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Effect of feeding gilthead seabream (Sparus aurata) with vegetable lipid sources on Cytokines gene expression: TNF α and IL-1β

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Trabajo realizado en el Instituto Canaria de Ciencias Marinas, España, bajo la dirección del Dr. Daniel Montero Vitores And Dra Silvia Hildebrandt

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I Introduction

I.1 Introduction

Nowadays, aquaculture provides about 43% of the food obtained from marine and continental waters, and the figure is on the rise especially with the increasing of the world population coupled with the growing per capita demand for fish (from 9 kg in 1961 to an estimated 16.5 kg in 2003)(FAO,2006). However, as in every form of intensive culture where single or multiple species are reared at high densities, infectious disease agents are easily transmitted between individuals, which makes aquaculture an important vector in the introduction, transfer and spread of aquatic pathogens and parasites (Acosta *et al.*, 2004).

Among several bacterial diseases that dangers the welfare of cultured fish, vibriosis and photobacteriosis are a disaster pioneers, and possess a wide distribution causing great economic losses in a variety of warm and cold water fish farm (Toranzo *et al.*, 2005). Gilthead seabream and seabass are very important economic species that are victims for such infection. Nevertheless, the appearance and development of a fish disease isn't only a result of the pathogen assault but is the consequence of simultaneous interaction among pathogen, host and environment (figure 1) nutrition and unbalanced dietary fish requirement enhance bacterial invasion and make difficult effective intervention.

In fact, Nutritional modulation of resistance to infectious diseases is based upon the following topics:

- ✓ The proper balance of macro- and micronutrients, including amino acids, polyunsaturated fatty acids (PUFA), vitamins and trace elements, which are essential for the development of immune system starting at the larval stage. Deficiencies in these nutrients may impact several development events including the proper development of lymphoid organs. Marginal deficiencies may negatively affect the immune system at later stages of life.

Severe deficiencies will increase susceptibility to disease and may result in the death of the animal.

- ✓ Adequate nutrition is essential for cells of the immune system to divide and synthesize effector molecules. The diet supplies the immune system with the amino acids, PUFA, enzyme co-factors and energy necessary to support lymphocyte proliferation and the synthesis of effector (e.g. immunoglobulins, lysozyme and complement) and communication molecules (e.g. cytokines and eicosanoids). The quantitative need for nutrients to maintain a normal immune function is relatively small compared to the requirements for growth and reproduction.
- ✓ It is important to consider that some nutrients provide essential substrates for the proliferation of pathogens (e.g. iron) and their presence at low concentrations in body fluids may limit the growth of pathogens within the fish.
- ✓ The fourth mechanism may include the indirect regulatory effects of diets on the immune system that are mediated through the endocrine system. The regulatory action of PUFA and other nutrients (vitamins A and E) on leucocytes has been demonstrated.

  Eicosanoids produced from PUFAs, especially arachidonic acid, are a major component of the humoral immune system.
- ✓ Finally, diet composition and physical characteristics of the diet may modify the micoroganisms in the gastrointestinal tract and the integrity of intestinal epithelium. The presence of oxidized lipids, plant anti-nutritional factors (e.g. lectins, protease inhibitors, and oligosaccarides) and fiber can affect the gut physiology along with the makeup and size of the gut microfloral population and thus aspects of the nonspecific immune response (Lall, 2000).

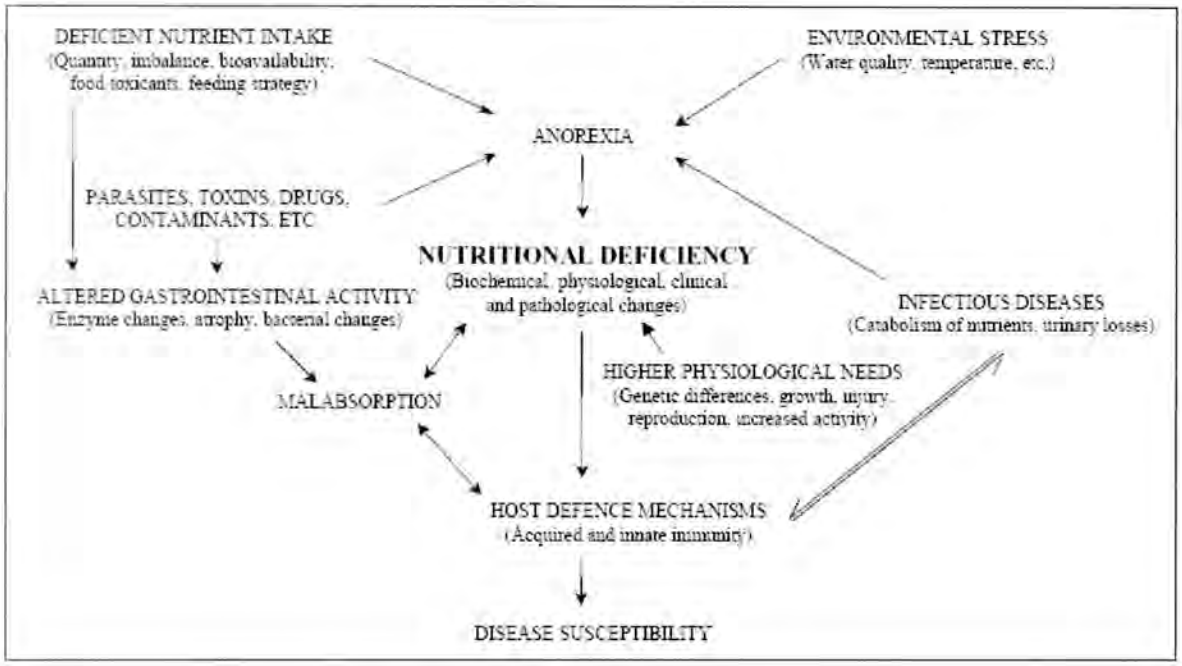


Figure 1: Factor influencing status health and immune functions of fish (Lall, 2000)

Among all nutrients, fatty acids and fats en general, have been widely took by investigation in the mammalian world to elucidate the tightly relationship between lipids and immune system. Few attempts are reported in the aquatic filed.

Fish tissues contain relatively higher concentrations of polyunsaturated fatty acids (PUFA) than found in mammals. PUFAs are important components of all cell membranes which makes fish tissue highly vulnerable to lipid peroxidation. Generally, essential fatty acids (EFA) requirements of freshwater fish can be met by the supply of 18:3n-3 and 18:2n-6 fatty acids in diets, whereas the EFA requirement of marine fish can only be met by supplying long-chain PUFA, 20:5n-3 and 22:6n-6 (NRC, 1993). Freshwater fish are able to elongate and desaturate 18:3n-3 to 22:6n-3, whereas marine fish, which lack or have a very low activity of Δ5-desaturase, require the long chain PUFA, eicosapentaenoic acid (20:5n-3; EPA), and docosahexaenoic acid (22:6n-3; DHA).

The quantitative requirements and deficiency signs of EFA in several freshwater and marine fish have been documented (NRC, 1993), however, the functional role of n-3 and n-6 PUFA in nonspecific and specific humoral and cellular immunity is not clearly defined (Bell *et al.* 1996; Secombes, 1996; Lall, 1998).
Such ambiguity makes more difficult assays of substitution of fish oil in the diet by vegetable oil. This behavior appears an urgent necessity nowadays to solve in part the leveling off of wild fish stocks.

In 2004, about 75% (105.6 million tons) of estimated world fish production was used for direct human consumption. The remaining 25% (34.8 million tons) was destined for non-food products, in particular the manufacture of fishmeal and oil.

Front of the continuous climb of fish food consumption, especially in affluent developed nations, the FAO's report estimates that an additional 40 million tons of aquatic food will be required by 2030, just to maintain current levels of consumption.

Aquaculture now provides 43% of the fish consumed by humans, compared to just 9% in 1980. The main question which arises is, if aquaculture sector will be able to make up the shortfall or not. Among several challenges, encounter solutions to fish meal and oil shortfall was a serious problem (FAO, 2006).

For the last decade, a lot of studies focused on such topic and proved that partial vegetable oil substitution for finfish might resolve such deficit en fish nutrition.

In fact, vegetable oil of many plant´s seeds were been demonstrated a good candidate to replace partially fish oil in the fish diet. They are rich in polyunsaturated fatty acids and particularly those which seem to be precursors to the synthesis of fish essential fatty acid.

Indeed, linseed oil is rich for 68% of polyunsaturated fatty acids of which 58% are linolenic acids (omega-3). Nevertheless, soybean oil as the major of other vegetable oil is rich much more of linoleic acid (until 50%) y poor for linolenic acid (8%).

Recent studies of Izquierdo *et al.*, 2005, reported the inability of marine fish to effectively synthesize essential fatty acid (eicosapentaenoic acid (EPA), decosahexanoenoic acid (DHA) and arachidonic acid (ARA)) from their precursors linolenic acid (18:3n-3, LNA) and linoleic acid (18:2n-6, LA).

Such handicap, permit a limited threshold for vegetable oil inclusion in marine fish diet, at the best manner that not alter strict fish requirement for n-3 HUFA necessary for its normal growth and healthy statue.

Many studies carried out by Montero *et al*, 2003, on the gilthead seabream proved that low levels of n-3 HUFA compared to the normal fish requirement (1.6% for seabream) harm to the fish health and its normal growth. In food regimes shortened in n-3 HUFA, studies showed an apparent decrease in fish growth (Grasso, 2006; Izquierdo et al., 2003; Montero *et al.*, 2003) and a high storage of saturated fatty acid in the liver. Such alteration touches also the fillet quality that seems to be tightly dependant to the fatty acid profile of the diet. In fact, Izquierdo *et al.*, 2005, reported that vegetable oil in the diet reduces ARA and DHA in the fillet lower than their reduction in the diet.

As for fish health, unbalanced n-3 HUFA in fish diet may alter fish metabolism and affect their resistance front of environmental aggression.

An inadequate n-3 HUFA dietary supply, may reduce antibody production demonstrated for rainbow trout (Kiron *et al.*, 1995), depleted the alternative complement pathway activity in the gilthead seabream (Montero *et al.*, 1998; 2003;

2008) and alter eicosanoids production which may act as inmunomodulators and affect fish resistance (Ashton *et al.*, 1994).

Tremendous reviews mentioned the importance effect of unbalanced ratio omega-3/ omega-6 in the diet on the physical proprieties of the membrane phospholipids (permeability and fluidity) which could alter macrophage functions.

Nowadays, all interest was directed to study the effect of vegetable substitution on the physiologic alteration aspect in the fish, scarce are experiments which deal with genetic side and explain how such inclusion modify the expression of genes related with immune system.

I.2 Overview of the fish immune system

Significant difference exists between mammal and fish immune system including absence of marrow bone and lymphoid nodes in fish. The kidney, spleen and thymus are the principal lymphomyeloid tissues of teleosts. Liver, skin and intestine play important role in defense system.

Evolutional gnathostomes fish immune system can be classified into specific adaptive immunity and non specific innate immunity. Both allocate cellular and humoral mechanisms to defend organism against potential colonization by virus, bacteria, fungi, protists and metazoan parasites and to repair tissue damage.

In evolutionary term, acquired immunity comes to help innate system getting more specificity and effectiveness through pathogen memorization since the first pathogen exposure. Immunoglobulins are the famous effectors molecules used in such reaction that production depends from many factors such as type and duration of antigen stimulation, stress, age and water temperature (Tatner, 1996 *in* Ganga, 2004).

Innate immunity provides the first line of host defense, it includes many complex phenomena and involves both fixed and mobile cells and a large a number of dissolved molecules in the body fluid; it also provides essentials signals to ensure proper responses by the organism's adaptive immune system (Wen-Chao *et al.*, 2000). The non specific defense does not alter on repeated case of infection (Sirgun *et al.*,2001).

I.2.1 Nonspecific immune system

Broadly speaking, the innate immune system consists of multiple effectors including physical barriers, such as the skin and mucosal surface of respiratory and digestive system and low pH levels in the stomach.

The non specific humoral defense actes to get rid and/or limits the spread and growth of pathogen. It includes proteases, lysins and agglutinins (aggregate cells) as first line defense (Secombes, 1996). The second line is attributed to the mucosal lining cells. Blood cells especially granulocytes and monocytes clean the circulatory system from microbes and destroy them and occupy the third defense line. Finally, endocytically active cells such as macrophages and granulocytes in distinct type of organ and tissue may destroy and eliminate microbe and microbe products by the process of phagocytosis (Dalmo *et al.*, 1997).

Monocytes, macrophages, neutrophils, eisonopjils and thrombocytes all appear to be phagocytic in fish, but neutrophils seems to be the most active due to their high mobility (Secombes *et al.*, 1992). Unfortunately, such cells are little encountered in comparison with macrophage and monocytes (Figure 2).

