.06 (-4.6%)
Aquagest 2	1.98 (-7.5%)	2.00 (-7.4%)

Growth promotion based on optimized gut health and intestinal microflora

The ban on the use of antibiotic growth promoters in poultry and pigs, and the subsequent search for alternatives, has revealed the importance of gut health and the development of a stable, favorable gut microflora, on feed efficiency, overall performance and productivity. Fish and shrimp are highly exposed to exchanges of microflora between the environment and the digestive system. This increases the risk for the proliferation of an unfavorable gut microflora or frequent destabilization of the microflora, which can affect the optimal functioning of the digestive system. Furthermore, the digestive system of fish and shrimp is the main entry port for bacterial and viral infections, which remain a major risk for the profitability of aquaculture production.

Sustainable approaches to modulate the gut microflora in farmed animals include the use of selected bacteria to inoculate the gut (probiotics), specific nutrients promoting the development of selected bacterial strains (prebiotics), and specific natural compounds (mostly derived from yeast and herbal extracts, so called "phytobiotics") capable of modulating the microflora towards a favorable composition, favoring the development of beneficial bacteria and inhibiting potentially pathogenic microorganisms. The latter strategies have the advantage of being easily applicable at

the feedmill on large volumes of feed and avoiding major adaptations of the production protocols at the farm. A synergistic blend of phytobiotics was selected for their bacteriostatic and bactericidal properties against pathogenic and potentially pathogenic bacteria in vitro using the disk diffusion method. This blend was capable of promoting growth significantly in feeding trials with healthy specimens of different species of fish and shrimp growing under controlled lab conditions (Figure 7).

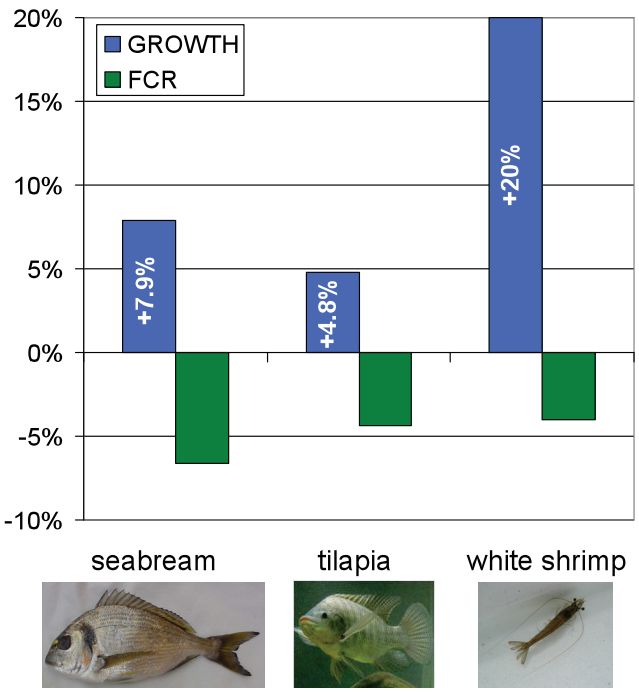


Figure 7: Percentage improvement of growth and feed conversion ratio (FCR) due to supplementing a phytobiotic growth promoter based on microflora modulation (SANACORE® GM) to a practical feed of different aquaculture species. Data show relative effect on growth (for fish : SGR, %/day; for shrimp g/week) and feed conversion ratio (FCR) relative to the performance of the non-supplemented control group in a feeding trial with healthy animals. Feeding trials were run in triplicate tanks for Gilthead seabream Sparus aurata (trial duration 56 days; starting from 70g); Nile tilapia Oreochromus niloticus (70 days; starting from 17g); and white shrimp Litopenaeus vannamei (56 days starting from 1g) (Coutteau et al., 2010)

Effect of optimizing gut health and intestinal microflora on productivity and economics of semi-intensive shrimp farming

The efficacy of phytobiotics was tested under the field conditions for shrimp production in Panama during the dry season (September 2009- February 2010) by Vaca et al. (2010). The dry season in Panama is characterized by unstable climatological conditions, resulting in strong temperatures fluctuations which in turn affect shrimp growth and increase the impact of outbreaks of white spot virus (WSSV). During the trial, two treatments were compared which only differed with regard to the supplementation or not of a phytobiotic growth promoter (Sanacore® GM, Nutriad, Belgium) to the standard feed used at the farm.

The supplementation of the phytobiotic feed additive resulted in improved values for all production parameters analysed in this study (Figure 8). Survival and processed crop yield (kg/ha) presented highly significant improvements ($p<0.03$), amounting to a relative increase with 24% and 35% compared to the control group, respectively. Although the other parameters did not show significant differences, important improvements were observed for the

Photo 5 Feeding manually from a boat and harvesting shrimp at the CAMACO farm, Panama (with courtesy of Jorge Cuéllar-Anjel)

treatment receiving the phytobiotic, including 5.8% larger average shrimp size at harvest and 12% better feed conversion compared to the control group. The addition of the phytobiotic reduced drastically the variability of production results among ponds fed the same feed (average coefficient of variation between ponds for the 6 production parameters: control 18% versus SANACORE group 10%; Table 7).

Table 7: Production results after processing for control ponds and treatment ponds receiving phytobiotic supplement after 141 days of culture

Treatment	Survival (%)	Shrimp size (g)	Crop Yield (kg/ha)	Feed (kg/ pond 3 ha)	FCR	Weekly Growth (g/wk)	Average coefficient of variation for parameters listed (CV%)
Sanacore® GM	55.5 ± 7.1 a	16.6 ± 1.5 a	735 ± 78 a	4,170 ± 338 a	1.91 ± 0.23 a	0.825 ± 0.075 a	10%
Control	44.6 ± 10.6 b	15.7 ± 2.9 a	543 ± 90 b	3,464 ± 396 b	2.17 ± 0.39 a	0.776 ± 0.137 a	18%
% change Sanacore vs control	+ 24.4%	+5.8%	+35.2%	+20.4%	-12.1%	+6.3%	-41%
P Value	0.034	0.4395	0.0004	0.0018	0.7130	0.3876	––

Average and standard deviation of 8 replicate ponds of 3ha per treatment; Vaca et al., 2010.

The drastic effects of the phytobiotic product on survival may be related to the fact that this study was performed during the worst farming cycle of the year in Panama, when shrimp ponds are exposed to severe transitional changes in weather at the end of the wet season and the beginning of the dry season. Natural White Spot Disease outbreaks were observed during shrimp farming in both treatments under similar frequency and severity; WSSV virus was confirmed by immuno-chromatography and nested-PCR tests. The presence of a synergistic blend of phytobiotics with antimicrobial activity, possibly protected the shrimp from co-infections with opportunistic bacteria, often the major cause of mortality in WSSV-infected shrimp. Higher crop yield per hectare due to better survival often results in slower growth due to reduced availability of natural food. Despite significantly higher survival and crop yield, a positive effect was observed of phytobiotics on growth rate and food conversion. This confirmed that the continuous dosing of natural antimicrobial activity in the feed results in beneficial physiological effects from stabilizing the microflora present in the digestive system.

Conclusion

Current aquafeed formulations are mainly focused on nutritional specifications and ingredient choice. The optimal utilization of the nutrients by the fish and shrimp and the health status of the digestive tract are two areas still under development in aquaculture. This is in strong contrast with the vast progress made in the agrifeed sector, where nutrient digestibility and gut health are regarded as two focus areas for technological feed development. The current paper illustrated with lab and field studies the potential benefits for aquaculture in terms of productivity and economics of specific feed additives developed to enhance the functioning of the digestive tract, either by improving the nutrient utilization or by stimulating the development of a healthy gut microflora.

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Plant protein isolates and hydrolysates as alternative to the animal protein in aquaculture diets

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Summary Aquaculture has been the bigger growing sector in food production over the last decade. To continue in this position, needs an optimization of the feeding and fattening systems of fishes, reducing the use of fish oil and meal, so dependent on extractive fishing, increasing more overexploited. The plant protein isolates and hydrolysates, from legumes and oilseeds, are attractive alternatives because of its availability and its low cost, being perfect candidates for this function, and consequently to achieve an increasingly sustainable and friendly environment aquaculture.

Introduction

In the last four years (2007-2011) and with a 20 million Euros budget, the largest aquaculture research project called "ACUISOST- Towards Sustainable Aquaculture" has been developed in Spain.

It has been supported financially by the Technology Development Centre (CDTI). This initiative was made possible thanks to the constitution of a consortium participated by 25 national companies, led by Dibaq Aquaculture and 20 public research organizations, which belong to the total of 17 participating research groups.

The purpose of ACUISOST has been to position the sector in an advantageous position by exploiting synergies and peculiarities of the Spanish production, cultural and geographical system,as well as generate new knowledge in areas such as new additives, encapsulation systems, application of new techniques and biotechnology in order to improve the functionality of aquaculture diets, implementation of new production and biosafety technologies, management of aquaculture waste or design of new and more attractive consumer products.

In this sense, one of the main activities in the project mentioned above has been the study from different points of view, such as nutritional, environmental, technical and economic aspects, several raw materials comparing with the traditional fish meal. After the production of protein isolates, protein hydrolysates were developed in a pilot-plant scale with the goal of being used subsequently in feed formulation and feeding on

industrial scale. Thus, commercial enzymes, like Alcalase and Flavourzyme, were used in a sequentially way in order to get protein hydrolysates with a high degree of hydrolysis.

Development

Traditionally, animal protein supplements were and are the main source of the aquaculture feed formulation. Thus, the diet provided in larval stages is based on live rotifer and artemia (micricrustaceans). Rotifers can be raised in ponds with algae, but is cumbersome, and the farmer has to have time, labour and place to grow them. More difficult is the case of Artemia due to the lack of an efficient culture system, because the artemia cysts are collected and commercialized in cans. A priori, the farmer only has to put those eggs to hatch. The problem is that there are few places worldwide where Artemia cysts can be harvested industrially, and subsequently, it is not only a scarce and expensive product but also this culture system has a high risk of overexploitation. Given the limited global supply and the increasing in prices of these products, aquaculture nutritionists are considering other protein resources. Moreover, these problems will beset in the future as aquaculture will take a greater role occurring a more competition for the same substrates and therefore less profits margins.

At the same time, other considerations, not just the lack and the price of animal protein foods in the future, are taken into account when alternative protein sources for aquaculture are looked for. One of the main current factors is the accumulation of contaminants in animal diets. Protein supplements based on fish meal are composed mostly of nitrogen. Those that are not eaten or digested are introduced into the environment and the production facilities, increasing nitrogen and phosphorus concentrations unnecessarily, causing eutrophication problems and excessive growth of algae.

