PROJECT FINAL REPORT

Grant Agreement number: FP7-288925

Project acronym: ARRAINA

Project title: Advanced Research Initiatives for Nutrition & Aquaculture

Funding Scheme: CP-IP Large-scale integrating project

Period covered: **from** 01 January 2011 **to 31 December** 2016

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1. Final publishable summary report

1.1 Executive summary

Traditionally fish feeds for cultured fish are based on fish meal and fish oil derived from capture fisheries. However, there is increasing pressure on these raw materials due to growing demands from a variety of users including the expanding aquaculture sector and the human health market (e.g. fish oil food supplements). Hence the sustainability and competiveness of aquaculture may depend on the replacement of fish meal and fish oil with alternative ingredients such as plant-based feeds. ARRAINA has been responding to this need by measuring the long-term effects that these changes in diet will have on the full life cycle of fish for which presently little is known.

By developing applied tools and solutions of technological interest to the European fish Feed Industry, in collaborations with SMEs, ARRAINA contributed to further strengthen the links between the scientific community and the EU feed industry and to increase the productivity and performance of the aquaculture sector leading to competitive advantage of the whole sector at a global level.

How nutrients or dietary components modulate fish performance and a wide range of biological processes (e.g. lipid and fatty acid metabolism, muscle growth, oxidative stress, health and immune response) affecting fish performance has been explored in ARRAINA project. Data generated in both salmonids and non-salmonid fish indicate that the most important European farmed fish are able to grow with plant-based diets without any or very limited supply (< 7%) of marine feed ingredients from first life stages to completion of sexual maturation, producing ova and viable alevins. Significant progress has also been made on the different metabolic pathways and processes affected by high dietary levels of plant proteins and/or vegetable oils.

ARRAINA project selected appropriate panels of measurements for routine and clinical assessment of fish performance. Examples include analyses of blood for electrolytes, enzymes that indicate liver function or serum levels of various metabolites or growth-promoting factors. An initial challenge has been not only to screen and make the best use of already available tools, but also to integrate new insights arising from transcriptomics (gene expression analysis), proteomics (protein expression analysis), metabolomics (metabolite profiling) and metagenomics (microbiota genome analyses) in order to define a reliable and healthy phenotype of fish.

Of particular value are biomarkers that precede the onset of metabolic disturbance or predict the capacity of the animal to cope with dietary, environmental and age-related stresses. At the present stage, we are still far from establishing the normal range of variation for most valuable biomarkers through all the productive cycle, but ARRAINA project contributed significantly on this.

It is difficult to generalize due to differences in growth performance, nutrient requirements and life cycle of the main five species of the European aquaculture, although important advances have been made in the development of interactive tools, including new genomic resources, such as sea bream and sea bass transcriptomic databases with a high coverage of protein transcripts.

1.2 Summary description of the project context and objectives

Today's fish feeds are produced from a large variety of feed ingredients that have different nutritional and physical properties, but sustainable fish feeds manufactured on the basis of a solid scientific knowledge and reliable raw materials contribute to make the aquaculture an environmentally-friendly industry that produces highly nutritious quality food for humans.
The production of environmentally sustainable aquaculture feeds starts with the selection of high quality raw materials that need to be supplemented with Amino acids, Phospholipids and Micronutrients to support maximum growth. The micronutrients are added as a premix of Minerals

and Vitamins, but the nutrient profile of marine feedstuffs and plant protein-sources is quite different. Thus, one of the aims of the ARRAINA project was to identify which micronutrients, and in which form, need to be added to plant-based diets to support maximum growth of farmed fish from early life stages to completion of production cycle and sexual maturation.
To refine our knowledge on the nutritional requirements and the long-term effects of new fish diet formulations on growth performance, physiological wellbeing and health, ARRAINA project brought together different competences for developing tools and methodologies based on targeted and non-targeted biomarker approaches in the five species of the project. Additionally, the project unravelled the potential presence and prevalence of contaminants associated to plant-based sources in fish fillets for human consumption, developing targeted and wide-screening methods for fast and sensitive analyses.

Nutrients exert their actions at various molecular and cellular levels ranging from direct effects in transcriptomic and protein expression processes to their roles as enzyme cofactors or substrates in biochemical pathways. How nutrients or dietary components modulate fish performance and a wide range of biological process affecting fish performance have been already explored, although remains mostly unexplored how quantitative changes or graded levels of dietary nutrients assist in discovering new biomarkers. In this sense, the overall objective of WP1 was to identify and validate promising panels of biomarkers to be used as integrative tools to accurately measure and predict growth responses, metabolic and health effects of plant-based diets for their application in other WPs of ARRAINA project.

The main objective of WP2 was to develop and validate adequate and innovative delivery vectors for macro and micronutrients and dietary supplements (including essential trace elements, vitamins) that will allow producing optimised alternative aquafeed formulations for the different farmed species over their life cycle, particularly during early stages. To achieve this goal several technical objectives were planned: to develop delivery vectors for nutrients during very early life stages; to validate newly developed delivery vectors for nutrients at early life stages; to validate newly developed delivery vectors for nutrients in juvenile stage; and to develop larval inert diets as a complete delivery vector for nutrients.

When dietary protein and lipid sources are changed from fish-based to plant-based ingredients, there is a concomitant change in a range of dietary nutrients, especially micronutrients. Large variation is found in phospholipids, vitamin and mineral contents of FM, corn gluten meal and dehulled and solvent extracted soybean meal, the most commonly used plant ingredients in fish feeds. WP3 aim was to provide new knowledge was needed to secure the fishes' requirements in micronutrients when using feeds with low levels of FM, to ensure their supply and bioavailability. This was done through a meta-analysis of available data on nutrient requirements with a follow-up review on nutrient levels and availability from feed ingredients available for EU fish feed industry, analyses of requirements for specific nutrients deficient in a diet low in marine ingredients, and analyses of the effects of nutrient interactions on quantitative nutrient requirements.

The aim of WP4 was to determine and critically assess the effects of long-term feeding of diets in which the traditionally used marine ingredients (FM and FO) were replaced at high levels with alternative, largely plant-based, ingredients in the five target species. Thus, rainbow trout, Atlantic salmon, gilthead sea bream, European sea bass and common carp were fed from first feeding, or soon after, throughout their entire life/production cycles to harvest and, when possible, to reproduction, and effects on growth performance, feed efficiency, nutrient retentions, biochemical composition, metabolism, fish health, reproductive performance, product quality, and food safety determined.

Using alternative raw materials in fish diets introduces together with possible unbalances in nutrients, a number of anti-nutrients like NSP, oligosaccharides, tannins, phytic acid etc, which may affect diet assimilation. Nutrient release as N and P by the fish to the environment can be increased due to the inclusion of plant raw materials to their diets leading to environmental burden, while except of changes in the nutritional value of the feeds their efficacy relies strongly on a series of technological criteria such as pellet durability or water stability which may be affected when plant raw materials are included. The objectives of WP5 were a) to evaluate and predict the impact of plant raw materials in the physical properties of produced pellets, b) to provide information on the environmental impact of feeds based on low Fish meal/Fish oil content for the species of interest, c) to evaluate the potential for decreasing this impact through dietary and technological manipulations and d) to provide a model of waste output when using new formulations and test produced environmental load in long term experiments.

Nutritional programming was a new concept to be tested in fish nutrition. Our hypothesis was that early nutritional stimulus was able to induce durably modification of use of sustainable diets through action on metabolism mainly. WP6 objectives were to test the nutritional stimuli at 3 developmental stages: at the broodstock levels; at the egg levels and at the larvae/alevins level. The existence of nutritional programming was tested in adult fish using challenges with plant-based diets. Rainbow trout, Atlantic salmon, gilthead seabream, European seabass and common carp were fish species tested for the nutritional programming.

ARRAINA dissemination aimed to raise awareness of the project and to communicate to the relevant stakeholders and wider public. WP7 objectives were to 1) disseminate knowledge to end-users on alternative feeds for the most important European farmed fish species and their respective industries in Europe; 2) create a platform for co-innovation where European scientists and stakeholders share the responsibility for a joint RTD and innovation agenda; 3) stimulate the West-East-South cooperation within Europe, with a particular focus on the new EU member – and associated states; 4) train junior professionals and scientists in novel methods for research and technology development as developed in ARRAINA; and 5) stimulate the societal awareness and acceptance of aquaculture in Europe.