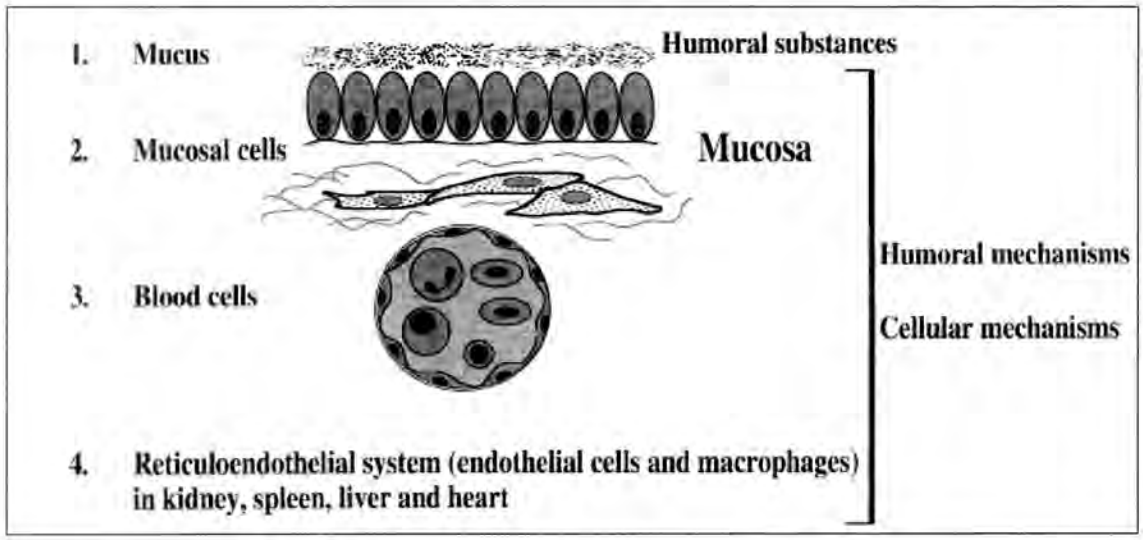


Figure 2: Simplified overview of non specific line defense (Damol *et al.*, 1997)

Phagocytosis is initiated with a passive adhesion to the pathogen o the strange particle, followed by the ingestion and ended by the digestion which can involve both oxygen dependent and oxygen independent processes (Secombes, 1996). Any defect or alteration in any phase may be condemning all immune response (Grasso, 2006).

Potentiation of non specific defense may occur during microbial invasion, injury and trauma through inflammatory response. Microbial product alters cells function (macrophages, monocytes) inducing an enhancement of antimicrobial secretion and a good cleanness. Such physiologic change is called acute phase response (APR) (Kushner, 1982). The APR is induced by plasma- borne signals called pro-inflammatory cytokines (Bayne *et al.*, 2001) which initiate and regulate this process.

I.2.1.1 Cytokines

Cytokines are simple polypeptides or glycoproteins released from macrophages or monocytes during acute inflammation to facilitate cells immune interaction and

modulate their secretion (Secombes, 1996). Their constitutive production is usually low and may acts as autocrine (signals leading to changes in the same cell that secreted the respective signal) paracrine (changes occurred in distinct cell closed to the releasing cell signal) or endocrine signaling through the blood stream. Cytokines effects can often be synergistic but antagonist case exists with scarcity (Vilcek *et al.*, 1994).

A significant number of cytokines are described for teleosts but little data are available compared to mammals. They are classified en families such as Interleukin family, Tumor necrosis factor family ect. (Tort *et al.*, 2003).

I.2.1.1.1 Tumor Necrosis Factor α (TNFα)

TNFα is a member of the β jellyroll family of cytokines. It augments responsiveness of fish head kidney cells and activates macrophages with macrophage-activating factor (MAF) (Secombes *et al.*, 2001). It is well defined as an important mediator in resistance against parasitic, bacterial and viral infections (Goldfeld *et al.*, 1996). Some of its proprieties observed in vitro are growth promoting activity of fibroblaste and capacity to induce bone resorption or the secretion of other cytokines in cascade such as IL-1β then IL-6 and others that serve as chemoattractants (Piguet *et al.*, 1992; Secombes *et al.*, 2001;). Furthermore, new researches demonstrate its fundamental neuroimmunoendocrine role and proved TNF neurotoxicity as well as its neuroprotective proprieties (Barbers and Nottet, 2006). Sleep regulation (Krueger *et al.*, 1998), embryonic development (Wride and Sanders, 1995 *in* Castillo *et al.*, 2002) and lipid metabolism are other features of its multifunction mentioned.

TNFα exerts many of these effects by binding, as a trimer to M_r 55.000 cell membrane receptor termed TNFR-1 or to a M_r 75.000 cell membrane receptor termed TNF-2. Both receptors are present in great numbers on most cells

(Loetscher *et al*, 1991). However, soluble form also exits having possibly distinct physiologic role (Watts *et al.*, 1997).

TNFα gene has been cloned in different fish species. The first two non mammalians TNFα gene sequences were reported from Japanese Flounder *Paralychthys olivaceus* (Hirono *et al.* 2000; Bobe and Goetz, 2001) and rainbow trout Oncorhynchus mykiss. (Laing *et al.* 2001). In 2002, Castillo *et al.* isolated successfully the TNFα from the marine fish gilthead seabream (*Sparus aurata*).

I.2.1.1.2 Interleukin-1β

Interleukin-1 β is a member of the IL-1β cytokine family having a β-trefoil structure (Hughes, 1994 *in* Pelegrin, 2001). It is one of the earliest pro-inflammatory cytokine expressed enabling organisms to respond promptly to infection. It plays a pivotal role in the maturation and proliferation of many immune cell types (T and B cells) (Dinarello, 1997; Apte et al., 2006). It is mainly produced by macrophages and monocytes (Bird *et al.*, 2002b). IL-1β is a pleiotropic molecule (influences multiple phenotypic traits) and shows a wide range of biologic activities. As other cytokines, IL-1 β is involved also in embryonic development, implantation, birth and neonatal development of mammals (Dinarello, 1997).

IL-1 β is synthesized as a 31 KDa inactive precursor (pro-IL-1 β) and stored in the cytoplasm. IL-1 β converting enzyme (ICE) gives it its active form of 17 KDa through cleaving Asp-X bonds (X designates a small hydrophobic residue) (Dinarello, 1997). Under this form IL-1 β is called mature IL-1 β and is released out of the cell cytoplasm to seek respective cell surface binding protein (Figure 3). There are two types interleukin receptor: the first is IL.1RI that transduces signal whereas the second (IL-1RII) binds IL-1 but does not transduces signal.

The active IL-1β mediates its effects through a complex containing IL-1β bound to the respective receptor IL-1RI; all are bound to the receptor associated protein (IL-

1RAcP), which leads to the activation of the nuclear factor kappaB (NF-$_K$B) and the mitogen-activated protein Kinase (MAPK) cascades (p38, JNK and ERK) to initiate expression of a series of genes in the nucleus (Auron, 1998; Dinarello, 1997) (Figure 4). For instance, IL-1β releasing causes up or down regulation of other cytokines and chemokines such as IL-8 important in recruiting leukocytes to site of inflammation (Laing and secombes, 2004). These later are tightly regulated by the cytokine IL-10 (Mege *et al.*, 2006).

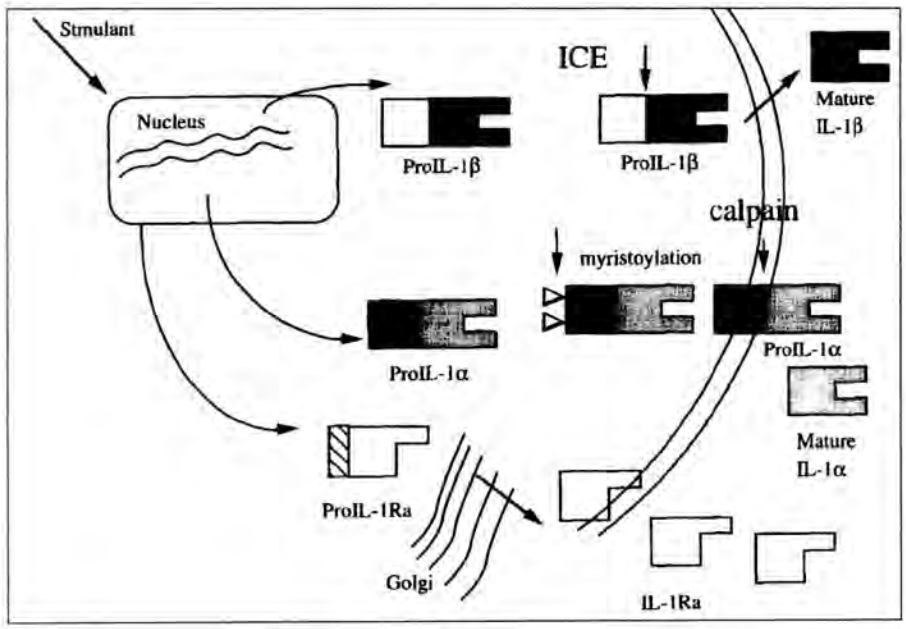


Figure 3: Schema for synthesis and secretion of IL-1β IL-1α and IL-1R (Dinarello, 1997).

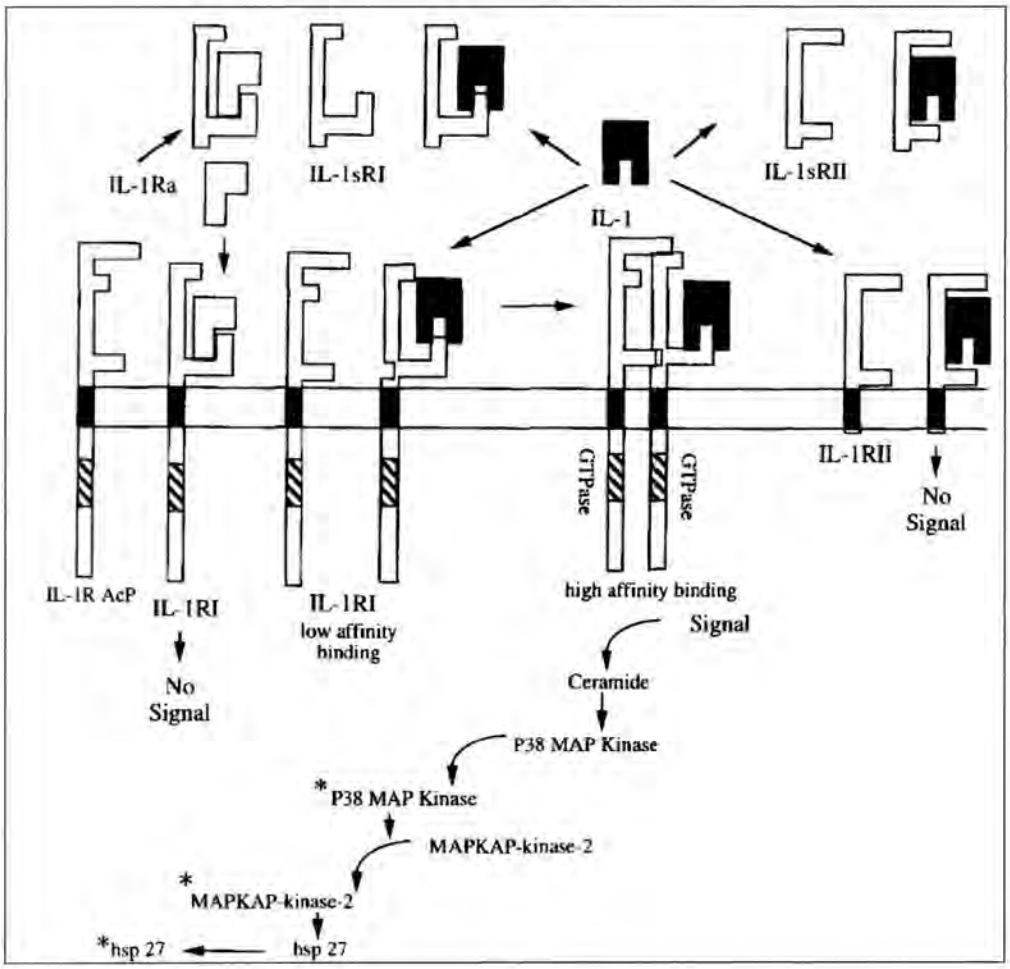


Figure 4: Different IL-1β binding possibilities and cascade effects releasing (Dinarello, 1997).

Recently, many studies have proved the existence of functional homologues IL-1β within a large number of vertebrates and sharing the fundamental characteristics of mammalian IL-1family. Homology cloning led to discoveries of IL-1 β of many teleosts species (Lu *et al.*, 2008).For instance, rainbow trout (Zou et al., 1999a), sea bass (Scapigliati *et al.*, 2001; Buonocore *et al.*, 2004), gilthead sea bream (Pelegrin *et al.*, 2001), Atlantic cod (Seppola *et al.*, 2008) and orange spotted-grouper (Lu *et al.*, 2008). All studies in vitro as well as in vivo showed involvement of IL-1 β in viral and bacterial infection and the up regulation of gene expression.

Even, Buonocore *et al.*, 2004 demonstrated that recombinant IL-1 β could have immunoadjuvant effect in sea bass vaccination experiment.

I.3 Nutrition and Cytokines

The relationship between non-specific immunity system in fish and diet has been reviewed immensely during lasts decades. Nevertheless, scarce are the studies dealing with nutrition effect on cytokines and their related gene expression. An early interest on this topic emanated in researches dealing with human health and tremendous are reviews which treat effect of n-3 PUFA in the profile of interleukins and tumor necrosis factor on mammals' subject of experiment. To overcome these gaps of information, following the eicosanoids pathways seem to be the unique solution. Since cytokine production is regulated by eicosanoids and since dietary lipids affect eicosanoid production, it might be expected that dietary lipids, especially those containing *n*-3 PUFAs, will affect cytokine production.

I.3.1 Precursors and pathways of eicosanoid synthesis.

Eicosanoids are a family of oxygenated derivatives of dihomo-y-linolenic and arachidonic acids and EPA. Eicosanoids include prostaglandins (PG) and thromboxanes (TX), which together are termed prostanoids, and leukotrienes (LT), lipoxins(LX), hydroperoxyeicosatetraenoicacids (HPETE) and hydroxyeicosatetraenoic acids (HETE).

In most conditions the principal precursor for these compounds is arachidonic acid and the eicosanoids produced from arachidonic acid appear to have more potent biological functions than those released from dihomo-y-linolenic acid or EPA (Calder *et al.*, 1996).