On the other hand, the wild fishes would eat also contaminants that have been accumulated in different prey in the food chain. Currently, in this case, the aquaculture fishes have the same peculiarity because it is fed by fishmeal and fish oil mainly. For these reasons and because it is not possible to control the diet in wild fish, the only option is to control the diet of farmed fish.

The contribution of alternative protein sources in aquaculture feed instead of the current animal protein, would decrease the wild fish catches, would improve water quality and would reduce pollutants in the diet derived from fish food. It is an urgent need that protein resources do not interfere with food safety in humans. Recent disease outbreaks or epidemics such as bovine spongiform encephalopathy transmitted through animal feed including animal protein, have led to a cascade of questions about if it is convenient to feed vegetarian species with animal protein sources. One of the alternatives could be found in plant protein sources as these are available in greater quantity and the cost is lower than the fish meal or animal protein. In fact, protein supplements derived from plant sources as protein resource are being incorporated into diets for aquaculture as a supplement of the animal protein, although this has been done in low rates of substitution. Some of the causes of its total replacement has not been

carried out may be the presence of antinutritional factors in vegetable meals, his low digestibility especially in the larval stage of fish, because they have not developed an efficient digestive system yet, and its lower contribution to energy intake and some minerals such as phosphorous. While this last point is easily solved by an optimal formulation of the final product, the first two reasons are attractive targets for further and deeper research.

Therefore, the biotechnology has much to say in this regard and could be a suitable instrument for solving these problems. Plant seeds and meals have a very heterogeneous composition, thus it is not easy to include them in the aquaculture formulation. The replacement of fish meal with plant protein concentrates is associated with antinutritional compounds in plant proteins presenting a mixed picture on antinutrient (Francis et al., 2001). Generally, these protein concentrates produced from oilseed contain antinutrients that affect the aquaculture nutrition more than those that come from the cereal process.

Some of these antinutritional factors are destroyed or inactivated (with or during the processing of vegetal material) by the pelletization and extrusion process. Others, however, remain unchanged and should be removed or reduced to eliminate the detrimental factor for the optimum growth of the fish. Among the antinutritional factors are the phytic acid, glucosinolates, saponins, tannins, carbohydrates or polysaccharides insoluble, gossypol and phytoestrogens. Therefore, a purification of the proteins in these seeds and meals not only would solve the problem derived from the formulation but also the removal of different compounds that could have antinutritional factors. Thus, the plant protein isolates obtained by alkaline solubility and subsequent precipitation of solubilized proteins at his isoelectric point, is a process of great performance and very feasible economically, where the final result is a product with a high protein content, more than the 70 - 80% in dry matter, and therefore with a great decreased of antinutritional factors that may be included in the starting vegetable meal (Pedroche et al., 2004, Rodríguez-Ambriz et al., 2005; Taherian et al., 2011 ; Rodrigues et al. 2012).

Nowadays, there are a number of experimental assays carried out with plant proteins, trying to achieve a possible substitution of the animal protein and many of them have a high digestible plant protein similar to the levels found in animal protein (Tibbetts et al., 2006; Hardy, 2010, Cabral et al., 2011, Enterria et al., 2011, Richard et al., 2011). Most of these tests were made from plant protein meals or concentrates where the percentage of antinutritional factors may be excessive for efficient growth of fish (Francis et al., 2001). Furthermore, recent studies have shown that the essential amino acid content and the source from which these amino acids come from are an essential requirement when we need to optimize the percentage of fish meal replaced by plant protein (Larsen et al., 2012). Thus, corn gluten meal is usually found as a high digestible protein source in Teleosts as tilapia (Wu et al., 1995), rainbow trout (Gaylord et al., 2010), Atlantic cod (Tibbetts et al. , 2006) or sargo/white seabream.

(Pereira and Oliva-Teles, 2003). In turbot, however, the inclusion of corn gluten meal causes a decreased of the total protein digestibility as well as somne amino acids as leucine, decreasing from 94% to 68% when the fish meal is totally replaced by corn gluten meal (Regost, 1999). It is important to know that most of these experiments have been carried out in stages with fish juveniles which have an efficient and fully developed digestive system in contrast to the larval stage which does not present a functional stomach and hence the protein digestion may vary according to the source from which comes the protein and the process that the ingredients have suffered during the manufacture of the food (Garcia, 2000). In this way, the protein isolates could present a major disadvantage as the low digestibility since they are composed by intact proteins with high molecular weights and aggregations in some cases. That is why these protein isolates are the ideal substrate for the production of plant protein hydrolysates which have a higher digestibility than the intact starting proteins.

Table 1. Chemical characterization of meals, protein isolates and hydrolysates from chickpea and lupin

	Chickpea Meal	Chickpea Isolate	Chickpea Hydrolysate	Lupin Meal	Lupin Isolate	Lupin Hydrolysate
Protein	26.2 ± 3.1	74.98 ± 0.12	62.65 ± 0.83	33.8 ± 6.9	85.17 ± 0.75	68.94 ± 0.05
Ash	2.8 ± 0.3	2.71 ± 0.04	8.21 ± 0.05	2.1 ± 0.1	2.39 ± 0.07	8.78 ± 0.05
Moisture	8.9 ± 1.6	1.54 ± 0.19	3.82 ± 0.06	7.9 ± 0.6	0.54 ± 0.20	4.00 ± 0 05
Fat	4.7 ± 1.1	14.94 ± 0.04	14.28 ± 0.20	13.6 ± 2.0	11.04 ± 0.18	8.62 ± 0.23
FIber	10.7 ± 2.0	1.65 ± 0.25	2.40 ± 0.54	39.9 ± 6.1	0.25 ± 0.20	2.59 ± 0.33
Soluble Sugars	1.9 ± 0.6	0.49 ± 0.01	0.28 ± 0.01	2.7 ± 0.1	1.42 ± 0.10	0.14 ± 0.01
Polyphenols	0.1 ± 0.0	0.03 ± 0.01	0.20 ± 0.01	0.1 ± 0.0	0.27 ± 0.01	0.04 ± 0.01
D.H.	-------	-------	44.26 ± 0.32	------	------	40.42 ± 0.79
FAA	<0.01	<0.01	14.65±0.21	<0.01	0.09±0.01	14.31±0.52

Results are expressed in percentage as mean ± standard deviation of three different determinations
D.H.: Degree of hydrolysis
FAA: Free amino acids

Based on the characterization of different plant protein products (meal, isolates and hydrolysates), protein enrichment and the decreased in non-protein components of protein isolates and hydrolysates are the biggest differences comparing with the starting meal (Table 1). The fat content is also higher than the starting raw materials. Firstly, this is because there is no a process of extraction of the oil phase of the meal trying not to increase the global operational costs by adding a defatting process, especially when the percentages of fat given in the chickpea and lupin meal are not a factor too limiting to solubilize and purify the protein fraction. Secondly, a significant proportion of fat, during the alkaline solubilization and subsequent precipitation of proteins at their isoelectric point for obtaining

the protein isolates, must interact with the soluble proteins to remain until the final stages of that process, accumulating in the final atomized product. Anyway, depending on the values observed of the fatty acid composition of these protein products, these may be supplemented with the needs of aquaculture diets formulation and reduced the inclusion of fish oil, making the final product formulation more profitable from an economic point of view (Table 2) (Nasopoulou and Zabetakis, 2012).

Table 2. Fatty acid compositions of protein isolates and hydrolysates obtained in pilot plant from chickpea and lupin

	Chickpea Isolate	Chickpea Hydrolysate	Lupin Isolate	Lupin Hydrolysate
16:0 (Palmitic acid)	14.95 ± 0.07	13.20 ± 0.04	13.33 ± 0.01	13.15 ± 0.03
16:1 (Palmitoleic acid)	0.24 ± 0.01	n.d	n.d.	n.d.
18:0 (Stearic acid)	1.56 ± 0.05	1.68 ± 0.00	6.80 ± 0.14	6.70 ± 0.01
18:1 (Oleic acid)	23.15 ± 0.08	24.48 ± 0.12	37.05 ± 0.27	35.02 ± 0.02
18:2 (Linoleic acid)	57.36 ± 0.02	55.48 ± 0.18	38.45 ± 0.42	38.08 ± 0.09
18:3 (Linolenic acid)	2.37 ± 0.05	2.36 ± 0.01	3.74 ± 0.05	3.84 ± 0.01
20:0 (Arachidic acid)	0.36 ± 0.03	0.62 ± 0.01	0.09 ± 0.02	0.70 ± 0.01
22:0 (Behenic acid)	0.07 ± 0.00	0.60 ± 0.11	0.17 ± 0.05	1.66 ± 0.09

Results are expressed in percentage as mean ± standard deviation of three different determinations
n.d.: no detected

It is also noted that due to the hydrolytic process developed, a free amino acid fraction around 15% is obtained. In this sense, a high free amino acid content between 10 and 20% of the total protein amino acids is found in natural marine environments (phytoplankton and zooplankton), which appears to be important in the initial stages of growth of marine fish since some of them have an attractant function (Ronnestad et al., 2003). In freshwater environments the free amino acid content, (although) even though is smaller, is important as well, being about 5% of total amino acids (Ronnestad et al., 2003). In this sense and considering the levels of free amino acids found in different hydrolysates, they may have an advantage of applicability comparing with the protein isolates from which they come from and, attending the results obtained, the hydrolytic process could be modulated depending on the final application. Few studies have been made in this regard and there is no general pattern, being the free amino acid stimulation-dependent of each species (Kasumyan and Doping, 2003). Non-polar amino acids seem to have a greater preference for a wider range of species. amino acid as leucine and phenylalanine are in a high concentration in the chickpea and lupin protein hydrolysates due to the exoprotease used (Flavourzyme), and it could be beneficial for this purpose.

The degrees of hydrolysis obtained in both cases by the sequential hydrolytic process using Alcalase and Flavourzyme as proteases is more than 40%, which implies a change in the protein molecular profile of protein hydrolysates, presenting mainly low molecular weight peptides (Figure 1). Thus, some studies on the proteolytic capabilities of fish larvae have suggested that the incorporation of small peptides or protein hydrolysates in diets for larvae improve development processes in the digestive tract of the larvae (Kotzamanis et al., 2007; Kolkovski, 2008; Hermannsdottir et al., 2009). However, the majority of these trials have been made from fish or animal origin hydrolysates and never from plant protein hydrolysates. Therefore, and although the fish or animal protein source has been the most used in the development of aquaculture diets, due to the above mentioned reasons, there is currently a great demand for the production of food formulations from vegetable protein sources to replace the animal.