1.3 Description of the main Scientific and Technical results/foregrounds

Progress made within ARRAINA are indeed remarkable and tangible. Exploitable predictive biomarkers have been developed as part of project activities in WP1; novel data on nutrient requirements have been obtained as part of WP3; nutrient delivery vectors to modify egg composition or to supply specific micronutrients to first feeding larvae in WP2; long-term growth studies undertaken with all the five species as part of WP4; tools to assess environmental impacts in WP5 and progress in demonstrating the concept of nutritional programming in WP6.

WP1:

A key achievement of WP1 is the **on-line tool of ARRAINA biomarkers** (www.nutrigroup-iats.org/arraina-biomarkers), which was designed to identify easy and highly informative nutritionally-regulated biomarkers in the five fish species of the Project. Final integration of new tools and biomarker approaches was done by means of the Technical Bulletin of ARRAINA biomarkers. The objective of this booklet (available at www.arraina.eu) is to provide examples of reliable sets of biomarkers and associated methodologies to assess the nutritionally mediated effects on growth performance, metabolic homeostasis, stress responsiveness and health condition of fish fed new diet formulations from early life stages to completion of production cycle and sexual maturation.

Biomarker attempts **in salmon** were focused on micronutrient requirements, which included classic tissue measures of vitamin and mineral status in combination with other surrogate markers. For instance, measurements of muscle vitamin B6 status were related to the activity of aspartate amino transferase (asat), a metabolic enzyme that uses vitamin B6 as cofactor. Alternatively, vitamin B6 takes part in the 1-C metabolism through the transsulfuration pathway, and the resulting metabolic outcome (organ lipid accumulation, metabolite profiles and gene expression patterns) should be used as a marker of vitamin B6 deficiency. Likewise, regarding Zn-dependent enzymes, vertebral Zn-content and Zn-requirements are negatively correlated with the bone activity ratio of ALP (alkaline phosphatase)/TRAP (tartrate resistant acid phosphatase).

In trout, biomarker attempts were focused on methionine requirements and blood and tissue metabolomics profiling using NMR approaches. Feeding trials conducted within WP3 clearly indicated that methionine deficiency has opposite effects on the activation of mTOR and CGNA signalling pathways, regulating also the ubiquitin-proteasome proteolytic system as indicated the enhanced expression of E3 ubiquitin ligases and znf216, which is involved in the delivery of ubiquitinated proteins to the proteasome. Otherwise, fatty acid synthase can be used as a biomarker of enhanced lipogenesis under methionine deficiency. Changes in cholesterol metabolism are highlighted by changes in the expression of the transcription factor lxr. Additionally, glutamate dehydrogenases (gdh1, ghd2) and hydroxyacyl-CoA dehydrogenase (hoad) emerged as indicators of energy metabolism. The same for glutathione S- transferase as a marker of antioxidant defence and insulin-like growth factor-I (igf1), myosin heavy chain (myhc) and myogenin as markers of muscle growth. Regarding, omics approaches, 1H-NMR and 13C-NMR metabolomic profiles were investigated in biological fluids (blood) and relevant tissues (liver, muscle) for polar and liposoluble compounds. In all cases, the metabolomic profiles were discriminant for fish fed from the first feeding with complete marine diets, a mixed commercial diet or complete basal diets. Such analysis helps to draw a comprehensive overview of the diet footprint on fish metabolism, corroborating the suitability of blood as a surrogate marker of overall metabolic status.

In common carp, rates of FA oxidation and LC-PUFA biosynthesis have been studied in fish fed with varying LA/LNA ratios, but data obtained in radio-gaschromatographic analyses are not sufficient to select validated biomarkers of fatty acid oxidation and elongation and desaturation of C18 PUFA.

In European sea bass, high DHA contents in diets of sea bass larvae lead to an alteration of the oxidative status and to the appearance of muscular lesions. Molecular pathways underpinning regeneration processes were analysed and the optimum range of variation for a set of selected markers, including catalase (cat), glutathione peroxidase (gpx), superoxide dismutase (sod), igf1 and igf2, s, alpha-actin, myhc and calpain (calp) were reported on muscle regeneration. The induced cortisol release from interrenal cells by dietary fatty acids is mediated by different pathways and genes, and the opposite regulation of cortisol-synthesis (cyp11β) and glucocorticoid-related genes (gr and hsp90) improves the ability of fish to cope with a stress-related increase of circulating glucorticoids. Based on this, reference values for selected stress molecular markers and in vitro cortisol release from interregnal cells after ACTH stimulation is established. Additionally, the intestine is highly responsive to changes in diet composition, and a histological scoring system based on the morphological changes induced by fish fed plant-based diets was proved as a highly feasible tool to assess intestinal health. Biomarker approaches in European sea bass also included the validation of histone acetylation and gene expression changes on methyltransferases and deacetylases as epigenetic target markers of butyrate.

In gilthead sea bream, clinical blood biochemistry in combination with organosomatic indexes and histopathological scoring of liver and intestine were functionally validated for the routine diagnosis of common nutrient deficiencies in fish fed free-FM diets. The diagnosis outcome might require confirmation by more specific assays, but the generated information is very useful for the overall assessment of fish performance and metabolic condition. The definition of the normal range of variation for most of the analysed parameters was done combining data from short (WP1) and long-term trials (WP4) through the production cycle. The tissue-specific regulation of 40 selected markers of lipid and lipoprotein metabolism has been reported in juvenile fish according to the different

metabolic capabilities of each tissue and vital functions for life, which makes the brain highly refractory at the transcriptional level to changes in nutrient and energy availability. In contrast, both in sea bass and sea bream, the liver is clearly a highly reactive tissue, with changes in gene expression affecting not only elongases and desaturases, but also to a high extent to the "lipolytic machinery". Likewise, co-expression analyses of more than 100 growth-rate regulated genes, including markers of GH/IGF system, muscle growth and cell differentiation, proteolysis, protein folding and assembling, inflammatory/anti-inflammatory response, energy sensing, oxidative phosphorylation, antioxidant defence and respiration uncoupling showed tissue-specific patterns of regulation, becoming skeletal muscle specially sensitive to changes in vitamin status. The usefulness of a targeted approach for the fine assessment at the molecular level of intestine architecture and function has also been proved in fish challenged with different additives and diet compositions. These pathway-focused PCR array approaches were combined with wide-gene expression analyses using customized microarrays with more than 15,000 unique genes, which highly revealed a high specialisation across the intestine of both sea bream and sea bass. All this contributed to establish a characteristic gene expression pattern for a healthy intestine and nutritional condition in marine farmed fish.

Another key achievement of WP1 is an **on-line database** (www.nutrigroup-iats.org/aquafat) **for predictive modelling the nutritionally tailored fillet FA composition of marine fish**, and sea bream and sea bass in particular. This is an easy-friendly tool to produce more tailored and healthy seafood products according to the guidelines of essential fatty acid requirements in humans.

WP2:

In WP2, a **range of novel delivery vectors for micro and macro-nutrients have been developed** and validated using innovative technologies such as sonophoresis, nanoparticles, micro-liposomes, micro-encapsulation, etc. These tools facilitate the availability of micronutrients in low FM/FO feeds along the whole life cycle, from early larval stages to juveniles and broodstock and these tools have allowed tailoring the nutritional composition of eggs and embryos. Besides, in cooperation with the industrial partners early feeding feeds have been improved by including optimised levels of nutrients through different delivery vectors, promoting larval growth and organ development.

Thus, in Task 2.1 **an efficient sonophoresis based-nutrient delivery method was developed and validated to enrich fish eggs during early ontogenesis**. For that purpose, a sonophoresis protocol was developed and optimal application conditions determined for the target species. This protocol was successfully applied in carp, in gilthead seabream to enrich eggs with methionine, leucine or betain and in rainbow trout with biotin or glucose. This sonophoresis device and the practical operational procedures developed allowed the controlled release of nutrients for nutrient fortification of batches of fish eggs, embryos and yolk-sac larvae, which was effectively applied in WP6 to perform a direct intervention for nutritional programming of the target species.