The precursor PUFA is released from membrane phosphatidylcholine (PC) by the action of phospholipase A_2, or from membrane phosphatidylinositol-4,5-

bisphosphate(PIP_2) by the actions of phospholipase C and a diacylglycerol (DAG) lipase.

The pathways of eicosanoid synthesis begin with cyclooxygenase, which yields the PG and TX, or with the *5-*, 12- or 15-lipoxygenases, which yield the LT, HPETE, HETE and LX (Figure 5). A third pathway which operates through the microsomal cytochrome P-450 results in formation of epoxides which are converted to HETE.

The amounts and types of eicosanoids synthesized are determined by the availability of arachidonic acid, by the activities of phospholipase A_2, and phospholipase C and by the activities of cyclooxygenase and the lipoxygenases (Kinsella *et al.*, 1990).

The major biologically-active products of the cyclooxygenase pathway are PGA_2 PGE_2 PGI_2, (prostacyclin), PGF_2 and TXA_2 although these are produced in a cell specific manner (calder *et al.*, 1996). These compounds usually have a short half-life and act locally to the cell from which they are produced (Rowley *et al.*, 1995).

Their production is initiated by particular stimuli such as cytokines, growth factors, endotoxin, zymosan, oxygen free radicals, antigen-antibody complexes, bradykinin, collagen, thrombin...; and, once produced; they themselves are able to modify the response to the stimulus. Different prostanoids have different, sometimes opposite, effects; for example, TXA, increases platelet aggregation whereas PGI, inhibits platelet aggregation.

The *n-3* PUFA, EPA and DHA, competitively inhibit the oxygenation of arachidonic acid by cyclooxygenase. In addition, EPA (but not DHA) is able to act as a substrate for both cyclooxygenase and 5-lipoxygenase ( ). Ingestion of FO will result in a decrease in membrane arachidonic acid levels and a concomitant decrease in the capacity to synthesize eicosanoids from arachidonic acid, EPA gives rise to the 3-series PG and TX and the 5-series LT ( ). The eicosanoids

produced from EPA do not always have the same biological properties as the analogues produced from arachidonic acid.

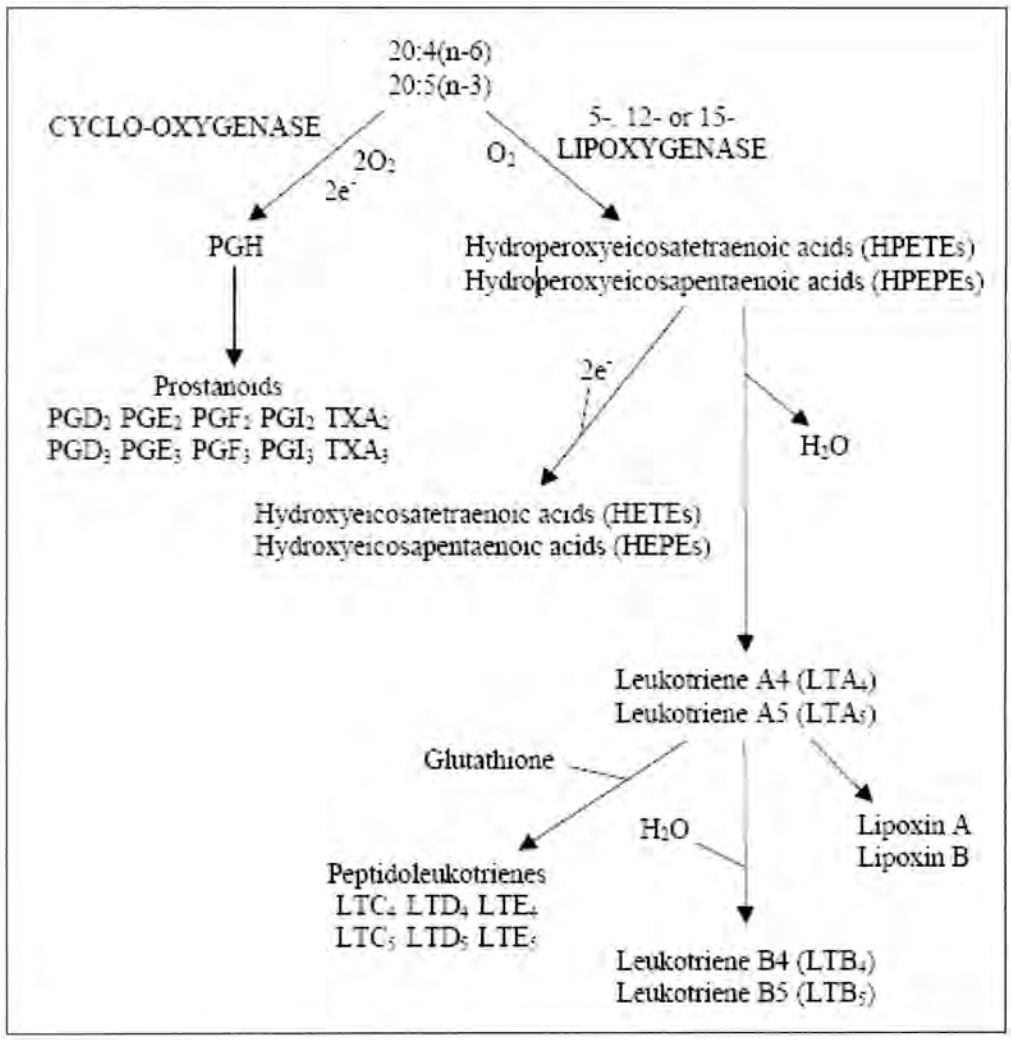


Figure 5: Synthesis of eicosanoids from arachidonic and eicosapentaenoic acids (Iall, 2000)

I.3.1.1 Eicosanoids and immune system

Eicosanoids are important intercellular signaling agents. They modulate secratory, smooth muscle and cascade type reaction which are essential to normal health (Kinsella *et al.*, 1990).

Several studies proved that macrophages are the principal immunocompetent cell among all other types of immune cell that synthesize eicosanoids (Hwang, 1989; Blazer, 1992), although granulocytes, monocytes and thrombocytes also contribute significantly (Rowley *et al.*, 1995). This statement not denied the probable participation of lymphocytes in such production (Rowley *et al.*, 1995; Calder, 1996).

Not only are immune cells a source of eicosanoids, but they are subject to their regulatory effects; the most-well-documented effects are those of PGE_2. *In vivo*, PG are involved in modulating the intensity and duration of inflammatory and immune responses and acted differently if it is about a chronic or an acute case.

PGs appear to play a role in regulating the differentiation of both T and B lymphocytes; for example, PGE_2, induces immature thymocytes to differentiate into mature T-cells. In addition, the functions of T-cells, B-cells, NK cells and macrophages are modulated by eicosanoids.

T-lymphocytes have receptors for PGE_1, and PGE_2, and these compounds suppress T-lymphocyte proliferation, T-cell-mediated cytotoxicity, IL-2 production and NK cell activity in *vitro*.

PG inhibit production of IL-1 and TNF by macrophages (Kunkel *et al.* 1982) LTB, and LTC, enhance IL-I production by macrophages (Kunkel *et al.* 1982) and LTB, enhances IFN-δ production by lymphocytes (Rola-Pleszczynski *et al.* 1983).

As it is shown, a same eicosanoid could play antagonist effect on the same target cell. Kinsella *et al.*, 1990 reported that effect and activity of eicosanoids depends on their relative concentration, for example PGE_2 at low concentration ($<10^{-9}$) activate same immune cell response whereas high concentration ($> 10^{-8}$) suppress it.

I.3.2 Modulator effect of dietary fatty acid on eicosanoids and cytokine profile.

Since cytokine production is regulated by eicosanoids and since dietary lipids affect eicosanoid production, it might be expected that dietary lipids, especially those containing *n*-3 PUFAs, will affect cytokine production. The effects of *n*-3 PUFAs on cytokine production have been reviewed several times in recent years, but before dealing with this topic a review about effect of n-3 PUFA on eicosanoids appears fundamental to a good understanding.

I.3.2.1 Modulation of eicosansoid synthesis by n-3 polyunsaturated fatty acids.

In vitro experiment carried on the culture of macrophages or lymphocytes with n-3 PUFA results in replacement of arachidonic acid in phospholipids by the n-3 PUFA provided (Calder *et al.* 199Oc). As a result of this modification, less arachidonic acid derived eicosanoids are produced by these cells.

Tremendous studies dealing with the same purpose but with in vivo design reported that dietary lipid modulation also results in significant modification of the fatty acid composition of macrophages isolated from several tissues and multiple rodent species (Surette *et al.* 1995), even human subjects, (Endres et *al.* 1989).

In the same way, Bell *et al.*, (1993) demonstrated that Atlantic salmon fed a diet with high contain of linolenic acid leads to the suppression of the amount of arachidonic acid-derived eicosanoids which is mirrored by an elevation in the level of EPA-derived eicosanoids (Chapkin *et al.* 1990). Thus, the decreased levels of ARA-derived PGE2 and TXB2 result in the increase of anti-inflammatory reactions.

James *et al.*, 2000 proved that the n-3 fatty acids such as EPA, a precursor of DHA, reduce the synthesis of antibodies and proinflammatory cytokines, and suppress inflammatory responses by reducing membrane AA and eicosanoid synthesis. Recently, considerable evidence says that several unsaturated fatty

acids including ALA, EPA and DHA act by blocking Ca entry into the cells causing a change in the concentration intracellular of free Ca. Such modification alter signaling pathway which follows the stimulation of lymphocytes, macrophages by cytokines and antigens (Chow *et al.*, 1990).

I.3.2.2 Effects of dietary n-3 polyunsaturated fatty acids on cytokine production

I.3.2.2.1 Macrophage-derived cytokines

A number of studies have reported that feeding rodents *n-3* PUFA-containing oils results in enhanced production of TNF, although there are reports of decreased production or no effect following fish oil feeding. A recent study showed that dietary fish oil increases IL-6 production by rat peritoneal macrophages (Tappia and Grimble, 1994).

However, there are contradictory reports. The most likely reason for the variations in experimental observations is the differing protocols used: studies have differed with respect to species of origin of the cells studied (mouse, rat, pig), the anatomical site of origin of the cells (liver, lung, peritoneal cavity), the state of activation of the cell (resident, inflammatory, activated), the stimulus used to elicit cytokine production (LPS, another cytokine), the nature of the culture conditions used(presence or absence of serum, serum source, duration of culture etc.), the level of oil added in the diet, the duration of feeding and the method used to quantify cytokine concentrations.

In a recent study in which all experimental procedures were standardized, it was found that feeding weanling mice on a 200 g FO/kg diet for 8 weeks resulted in diminished ability of thioglycollate-elicited peritoneal macrophages to produce TNF-a and IL-6 in response to LPS; IL-1α production was also decreased by FO feeding, but not significantly (Yaqoob & Calder, 1995).

In agreement with some of the animal experiments, Endres *et al.* (1989, 1993), Meydani *et al.*,(1991, 1993) and others have found that supplementation of the human diet with *n-3* PUFA results in a significantly diminished ability of peripheral blood monocytes to produce TNF, IL- 1α, IL-1β and IL-6 *in vivo*.

I.3.2.2.2 Lymphocyte-derived cytokines

In contrast to the large number of studies of the effects of FO feeding on the *ex vivo* production of macrophage-derived cytokines, there have been few studies on lymphocyte-derived cytokines. Three studies have reported that supplementation of the diet of healthy human volunteers with *n-3* PUFA significantly lowers IL-2 production by PBL (Meydani, 1992). Gallai *et al.* (1993) also reported diminished of IFN-δ production.

One animal study has reported diminished *in vivo* production of IL-2 (by pig alveolar lymphocytes) following LO and FO feeding.

It is now well documented that dietary fatty acid has profound effects on gene expression, leading to changes in metabolism, growth, and cell differentiation. The effects of dietary fat on gene expression reflect an adaptive response to changes in the quantity and type of fat ingested (Jump *et al.* 1995; Jump, 1999). In human studies, Plat *et al.*, (2002) have found that plant stanol ester consumption increased LDL receptor mRNA concentrations in mononuclear blood cells.

Several studies carried on the Atlantic salmon put in evidence the effect up regulating of Δ6 and Δ5 desaturase gene expression involved in hepatic fatty acid metabolism, after dietary PUFA administration (Zheng et *al.*, 2004, 2005; Jordal *et al.*, 2005; Leaver *et al.*, 2006). Seiliez et *al.* in (2003) identified successfully the Δ6 gene sequence in gilthead seabream and proved that this later is highly expressed in fish fed HUFA free diet and slightly expressed in fish fed on HUFA rich diet.

Little is known about effect of vegetable oil inclusion on the expression of genes related with immune system. However, a recent study showed that inclusion of Fish oil in the diet of autoimmune-disease-prone mice results in elevated levels of mRNA for IL-2, IL-4 (Fernandes *et al.* 1994) (Figure 6). The same workers showed that dietary FO completely abolished mRNA production for IL-IB, IL-6 and TNFα in the kidneys of these animals (Chandrasekar & Fernandes, 1994). Robinson *et al.* (1995) reported that feeding mice on a FO-rich diet significantly diminished in vivo 1L- B mRNA production and basal TNFα by LPS- or PMA-stimulated spleen lymphocytes. Sijben et al., (2003), proved that dietary oil (5% inclusion of FO, CO, LO and beef tallow) did not affect the spleen expression level of IL-6, IL-8, IL-18 and IFN-g mRNA obtained from chicken challenged with *salmonella typhimurium* lipopolysaccharide.

These studies suggest that n-3 PUFA might affect immune cell functioning by control at the transcriptional level.