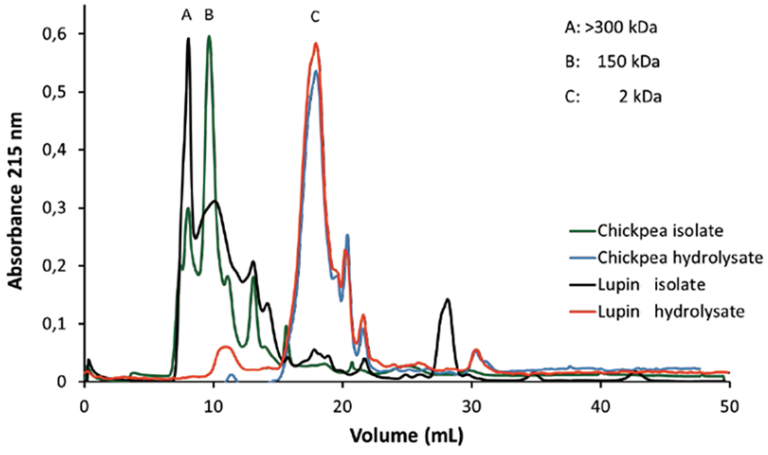


Figure 1: Molecular profile by gel filtration chromatography of chickpea and lupin hydrolysates and isolates obtained in trial facilities

This new molecular profile observed in plant protein hydrolysates also affects the solubility of proteins according to the pH of the medium compared to the intact native protein in the protein isolates (Figure 2). As shown, while the starting protein presents maximum solubility at basic pHs and minimum (isoelectric point) at acid pHs, the protein hydrolysate has a high and greater solubility in the whole pH range studied (pH 2 to 12) as a result of the decreasing in the size of the peptides and the increased presence of ionic groups (COO-and NH3+) that promote greater interaction with water molecules. It has been observed that the use of native casein as the only source of nitrogen in food supplements in the larval stages of common carp is not enough to survive and grow satisfactorily, contrary to the juvenile stages that seem to use effectively this protein (Sen et al., 1978). However, the partial substitution of the native casein by casein hydrolizate (Szlaminska et al., 1993) or sodium caseinate, a soluble form no hydrolyzated of casein, improves the larval performance (Radünz-Neto et al., 1993). As both products are soluble in water, unlike native casein, these results suggest that the solubility may be an important criterium of using protein by fish larvae. In this way, studies by Carvalho et al., (2004) have shown that the total or partial

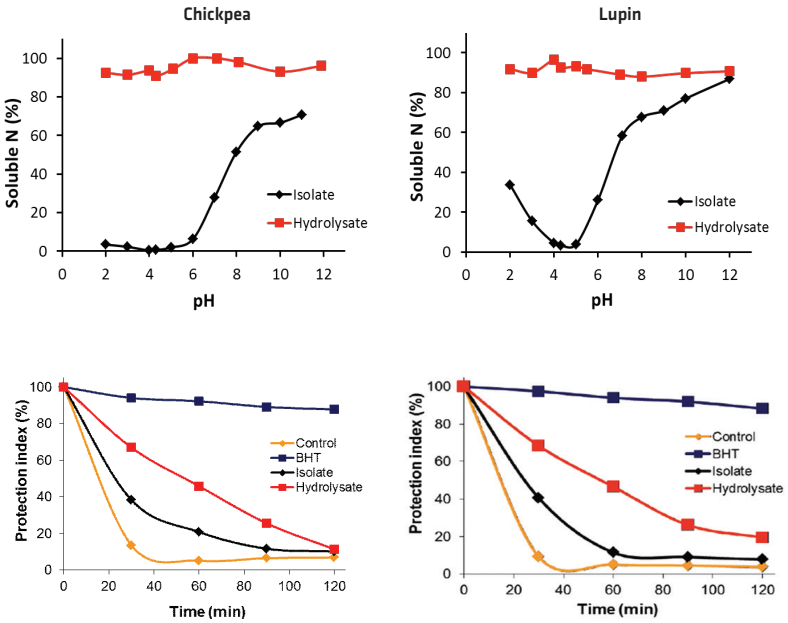


Figure 2: Protein solubility curves as a function of pH of the chickpea (left graph) and lupin (figure right) hydrolysates and isolates obtained in a plant pilot scale

replacement of insoluble casein in water to soluble casein, improved survival and increased growth of common carp larvae, being of great importance the solubility and the hydrolysis degree of the products compared. Also, Tonheim et al. (2007) in a study on the nitrogen content and digestibility of foods and food ingredients in nutrition of larvae of both soluble and insoluble fractions in water, concluded that the protein bioavailability of dietary sources in fish larvae depends on a large number of factors such as particle size of food, its digestibility, rate of digestion and absorption rates.

The amino acid composition of both protein isolates as hydrolysates obtained in trial plant it is shown in Table 3. As it is shown, the values are very similar between the two products which confirms the goodness of the hydrolytic process conditions used by using commercial proteolytic enzymes. In terms of quality, regarding the addition of the essential amino acids as a nutritional supplement depends entirely on the individual requirements of each species. Thus, it can be seen that the sulfur amino acids (methionine and cysteine), tryptophan and lysine may be limiting factors in some cases when using such products. However, the usual practice will lead to a certain percentage within a complete feed formulation and considering that the differences observed respect the nutritional requirements of essential amino acids are not overly significant, their inclusion in such formulation would not be a nutritional imbalance in the required protein intake.

There have been described antioxidants peptides that have the ability to scavenge free radicals and to form complexes with metal ions which catalyze the reactions of free radicals (Chen et al., 1998). Furthermore, it has been reported that hydrolysis of proteins from egg yolk with food grade proteases provides protein hydrolysates with antioxidant activity (Sakanaka and Tachibana, 2006).

Table 3. Amino acid composition of protein isolates and hydrolysates from lupin and chickpea

	Chickpea Isolate	Chickpea Hydrolsyate	Lupin Isolate	Lupin Hydrolysate	Gilthead seabream[c]	Tilapia[d]	Carp[d]	Catfish[d]
Asp[a]	13.86±0.20	13.21±0.42	12.75±0.05	11.95±0.35				
Glu[b]	18.26±0.14	17.57±0.30	23.20±0.14	20.81±0.87				
Ser	5.98±0.04	6.40±0.08	6.00±0.00	6.12±0.28				
His	2.75±0.08	2.99±0.04	2.40±0.00	2.96±0.23	*1.7*	*1.5*	*1.7*	*1.5*
Gly	3.86±0.02	4.41±0.03	4.50±0.00	4.45±0.08				
Thr	3.72±0.16	3.92±0.08	3.90±0.00	4.00±0.07	*2.8*	*3.8*	*3.8*	*2.0*
Arg	9.60±0.12	9.71±0.02	11.25±0.05	11.24±0.19	*5.4*	*4.4*	*4.2*	*4.3*
Ala	4.35±0.01	4.65±0.04	3.75±0.07	3.83±0.07				
Pro	2.80±0.03	2.35±0.20	1.75±0.07	2.52±0.24				
Tyr	2.16±0.01	2.97±0.14	3.15±0.05	4.12±0.11				
Val	4.51±0.06	4.72±0.11	4.00±0.10	4.35±0.09	*3.0*	*3.4*	*2.8*	*3.0*
Met	1.22±0.04	0.33±0.21	0.20±0.00	0.21±0.12	*2.4[e]*	*2.7[e]*	*3.2[e]*	*2.3[e]*
Cys	0.91±0.04	0.74±0.21	1.00±0.00	0.99±0.04				
Ile	4.16±0.02	4.12±0.04	3.90±0.00	4.44±0.06	*2.6*	*2.6*	*3.1*	*2.6*
Trp	0.73±0.03	0.94±0.08	0.75±0.05	0.85±0.04	*0.6*	*0.8*	*1.0*	*0.5*
Leu	8.39±0.03	8.39±0.05	7.90±0.00	8.01±0.12	*4.5*	*4.8*	*3.4*	*3.5*
Phe	6.30±0.02	6.42±0.11	4.65±0.07	4.60±0.09	*2.9[f]*	*5.7[f]*	*5.5[f]*	*5.3[f]*
Lys	6.46±0.03	6.58±0.03	4.90±0.00	4.94±0.12	*5.0*	*6.0*	*5.1*	*5.1*

Results, expressed as g amino acids/100 g amino acids, are the mean ± standard deviation of three determinations.
a: Aspartic + Asparagine
b: Glutamic + Glutamine
c: Gómez-Requeni et al., 2004
d: Monetcham et al., 2010
e: Methionine + Cysteine
f: Phenylalanina + Tyrosine

Lipids from marine origin, with a high content in polyunsaturated FA, are especially rich in FA of group w3; they have essential FA that allow the proper growth and development of these animals. However, these lipids are very susceptible to the oxidation process due to the exposition of the atmosphere oxygen. If the preparation and storage conditions of animal food are inadequate, this positive nutritional value becomes a negative factor. The oxidation products of lipids can react with other dietary nutrients (proteins, vitamins, etc.) and reduce the nutritional value of lipids and proteins (decreases its digestibility). In addition to this, oxidized lipids are well known to be toxic, causing severe diseases such as muscular dystrophy, liver and kidney damage, inactivation of enzymes, etc.

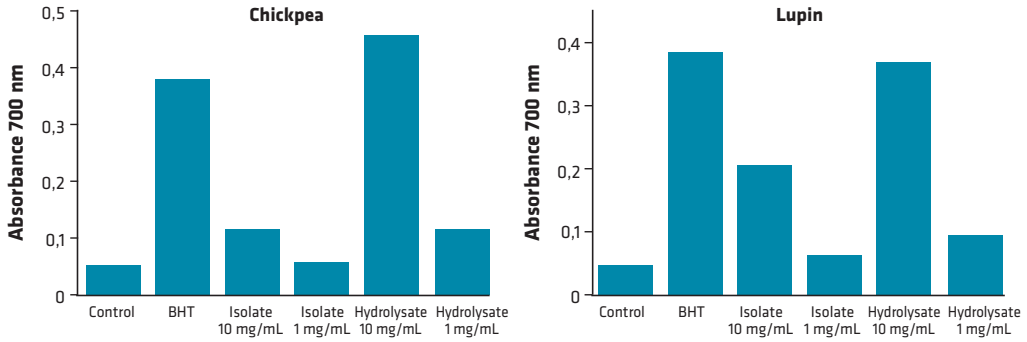
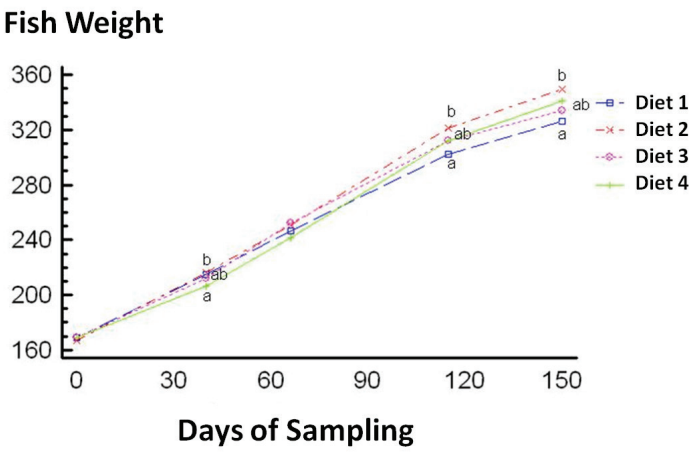


Figure 3. Antioxidant activity of the chickpea and lupin hydrolysate and isolated of b-carotene bleaching by oxidation products obtained in plan pilot scale

This critical problem can be resolved by adding substances that inhibit oxidation, synthetic antioxidants usually, and there is a wide variety in the market. The antioxidant effect may be enhanced by synergistic products (ascorbic, tartaric and citric acid, phosphates and phospholipids). From the text mentioned above, we can conclude that it is very important to control the storage conditions and the expiration dates of food (Castelló, 1993).