In Task 2.2, the **efficiency of different delivery vectors and molecular forms of selected minerals** (i.e. Se, Zn, Mn, Cu), **and vitamins** (i.e. vitamin C, vitamin E, vitamin D, vitamin K, etc.) **were tested in diets for larval stages** of seabream and carp, comparing novel and traditional methods. In seabream encapsulated, nano-particulated, inorganic or organic minerals (Fe, Zn, Mn and Se) and combinations of Zn and Mn were delivered through microdiets in several trials and their effect on larval biochemical, histological and molecular parameters. Besides, the chemical properties and in vitro cell up take of the nanoparticles were tested and their effect on larval gut integrity and expression of selected genes was evaluated. Se and Zn were more efficient delivered in organic or inorganic forms, respectively. Mn was toxic regardless the delivery form. Nano-particulated minerals were rapidly up-taken in vitro and did not negatively affected gut morphology. Dietary minerals affected gene expression of oxidative stress- related enzymes, glucocorticoid receptors and bone development-related proteins. In carp, delivery vectors for effective transfer of nutrients to carp larvae were tested with live preys and microdiets with non-encapsulated, protein encapsulated and oil encapsulated vitamins were tested. Protein encapsulation was more efficient

to delivered water-soluble vitamins for carp larvae. The results allowed selecting effective methods for delivery of nutrients in fish diets and live food enrichments. The most effective delivery forms were used to determine nutritional requirements in WP3.

In Task 2.3, several studies were conducted to **determine the effect of several specific and general delivery vectors for nutrients** on gilthead seabream juveniles' growth, survival, health status and bone development in diets where FM/FO was replaced by plant ingredients. Effects on feed intake, juvenile growth, feed and nutrient utilization and organ development was studied. Gene expression of nutrient-related enzymes and bone development related proteins were studied. The results showed that dietary treatments had a higher effect on the biochemical composition and morphology of posterior vertebrae than that of anterior vertebrae. Supplementation of plant-based diets with inorganic forms of the target minerals significantly promoted growth and increased the vertebral weight and vertebral content of ash and Zn. Supply of inorganic minerals enhanced bone mineralization, up-regulating osteocalcin, and produced vertebrae with a larger length in relation to height. Encapsulation of inorganic minerals reduced fish growth and increased oxidative risk. Reduced availability of encapsulated Zn, Mn, and Se lowered vertebral mineral content. Supplementation with organic minerals down-regulated the expression of glutathione peroxidase 1, suggesting that dietary Se supplemented in the organic form is more effective than the other forms of supply. Results were applied in the requirement studies conducted in WP3.

The main aim of Task 2.4 was to **develop microdiets as delivery vectors of nutrients for fish larvae**. For that the efficiency of different microdiet production technologies on the nutrient stability and availability was studied. After reviewing microdiet production methodologies, two encapsulation methodologies, internal-external gelation with alginate (IEG) and encapsulated protein (EP), were compared to a microbounded (MB) method. Ingestion rates were lower for IEG in comparison to MB and EP, but Artemia co-feeding increased microdiets consumption. Survival, stress resistance and growth was similar among larvae fed the different diets and not different from Artemia feeding. Trypsin activity was significantly higher in MB larvae as well as trypsinogen gene expression. Considering the delivery vectors selected from Task 2.2, microdiets were then produced by the industrial partners and tested by the scientific partners, allowing long term feeding with alternative feeds along the whole life cycle. Studies on diet performance examined larval behaviour, development, growth and survival. The effect of the diet on digestive, neural, skeletal and immune systems was also considered. Delivery of Tau in liposomes in MB diets for gilthead seabream interacted synergistically with other antioxidant nutrients to reduce oxidative stress risk, enhancing larval growth, survival and skeletal system development. Optimum dietary levels of Mn and Zn in semi-commercial microdiets for seabream were identified. Vitamin C, B and E levels in semi-commercial microdiets for common carp were found to be at least 3 times higher than NRC recommendations.

Semi-commercial microdiets produced by the industrial partners with nutrients provided by delivery vectors were successfully validated under experimental conditions.

WP3:

Meta-analyses revealed a need for renewed recommendations of vitamin and mineral levels in fish diets. A multivariate approach, utilizing early biomarkers (from WP1), made the basis for several regression trial in salmon, seabass and seabream. For the salmon trials significant regressions were obtained in body compartments for several of the B-vitamins. Whole body biotin concentration was unaffected by micronutrient premix level, and mRNA expression of the enzymes dependent of biotin showed only weak increases with increased biotin, earlier recommendations are therefore valid also for plant diets. Muscle thiamine plateaued at a diet level similar to NRC (2011) recommendation in freshwater, and showed stable values independent on premix addition in seawater. The mRNA expression of the enzyme G6PDH (glucose-6-phosphate dehydrogenase) is sensitive to thiamine availability; results did not indicate any need to add thiamine above levels recommended for fish in general. Niacin showed a steady increase in whole body concentrations as feed niacin increased. Muscle riboflavin peaked at a diet level of 12.4 mg/kg. Sufficient riboflavin is important to avoid e.g.,

development of cataract, which was not developed, and NRC 2011 recommendations for fish in general are therefore valid. Cobalamin (B12) in muscle and liver was saturated at 0.17 mg/kg diet. Muscle pyridoxine showed a dose dependent level in muscle, and peaked around 10 mg/kg diet. White muscle ASAT (asparagine amino transferase) activity steadily increased, with indications of stable values when dietary pyridoxine was around 10 -16 mg/kg diet. Pantothenic acid increased in gill tissue up to a level of 5.5 mg/kg soft gill tissue; at a dietary level of 22 mg/kg. Improved performance, and coverage of metabolic need for niacin was at a dietary level of 66 mg/kg, riboflavin 10-12 mg/kg, pyridoxine 10 mg/kg and panthotenic acid 22 mg/kg. **Based on these results, new recommended B-vitamin supplementation in plant based diets are made and published.**

The antioxidant nutrients Vitamin C, E and selenium (Se), affects tissue redox status. Diets high in plants did however appear to contain sufficient levels of vitamin E and Se to cover the requirements to prevent clinical deficiency symptoms. The body levels of vitamin E in salmon parr and that of Se in parr and post-smolt showed a linear relationship with dietary concentration, while vitamin E in post-smolt seemed to be saturated with a breakpoint near 140 mg/kg. Vitamin C concentration in the basal feed was below the expected minimum requirement, but the experimental period was probably too short for the fish to develop visible deficiency symptoms. Vitamin C was saturated in both parr and post-smolt whole body at dietary concentrations of 190 and 63–89 mg kg-1, respectively. Maximum whole body Vitamin C concentration was approximately 40 mg/kg in parr and 14 mg/kg in post-smolt. Retention ranged from 41 to 10% in parr and from -206 to 12% in post-smolt dietary level. This indicates that the post-smolt had an extraordinarily high consumption of vitamin C. Analyses of glutathione (GSH), glutathione disulphide (GSSG) concentrations and the calculated GSH based redox potentials in liver and muscle tissue, indicated only minor effects of diets on redox regulation. **Based on the present trials, the recommendations for supplementation of vitamin C and E in diets for Atlantic salmon are similar to current practices but higher than NRC 2011 recommendations.** In detail, 150 mg/kg vitamin E and 190 mg/kg vitamin C are recommended in plant-based diets. These recommendations are higher than the levels necessary to prevent clinical deficiency symptoms, but are necessary to protect fish against incidents of oxidative stress and to improve immune and stress responses. There were no indications that the Se requirement exceeded the current recommendation of 0.3 mg/kg.

Plant based diets need higher additions of methionine than FM based diets, the level of methionine affected growth and reproductive performance in rainbow trout.

In seabass better growth and overall feed utilization was obtained by the inclusion of minerals 1.5 times NRC 2011, a further increase did not ameliorate these parameters. In seabass dietary level of Fe, Zn, Mn and Se, affected GSH-related antioxidant enzymes activities and GSH content in fish. There was a stimulation of the respiratory burst and ceruloplasmin activities at intermediate dietary concentrations of minerals (0.5-2 times). Significant immunosuppression was obtained at both lower and higher mineral levels (respiratory burst activity in the depleted diet, lysozyme at 1.5-4 times, and antiprotease activities at 1.5 times NRC 2011

Variations of lipid peroxidation and vitamin E contents in tissues of rainbow trout confirmed that dietary supplementation of vitamin E had a protective action against lipid peroxidation. This was reflected in the fatty acid profile of the whole fish. The effect of dietary vitamin E supplementation on the flesh fatty acid profile in fish fed vegetable oil suggests another effect of vitamin E on PUFA synthesis, even if there was no significant effect on gene expression for PUFA synthesis in the liver of rainbow trout.