Until now, the unique study dealing with the possible modulator effect of dietary vegetable oil in immune gene expression is advanced by Montero *et al.*, 2008. They reported that seabream fed PUFA rich diet manifested an elevated basal constitutive level of Mx transcript hepatic expression after a challenge with *photobacterium damselae* subsp *piscicida*.

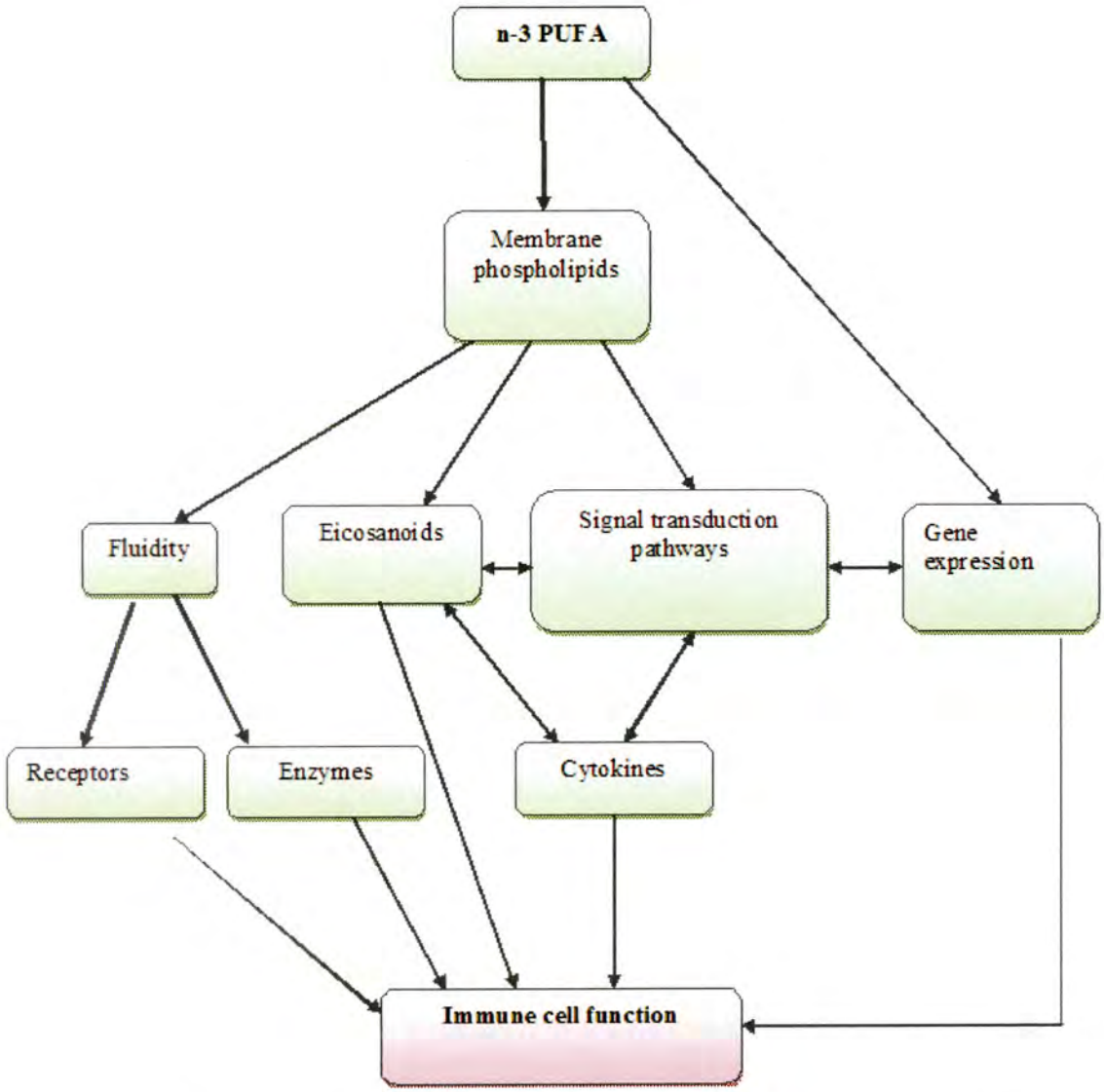


Figure 6: Mechanisms by which n-3 polyunsaturated fatty acids (PUFA) could exert immunomodulatory effect (Calder, 1996)

I.4 Objectives

- The aim of the present study was to investigate the effect of different levels substitution of fish oil with vegetable oils (rich in LA and LNA) on cytokines gene expression.
- To achieve this purpose following objectives were addressed:
- To study the vegetable oil inclusion effect on the basal level expression of TNFα and IL-1β.
- To study the pattern expression of these target gene during post bacterial infection.
- To assess the effect of vegetable oil substitution in diet on gene expression during post infection.

II Materials and Methods

II.1 First Experiment

II.1.1 Maintenance of animals and experimental design

Two thousand juvenile gilthead sea bream (*Sparus aurata*) of Atlantic origin were obtained from a Spanish local farm (ADSA, San Bartolomé de Tirajana, Canary Islands, Spain). Upon arrival to the experimental facilities of the Canary institute of Marine Sciences (ICCM), fish were initially weighted; having an average weight of 35 g. Fish were maintained in four polyethylene circular tanks of 1000 l × 2 and 500 l × 2 and fed to visual satiety with a control feed for 2 weeks.

After the acclimation stage, fish were first weighted and sampled have an average weight between 28 and 47 g and then located randomly and equally into polyethylene circular tanks of 500 l. Experimental design was applied for 50 fish in each tank in quadruplets for each experimental diet. Thus 24 tanks in total were established for the experiment.

Experimental tanks were located into an isolated area far away from other installations and each one was supplied with a hood structure in order to minimize any disturbance or interference source with others experiments. Tanks were supplied with well aerated running seawater (30–40 l/min). Water temperature and dissolved oxygen during experimental period ranged between 21.4–22.8 ºC and 5–6.5 ppm, respectively.

Fish were fed under natural photoperiod (approximately 12:12 l/d) at 1.7% of the initial biomass. The experimental diets were hand fed until apparent satiety three times a day at 8:30, 11:30 and 14:30, six days per week for two months and a half.

All individual fish in each tank were weighted per month and the feed intake was determined daily. Conversion index (CI) and specific growth rate (SGR) was calculated according to the following formulae:

- *CI= Feed intake/weight gain*
- *SGR=[(ln Final weight−ln Initial weight) /t]*100*

with t: experimental period (days)

Six iso−energetic and is iso−nitrogenous diets with a lipid content of about 22% were formulated by Proaqua farm (Spain). Anchovy oil was the only added lipid source in diet FO (fish oil). All other diets contained vegetable oils. 100 % of the anchovy oil was substituted either by soybean oil (SO) in diet 100 SO or linseed oil (LO) in diet 100 LO. In the other diets, fish oil was substituded by a mix of linseed and soybean oil. Percentages can be seen in (Table I).

Table I: Type and percentage of oils included in the experimental diets

	FO	100S	100L	70S	70L	50S:50L
Fish oil	100	0	0	30	30	0
Linseed oil	0	0	100	0	70	50
Soybean oil	0	100	0	70	0	50

A strong hygienic behavior was taken during the experimental process to avoid any type of bacterial contamination or a favorable environment for the

enhancement of one stage of the biological cycle of parasites or bacteria. So, every day tank walls were rigorously cleaned and one day per week, the entire tank was washed.

To ensure a good water renovation per night, water volume per tank was lowered by 100 m^3 at the last feeding.

II.2 Second Experiment

II.2.1 Infection Experiment

II.2.1.1 Bacterial assay

In this experiment, the bacterial strain 94/99 *Photobacterium damselae subsp. Piscicida,* was provided by the Infectious Diseases laboratory of the Instituto Universitario de Sanidad Animal y Seguridad Alimentaria (IUSA) (Canary Islands) from diseased gilthead sea bream.

The lyophilized 94/99 stock was processed by the laboratory personal in order to get the bacteria with its original virulence and ready to be used with a concentration of 1.2 10^9 cfu ml^{-1}.

II.2.1.2 Infection procedure

After 10 weeks of feeding trial, 120 fish per diet were randomly selected and transported to a pathogen room. Fish that underwent the soybean treatment were transferred first and in the following order: the first day fish of the 50 SO: 50 LO diet, the second day those of the 70 SO diets and finally those of the 100 SO diet. The following week, as tanks started to be released, fish fed linseed diet were passed into; firstly, the control diet (100 F), then the 100 LO and finally the 70 LO.

In the pathogen room, transported fish were put into a square well oxygenated polyethylene tank of 500 l. Three groups of 24 fish from each dietary treatment were inoculated via anal canalization with *Photobacterium damselae* ($1.2\ 10^9$ cfu ml^{-1} per fish). Another three groups of 24 fish per treatment were inoculated via anal canalization with 1 ml of sterile saline solution; these later were useful like control (figure 7).

Figure 7: Inoculation of *Sparus aurata* via anal canalization

Each group of manipulated fish was allocated in its correspondent cylindrical 500 l fiberglass tank, equipped with a good aeration (Figure 8).

During the trial water temperature ranged from 22 to 23 ºC and the artificial light photoperiod was adjusted to 12L: 12D. Fish were fed manually with their respective experimental diet twice a day.

Figure 8: Labeled tanks in the pathogen room

After four hours and one, three, five and seven days post inoculation, four fish were randomly selected and sacrificed in a bath of high concentrated anesthetic solution. These fish were randomly ordered in a plate and covered with ice. At the same moment fish were transferred to the pathogen room, 12 individuals of each diet were sacrificed as control samples of 0 hours.

At this stage, the spot was yielded to another person who was outside the pathogen room to guaranty more safety and security to the following process and not allowing pathogen propagation.

In another laboratory, a second team was working online to get the needed information about of each fish for every sample range (weight, blood sample and respective tissues of interest) in order to be able to define the gene expression profile after a challenge with a pathogenic strain of *Phobacterium damselae*.

II.2.1.3 Sample collection

II.2.1.3.1 Preparation of tissue sample

All samples were well labeled. Each Eppendorf tube was provided with a colored label corresponding to each diet, the date, the fish number, the tank number and its respective group (control or infected).

To avoid any way of contamination, the dissection material was autoclaved every day and always disinfected after each use with a Diethyl piro - carbonate (DEPC) solution. Even the working surface was disinfected daily with a hypochlorite solution (Figure 9).

Figure 9: Essential Sampling material

Tissue samples of proximal and distal intestine were collected from each fish. Head kidney, spleen and liver were also collected to assess the effect of contamination on systemic immune tissues. Each sample was first put on a numerated aluminium support, then well washed with a DPEC solution and finally a piece of 50 to 100 mg was put into its corresponding tube.

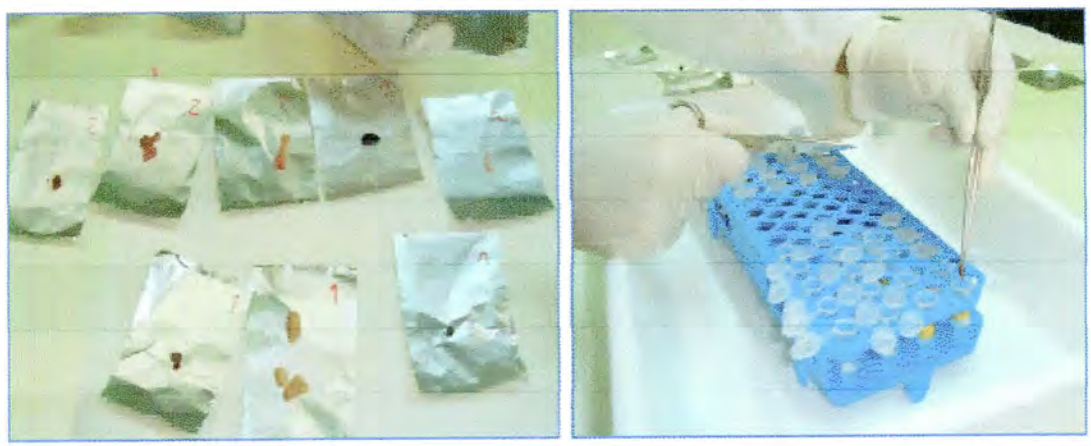


Figure 10: Sampling technique

Tissue was conserved into RNA Later (SIGMA), a product that rapidly allows tissue to stabilize and protects cellular RNA in situ in unfrozen samples and permits us to postpone RNA isolation for a more suitable moment even after months. So each Eppendorf tube was filled with 300 to 500 µl of the tissue storage reagent to ensure that the fresh tissue was submerged into 5 volumes of RNA Later (Figure 10).

Samples were stored overnight at 4 °C. The next day the RNA Later was removed from the tubes before being stored at - 80 °C to prevent the formation of salt crystals.

In some case, when RNA Later was not available, samples were rapidly frozen in liquid nitrogen for a short moment before being stored at – 80 °C.

II.2.2 Gentic Study

II.2.2.1RNA isolation and cDNA synthesis

II.2.2.2RNA extraction

RNA isolation from sample tissue was performed using TRI Reagent (SIGMA-Aldrich registered trademark of Ambion, the RNA Company, United State) according to the manufacturer's instructions. TRI reagent is a mixture of guanidine thiocyanate, a strong choatropic protein denaturant agent, and phenol. There are combined in a monophasic acid solution to inhibit rapidly RNAase activity and causing the lysis of the sample. (Chomczynski *et all.*, 1987). TRI-reagent on its own is incapable to disrupt cells and isolate RNA, therefore a physical disruption is needed. This step is the most crucial one and determines the yield and the quality of RNA. This process of cell disruption must be fast as possible to avoid RNA degradation by endogenous RNAse released internally, yet still inaccessible to the protein denaturant solution. There should be no visible particulates after homogenization and at the same time avoiding disruption of the macromolecule [1]. For this purpose, we have tested tow different mechanical techniques of cell disruption: homogenization through pellet pestels (Biosigma) and through a sonicator "Ultraschall-homogenisator Labsonic M"(Figure12)

In the same working conditions, the same tissue and same denaturant solution we have obtained the following results resumed in the following table (table II).