In this sense, it was proceeded to evaluate the antioxidant activity of protein hydrolysates obtained from chickpea and lupin with the aim of using them, not only because of its nutritional properties but because of its antioxidant activity. Two methods to measure the antioxidant activity were used for this purpose. The first one determines the decoloration of β-carotene by the products of lipid oxidation of linoleic acid. The second test determines the power of the hydrolysates to reduce metals. As it is shown in Figures 3 and 4, the antioxidative property of protein hydrolysates improves comparing with the starting isolates as a result of hydrolysis of the native protein. This could be explained by exposure of peptides with an amino acid composition with an antioxidant role recognized as in the case of methionine, tyrosine, histidine, lysine or tryptophan (Elias et al., 2008)

Figure 4. Fish growing afte 150 days under the challenge conditions in the trial



Future

According to the chemical characterization values observed, plant protein hydrolysates may have a promising future in aquaculture nutrition and not just because they possess an important nutritional role, but because today some of these hydrolysates peptides may provide certain benefits to the physiological functions of a living organism (antioxidant, chelator, antimicrobial, immunostimulant, etc). Thus, in recent years, there has been investigating the presence of certain dietary protein amino acid sequences, bioactive peptides, which could contribute with different beneficial functions for the consumer. Bioactive peptides can be defined as amino acid of small inactive sequences inside the intact protein but with the possibility of being released during the digestion of food in the organism or by a previous process like an enzymatic hydrolysis. Some of the bioactive peptides described are opioid peptides and antagonists of opioid peptides, antithrombotic peptides, inhibitors of angiotensin converting enzyme, hypocholesterolemic, antioxidant peptides and phosphopeptides.

Experimental trials

TRIAL 1: **SOYBEAN MEAL REPLACED BY RAPESEED HYDROLYSATE WITH LOW CONTENT IN GLUCOSINOLATES IN FATTEN DIETS FOR SEABREAM** *(Sparus aurata)*

Summary:

Rapeseed is one of the vegetal ingredients with less anti-nutrients, being the glucosinolates (highly toxic in the diet for its negative effects on thyroid function) those that can affect in higher way. Currently, the use of genetically improved seeds combined with the advances in-the technologies used in the meals production, allow having rapeseed meals and hydrolysates with very low levels of antinutrients.

Material and Methods:

We used seabream with initial weight of 160-170 g about 5 months to duplicate theirs weights. These fishes were divided into 3 circular tanks of 500 L with 20 fishes per tank. Approximately every 40 days from the beginning of the experiment and until the end of it, each fish from each tank were weighed to calculate the different growth rates and food conversion. At the beginning and end of the test, different measures of length, weight, viscera, visceral fat and whole fish sample and steak for the calculation of the parameters for biological and biochemical analysis were made (Photo 1). The start trial conditions were as follows:

Temperature (23-24°C);
Feed Intake rate (1.5-1.6%);
Intakes/day: 2;

It was developed and produced a total of 4 diets (Diet 1 - Diet 4) based on a previously tested control formula, with a level of protein/lipids 42/21. They were designed with the aim of including increasing levels of rapeseed hydrolysate with low glucosinolate content (from the oil extraction), to replace soy meal 47%, both diets with a composition and content of nutrients similar between them (Table 4).

Table 4. Experimental diets composition (% wet weight)

	Diet 1 Control	Diet 2 *Rapeseed Hydrolysate (D.H.: 23,63%)*	Diet 3 *Rapeseed Hydrolysate (D.H.: 14%)*	Diet 4 *Rapeseed Hydrolysate (D.H.: 7%)*
Proteins	40.73	39.38	40.89	42.21
Fats	19.42	19.85	18.05	18.5
Ashes	8.9	6.95	7.83	7.73
Moisture	5.69	6.83	6.72	6.84
Fiber	1.97	3.76	3.44	2.57
P	0.95	1.18	1.1	1.05
Ca	2.58	1.66	1.78	2.01
Ca/P	2.72	1.41	1.62	1.91

D.H.: Degree of Hydrolysys

Table 5. Mean weight evolution

Weight (g)	Diet 1	Diet 2	Diet 3	Diet 4
Tanks	2-7-12	3-8-13	4-9-14	5-10-15
Initial	168.70 ±1.35	166.92 ±2.01	168.67 ±0.8	168.67 ±0.84
0-40 days	214.90 ±19.35^{ab}	216.33 ±22.53^{b}	211.79 ±14.29^{a}	206.48 ±17.51^{a}
40-66 days	246.84 ±25.46	250.84 ±25.12	252.36 ±20.71	241.83 ±21.79
66-115 days	302.54±43.47^{ab}	321.51 ±51.51^{b}	312.24 ±39.17^{ab}	312.39 ±44.75^{b}
115-150 days	326.07 ±45.47^{a}	349.42 ±52.01^{b}	339.95 ±44.49^{a}	341.05 ±50.54^{ab}

Values with different letter in same row differ significantly ($p<0.05$)

Photo 1: Viscera of the fish sampling for the purpose of testing

Results and discussion:

From the first sampling after 40 days of feeding, higher growth was obtained in fish fed with diet 2, with maximum levels of rapeseed hydrolysate, maintained in all sampling periods, from the diets with medium and minimum levels of rapeseed, we obtained the same weight as the control fish (Table 5).

Specific Growth Rate (SGR) obtained were similar in all diets and sampling periods, on the other hand, a higher condition factor (K) was obtained in diet 2 comparing to the control one during the entire experience (bigger proportion of weight for the same length, which indicates higher profits in fillet) (Table 6).

Table 6. Condition factor (K) evolution

K	Diet 1	Diet 2	Diet 3	Diet 4
Tanks	2-7-12	3-8-13	4-9-14	5-10-15
0- 40 days	2.21±0.19^{a}	2.28±0.20^{b}	2.22±0.16^{ab}	2.21±0.15^{ab}
40- 66 days	2.29±0.19^{a}	2.32±0.17^{b}	2.32±0.09^{ab}	2.31±0.16^{ab}
66- 115 days	2.34±0.18^{a}	2.44±0.18^{b}	2.44±0.20^{b}	2.46±0.23^{ab}
115- 150 days	2.32±0.16^{a}	2.40±0.22^{b}	2.37±0.15^{ab}	2.37±0.18^{ab}

Values with different letter in same row differ significantly ($p<0.05$)

Feed intake was the same in the different experimental diets, with normal behaviour. There was no negative effect of palatability of the diets by the inclusion of increasing levels of rapeseed hydrolysate and feed conversion values were similar between diets for all sampling periods.

Table 7 shows the summary of the entire experimental period.

Table 7. Parameters after 150 days of feeding different experimental diets

Tank	Diet	Fish	Weight	Length	K	SGR	Gain Weight (% Initial Weight)	Gain Weight (g)	Intake (g)	IC
2-7-12	*1*	60	326.07±45.47^{a}	24.10±1.11	2.32±0.16a	0.43±0.12	93.28±5.78	3147.4±201.07	7649.03±192.27	2.44±0.14
3-8-13	*2*	60	349.42±52.01^{b}	24.38±1.19	2.40±0.22b	0.49±0.13	109.35±8.43	3652.2±311.30	8537.90±604.67	2.34±0.06
4-9-14	*3*	40	333.95±44.49^{ab}	24.12±0.94	2.37±0.15ab	0.45±0.12	97.63±5.49	3302.4±187.53	8116.50±337.43	2.46±0.04
5-10-15	*4*	60	341.05±50.54^{ab}	24.27±0.93	2.37±0.18ab	0.46±0.13	102.16±10.75	3447.4±379.55	8150.87±428.20	2.37±0.13

Values with different letter in same row differ significantly ($p<0.05$)

Concerning the biometric parameters and the final composition of the fish, there were no differences between treatments, except for the percentage of the total ash, where the higher values were found in fish fed by rapeseed hydrolysate comparing to fish fed by control diet with soybean meal. The figures of Ca, P and the ratio Ca/P were similar in the ended fishes in the 4 diets.

Regarding the nutrient retention rates obtained from the composition of whole fish and the particular nutrient intake, it was found a nitrogen incorporation rate significantly higher in diets with maximum content in rapeseed hydrolysate. Nitrogen excretion rates showed a significantly lower value compared to the control diet 2 (Table 8), however, the retention/excretion of total P and total ash did not differ between treatments.

Table 8. Excretion rates of N and P

Diets	Diet 1	Diet 2	Diet 3	Diet 4
N Excretion (%N)	79.69±0.10^{b}	77.12±0.33^{a}	79.93±1.38^{b}	79.00±2.24^{ab}
P Excretion (%P)	71.30±11.38	76.92±9.23	74.13±2.86	63.97±15.59

Values with different letter in same row differ significantly ($p<0.05$)

At the end of the experiment, the composition of the fillets did not differ between diets, while in the case of fatty acids, it was observed a slight increase in some of them (w-3, DHA, EPA, for example) by the inclusion of rapeseed hydrolysate in the diets, the only statistical differences were found in the total w6 fatty acids (lower for all diets with rape compared to the control one) and 18:2 w-6 (lower for the diet with higher level of rapeseed comparing to the control one) (Table 9).

Table 9. Fatty acid composition in the fillets at the end of the experiment (% of total fatty acids)

Diets	1	2	3	4
Fatty Acids				
Total Saturates	23.96±1.22	24.41±0.78	24.43±0.42	24.61±0.30
Total Monoenoics	34.76±1.31	34.57±2.21	34.0±3.65	33.59±2.59
Total w-3	19.94±2.10	21.85±1.96	22.39±3.73	22.29±3.16
Total w-6	19.96±0.19^{b}	17.83±0.35^{a}	17.83±0.62^{a}	18.10±0.77^{a}
Total w-9	25.56±0.81	25.22±1.46	24.58±2.45	24.22±1.58
Total w-3HUFA	17.0±2.18	19.15±2.24	19.53±4.21	19.42±3.33
18:2 w-6	16.94±0.39^{b}	14.79±0.51^{a}	14.82±0.99^{ab}	15.03±0.99^{ab}
EPA	3.97±0.12	4.33±0.14	4.44±0.26	4.54±0.50
DHA	9.73±1.91	11.37±2.07	11.52±3.90	11.36±2.71

Values with different letter in same row differ significantly ($p<0.05$)

According to the oxidative state of the fillets as a measure of its quality, all diets with rapeseed provoked lower values comparing to the control one when the slaughter happened. The day 4, however, these differences disappear.