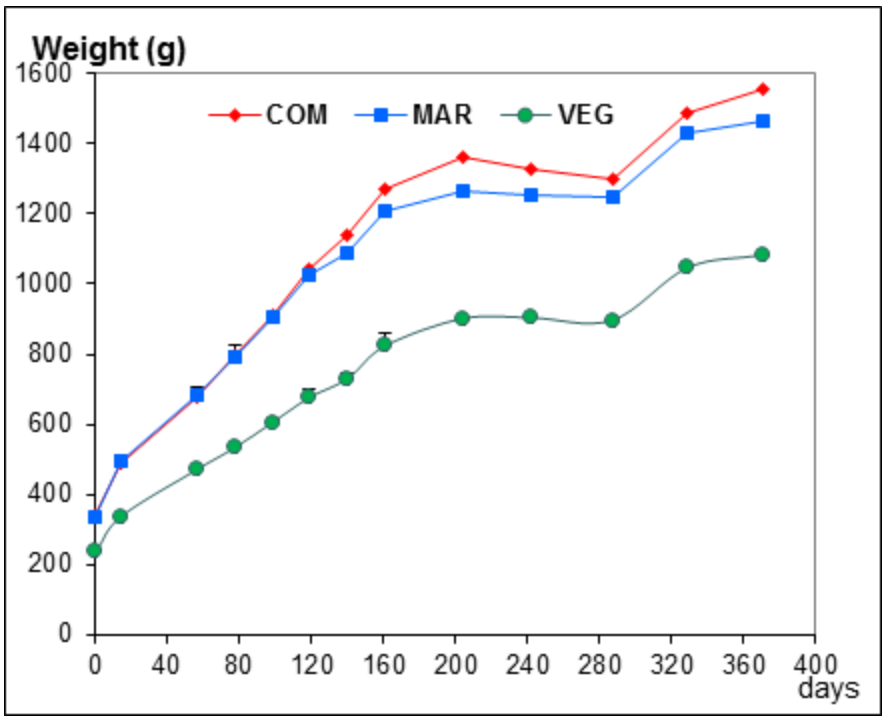
Polyaromatic hydrocarbons (PAH) contamination in fish diets reduces the storage of liver vitamin A in salmon. The supplementation of vitamin A to the diets restores partially the vitamin A status in PAH exposed salmon. The supplementation of vitamin A furthermore restores the reduced growth and energy metabolism in PAH exposed fish and enhances PAH detoxification mechanisms. The

presence of dietary PAH in plant based feeds will likely affect the requirement of vitamin A supplementation in plant-based feeds. **Reversely, vitamin A supplementation can counter the adverse effects of dietary PAH contamination.**

WP4:

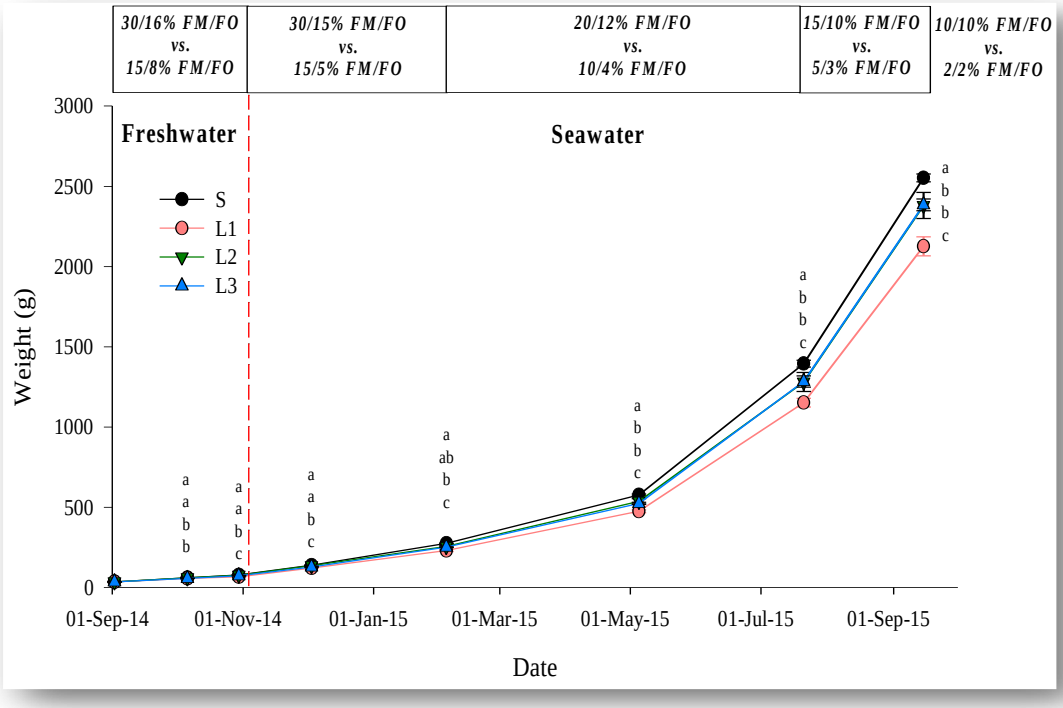
The long term trial conducted on rainbow trout showed for the first time that **rainbow trout reared on a plant-based diet completely devoid of marine ingredients throughout the entire life cycle** (from 1st feeding until reproduction) **was able to grow and to produce viable ova and fry**. **However growth was reduced (30%; Fig.1) and the global reproductive performance and survival during the first stages were lower**. This study showed that the totally plant-based diet has slight effects on metabolism after 7 months feeding, as shown by the relatively low proportion of metabolism-related genes found to be differentially expressed in intestine and liver transcriptome. Pathways involved in fatty acid bioconversion and cholesterol synthesis were up-regulated in fish fed plant-based diet, however, this regulation is not sufficient to counteract the absence of beneficial omega-3 and cholesterol inputs as shown by the modifications of tissue fatty acid profiles and plasma cholesterol levels. On the other hand, the activity of the "autochthonous" intestinal microbiota appeared only marginally dependent on the diet.

Fig. 1. Growth of rainbow trout fed diets with low or no FM/FO diets



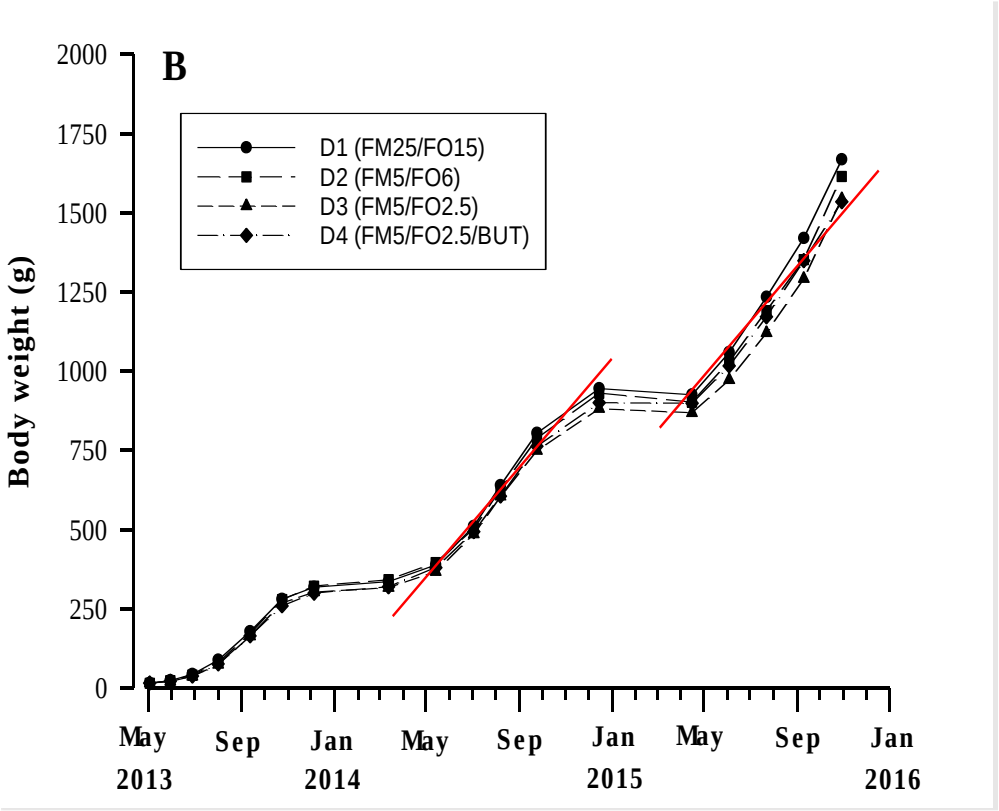
The long-term salmon trial used an entirely novel and previously untried multiplex screening approach, which increased the amount of data generated for the resources used. **The trial established that the currently accepted micronutrient requirement levels for Atlantic salmon are not all sufficient when feeds with very high levels of replacement of fishmeal and fish oil are used as evidenced by lower growth if fish fed L1** (Fig.2). Specifically, vitamin B3, B5 and B6 levels appeared to be sub-optimal and therefore they should be increased in nutrient premixes, whereas levels of vitamins B1, B7, B9 and B12 appear to be sufficient at current recommended levels and so no changes are recommended. However, there are still some micronutrients where no clear new recommendations could be given based on the studies performed in ARRAINA and, furthermore, all new recommendations should be validated in experiments targeting specific micronutrients.