Table II: Comparison results between tow cell disruption techniques

Characteristics	Pestel homogenization	Sonicator homogenization
Initial Sample weight (mg)	27.5	26.4
Time processing(min)	10	2
RNA concentration (ug/ul)	0.365	0.56
A 260/280	1.72	1.71
Sample contamination risk	--	++
Tool cleaning	autoclaved pestel	double cleaning of the probe with ethanol 75% and DPEC 0.1% solution

Obviously, except the sample contamination risk, all data showed the advantage of the sonication technique versus the traditional one. However, the quality of the checked RNA disproved this statement. The following ethitium bromide staining of RNA separated in denaturing agarose gel visualized differently the two predominant bands of small and large ribosomal RNA in distinct sample (Figure 11). It appeared clearly that samples homogenized with sonicator loosed its integrity. RNA quality is an exigent condition that determines the success of all following steps of real time PCR.

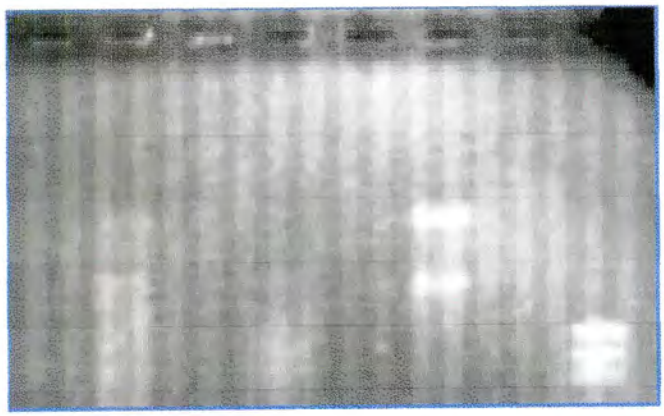


Figure 11: Denaturing agarose gel 1% showing integrity RNA difference between samples processed with pestel and those with sonicator ultrawaves. (P): with pestle. (S): sonication.

Figure 12: Homogenization process with pestel motor

The homogenization process was followed by a cold centrifugation to separate the homogenate into an aqueous and an organic phase (Fig 13). At this step, alcohol substances such as chloroform or bromochloropropane (BCP) helped by the centrifuge movement purify the aqueous phase into a RNA partition above the DNA film which appears as a white interphase separating all from the protein substrate. Then, the adding of isopropanol ensures a RNA precipitation which will be finally rinsed with ethanol.

Figure 13: Centrifugation conditions of RNA samples

RNA pellet is finally solubilized in a sterile water solution at a convenient volume. The following diagram describes in detail the extraction protocol: (Figure 14).

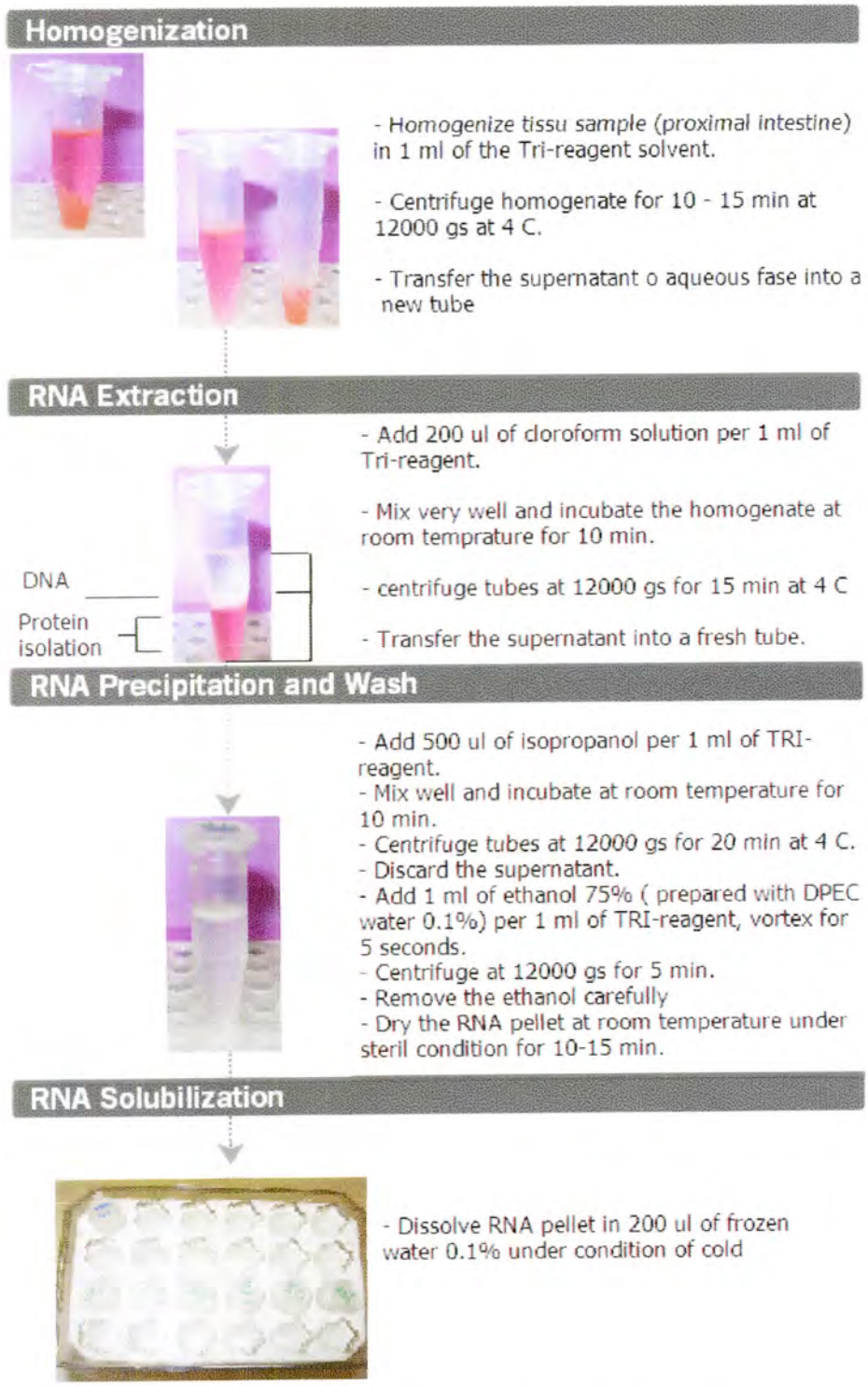


Figure 14: Extraction Protocol

Since it was already programmed to evaluate the genetic expression in terms of an average per tank instead of individual values for each fish per tank, each four samples per tank were mixed with the aim of having a final concentration of 0.1 ug/ul (Figure 15).

For this reason, 25 mg of each sample were weighted and then homogenized first in 50 ul and then in a total volume of 250 ul of TRI reagent.

During the whole treatment samples were kept on ice.

Figure 15: Pool sample preparation

II.2.2.3Quantification of the amount and the RNA quality

RNA concentration was estimated spectrophotometrically measuring the absorbance at 260 nm by means of an Eppendorf BIOPHOTOMETER spectrophotometer. Also the degree of purity of the RNA was considered by the ratio between the absorbance values at 260 and 280 nm and the quality of the same by means of an agarose denaturing gel. In the particular case of our spectrophotometry analysis, the method was the following:

98 ul of water treated with 0.1% DEPC was used as a blank to standardize the reading. Then 2 ul of the RNA sample was added and mixed well with the pipette. Concentration value was read at least five times.

The read values of absorbance should be between 0.05 and 2 to reflect with precision the RNA concentration, in the opposite case, if the value of 2 is exceeded the sample should be diluted (Sambrook *et al.*, 1989).

Concerning the RNA purity, the ratio A 260/ A 280 should have an inferior limit of 1.6 and superior limit of 2.

The RNA integrity was analyzed through the visualization of RNA ribosomic subunits in an agarose gel.

II.2.2.4 Electrophoresis RNAs

RNA is a delicate macromolecule susceptible to rapid degradation so a special care needs to be taken to avoid this risk through the following protocol.

- The electrophoresis bucket was well washed with Ultra pure water and immediately it went through sterile water treated with DEPC 0.1%.
- A fresh 0.5x TBE buffer was prepared for the electrophoresis bucket.
- Native agarose gel of 1.4% (weight/volume) was prepared with the same buffer used in the electrophoresis bucket.
- Samples to load were prepared with 5 µl of sample and 1 µl of 6X loading buffer (Sambroock *et al.*, 1989).
- Each 6 µl of sample was loaded into its correspondent well of the gel.
- The gel was ran at 80 V for 2 hours or until the tracking dye migrated about 80% of the distance to the end of the gel.

- Gel was dyed with a 0.5 µg/ml ethidium bromide solution for 15 min in a dark room and afterwards washed in a bucket with distilled water for 20 min.
- The gel was visualized under Ultra Violet illumination (UVP™) and photographed with the gel documentation sortware Imagestore 5000 (UVP™) (Figure 8).

Ribosomal RNA 28S

Ribosomal RNA 18S

Figure 16: Total RNA of intestine sample

II.2.2.5 cDNA synthesis

cDNA was synthesized using the iScript cDNA Synthesis Kit (BIO RAD). Manufacturer's instructions recommend using 1 µl of RNA (1µg) in 20 µl reaction containing 4 µl 5x iSrcipt Reaction Mix, 1 µl Script Reverse Transcriptase (BIORAD) and 14 µl Nuclease-free water. Complete reaction mix was incubated in the thermal cycler (iCycler) (Figure 17).

Reverse transcription was initiated at 25 °C for 5 min, followed by 60 min at 42 °C. The reaction was terminated by heating the samples for 5 min at 85 °C.

Figure 17: iScript cDNA Synthesis Kit

II.2.2.6PCR conditions

Quantitative PCR was performed using primers for the constitutively expressed b-actin as a positive control and subsequently used for sample normalization, and primers for cytokine genes of interest: Interleukin-1B and Tumor necrosis Factor.

Primer sequences of target genes and expected sizes of amplicons are shown in Table 1. They were designed by Generunner software on the basis of sequence deposited in Genebank respectively published for *Sparus aurata*. All primers were designed to anneal to the highly conserved region of the gene sequence respecting following guidelines:

✓ For amplicon:

- Amplicon design would be between 75-200 bp. It should be at least 75 bp to be easily distinguished from any primer-dimers that might form.
- Avoiding secondary structure that could be formed at annealing temperature.
- Maintaining a GC content of 50 to 60 %

✓ For primer:

- Design primers should content at least 50-60% GC.
- Maintaining a melting temperature (Tm) between 50 C and 65 C.

- Avoiding secondary structure.
- Avoiding repeats of Gs or Cs longer than three bases.
- Placing Gs and Cs on ends of primers.
- Checking sequence of forward and reverse primers to ensure no 3¨complementarity.

- Avoiding primer-dimer formation. [2]

First step to create a new dialog box under Gen runner menu, in order to find a pair of primer for target gene sequence, was to stick the oligonucleotide sequence from the bloc note archive into the respective window opened from file, new, nucleic acid sequence (Figure 18).

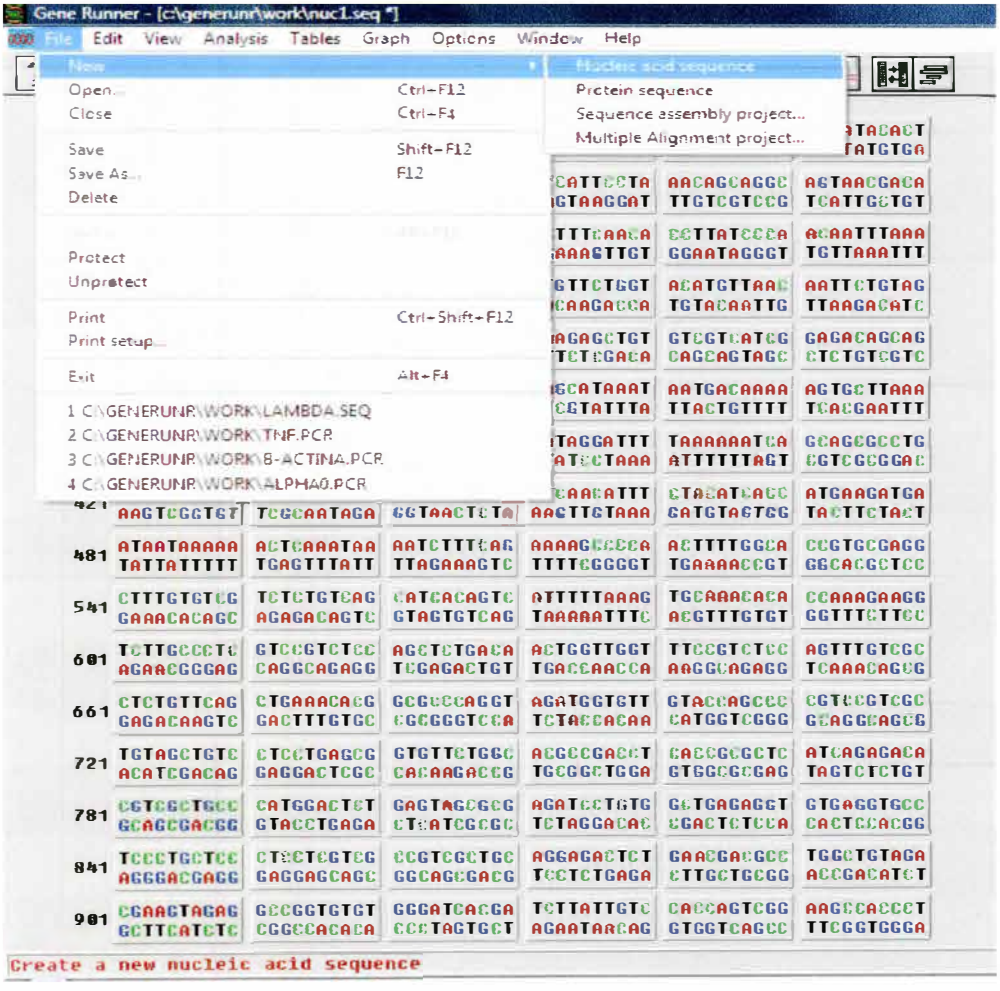


Figure 18: Creation of new nucleic acid sequence

Then, through the command Analysis, PCR analysis dialog box enabled us to specify the primer requirement and maximize its qualities (Figure 19).