At the end of the experience the external appearance of the fish did not differ between diets (Photo 2).

Photo 2. Sample of fishes of different diets after finishing the test

Conclusions trial 1

- Improves absolute weight and performance of the diet.
- Increases the condition factor with possible performance improvement of the fillet.
- Nitrogen retention increases and the excretion reduces.
- It does not affect the retention/excretion of phosphorus.
- It does not affect the relative proportions of viscera, visceral fat, liver and gonads.
- It does not affect the biochemical composition of whole fish or fillet.
- Oxidative state of the fillet at the slaughter moment is improved.
- It does not affect the external appearance of the fish.

TRIAL 2: **SOYBEAN MEAL REPLACED BY LUPIN AND CHICKPEA HYDROLYSATES IN FATTEN DIETS FOR SEABREAM** *(Sparus aurata)*

Summary:

Following with the aim of evaluating different innovative alternative raw materials from the ones with animal origin to use in the aquaculture nutrition in marine species, in this trial will be tested protein isolates from different sources against commercial control diet.

Material and Methods:

Sea bream with initial weight of 147 g to duplicate theirs weights were used. These fishes were divided into 3 circular tanks of 500 L with 18 fishes per tank. Approximately every 40 days from the beginning of the experiment and until the end of it, each fish from each tank were weighed to calculate the different growth rates and food conversion. At the beginning and end of the test, different measures of length, weight, viscera, visceral fat and whole fish sample and fillet for the calculation of the parameters for biological and biochemical analysis were made. The starting trial conditions were as follows:

Temperature (23-24°C);

Feed Intake rate (1.5-1.6%);

Intakes/day: 2;

A total of 2 diets (Diet A - Diet B) based on a previously tested control formula, with a level of protein/lipids 42/21 were developed and produced (Photo 3). Both diets were designed with an inclusion of 15% protein hydrolysate which is shown in Table 10.

Table 10. Experimental diets composition (% wet weight)

Diets	Diet Control	Diet A- Lupin Hydrolysate	Diet B- Chickpea Hydrolysate
Proteins	40.73	46.02	45.30
Fats	19.42	20.83	20.04
Ash	8.9	7.74	7.66
Moisture	5.69	6.45	6.52

Results and discussion:

From the third month, differences in the animal growth , with higher values for all diets compared to the control diet were observed; from this period, only fishes fed by Diets A and B are significantly bigger than fish fed by control diet (Table 11).

Table 11. Mean weight evolution

Weight (g)	Control	Diet A	Diet G
Tanks	1-6-11	2-7-12	5-10-15
Initial	146.84±9.40	147.03±8.47	146.68±8.29
0- 42 days	175.26±15.54	182.05±17.80	185.34±15.65
42- 91 days	210.76±28.35^{a}	234.60±29.65^{b}	237.08±22.74^{b}
91- 133 days	268.71±41.2^{a}	309.18±39.74^{b}	308.93±34.04^{b}
133-169 days	331.48±54.39^{a}	384.71±50.55^{c}	374.73±42.59^{bc}

Values with different letter in same row differ significantly ($p<0.05$)

Feed intake was the same, so the performance diet was better in the fishes fed by diets A and B.

Regarding the biometric parameters analyzed, it can be ruled out an accumulation of lipids in animals fed with diets A and B, as these fish increased proportionately in weight and length (similar K factor), as it is shown in Table 12.

Table 12. Condition factor (K) evolution

K	Control	Diet A	Diet B
Tanks	01/06/2011	02/07/2012	05/10/2015
0- 42 days	2.20±0.14	2.23±0.15	2.24±0.13
42- 91 days	2.31±0.17^{a}	2.39±0.19^{ab}	2.41±0.16^{ab}
91- 133 days	2.33±0.17	2.38±0.16	2.38±0.21
133-169 days	2.38±0.29	2.42±0.18	2.41±0.19

Values with different letter in same row differ significantly ($p<0.05$)

Photo 3. Sample of fishes of different diets after finishing the test

The complete carcass analyses of fish for the different diets were similar in the case of lipids. However, in the case of proteins, a higher proportion was found in the experimental diets than the control.

There were no differences between treatments for the parameters VSI (Viscerosomatic index), HIS (Hepatosomatic Index) and GSI (Gonado-Somatic Index), being higher the initial values of VSI and HSI comparing to the final results.

The fillets of fish fed by Diet B had the higher proportion of w-6 fatty acids and 18: 2n-6. The results of A and control diets were similar.

The different diets tested did not affect the oxidative capacity of the fillet in the slaughter of animals.

The external appearance of the fish at the end of the experience did not differ between diets.

Conclusions trial 2

- Diets composed of protein hydrolysates improves absolute weight and yield compared to control diet
- Same condition factor in fish (VSI, HSI and GSI) in the three diets
- Higher protein assimilation in fillet with diets hydrolysates diets
- It does not affect the oxidative state of the fillet at the slaughter moment
- It does not affect the external appearance of the fish

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Use of purified extracts from fish viscera as an enzyme additive in feeds for juvenile marine fish

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Summary Fish viscera rejected from processing fish captures represents an important amount of biological material with a very limited industrial utilization. The possibility of using this material as a source of enzymes to be included in aquaculture feeds was assessed in the present work. A preliminary evaluation of total activity, pH and thermal stability of proteases present in viscera of nearly 20 different species was carried out. Using this information, extracts of some of selected species were freeze-dried and included as additive in feeds for juvenile seabream (*Sparus aurata*). Growth and food utilization of fish receiving feed including 12 g/kg of the extracts obtained from preparation the viscera of black scabbardfish (*Aphanopus carbo*) was significantly improved. A financial approach of the potential use of this resource was also developed.

Introduction and objectives

The discards from fish processing, together with fish by-products, account for great amounts of biological material produced worldwide annually. Among these products, the internal organs of fish, which constitute approximately 7.5% of body weight, have been pointed out as interesting potential sources of different hydrolytic enzymes like pepsin, trypsin, chymotrypsin, gastricins and elastase. Digestive proteases obtained from marine organisms are especially interesting to the food and feed industry due to their unique properties, which include high catalytic efficiency at low reaction temperatures, lower thermostability, and cold stability (Sahidi and Janak, 2001; Klomklao, 2008). In addition, the extraction of enzymes from fish processing wastes and their utilization in the food and feed industry may contribute significantly to reducing local pollution problems (Blanco et al., 2007)

A great number of studies have demonstrated that marine fish have a limited activity of some of the main digestive enzymes during their initial developmental stages (Kolkovski, 2001). On the other hand, there is an increasing trend to reduce the dependence on live food and current feeding practices are based in early weaning using compound feeds as soon as possible. In spite of the good quality of the ingredients used in most commercial formulations, the net efficiency obtained from protein and lipids present in feeds provided during those stages is limited by the poorly developed

digestive function of the young fish. An interesting approach oriented to enhance the nutritional value of such feeds for very young fish should be the use of exogenous enzymes from marine origin that could increase the hydrolysis of substrates within their digestive tracts. Taking this into account, the main objective of the project ZIMOFEED (Cod. 08MMA003CT) financed by the applied research sectorial programme of the Galician Plan for R&D (INCITE) was to evaluate if the inclusion of partially purified enzyme extracts obtained from fish viscera in feeds provided to juvenile marine fish could result in an improvement in growth and feed efficiency. This objective was assessed through a work plan comprising the following steps:

Preparation of an active and stabilized enzyme extract from fish viscera

Nutritional evaluation of the product

Feasibility study on potential industrial exploitation

Preparation of an active and stabilized enzyme extract from fish viscera

Species selection

Almost any commercial fish species could be a source of visceral extracts, since all of them are subject to the evisceration process. Table 1 shows the average annual catches (in the period 2007-2010) of different commercial species by the Spanish fleet in the North Atlantic fishing grounds obtained from FAO FishStat Plus.

Table 1. Catches (tonnes/year) and estimated amounts of viscera (tonnes/year) by commercial species caught by Spanish fleet in the Atlantic fishing grounds

Common name	FAO 3 α code	Scientific name	Catches (tonnes/ year)	Viscera (tonnes/ year)
North Atlantic				
Redfish	RED	*Sebastes* spp.	4,180	334
Roughhead grenadier	RHG	*Macrourus berglax*	851	68
Greenland halibut	GHL	*Reinhardtius hippoglossoides*	4,897	392
Roughsnout grenadier	TSU	*Trachyrincus scabrus*	**	**
Atlantic horse mackerel	HOM	*Trachurus trachurus*	**	**
Northeast Atlantic				
Baird's slickhead	ALC	*Alepocephalus bairdii*	1,643	131
Black scabbardfish	BSF	*Aphanopus carbo*	260	21
Blue ling	BLI	*Molva dypterygia*	596	48
Haddock	HAD	*Melanogrammus aeglefinus*	167	13
Blue whiting	WHB	*Micromesistius poutassou*	19,868	1,589
Northeast and Middle East Atlantic				
Blackbellied angler*	MNZ	*Lophius* spp.	6,021	482
European hake	HKE	*Merluccius merluccius*	24,334	1,947
European pilchard	PIL	*Sardina pilchardus*	30,738	2,459

Common name	FAO 3 α code	Scientific name	Catches (tonnes / year)	Viscera (tonnes / year)
Middle East Atlantic				
Megrim	MEG	*Lepidorhombus whiffiagonis*	249	20
Southwest Atlantic				
Argentine hake	HKP	*Merluccius hubbsi*	13,421	1,074
Patagonian grenadier	GRM	*Macruronus magellanicus*	12,106	968
Longtail southern cod	PAT	*Patagonotothen ramsayi*	28,977	2,318
Pink cusk-eel	CUS	*Genypterus blacodes*	2,470	198
***Argentine shortfin squid	SQA	*Illex argentinus*	80,000	6,400
		TOTAL	248,614	19,889

*In the case of blackbellied angler, data from the whole group of monkfish (*Lophius* spp.) captured by the Spanish fleet in the Atlantic have been accounted.