Fig. 2. Growth of Atlantic salmon fed low FM/FO diets over the full cycle



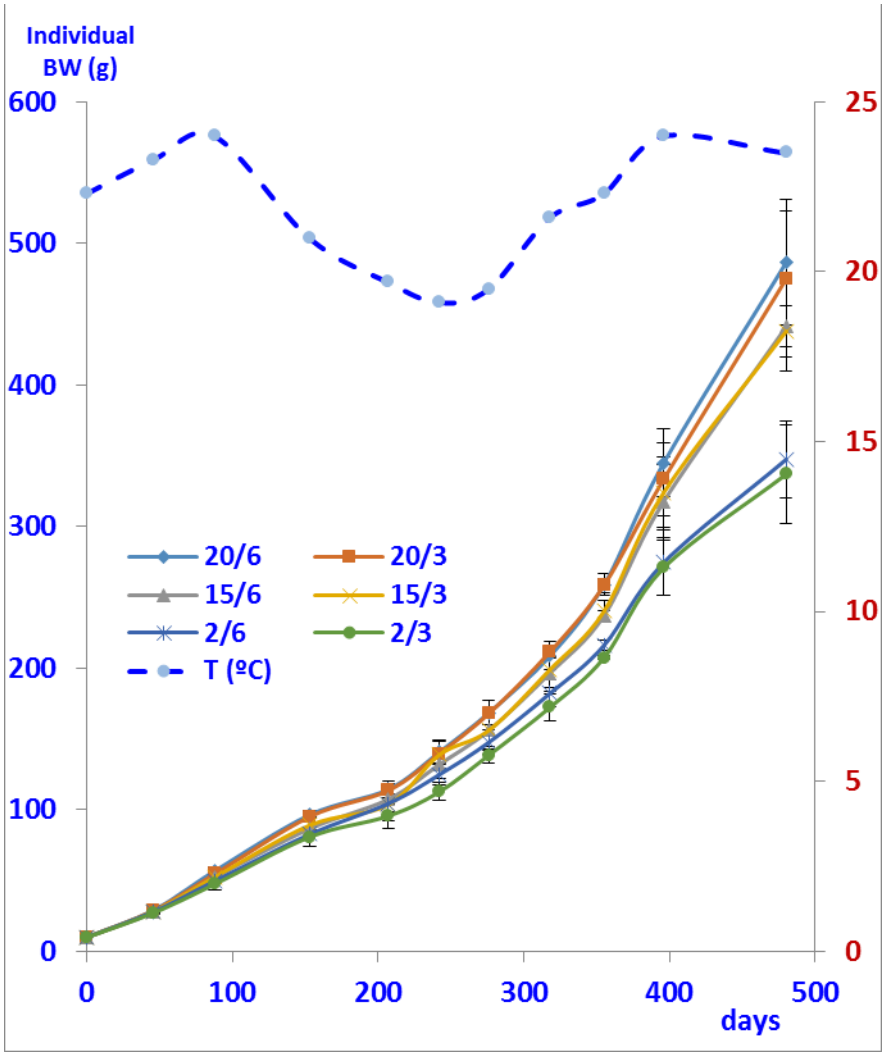
Low FM/FO diets were able to support maximum growth in gilthead sea bream, yielding safe seafood products (Fig.3). Major constraining factors were relatively minor health issues and reproductive performance (measured as the sex population ratio), although butyrate supplementation helped to restore the wild phenotype of fish fed the control diet. **Briefly, formulations with very low FM and FO required an adaptive period of 2-3 weeks to avoid transient detrimental effects on feed intake and feed efficiency. Dietary butyrate prevented clinical signs of anaemia and hypocholesterolemia, partially reversed the masculinization effect, and alleviated the slight pro-inflammatory status induced by diets with very low levels of FM and FO**. Sea bream fed low FM/FO diets had increased susceptibility to parasitic enteritis but, again, this was partly reversed by dietary butyrate supplementation. Levels of contaminants in the low FM/FO diets were far below the maximum legislated limits but still require to be monitored. Growth-related markers of the GH/IGF axis can be used to define normal and abnormal growth through the production cycle for fish fed different diet formulations, and the intestine was identified as a key tissue to assess the impact of diet formulations using gene expression analysis.

Fig. 3. Growth of gilthead sea bream fed low FM/FO diets over the full cycle



Feeding European seabass for the entire on-growing period with diets containing only 10 % FM and 3 % FO not only had no negative affects on fish performance, metabolism, health and fillet quality, but also reduced fillet contaminants. However, further FM and FO reduction to 5 % and 3 % respectively, reduced growth (Fig.4) and altered lipid metabolism by increasing lipid (fat) deposition and cholesterol synthesis in the liver, causing enlargement of hepatocytes and raising the hepato-somatic index. In addition, it negatively affected health parameters causing inflammation of the posterior gut with infiltration of immune cells, up-regulation of molecular markers of the immune system and increased sensitivity to experimental disease infections.

Fig. 4. Growth of European sea bass fed low FM/FO diets over the full cycle



The long-term trial from broodstock to broodstock of Common carp reared in pond conditions showed that production performance was comparable when fish were fed either moderate FO/FM, or entirely plant-based (PM/VO) supplementary feeds. Increased linolenic acid content in the supplementary feeds improved the essential fatty acid status of the fish, but increased deposition of linolenic acid rather than conversion to higher homologues. Fatty acid composition of muscle/flesh in market fish reflected feed composition. In the last stages of gonadal recrudescence, partial deficiency of beneficial omega-3 fatty acids was accompanied by hepatic steatosis. The feed polyunsaturated fatty acid supply was insufficient for normal lipid transport from the liver to other tissues resulting in fatty liver. In addition, oogenesis was seriously impaired and lack of ovary or milt was frequently observed. Furthermore, offspring from the few successful propagations showed decreased vitality.

WP5:

The study performed on the pellet quality containing plant protein rich ingredients showed that there is a need to standardize methods for a comparative assessment of the physical quality of fish pellets. The tested plant-based ingredients caused unique processing responses and physical properties when applied in fish diets and specific processing parameters should be adapted to diet ingredient composition. **Knowledge generated confirms that within the "commonly used" incorporation levels and with a careful adjustment of extrusion conditions, the use of plant ingredients results in fish pellets with high physical quality, without detrimental effects on the environmental loads associated to feed losses**.

In the study performed in order to provide experimental data on the impact of dietary composition on waste production (N, P solids) and characteristics of faecal waste (amount, chemical composition and physical property) in carp and sea bass **it was shown that in both species the inclusion of 30% of different test ingredients affected significantly nutrient digestibility**. The largest variability between diets was due to the carbohydrate fraction and in particular, diets rich in non-starch polysaccharides (NSP), wheat distilled grain from soluble (WDDGS) and sun flower meal (SFM) resulted in higher recovery percentage thus lower non removed faeces for both species. In carp a 6 week acclimation period of feeding with test diets is needed before evaluating effects of different diets on nutrient digestibility (ADC), faecal physical properties and recovery. Differences in faecal physical characteristics were related to different methods of faeces collection (stripping - settling) rather than to different diets in carp, whereas the differences observed in sea bass were attributed to different test ingredients. A strong effect of water temperature occurred for ADC of DM and starch, faeces recovery and thus solid waste, as well as faecal physical characteristics in sea bass. Sea bass and sea bream fed on diets from the long term experiments showed very low faeces recovery which leads to a higher waste production. It was suggested that the inclusion of raw materials high in soluble NSP's may contribute to the management of solid waste produced. Differences were evident between species in ADC for nutrients, P and faeces composition.