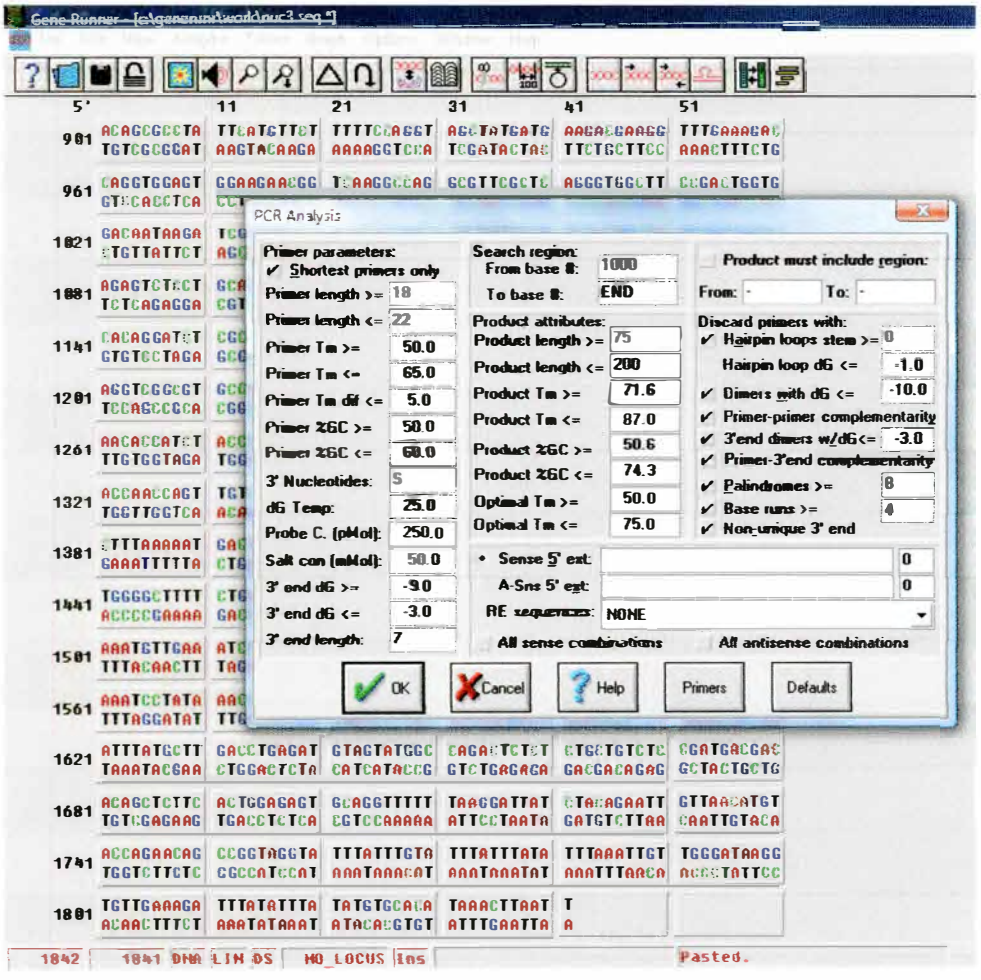


Figure 19: Primer specification design

As results of analysis execute, hundreds of choices were generated. The oligo function under analysis choice enabled us to control characteristics and secondary structure that could be formed for each oligonucleotide (primer) selected (Figure 20).

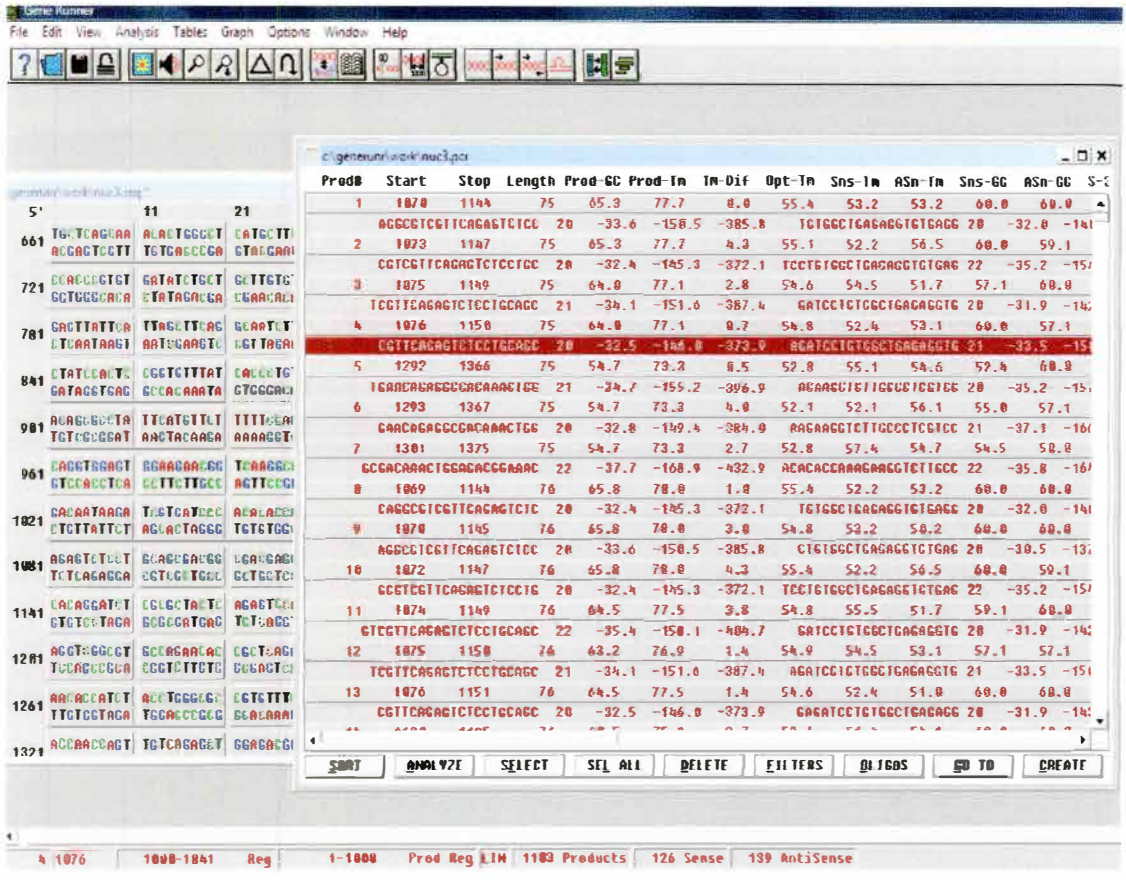


Figure 20: Variety of primers choice

At this stage, a hard work was allocated to detect the best pair of primer that respect at maximun anterior desribed guidelines.

After primer selection, a demand with product description according to manufacturer´s instructions was sent to TIB MOLBIOL Syntheselabor Gmbh (Berlin, Germany) in order to be designed.

Products reached us under lyophilized form and were diluted with respective volume of TE (1X) to be at a concentration of 100 pmol/ µl each.

TE product was new prepared to avoid any contamination risk of the primer stock. TE (1X) is a mix of 10 ml of TRIS (1M, ph=8) and 2 ml of EDTA (0.5M, ph=8).

Table III: Primers sequences used in PCR and real time PCR

Gen		Primer Sequence	P. length (bp)
B-actin	Forward	TCTGTCTGGATCGGAGGCTC	113
	Reverse	AAGCATTTGCGGTGGACG	
IL-1B	Forward	AGCGACATGGCACGATTTC	140
	Reverse	GCACTCTCCTGGCACATATCC	
TNF	Forward	CTCACACCTCTCAGCCACAG	195
	Reverse	TTCCGTCTCCAGTTTGTCG	

The PCR was performed in 25 µl reactions using the adjusted amount of template (1, 2, 5 µl). Reactions contained 2.5 µl forward and reverse primer (10 pmol/ µl), 2.5 µl NH4 buffer (10X), 0.5 µl dNTP mix (10 mM each), 1.5 µl MgCl2 (50 mM), 0.2 µl BIOTAQ (20U/µl) and adjusted water ULTRA PURE.

To find the optimal annealing temperature for each gene, a gradient of annealing temperature above and below the calculated Tm, was tested to enable us avoiding non specific products formation.

The polymerase chain reaction was performed using a specific amplification program established for each gene and described in the following tables (tables IV, V, VI):

Table IV: PCR condition for β-actin gen

	Step	Temperature	Time
Cycle 1 (1X)	1	94 °C	5:00
Cycle 2 (30X)	1	94 °C	00:15
	2	54.8-58.5 °C	00:30
	3	72 °C	00:05
Cycle 3 (1X)	1	72 °C	00: 10
Cycle 4 (1X)		4 °C	∞

Table V: PCR condition for IL-1β gen

	Step	Temperature	Time
Cycle 1 (1X)	1	95 °C	5:00
Cycle 2 (40X)	1	95 °C	00:15
	2	50-60 °C	00:30
	3	72 °C	00:30
Cycle 3 (1X)	1	72 °C	1:00
Cycle 4 (1X)		4 °C	∞

Table VI: PCR condition for TNF gen

	Step	Temperature	Time
Cycle 1 (1X)	1	95 °C	5:00
Cycle 2 (40X)	1	95 °C	00:15
	2	50-60 °C	00:30
	3	72 °C	00:05
Cycle 3 (1X)	1	72 °C	00:10
Cycle 4 (1X)		4 °C	∞

II.2.2.6.1 PCR control and quantification of amplified product

PCR product was checked with the common method of agarose gel. An aliquot of PCR samples was ran on agarose gel 2% and stained with ethidium bromide.

Gel was submerged with TBE 0.5X in the electrophoresis bucket and put under an electric tension of 80 V for 100 min.

Aliquot samples were prepared mixing 3 µl of samples with 2 µl of ultra pure water and 1 µl of loading buffer 6X. The φ 174 marker concentrated (0,4µg/µl) and 100 bp marker (0.2 µg/µl) were loaded next to samples to be able assessing product amount (table VII).

Table VII: Different bands φ 174 marker and its respective concentrations

Bands	Size	Concentration for 150 ng of φ 174
1ª	1353	37.68
2ª	1078	30.02
3ª	872	24.28
4ª	603	16.79
5ª	310	8.63
6ª	281	7.82
7ª	271	7.54
8ª	234	6.51
9ª	194	5.4
10ª	118	3.28
11ª	72	2

A second marker (100bp PCR Molecular Ruler) was used to ensure band size. In stained gel, three reference bands appear clearly: 3000, 1000 and 500. In total were 30 bands in exact incrementing by 100pb from each other.

II.2.2.6.2 Sequencing of amplified products

PRC products were been sequenced to ensure the correct amplification of the target genes following the BIG DYE V 3.0 sequencing protocol.

For a PCR product of 100-200 bp (5-10 ng), a mix product was prepared containing 0.5 µl of primer (10 pmol/ µl), 0.5 µl of SOLUTION 1, 1.5 µl of SOLUTION 2 and the adjusted volume sample for each cDNA respecting manufacturer instruction concentration. Finally adequate water volume was added to reach a final volume reaction of 5 µl (table VIII).

Table VIII: Respective volume reactive for sequencing reaction

cDNA	MIX(S1+S2) Vol (µl)	Sample vol (µl)	Primer Vol (µl)	H2OmqVol (µl)
IL-1B	2	1	0.5	1.5
TNF	2	0.5	0.5	2
B-actin	2	0.5	0.5	2

Once all was ready, samples were incubated in the thermal cycler programmed as follow (table IV).

Table IX: Program thermal cycler for sequencing reaction

	Step	Temperature	Time
Cycle 1 (1X)	1	94 °C	3:00
Cycle 2 (25X)	1	96 °C	00:10
	2	50 °C	00:05
	3	60 °C	04:00
Cycle 4 (1X)	1	4 °C	∞

After that, each 5 µl of product was precipitated into 14.83 µl absolute ethanol, 4.4 µl H2O ULTRA PURE and 0.75 µl sodium acetate (3M, pH 5). All reaction was well mixed and was incubated at room temperature for 15 min, then was centrifuged at maximum speed for 25 min. The supernatant was discarded and tubes were washed by 62.5 µl of ethanol 70% each one. After a new centrifugation of 10 min, ethanol was eliminated and samples were dried at room temperature for 15 min.

At this stage, samples were sent to genetic laboratory in Medicine University to be sequenced.

II.2.2.6.3 Real time quantitative PCR analysis

II.2.2.6.3.1 General design of qRT-PCR

Quantitative PCR assays for studied genes were performed using IQ^{TM}5 Real-Time PCR System (BIO-RAD). Multiple assays were been done to ensure suitable sample concentration and developing convenient range of sample dilution in order to be able defining the PCR efficiency for each primer set.