**For these particular species no data have been found in the FAO statistical program.

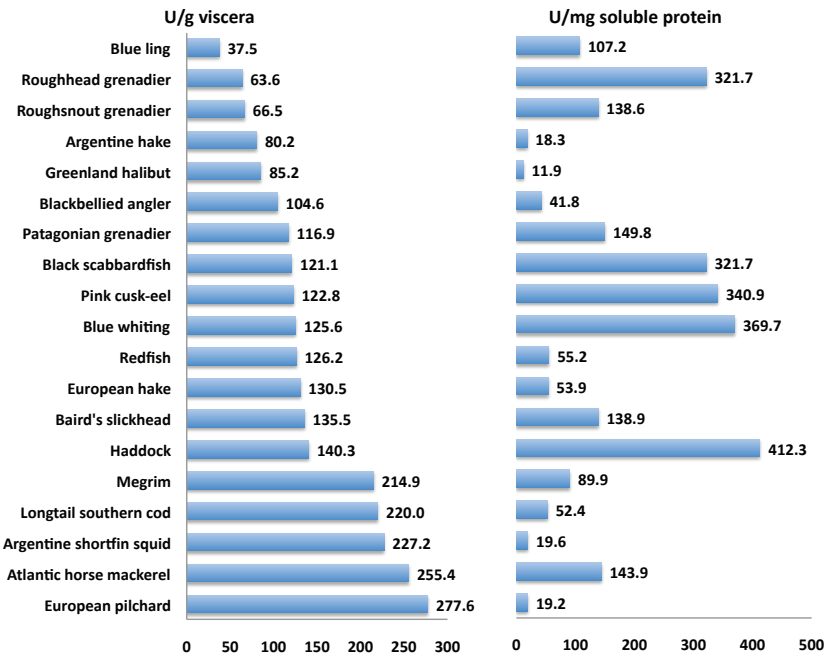
***Data of argentine squid catches are estimates based on consultations to the fishing sector.

All these species were evaluated as potential sources of proteases. The evaluation was oriented to assess the main types of proteases present in the viscera, as well as the amount of enzyme activity present per g of fresh tissue. The selection was focused to find sources of alkaline proteases, considering their potential use as additive to enhance protein digestion in the gut of juvenile fish, on which stomach digestion is still not well developed.

Preliminary evaluation of protease activity

Protease activities measured at pH 8.0 using haemoglobin as substrate in crude visceral extracts are resumed in Figure 1.

Figure 1: Protease activities in extracts



Values of protease activity showed a great variation within species, from 37 units/g fresh viscera in the blue ling (*Molva dypterygia*) to 278 units/g fresh viscera in the European pilchard (*Sardina pilchardus)*. The same variation was observed when activity was expressed in relation to soluble protein, with values ranging from 12 units/mg soluble protein measured in the Argentine hake (*Merluccius hubbsi)* to more than 400 units/mg soluble protein in haddock (*Melanogrammus aeglefinus).* The absence of correlation between both values reflects that the concentration of active enzyme in visceral samples is affected by a great number of factors (species, feeding status, procedures used for processing and storing of tissues, etc.). After this preliminary screening, some of the species were not considered in further steps of the study due to the difficulties to obtain samples in amount enough, or stored under the required conditions. This aspect was related both to the size of catches for a species and to the procedures followed in the vessels for evisceration of fish.

Preparation of active and stabilized products

The process followed in order to obtain a Purified Enzyme Extract (PEE) was characterized by three main stages; extraction, purification and stabilization,

Figure 2. PEE extraction, purification and stabilization

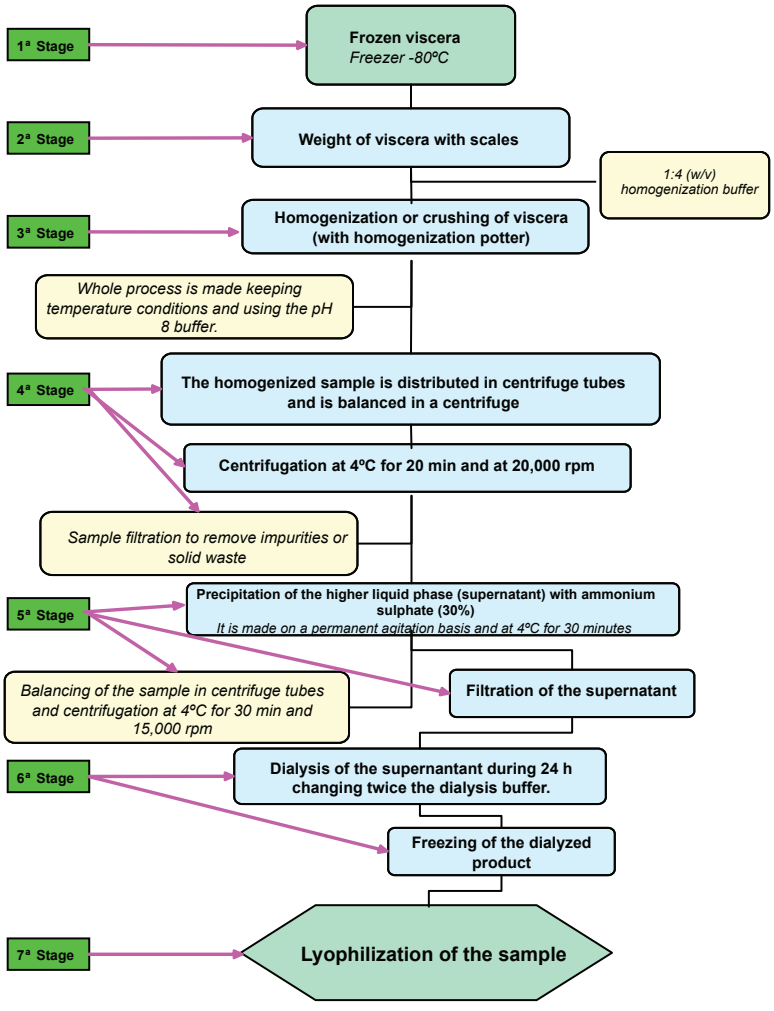
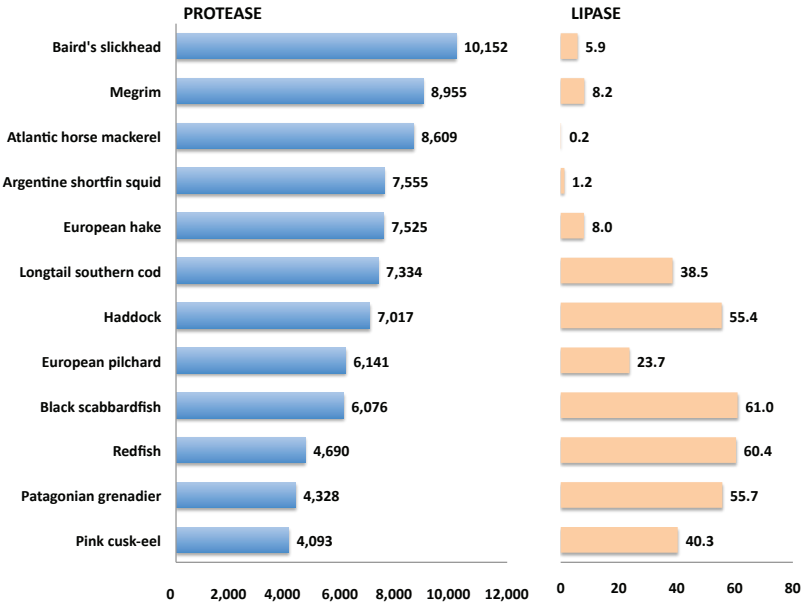


Figure 3: Protease and lipase activities in freeze dried extracts (U/g)

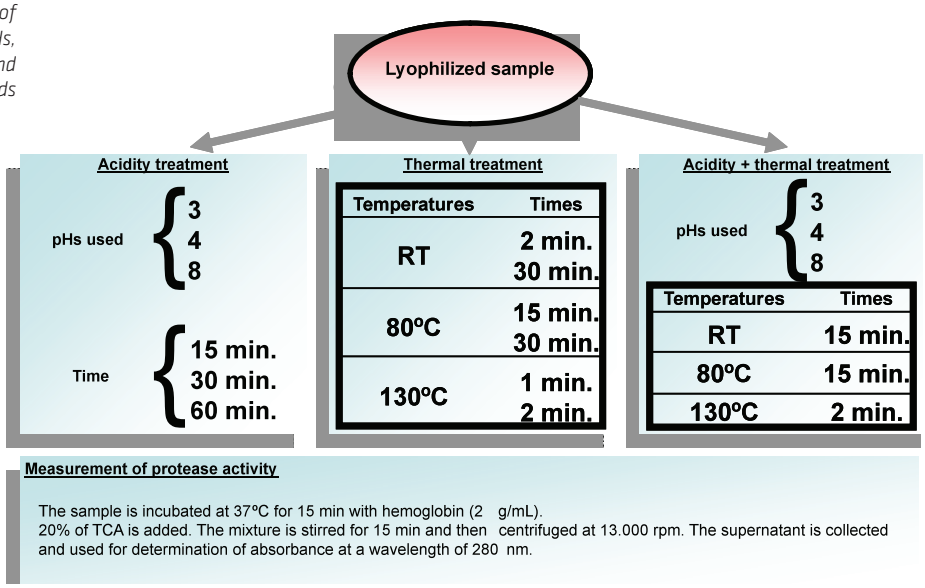


developed through seven different steps (Figure 2). In addition to the PEE, some extracts used in one of the experiments did not undergo purification/ concentration with ammonium sulphate and were identified as Crude Enzyme Extracts (CEE). The amount of fresh tissue required to prepare 1 g of PEE ranged from 22 g (European pilchard) to 75 g (Baird's slickhead). The activity of protease and lipase activities determined in PEE, which showed an almost inverse relationship, is detailed in Figure 3.

Characterization of protease activity

One of the main requisites for the use of PEE at industrial scale is that enzyme activities should resist both the thermal conditions used in the

Figure 4. Incubation of PEE at different pHs, temperatures and time periods



preparation of feeds, as well as the effect of acid pH during passage through stomach, before being active in the intestine of the targeted fish. For this reason, incubation of PEE for several minutes at different temperatures resembling those used during feed preparation (1-5 min at 130°C or 30-120 min at 80°C) was used as a preliminary evaluation of its stability. In addition, PEE were incubated at pH 3 and pH 4 during different time periods. Finally, a combination of thermal and pH treatments was also used to evaluate PEE, as resumed in Figure 4.

The highest activity after such treatments was obtained from PPE prepared with viscera of black scabbardfish, Baird's slickhead, Argentine shortfin squid and particularly of the redfish (*Sebastes sp*). Considering this result, as well as the local availability of fresh material, this latter species was selected to prepare the visceral PEE used to perform the first experiment of nutritional evaluation. In a second experiment the assayed products were obtained from black scabbardfish (*Aphanopus carbo)* and the Argentine shortfin squid (*Illex argentinus*).