From the review on anti-nutrient (ANF) effects on fish performed **it was concluded that pure protein concentrates are highly preferred and recommended for use in aqua-feeds due to their low amounts of ANFs and their better nutrient retention by fish and therefore less nutrient outcome in the environment.** However, since levels of NSP are still relatively high this constitutes potentially the major factor contributing to waste production from the aquaculture operations due to their low utilization by fish. Research efforts nowadays should focus on the use of NSP-degrading enzymes and improvement of carbohydrate utilization by fish as a major nutritional bottleneck for the sustainability of the sector.

A model simulating waste production with different feed formulation has been developed. The tool has been successfully implemented as a public web application (named wastEst), providing waste estimation from growth performance data (for gilthead seabream, European seabass and common carp). WastEst can be freely accessed at http://sparos.pt/wastest/. The model was implemented in the proof of concept project ASSESSFEED.

In order to reduce faecal waste produced and the impact on faecal waste quality when using NSP rich diets the potential use of specific supplement feed enzymes has been studied in sea bass and carp. **In both species the inclusion of an enzyme cocktail in diets containing different type of NSP insoluble (SFM) and soluble (WDDGS), leaded to an improvement of both growth and feed utilization**. Faeces recovery was affected by the enzyme supplementation in the case of sea bass resulting in less waste produced by the fish, while in carp this was affected only by diet. The enzyme addition didn't result in differences in the faecal physical characteristics in both species studied. Only the ingredient used in each diet had an effect and only in the case of sea bass faeces viscosity was decreased when the enzyme cocktail was added. The improved ADC of N and P led to a decrease of waste produced by the fish as solid and soluble wastes.

The SFM based diet was the most affected diet by the enzyme cocktail addition compared to the WDDGS diet in both species studied (carp and sea bass). No clear effects were evident in both species in the synergism of the enzymes phytase and xylanase when included in diets containing SFM.

The study on the impact of low FM/FO diets on soluble and solid waste during long term feeding and water and sediment quality in carp pond aquaculture reviled that formulated feeds with approx. 30% protein content increase nitrogen and phosphorus input to the ponds. Earthen fishponds retained considerable amounts of nutrients as sinks or decomposition “reactors”. Water quality and sediment chemistry parameters indicated only marginal differences between treatments which were not permanent throughout the experimental years. **Plant-based diet resulted in higher fish yields, and slightly elevated phosphorus and organic matter outflow and moderately higher nitrogen output than the control diet**.

The impact of low FM/FO diets on solid and soluble outputs from the culture of all species except carp from the long term experiments, showed differences between species as far as total waste, N and P waste (soluble and insoluble) is concerned. From the two marine fish taken into consideration, sea bass was characterized by higher waste emission than sea bream, while the use of plant raw material in the diets resulted in significantly higher waste output than the FM/FO diets only in sea bass and not in sea bream. The two salmonid fish, Atlantic salmon and rainbow trout were characterized by similar waste emission at the first growth stage while in the last period salmon gave 3 times less waste that rainbow trout. The vegetable diets affected the waste output in the first stage for both species (higher waste output). **No differences were observed afterwards for salmon while in rainbow trout the vegetable diet resulted in lowering the waste output.**

WP6:

After parental nutrition, broodstock feeding can have long term effects on progenies in rainbow trout, common carp and gilthead seabream. Indeed, feeding broodstock gilthead seabream with different dietary lipids (i.e. vegetable oils) gave birth to progenies with different growth performance and improved use of plant-based diets (ULPGC). Feeding parent carp (from first feeding up to reproduction) with plant-based diets gave birth also to progenies with different capacities to resist to hypoxia challenges as well as different immunological capacities (HAKI). Finally, after feeding rainbow trout from first feeding up to reproduction, very few effects related to nutritional history have been detected in contrast to the drastic effects (at a metabolic level in alevins) observed after modification of feeding rainbow trout broodstock with different levels of methionine (INRA). Action at the level of broodstock feeding seems to be very interesting in the future for programming fish.

Regarding the direct egg supplementation with amino acids, **the modification of egg composition was a success in gilthead seabream using new techniques such as sonophoresis** (CCMAR). Even though all the analysis have not been finished at the end of the project, it seems that the nutrient supplementation in eggs (with methionine for example) can affect the metabolism at long term in adult fish. This approach is promising but needs clearly more researches to be easily applicable in aquaculture in the future, modification of egg composition through direct interventions staying always a challenge.

Studies of the programming linked to the first feeding stage were numerous in our project for the five fish species: Atlantic salmon, rainbow trout, gilthead seabream, European seabass and common carp. Indeed, first feeding rainbow trout (INRA), Atlantic salmon (UoS) and common carp (HAKI) with plant based diets during 3 weeks were linked in adult fish to different use of aquafeeds at the level of growth performance, metabolism, transcriptomics and feed intake for example. Specific feeding at early life with diets composed of specific nutrients (high levels of carbohydrates, low levels of lids in diets) were also associated with different metabolism and microbiota in adult life in rainbow trout (INRA) and – even though at a lower level - in European seabass also (INRA-IFREMER) and gilthead seabream (CCMAR-INRA). In contrast, first feeding with stimuli diets composed of low levels of fish oil in gilthead seabream larvae (ULPGC) were very difficult in term of survival and were not successful to test the programming in this fish species linked to the first feeding. **Globally, the first feeding stage seems to be a very promising stage for inducing a nutritional programming for all the fish species.**

In conclusion, our data in the WP6 prove clearly for the first time in fish the existence of a nutritional programming in the different fish species linked to nutritional stimuli at different moments of the development. These finding opens new promising strategies for the development of new aquafeeds in the future for aquaculture.

WP7:

The ARRAINA website, newsletters and a total of 112 scientific publications were used as central channel for communication.

The training (workshops and training courses), 3 technical booklets and a total of 337 presentations through several (annual) meetings and reports, (EAS and its annual conferences, EATiP (together reaching farmers, feed producers, scientists and consumers), FEAP and FEFAC (both reaching fish producers and the European feed producing industry) were used as a strong channels for spreading research outcomes of ARRAINA.

Within ARRAINA, specific distributional channels were set up like workshops, training courses and technical booklets, to reach targeted groups of stakeholders and end-users. These instruments were used to guarantee that the new knowledge, acquired through ARRAINA, is widely shared. Further, a stakeholder platform for cooperation was created.

To improve the research capacity across Europe (in particular new member states and candidate countries) a mobility programme was set up funding 35 mobility stays during the project period. This programme aimed specifically at junior professionals and young scientists, such as PhD students, and employees working in the in the field as RTD or as nutritionists. Candidates for the mobility programme were selected via a dedicated call.

Links to the three technical ARRAINA booklets:

- "Feed Ingredients in Aquaculture" (May 2015). LINK

-"Understanding Vitamins, Minerals and Other Nutrients in Fish Feed Diets Based on Plant Derived Ingredients" (September 2016). LINK

-"Understanding Biomarkers in Fish Nutrition" (December 2016). LINK

- Project Video: LINK

- Key Achievements Booklet: LINK

1.4 Potential impact and main dissemination activities and exploitation of results

A constant goal all along the duration of ARRAINA has been to make its results easily available for the partners of the project and, at longer term, to all the stakeholders. This has been a general effort from all the WPs to keep this trend and to make this project as useful as possible to the whole aquaculture community.

WP1:

The ARRAINA Project selected appropriate panels of measurements for routine of fish performance. The multi-nutrient dietary approach chosen in ARRAINA dictates an organismal approach on fish health and fish performance, integrating both classic measurements of single nutrient status (e.g. tissue nutrient levels) with measurements of physiological status. This approach has delivered new insights into the assessment of nutrient requirements and long-term physiological consequences of low FM/FO diets. A challenge has been not only to screen and make the best use of tools available already, but also to integrate new knowledge arising from transcriptomics (gene expression analysis), proteomics (protein expression analysis), metabolomics (metabolite profiling) and microbiota analyses in order to define a reliable and healthy phenotype of fish.