In this study, samples were been diluted 1/10 and standard dilution aliquot was performed in duplicate using serial dilutions from a pool of all cDNA samples. The gradient used was as follow:

1:1 1:2 1:4 1:8 1:16 1:32 1:64

Every designed plaque was performed with special care to avoid sample contamination and pipetting error (fig 21.). Control reactions without template were incubated in duplicate for each primer combination in order to control dimer product formation. Furthermore, a same sample chosen arbitrarily was performed in duplicate and separately in each plaque in order to be able unifying the threshold value (C_t) between all plaques even elaborated in distinct days and for different target genes.

For this purpose, this elected sample was the 50 D1C1 with the specific IL-1β primer and the chosen (C_t) was established at 22.83, so for all following assays the baseline was adjusted manually at this value.

Eventually, 6 plaques en total were designed twice per gene. Firsts were elaborated for control and infected samples of 1 day and 7days post infection. Seconds were samples of 0 hour and 3 days.

The software program permitted us to describe the repartition of samples in wells and specify the nature for each (sample, standard or positive control) through the various geometric shapes. Moreover it allocated for each plaque its specific spreadsheet that describes specific parameters of each template (Name, localization, concentration, C_t value) (fig 22, 23).

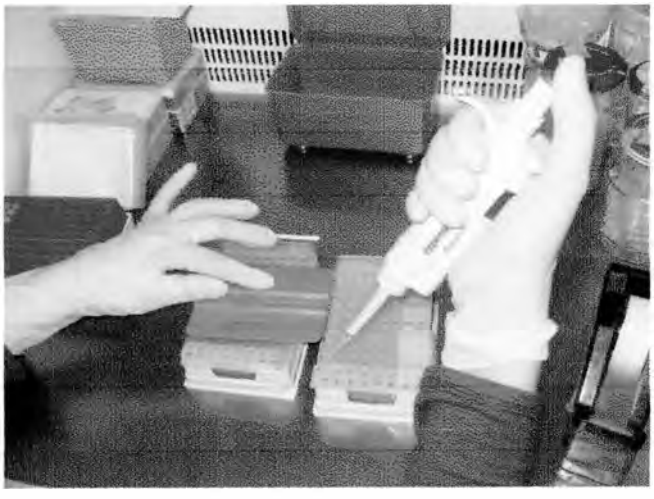


Figure 21: Preparation of PCR plaque

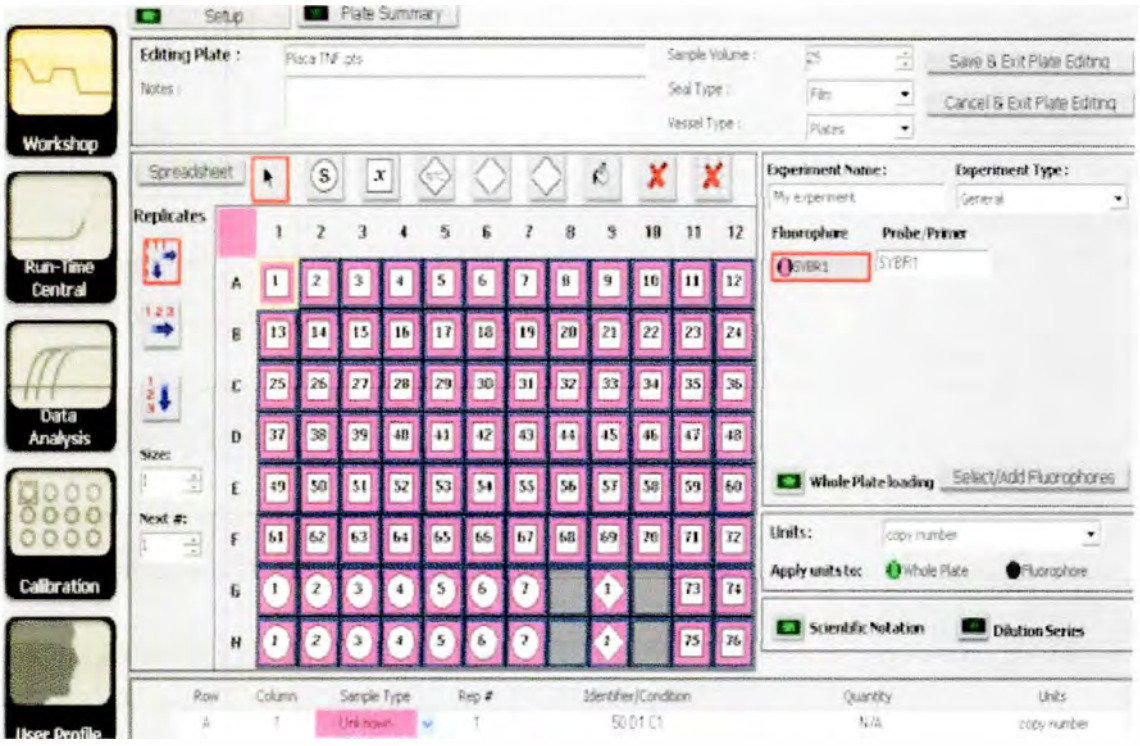
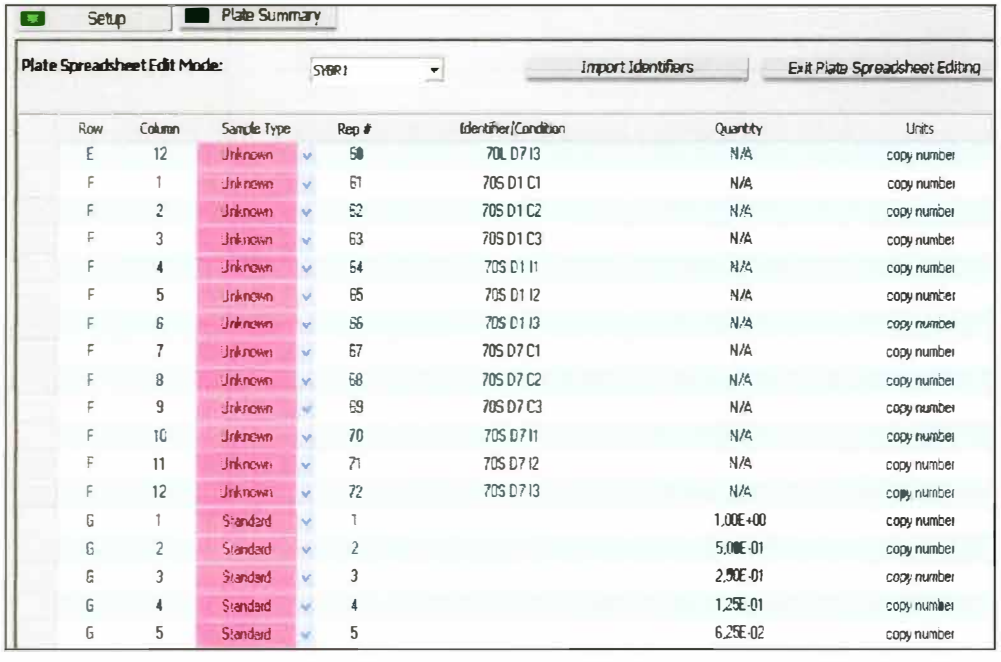


Figure 22: Plaque design for TNF qRT-PCR



Row	Column	Sample Type	Rep #	Identifier/Condition	Quantity	Units
E	12	Unknown	60	70L D7 I3	N/A	copy number
F	1	Unknown	61	70S D1 C1	N/A	copy number
F	2	Unknown	62	70S D1 C2	N/A	copy number
F	3	Unknown	63	70S D1 C3	N/A	copy number
F	4	Unknown	64	70S D1 I1	N/A	copy number
F	5	Unknown	65	70S D1 I2	N/A	copy number
F	6	Unknown	66	70S D1 I3	N/A	copy number
F	7	Unknown	67	70S D7 C1	N/A	copy number
F	8	Unknown	68	70S D7 C2	N/A	copy number
F	9	Unknown	69	70S D7 C3	N/A	copy number
F	10	Unknown	70	70S D7 I1	N/A	copy number
F	11	Unknown	71	70S D7 I2	N/A	copy number
F	12	Unknown	72	70S D7 I3	N/A	copy number
G	1	Standard	1		1.00E+00	copy number
G	2	Standard	2		5.00E-01	copy number
G	3	Standard	3		2.50E-01	copy number
G	4	Standard	4		1.25E-01	copy number
G	5	Standard	5		6.25E-02	copy number

Figure 23: Spreadsheet design of TNF plaque

II.2.2.6.3.2 qRT-PRC reaction

All PCR reaction were performed using 10 µl Brillant SYBR Green QPCR Master Mix (BIO-RAD), 1 µl of cDNA, 1 µl of forward and Reverse primer (4 pmol/ µl), filled up with ultra pure water to final volume of 25 µl. To guaranty the same volume of reactive for each sample, a mix of all components except cDNA sample was prepared and 24 µl of it were put into its respective wall with the means of automatic pipette. Finally, each 1 µl of respective cDNA was added with a normal pipette to avoid as possible pipetting error.

After choosing adequate protocol and respective plaque selection, the thermal cycler was put to heat for ten minutes before incubating plaque sample.

II.2.2.6.3.3 Cycling conditions

Common PCR was been a fundamental step to define some parameters in order to economize time and reactives when manipulating with qRT-PCR, however doing again temperature gradient for each gene was essential to limit exactly the melting point for each one and discarding possibilities of primer dimer products.

- ✓ Interleukin protocol

The cycling conditions were: 95ºC for 5 min, followed by 40 cycles consisting of 95ºC for 15 s, 55.6ºC for 30 s, and 72ºC for 30 s. Amplification program was followed by three cycles more starting again with one cycle at 95 ºC for 1 min, another at 70 ºC for 1 min, followed by 81 cycles at 55 ºC for 10 s. Last program allowed us to corroborate the specificity for PCR product (Figure 24).

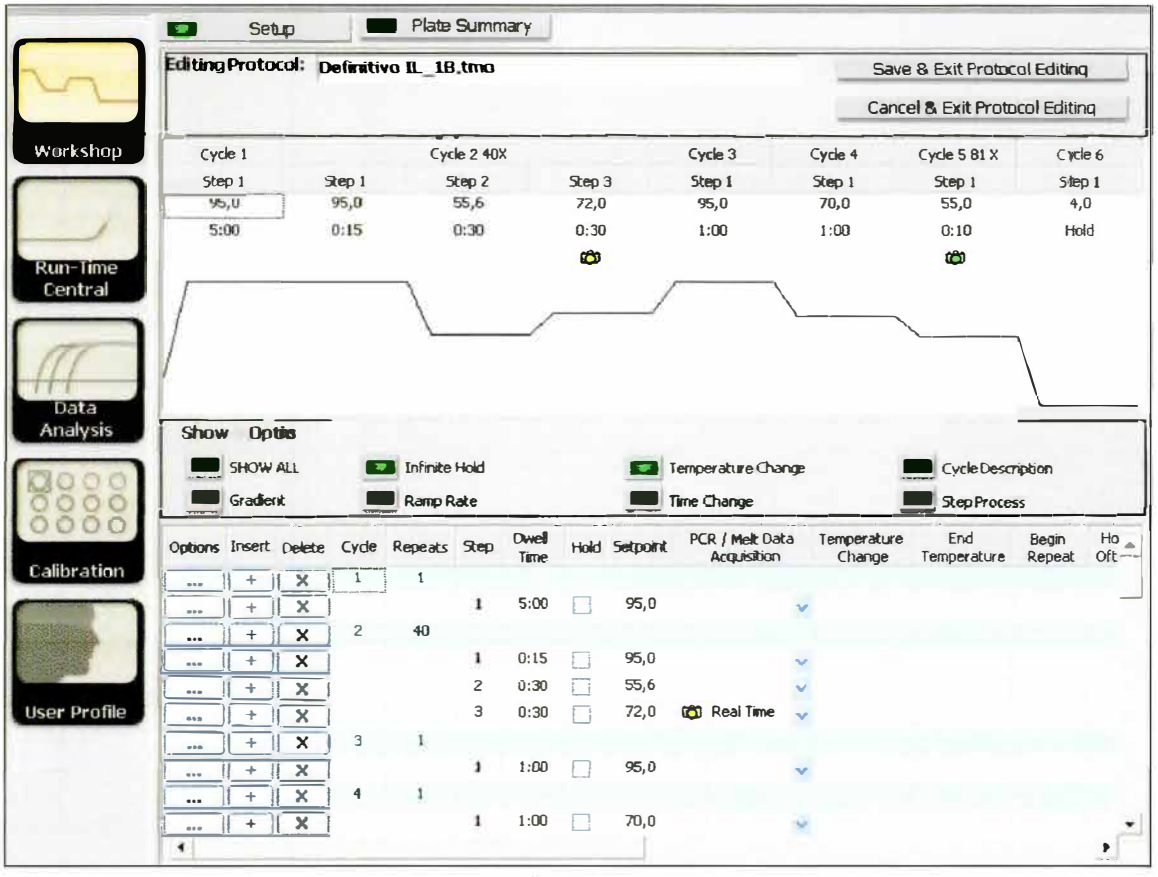


Figure 24: Cycling program of Interleukin gene

- ✓ Tumor necrosis Factor

Templates were incubated 1 min at 94ºC, following by 39 cycles consisting of 15 s at 94ºC, then 30 s at 62.4 ºC, finally 12 s at 72ºC. As usual, amplification cycles were ended by three other to test no specific product formation. They were been established like that would be described for anterior gene (Figure 25).