Nutritional evaluation of feeds including enzyme extracts obtained from fish viscera

The nutritional evaluation was carried out in two different experiments. As indicated previously, in the first one the assayed PEE was obtained from the viscera of the redfish (*Sebastes sp*) and showed a total protease activity of 3,400 U/g. Preliminary assays showed no reduction in the activity after incubation at 80 °C for 30 min at neutral or alkaline pH, although it was affected to a great extent by acid environment; it showed a reduction by more than 90% after incubation at pH 3.0. Nevertheless, due to the absence of a completely functional stomach in the juvenile fish on which feeds should be used, this was not considered a problem. The PEE was included at 10 g/kg (34,000 U/kg feed) in a commercial feed for juvenile sea bream (*Sparus aurata*) of 4 g initial weight. The feed was finely grounded and after addition of the product, which was carefully mixed into the mass, it was re-pelletized into the suitable size. The feed used as control was processed in the same manner. The conditions of the biological test are detailed in Table 2.

Table 2. Conditions of the biological assay used to test the nutritional effects of using PEE from viscera of redfish as nutritional additive in feeds for juvenile seabream

Biological material	Gilthead seabream juveniles 4 g initial weight
Aquaria	80 L tanks in open circulation of seawater 3 tanks/treatment and 25 fish/tank
Feeding protocol	3 moments food supply/day at 9:00, 12:00 and 15:00 PM. Ration adjusted to fish weight
Controls	Temperature and oxygen (daily) Weight (each 7-10 days) Feed consumption (daily)
Duration of the assay	50 days (including 10 days of acclimation)

In this first experiment, the addition of the PEE did not result in a significantly positive effect on growth or feed efficiency of the fish. In a second experiment the products were obtained from black scabbardfish (*Aphanopus carbo)* and Argentine shortfin squid (*Illex argentinus*) and some of experimental parameters were modified. The products were included at a higher level (12 g/kg) and two different types of products were evaluated; the PEE obtained after concentration with ammonium sulphate and the crude extract obtained prior to this step (CEE). The initial evaluation showed a much higher activity in the products obtained from black scabbardfish than from Argentine shortfin squid, as well as the presence of activity at both acid (6,5) and alkaline (8,0) pH (Table 3).

Table 3. Protease activity (U/g) measured in the extracts prepared from viscera of *Aphanopus carbo* and *Illex argentinus* at two different pH

	pH 6.5	pH 8.0
CEE *Aphanopus*	1,318	3,194
PEE *Aphanopus*	2,000	3,146
CEE *Illex*	539	265
PEE *Illex*	201	424

The evaluation of the protease activity present in the feeds after inclusion of the different products showed is detailed in Table 4. Results evidenced a basal protease activity at both pH in the control feed, probably due to the residual activity in some of the ingredients used (e.g. fish meal) and also the significant increase of such activity after addition of the products. The higher increase at acid pH was obtained when using the products obtained from squid, while the higher activity at alkaline pH was measured when using those from scabbard fish.

Table 4. Protease activity at pH 8.0 measured in the experimental feeds. The differences in relation to the control feed are shown as percentage increase

Feed	Protease activity (U/g feed)	% increase over control	Protease activity (U/g feed)	% increase over control
Control	4.0	-	8.0	-
CEE *Aphanopus*	3.7	0.0	11.1	39.1
PEE *Aphanopus*	4.8	22.0	10.7	34.2
CEE *Illex*	5.8	45.8	9.0	12.8
PEE *Illex*	5.6	41.2	8.6	7.2

The experimental design and conditions were similar to those used in the previous experiment (Table 2). No direct correlation between the total enzyme activity in the feeds and the final growth or feed efficiency was observed, but after 40 days, fish fed on the feed including the CEE of black

Figure 5: Growth of seabream juveniles fed on feds including diferent enzyme extracts obtained from the viscera of black scabbardfish and Argentine shortfin squid. Each point is the mean of three replicates. Values showing a different letter are statistically different at p<0.05

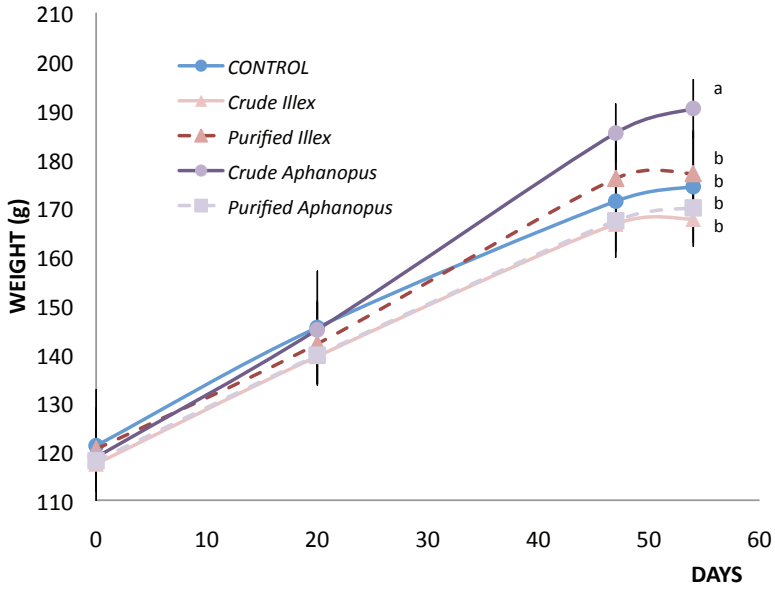
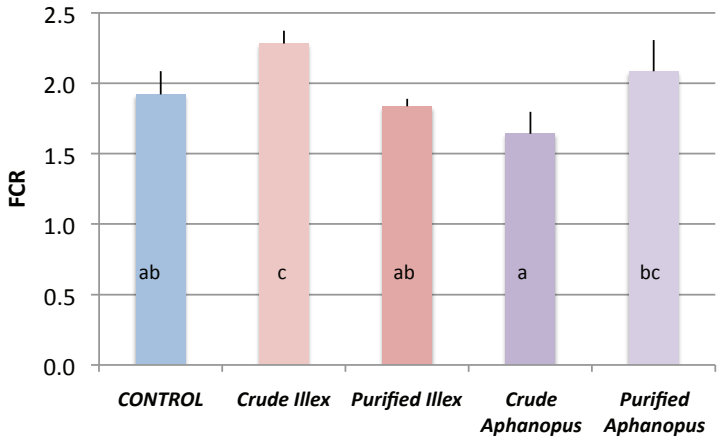


Figure 6: Values of feed efficiency measured in seabream juveniles fed on feeds including diferent enzyme extracts obtained from the viscera of black scabbardfish and Argentine shortfin squid. Each point is the mean of three replicates. Values showing a different letter are statistically different at p<0.05. FCR: Food Conversion Rate



scabbardfish showed a 10% increase in weight over those fed on the control feed (Figure 5). In addition, they also showed a better efficiency in the use of the feed, with a food conversion rate (FCR) of 1.64 vs 1.92 (Figure 6).

It was concluded that the inclusion of a freeze-dried product, obtained from crude extracts of the viscera of *Aphanopus carbo*, at 12 g/kg feed could result in a significant improvement in growth and FCR in juvenile marine fish.

Feasibility study on potential industrial exploitation

Initial assumptions

The obtaining of enzyme fractions from viscera of different fish species and its use as an additive in aquaculture feeds to enhance digestibility has been suggested by several authors (Kollowski, 2001), although results showed in the present work are the only published evidence of this possibility. In addition to the biological evaluation, a prospective on the real applicability of this product should consider financial cost of production at industrial scale. This aspect is developed in the following section. The main elements to evaluate are:

- **Availability:** estimation of the amount of viscera to be obtained from each fish target species according to their percentage over the weight of the animal and based on the available statistical data on captures.
- **Cost of raw material (viscera):** the cost of this by-product should be estimated like the rest of products exploited by a shipping company.
- **Cost of enzymatic extract:** it may be estimated considering the yield per kg of viscera and the costs from the extraction process. Scaling up and extrapolation must be done to estimate production at industrial scale.
- **Impact on the feed price:** in terms of relative percentages and depending on the proportion of enzyme to be added to the feed, it is possible to estimate the final increase in price of the product. This price increase greatly depends on the beneficial effects that the modified feed may have on fish growth and food conversion.

Also, some initial assumptions must be considered:

The starting material to obtain enzymes are the viscera separated on fishing vessels; more specifically trawl freezer factory vessels working in multispecific fisheries which systematically gut their catches. Since they have freezing facilities, they can keep these products under suitable conditions for further valorisation.

Some species from Great Sole fishing ground could be exploited to a lesser extent (not all ships have freezing facilities), as well as others from small-scale fisheries (sardine, hake, horse mackerel and blue whiting), which are eviscerated by inland processing companies.

The weight of viscera as a percentage of the whole fish varies with the species from 5% to 12%, so, an average value of 8% was used in the present study. On the other hand, an average yield of 0.15 kg of CEE or 0.025 kg of PEE may be obtained from 1 kg of fresh viscera. The estimated annual production of both products from the viscera of different species is detailed in Table 5.

Results obtained in the nutritional evaluation have proved that similar amounts of either purified enzyme extracts (PEE) or crude enzyme extracts (CEE) may result in equal improvement on nutritional efficiency. Since the estimated cost of the CEE is much lower (Table 6), it will be considered in the present study as the most suitable for inclusion in the feed.

The theoretical industrial process to prepare crude enzyme extracts differs from that commercially developed to obtain purified enzymes. Hence, it was designed using as a reference the process developed at laboratory scale.

The final product is a dry powder obtained after freeze-drying. Although stabilization of the CEE could be achieved using a different and probably less expensive procedure, this has been the selected method considered in the study.

Table 5. Estimated amounts of enzyme extracts that could be obtained from the viscera of fish species caught by the Spanish fleet (tonnes/year)

Species	Catches	Viscera biomass	Crude enzyme extract (CEE)	Purified enzyme extract (PEE)
Redfish	4,180	334	50.10	9.02
Roughhead grenadier	851	68	10.20	1.15
Baird's slickhead	1,643	131	19.65	1.71
Black scabbardfish	260	21	3.15	0.41
Blue ling	596	48	7.20	0.95
Greenland halibut	4,897	392	58.80	8.23
European hake	24,334	1,947	292.05	33.09
Megrim	249	20	3.00	0.48
Haddock	167	13	1.95	0.27
Blackbellied angler	6,021	482	72.30	10.12
Blue whiting	19,868	1,589	238.35	33.38
European pilchard	30,738	2,459	368.85	110.66
Argentine hake	13,421	1,074	161.10	31.14
Patagonian grenadier	12,106	968	145.20	26.15
Longtail southern cod	28,977	2,318	347.70	69.54
Pink cusk-eel	2,470	198	29.70	5.93
Argentine shortfin squid	80,000	6,400	960.00	217.60

Exploitation costs

The price of a kg of viscera was calculated applying the methodology followed by ship owner companies. This presents some particular features when compared to land-based companies since:

- The labour cost of sailors is not fixed; it is calculated as an agreed percentage (around 25%) on the global amount obtained with the turnover of the working period. In the present study the working period was considered the time lapse between the ship departure and arrival to port. Therefore, the tonnes captured, the composition of the captures, as well as the average selling prices, were used to determine the wages of the crew in absolute terms.
- There are fixed costs in each trip regardless its success. Such success is measured by the size and quality of the catches, as well as by the setbacks happened during the fishing period.