Of particular value are biomarkers that precede the onset of metabolic disturbance or predict the capacity of the animal to cope with dietary, environmental and age-related stresses. Currently, we are still far from establishing the normal range of variation for most valuable biomarkers through the full productive cycle, but the ARRAINA project contributed significantly to progress in this area

combining targeted and non-targeted approaches with focus on the five species studied in the Project (Atlantic salmon, rainbow trout, carp, European sea bass and gilthead sea bream).

Key achievements are the on-line tools, including searchable databases of biomarkers, predictive on-line tools of fillet fatty acid composition and updated sea bream and sea bass transcriptomic databases, which have been proved very useful as a reference library in proteomic studies and wide-genomic studies, helping also to define the molecular identify and the tissue specific regulation of a wide-range a new sequences (more than 500) which have been uploaded to public databases repositories.

These new tools and knowledge has been initially exploited in other WPs of ARRAINA Project, but they are coming fully exploited in other EU projects of the H2020 framework (e.g., AQUAEXCEL2020, PARAFISHCONTROL, PERFORMFISH, WISEFEED) in which several ARRAINA partners are involved. This will contribute to generate more integrative tools for quick screening and prediction of effects of feed formulation and additives. **It will also allow fish farmers to early predict changes in fish performance** as it has been tested in ASSESSFED Project (ARRAINA Proof-of Concept Project). The envisaged impact for the industry is therefore high, but also for the scientific community with more than 25 peer-reviewed articles of the first quartile.

WP2:

WP2 has applied innovative approaches such as sonophoresis to develop protocols to conduct metabolic programming and provided new tools such as novel vectors (nano-minerals, micro-liposomes, micro-encapsulation…) to deliver nutrients to embryos, larvae and juveniles to complement existing knowledge on nutrient requirements. Thus, WP2 build up on previously existing knowledge to provide basic information on comparative aspects of fish biology, demonstrating for instance: the beneficial effects of nano-minerals for skeleton development and bone mineralization; the importance of Se in its organic form and Cu in its inorganic form for larval growth and survival; the negative effects of Zn and Mn at commonly used levels in commercial early weaning diets on larval survival; the importance of Zn levels and delivery form for skeleton development in juvenile stages; the importance of Tau-protected delivery in larval diets and its synergistic effect with other antioxidant nutrients; the convenience micro-binding diets during the early larval stages but micro-encapsulated and protein-encapsulation during late larval development; the importance of microdiet water content and hydration; the determination of mineral and water soluble dietary levels for carp and seabream larvae; etc. These basic knowledge will strengthen the European research area's knowledge based bioeconomy applied to the aquaculture sector, since **it will facilitate the development by feed producers of better adapted feeds and larval diets improving feed effectiveness**. These feeds, with optimal levels of micronutrients, delivered in an easily assimilated form and with cost-effective techniques, will promote the proper development of larval organs during early life stages and will therefore increase the survival rate of the larvae in commercial hatcheries. To achieve that, the information generated by WP2 not only has been disseminated through scientific symposiums and publications, but **it has been directly developed together with the industrial partners participating in ARRAINA, particularly feed producers and hatcheries**.

Most of this information has been produced by the close cooperation of several partners promoting exchanges of knowledge and know-how that have strengthen European cooperation and communication that are also of interest to academia throughout the world. Besides, this exchange of expertise and skills has contributed to improve human resources.

Thus, these improved and innovative sonophoresis methods, nutrient delivery tools, nutrient requirements and microdiets production information produced by WP2 are being delivered to farmers, industry and the scientific community to ensure increased use of sustainable feeds in aquaculture and profitability of fish production.

The active involvement of SMEs in WP2 RTD activities has been particularly promoted. Therefore, feed producers and other industrial stakeholders participating in WP2 have been involved in

experimental design, formulation, feed and fish production from the beginning of the project promoting dissemination and practical application of the results. This active involvement has allowed to apply the results and to transmit them to the industrial sector with a realistic approach.

The inclusion of several species in the WP (trout, carp and seabream) ensures the wide diversity of farmers and feed producers that may benefit from the information provided by these WP. Moreover, the differences among the selected species (marine-freshwater, cold-warm water, oligolecytic-polilecytic eggs…) and comparative studies facilitate that the information produced would be also useful also for other researchers and farmers.

Finally, the participation of academic partners in WP2 has allowed the participation of technicians, MSc and PhD students, post-doctoral researchers that have complete their training as skilled workers, producing several PhD and MSc Thesis as results of their investigations in WP2. In this way, WP2 has also contributed to help the labour force to adapt to changed skill demands, facilitating their incorporation into labour market and contributing to create jobs opportunities. By this means, dissemination of new knowledge and techniques will be also more easily transferred to the industry.

WP3:

The results from WP3, including peer-review publications, has made it possible to produce fish diets with appropriate levels of B-vitamins, Vitamin C and E, and minerals. The lack of knowledge of nutrient requirements, before the ARRAINA project, was a challenge when FM and FO resources became limited. Fish performance suffered. Knowledge on which level of vitamins, minerals, and marine specific nutrients like phospholipids, methionine, and more, and how to balance vitamin C and E (the anti-oxidants) is a pre-requisite for a healthy aquaculture production. **In this context the results from WP3 have played an important role, and results are already implemented by the industrial partner BIOMAR, and are asked for by other fish feed companies.**

The necessity to balance the level of vitamin E dependent on PUFA level in diet to avoid negative immune and stress responses in fish, was confirmed in studies on rainbow trout, also expected to be utilized by fish feed producers.

Earlier the FM part of the diet secured proper mineral balance and availability of essential minerals. Turning to plant based diets challenged the lack of knowledge on bioavailability, the effect of fibre/ phytate in plants. WP3 results on minerals laid the basis for input to EFSA and the EU-commission when upper limits for minerals were discussed, like Se upper limit in fish feed. If the EU-upper limit were too low, this would negatively influence fish requirement for this mineral, which would again challenge fish health and production. ARRAINA results have been an important input to secure and discuss these upper limits, with input of publications from this (and other) projects. Mineral additions are identified necessary to increase when fish are given plant-based diets, the detailed consequences on fish performance and health when fish feed contained too low levels were identified for seabream, seabass and salmon. Se was in focus in the context of upper-limits, it will be necessary to continue a focus on the requirements versus upper-limits, as plant based diets challenge bioavailability differently than FM based diets. Results from WP3, in close collaboration with WP4, lay the basis for healthy diets for salmon, seabream, seabass, rainbow trout and carp.

Methionine in rainbow trout diets highly influence fish performance, reproductive performance, and the next generation; and should carefully be considered in plant-based diets. .

For a healthy aquaculture production, where feed ingredients are mainly based on plants, the new recommendations from the ARRAINA project, especially WP3, are a prerequisite.

The world lack fish (CFS, HLPE 7th report on fish and fisheries, 2014), and FAO concludes that the major increases will depend on a healthy aquaculture production, with a 3-5% yearly increased production. If aquaculture production aims to be sustainable, feed must support nutrient requirements. A well balanced diet is a key to achieve good growth, feed utilization, general fish health, to avoid anomalies, and to secure reproductive performance. **WP3 results show how to**

make sustainable and healthy aquaculture feed for salmon, rainbow trout, seabass, seabream, and partly carp, utilizing new feed ingredients, especially plants.

Major results from WP3 was published in 2016 as a booklet on nutrient requirements; this is open and available to all interested parties.

WP4:

WP4 carefully studied and determined the detailed impacts of long-term feeding of diets with very high replacements of the marine ingredients, fishmeal and fish oil, with terrestrial-sourced plant-based ingredients from first feeding through to reproduction in the major fish species currently farmed in Europe. The precise physiological and metabolic impacts of specific micronutrients and/or feed supplements and their consequences for growth and health of the fish have been defined. These data have greatly contributed to our knowledge of five major farmed fish species, which will improve the performance of the fish when being fed modern, sustainable feeds formulated with high levels of alternative ingredients such as plant meals and vegetable oils.