Therefore, the price of viscera arriving to port will be at least equivalent to that of the cheapest raw material. Yet, that price would be acceptable while storage of the viscera does not result in an obstacle to fill the hold with more valuable captures. Prices of raw material may show a great variation depending on the fishing ground, species and current demand, from 0.50 to 4.00 €/kg. The price of viscera would be at least 0.5 €/kg for the NAFO area (rabbit fish, black cardinal fish, king of herrings, Portuguese dogfish) and 0.8 €/kg for other areas requiring a more complex logistics.

Table 6. Total cost of the process required to obtain extract enzymes: equipment, materials and reagents

Phases	Equipment	Materials & Reagents	Costs (€)	
			CEE	PEE
1	Freezer -80°C		9,000	9,000
2	Scales with cabin		850	850
	Cutter		1.50	1.50
		Aluminium paper	0.15	0.15
3	Potter homogenizer		155	155
	Drill		70	70
		Test tubes	60	60
		Homogenization buffer*	1.04	1.04

Phases	Equipment	Materials & Reagents	Costs (€)	
			CEE	PEE
4	Two-plates scale		79	79
	Centrifuge		2,200	2,200
		Centrifuge tubes	40	40
		Filtration material	500	500
5		Glassware		
	Magnetic stirrer		-	180
		Ammonium sulphate	-	26
6		Membrane dialysis	-	103
		Homogenization buffer*	-	20.8
7	Freeze-dryer		1,500	1,500
		Falcon tubes	<1	<1
		TOTAL	1.04 €	47.84 €

*Calculation of the homogenization buffer required for processing 1 kg of viscera.

Although Table 6 shows in detail the prices of all elements, when calculating the totals only the reagents must be considered because the equipment must be included into the amortization concept and materials would be included into the operational costs. Also, it must be taken into account that this cost would never be similar to that existing at industrial scale. The total amounts represent the invested quantity, regarding reagents, by kg of viscera or raw material used.

The estimated average yield of crude enzymatic extracts was 150 grams per kg of viscera and the cost of the extraction process per gram was 0.007€. In the case of purified enzyme extracts, the yields greatly varied with species (Table 5), but considering an average value of 25 g/kg of viscera, the cost would be increased up to 1.91 €/g. This great difference is the main reason that oriented the use of CEE for the *in vivo* experiment.

Estimated cost of the freeze-dried extracts produced at industrial scale

Most companies work in the enzymes extraction via fermentation through bacteria and fungus with terrestrial and marine origin like Novozymes, Larbus, AddFood Biocon and Aqua Biotechnology, among others. Very few companies are dedicated to enzymes extraction from fish raw material: in the case of Arcticzymes, they use processed fish from land industry for applications in food industry and other fields. Zymetech, in contrast, use viscera from on board processing since 10 years ago with applications in natural, cosmetic and medical products.

So at present there is not any well established industrial process oriented to the production of enzyme extracts from fish viscera, so, cost calculation has been based in the steps described in Figure 3 for the lab process, as well as the costs of the extraction process and average yields described on the previous section. Taking this into account, the approximate cost would be:

Concept	Price / gram	
	CEE	PEE
Raw material*	0.003	0.02
Reagents	0.007	1.91
Direct labour cost	0.300	0.300
Packing and packaging	0.050	0.050
Energy	0.300	0.300
Other operational costs**	0.050	0.050
Production direct cost	*0.71 €*	*2.63 €*
Company benefit 20%	0.142	0.526
Selling price	*0.85 €*	*3.156 €*

Remarks:

* Initial cost of viscera: 0.5 €/kg. Yields of 150 g/kg and 25 g/kg for CEE and PEE, respectively.

**Operational costs would include: commercial labour, maintenance and repair labour, accountancy labour, quality labour, logistics, spare parts, amortizations, etc.

Of course, a company should not be profitable if producing only this kind of products. This would be only feasible in a well-established company producing a wide range of other additives.

Applicability. Calculation of the increase in cost and return

The amount of product to be included in a commercial feed is limited to a certain extent by its consideration as an additive, not an ingredient. These kinds of products (acidifiers, probiotics, enzymes) are commonly included in very small amounts ranging from 0.5 to 5 g/kg. The amount of enzyme extract included in a commercial feed to produce a significant improvement on growth and feed efficiency will depend on its concentration of active product (UA/g). Considering the relative low concentration of protease activity in enzyme extracts used in the present study, they were included at a quite high level (12 g/kg).

The calculations were made considering the following assumptions:

Inclusion of the additives in feeds should not result in a modification in the cost or main features of the production process.

All calculations will consider only the production of CEE, taking into account its comparative lower cost and positive biological effect.

The average market price for commercial feeds provided to juvenile fish is around 1.20 €/kg.

Addition of 12 g CEE per kg of feed (with a cost of 0.85 €/g) should result in a selling price for weaning feed of 1.20 + 10.20 = 11.40 €/kg.

Such a high value, which represents nearly 10 times current market price of feeds, points to the unviability of the addition, at least under current operational costs. Nevertheless, a complete financial evaluation requires also making an estimation of the expected return. The following assumptions were considered to make such estimation:

The information used is based in average data from the more commonly cultured species in the Mediterranean, the gilthead seabream (*Sparus aurata*).

Three different stages in the production cycle were considered: juveniles (post-weaning to fingerling; 0.5-15 g), medium grower (15-150 g) and market size (150-300 g).

FCR in each stage is different; juvenile fish presents better values, although they get worse with age. In this example, values of 2.0, 2.3 and 2.6 were considered for each of the three growth stages. Although in the present work (section 3) the improvement in FCR was only demonstrated in juvenile fish, it was also applied linearly to older fish for this estimation.

Calculation of the feed consumed in each growth stage, as well as total consumption, was made using average values of FCR for this species.

The composition and cost of feed used in each growth stage are different. Although market prices of feeds are continuously changing, in the present example the values considered were 1.2, 1.0 and 0.85 €/kg.

The estimated total saving in feed costs potentially derived from the inclusion of the CEE in feeds, once a 30% improvement in FCR was applied, is detailed in Table 7.

Table 7. Estimated consumption and cost of feed under normal conditions or improved after addition of CEE

Size range (g)	Weight increase (g)	FCR	Feed cosumed (g)	Feed cost (€/kg)	Feed cost (€)	FCR improved	Feed cosumed (g)	Feed saving (g)	Cost saving (€)
0.5-15	14.5	2	30	1,2	0.04	1.71	25	5.23	0.01
15-150	135	2.3	320	1	0.32	1.96	265	54.8	0.05
150-300	150	2.6	400	0,85	0.34	2.22	333	66.9	0.06
TOTAL									
by fish	299.5		750		0.696		623.1	126.9	0.1
by kg	998.3		2,500		2.32		2,077	423	0.39

On the other hand, an increase of growth rate by 10% should represent an equivalent reduction in the time required by fish to reach market size. If an average production cycle lasts 330 days (11 months), this should mean nearly one month (33 days) less time and hence an additional saving of monthly costs. Considering than average total production cost are nearly 3.3 €/kg, monthly cost may be estimated in 0.3 €/kg, this being the additional saving derived from the use of CEE in feeds.

As a resume, total saving in production cost should be 0.39€ (feed) + 0.3€ (cycle shortening) = 0.69 €/kg of fish produced. Clearly, the estimated cost of including the enzyme additive (11.40 €/kg) exceeds by far the expected benefits. In other words, the potential use of the CEE should be only profitable if its inclusion in feeds do not increase cost more than 0.5-0.6 €/kg. This means a reduction by 20 times of the costs estimated in the present study.

Conclusions

Fish viscera can be used to obtain and prepare a product with enzymatic activity that can be included as additive in fish feeds.

The selection of the most suitable species as a potential source of enzymes should consider both its availability (regular supply) as well as biochemical aspects (stability of the enzymes under a wide range of pH and temperature).

After evaluation of 19 species, enzymes obtained from the viscera of *Sebastes* spp., *Aphanopus carbo* and *Illex argentinus* seem to be potential candidates for their inclusion as additives in fish feeds.

The use of a semipurified extract obtained from viscera of *Aphanopus carbo* significantly improved growth and food conversion in juveniles of gilthead seabream (*Sparus aurata*) when included at 12 g/kg feed.

Nevertheless, the expected biological advantages obtained by the inclusion of enzyme extracts obtained from fish viscera (reduction of the fish production cycle or increase of the food conversion efficiency) are not good enough to counteract the high increase in feed cost derived from their inclusion.

There are still many questions regarding real cost of production of enzyme extracts at industrial scale. The scaling up study has been developed as detailed as possible, but a massive production would probably decrease the costs calculated here. In this sense, perhaps the main factor to consider should be the substitution of the highly expensive stabilization of final product by freeze-drying by alternative procedures like (J Sineiro 2012, pers. comm., 20th of March):

Vacuum drying at low temperature; the final product is not a freeze-dried but an aggregated powder with low moisture. Equipment for this processing is routinely used in food industry.

The use of cold acetone for precipitation of the enzymes before the second centrifugation would allow to obtain a semi solid material not requiring further freeze drying and hence reducing production cost. The product could be dried using cyclonic dryers operating at low temperature and not complete vacuum (J A Vázquez 2012, pers. comm., 27th of March).

Ultrafiltration followed by stabilization by adsorption in chitin or chitosan. In this case the adsorbent is a cheap product with a not great purity, what also implies a price reduction compared to the initial procedure.

Ultrafiltration and further freezing in liquid phase. In this case, the suitability of using a liquid additive by feed producers should need to be assessed.

Microencapsulation in cyclodextrin. It is routinely used in the elaboration of pharmaceutical/cosmetics, although it is also quite expensive.

Although at a first glance the high production cost enzyme extracts from fish viscera may be discouraging, some additional points should be considered in a future prospect, i.e.; the biological evaluation using a different species or the combination of extracts obtained from different sources which can provide an enrichment in both protease and lipase activities.

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