Minimum levels of marine ingredients required to maintain high growth rates, feed efficiency, fish health and reproductive performance have been defined or partially defined in all species. Specific limiting factors in alternative sustainable feeds, and various mitigating strategies including optimising micronutrient (vitamin and mineral) levels and nutritional supplements, were thoroughly researched and a set of key recommendations, tailored to the specific physiology and current culture/farming practices in all species were produced. The information gained through the long term trials conducted in rainbow trout, Atlantic salmon, gilthead sea bream, European sea bass and common carp opens up avenues for further reduction of marine ingredients in aquaculture feeds, specifically optimised for different phases/stages of the rearing cycle from egg to broodstock. However to optimise growth, reproductive performance and flesh composition, further fine adjustments of feed formulations are required and it is recommended that small proportions of marine ingredients, fish meal and/or fish oil, should still be included in diets for all species.

The results of ARRAINA Workpackage 4 will have major beneficial impacts for the aquaculture industry, fish consumers and the general public. **Providing ideal feed formulations including optimal micronutrient levels and key nutritional supplements will improve the efficiency and economics of fish farming in Europe, ensuring optimal growth and health of the farmed animals**. The latter issue (farmed animal health) is a key driver for the public now. In this respect, ARRAINA has the potential to contribute very positively to the public perception of fish farming. Furthermore, in this respect, the project has provided data that will maximise the use of plant meals and vegetable oils derived from terrestrial agriculture, and minimise the use of marine derived meals and oils in aquaculture, increasing environmental and economic sustainability of fish farming. Again, these are issues that are high on the public agenda in Europe and will be key to improving public perception of aquaculture.

Timely dissemination of the WP4 data to the scientific community, aquaculture industry, especially production and feed sectors, and the general public is currently the major focus. As of February 2017, it is projected that WP4 will result in a total of 30 publications in the scientific peer-reviewed literature, many open-access, with 9 papers already published, 6 more submitted and in review, and a further 15 papers in preparation. In addition, 28 abstracts representing 12 oral and 16 poster presentations from the various studies constituting Workpackage 4 were presented at International and national conferences held between 2014-1016. In addition, studies in WP4 have resulted in 1 PhD and 2 Masters Theses.

WP5:

Extrusion tests with different raw materials showed that extrusion technology is an efficient feed manufacturing process and plays an essential role in ensuring competitiveness of the European aquaculture industry. Advantages of extruded aquaculture feeds include enhanced feed efficiency, a

better control of pellet density and water stability, all aspects that contribute towards a reduction of feed wastes and a minimisation of the environmental impact of farming systems even if plant raw materials are used in the formulations.

An online web application that allows estimating the environmental impact based on growth performance measurements (initial body weight, final body weight, food conversion ratio) and basic information on feed composition, providing confidence intervals that reflect the amount of information provided, was developed. **Fish farmers, feed producers and researchers may now use this tool to better assess and compare feeds / feed formulation scenarios in terms of environmental impact.** Immediate impacts:

- Industry as a whole: Increase awareness and concern about estimation of waste loads and environmental impacts.
- Farm level: Estimations to assess compliance with regulatory frameworks addressing environmental impacts.
- Research and feed mill levels: Estimates of waste load assessments with different feed formulations, so to reduce scenarios to test in growth trials – fast track of innovation.

The combined experiments done in carp and sea bass showed that regarding faecal waste management, the faecal pellet stability is very high in general and specifically when using raw materials high in NSP content. The faecal recovery with settling was almost always superior to 70%. It was demonstrate that the major way to steer faecal waste management is to alter the nutrient digestibility in both species. The ingredient composition of the diets is a major issue. However, it was shown that inclusion of enzymes targeting the non-starch polysaccharide fraction can strongly reduce the total amount of faecal waste produced. Also, the application of enzyme to feeds showed to have strongly variable results: from no impact to a reduction of about 20% of DM faecal waste produced.

When compared to the fish meal and fish oil based diet or to the traditional grain feeding the plant based feed provided similar environmental effects in pond experiments with common carp. The few significant differences between diets in water and sediment samples were not consistent through the years. From environmental aspects, our long-term results support the viability of plant based feed ingredients in freshwater pond aquaculture.

<u>WP6:</u>

Potential impacts of the WP6 of the ARRAINA project are the following:

- Nutritional programming can help reduce some of the potential problems associated with using alternative (especially non-marine) raw materials in aquafeeds.
- The strategy of programming could be used not only with nutritional factors but also with abiotic factors (temperature, hypoxia etc.) in order to better adapt fish to aquaculture.
- The strategy of programming could be also done in order to improve fish in aquaculture for other targets than fish nutrition (fish health, fish performance).
- The interaction between programming and genetics must be performed in the future as a new tools in fish selection.
- **For an economical point of view, the possibilities of acting in fish through early stimuli could be very easily transferred into aquaculture farms without any strong investments**.
- From a societal point of view, this new strategy of programming has to be well explained to the public because the main idea is to do an early stimuli which permit fish to react but these stimuli must follow the ethical recommendations. It is clear that during the stimuli stage, fish must not suffer.

Regarding the dissemination, results about nutritional programming (WP6) in the ARRAINA project have been widely presented in national and international symposium as well as in papers accepted in international scientific journals. The professionals in aquaculture have been also largely informed of the nutritional programming concept and were really interested by the idea and the potential applications. A training in Las Palmas in 2016 was done with a specific course about nutritional programming (S Panserat).

WP7:

Outputs of the ARRAINA project were presented in booklets, congresses, publications, database, tools and the website. In the plenary sessions of the various scientific conferences, the research outputs, future research demands and views on the future of aquaculture in Europe was discussed among the scientific and industry participants. Hence, the ARRAINA sessions at the conferences were efficient to share, disseminate and discuss ARRAINA research results but also to create exchange with stakeholders from the aquaculture industry. These actions will also lead to the constructions of further collaborative research projects between industrials and partners of the ARRAINA project.

Targeted ARRAINA dissemination activities such as hosting nutrition sessions took place at annual conferences such as Aquaculture Europe. ARRAINA partners also presented results and shared knowledge through attendance at worldwide events which is expected to enlarge the impact of the research results of ARRAINA to other regions outside Europe. **A highlight was the special invitation to ARRAINA to attend the Blue Economy Business and Science Forum organised by the EC in Hamburg in September 2016. This event allowed ARRAINA to rise interest of private investors in aquaculture at large and therefore contributed to the continuous growth of this sector. ARRAINA also participated in dissemination activities such as involvement in the COMMBEBIZ 'Marine Testinar - Unlocking the blue potential" on 29 November 2016.**

News of the project was disseminated regularly, making use of a range of publications and services. Press releases were issued to appropriate media outlets (trade press, journals, web portals) to ensure that industry, civil society organisations, policy-making authorities, research community and the wider community are aware of the project, its objectives and its outcomes. The strategy was intended to ensure that there is publicity and media coverage at local, regional, European and even international levels. ARRAINA press releases were distributed through the following channels:

- AquaTT Training News newsletter (over 5,500 subscribers);
- Technology platforms (i.e. EATIP and EFTP);
- Aquaculture focused Media mailing lists, contacts and distribution lists (i.e. "World fish and Aquaculture Magazine", "Intrafish", "EATiP", "EAS "EAS-Student Group", "Fisheries and Aquaculture Europe", "Mispeces.com", "Sea food Source" etc,);
- General marine and science focused publications (i.e. "Science.ie", "Megapesca Fishfiles", "NERC Planet Earth Magazine", "World-science.net" etc.);
- LinkedIn groups.

Examples of press releases picked up by European and International organisations:

- http://www.aquafeed.com/technical-center/papers-abstracts-article/6990/Aquaculture-Nutrient-Requirements/
- http://www.bedsonnews.com/our-projects/arraina-sustainable-european-fish-nutrition/
- http://m.2lua.vn/article/new-publication-addresses-biomarkers-in-fish-nutrition-585746ede4951979408b456b.html?hl=en
- http://www.iffo.net/system/files/Update%20-%20July%202015%20-%20271.pdf (page 13)

Other partners and the Stakeholder Advisory Board were encouraged to publish articles and press releases at regional, national and international level, making use of their own communication networks and channels.

Furthermore the Proof of concept project 'ASSESSFEED' was successfully conducted and the Portuguese SME SPAROS has the intention to exploit the technology developed in-house.

1.5 Project public website

The website address is the following: http://www.arraina.eu/

1.6 Relevant contact details

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1.7 Project logo