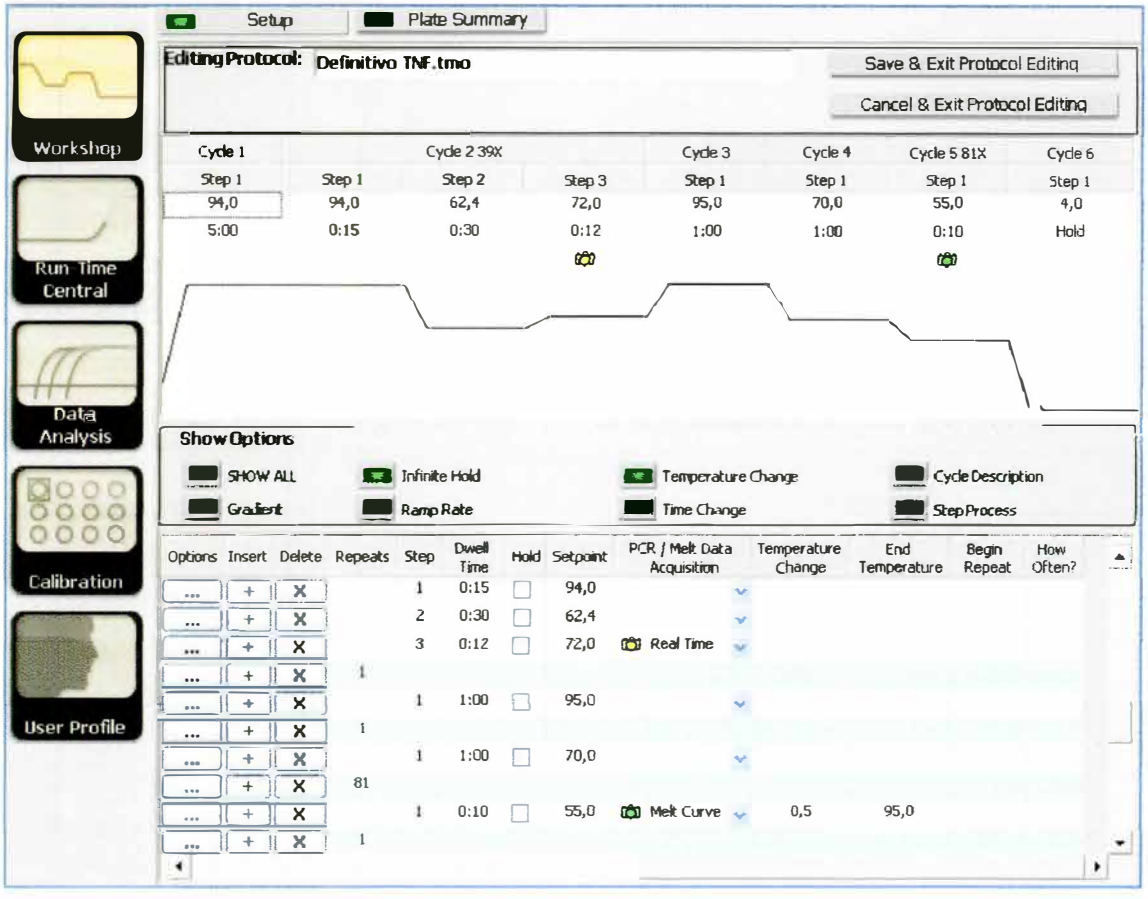


Figure 25: Cycling program of Tumor Necrosis Factor gene

✓ B-actin

Templates were incubated using the same Tumor Necrosis factor cycling program except the number cycle for amplification step which was performed just for 31 cycles.

II.2.2.6.3.4 Expression gene analysis

The comparative CT method (2-ΔΔCT) was used to determine the expression level of analyzed gene. (Livak and Schmittgen, 2001). This method is commonly used

and assumes that both target and reference genes are amplified with efficiency near 100% and with 5% of each other.

First step consist on normalization of the C_T of the target gene (IL_1B or TNFα) to that of the reference gene (B-actin), for both the test sample (Infected) and the calibrator (control) sample:

$$\Delta C_{T\,(test)} = C_{T\,(target,\,test)} - C_{T\,(reference,\,test)}$$

$$\Delta C_{T\,(calibrator)} = C_{T\,(target,\,calibrator)} - C_{T\,(reference,\,calibrator)}$$

Second step consist on normalizing the ΔC_T of the test sample to the ΔC_T of the calibrator:

$$\Delta\Delta C_T = \Delta C_{T\,(test)} - \Delta C_{T\,(calibrator)}$$

Finally, calculating the ratio $2^{-\Delta\Delta CT}$ gives us the normalized expression ratio that translates the folds increase or decrease of target gene in the test sample (infected) compared to the calibrator (control).

II.2.2.7 Statistical analysis

Data were compared using general linear model univariate (ANOVA) followed by Duncan multiple comparison test. Analyses were performed using SPSS software (SPSS for windows 11.0).

III Results

III.1 First experiment

III.1.1 Growth

All experimental diets were very appreciated by seabream. Individual weight of seabream fish of each diet are presented in the Annex I. All analysis were been done with mean values.

The statistical analysis (One Way ANOVA) not proved significant difference between different experimental diets during the first feeding month. All growth parameters were similar during this phase. Same statements are deduced for the second feeding month. Although a slight difference persists between the 100 soybean oil diet and this of 70% inclusion of soybean oil (Duncan test). This effect reveals significant in the relative growth parameter ($p<0.05$) between the same diets mentioned above. Concerning the conversion index, the feed intake, specific growth rate and weight gain, all seems to be similar between diets except the fish fed partial and total soybean oil. Such difference appears clearly when comparing individual final body weight between different diets (Table XII).

Except final mean weight, all calculated parameters seem to be in decrease. It s clearly apparent, that after 70 days of feeding, all experimental fish were been able to double its weight. All parameters values are recapitulated in the table (X, XI) and illustrated in the following (figures 26).

Table X: relative growth rate between initial and final weight during experimental period

Diet	100F	100L	100S	70L	70S	50S50L
%Growth_T	2,123	1,947	2,00	2,149	2,190	2,076

Table XI: Conversion index (CI), Specific Growth Rate (SGR) and relative growth of seabream during 70 days of fattening

		100F		100L		100S		70L		70S		50S50L	
1st month	CI	1,03	± 0,03	1,077	± 0,04	1,057	± 0,04	1,019	± 0,05	1,050	± 0,08	1,037	± 0,05
	SGR	1,86	± 0,09	1,75	± 0,13	1,74	± 0,07	1,86	± 0,11	1,82	± 0,15	1,82	± 0,13
	% Growth	0,92	± 0,06	0,85	± 0,08	0,84	± 0,04	0,92	± 0,07	0,90	± 0,10	0,89	± 0,08

		100F		100L		100S		70L		70S		50S50L	
2sd month	CI	1,32	±0,07	1,34	± 0,02	1,41	± 0,05	1,32	± 0,07	1,3	± 0,03	1,32	± 0,04
	SGR	1,290	± 0,09	1,260	± 0,06	1,190	± 0,05	1,270	± 0,08	1,330	± 0,05	1,250	± 0,03
	% Growth	0,59	± 0,05	0,58	± 0,03	0,56	± 0,03	0,61	± 0,04	0,66	± 0,03	0,61	± 0,02

Table XII: Initial and final mean weight of seabream during 70 days of fattening experimental diets

DIET	Initial_Mean Weight	Final_Mean Weight
100F	36,31±1,25	113,44± 6,04 (ab)
100L	35,96± 0,62	106,01± 6,14 (a)
100S	36,99± 0,20	111,01± 4,88 (b)
70L	36,25± 1,23	114,18± 7,60 (ab)
70S	36,53± 1,31	116,56± 6,68 (c)
50S50L	36,13± 1,24	111,17± 7,33 (b)

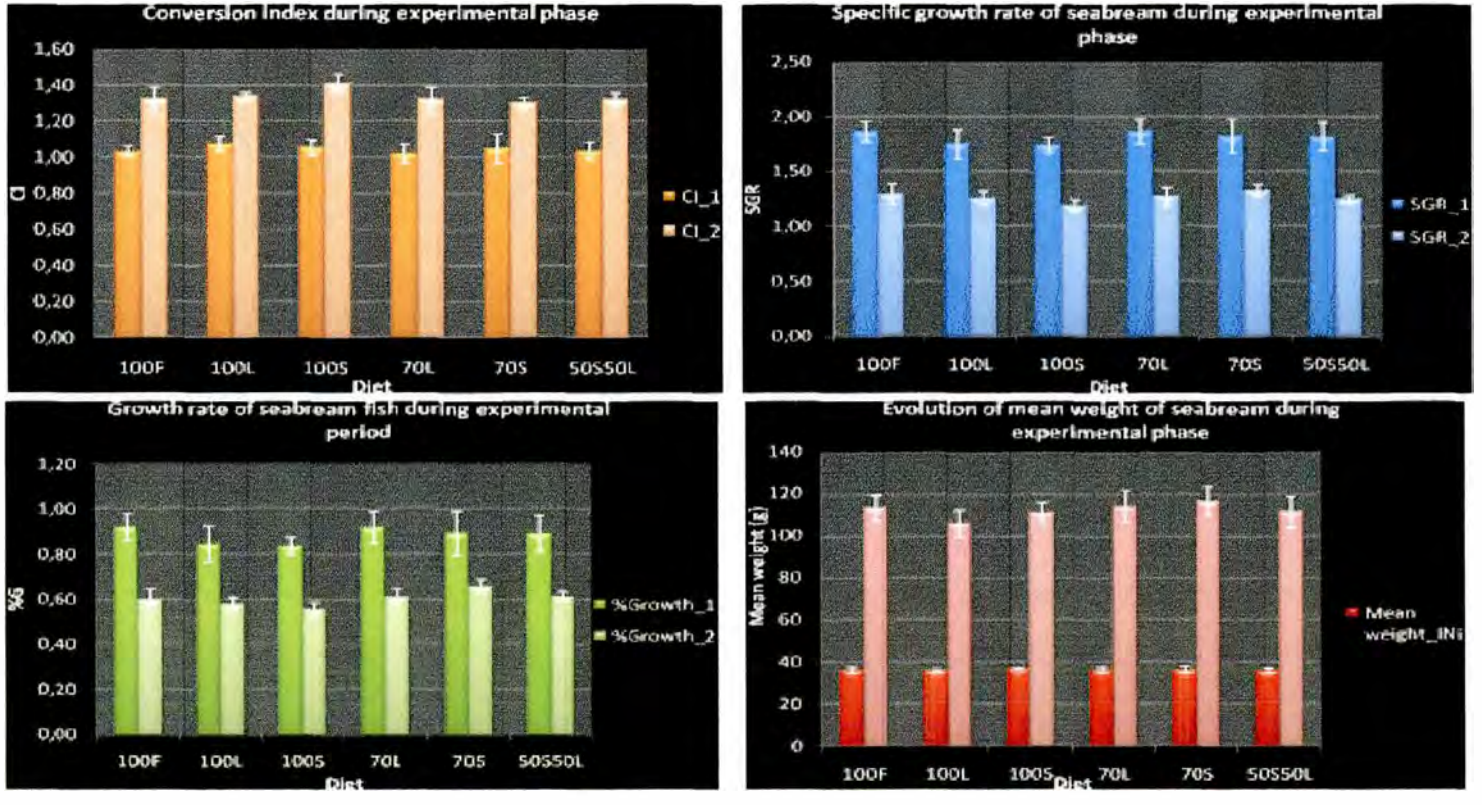


Figure 26: Illustration of the evolution of different growth control parameter during the experimental phase

The following table XIII, shows the Fatty acids profile of each experimental diet.

Control diet (100F) appears the richest on total saturated fatty acids, followed by diet with half inclusion of linseed oil and soybean oil and the poorest are diets with total inclusion of vegetables oil. Amounts range from 44% to 21%. With the regard to Linoleic acid (LA), diets with partial and total substitution by soybean oil present highest amount about 40%, although diets with total and partial substitution with linseed oil are leader in linolenic acid (LNA) with a percentage oscillating between 34% and 1.4% in fish oil diet. The highest level of EPA and DHA were attributed to fish oil diet (respectively 7.5% and 5.27%) following by diet with 70 % of vegetable oil inclusion with the half amount encountered in the control diet. Other diets reflected a content of only 1%. Therefore, the polyunsaturated fatty acids n-3 HUFA are more accumulated in control diet (12.78%) decreasing from diets with partial inclusion (6.9%) to reach 2% in 100L, 100S and 50S50L diets. Finally, comparing ratio omega 3/omega 6 reflects high level in control diet and the 70 L (3.47%) followed by the 100L diet whereas diets of soybean oil inclusion presented the minor value about (0.4%).

Table XIII: Diet fatty acids composition (g/100g AA)

Fatty acids	100 F	70 L 30F	70S 30F	50 S 50L	100 L	100 S
12	0,1373	0,0503	0,0492	2,4585	0,0235	
14	10,5071	3,5314	3,5459	6,5305	1,7404	1,3419
14:1n7	0,2941	0,1123	0,1124	0,8084	0,0735	0,0545
14:1n5	0,0930	0,0338	0,0332	0,1858		
15	0,8353	0,3245	0,3218	1,2666	0,2091	0,1525
15:1n5	0,1236	0,0480	0,0486	0,1134	0,0306	
16:0ISO	0,1317	0,0627	0,0573		0,0734	0,0528
16	24,7202	13,2084	16,4357	24,8909	11,7785	14,4574
16:1n7	0,0554	0,0418	0,0414	1,7376	0,0424	0,0395
16:1n5	12,1028	4,2213	4,2681	21,3951	2,0284	1,5967
16:2n6	0,4111	0,1524	0,1365	0,4401	0,0602	0,0484
16:2n4	1,8758	0,6652	0,6734	0,2281	0,2765	0,2186

17	0,9943	0,4094	0,4210	0,5857	0,2951	0,2563
16:3n4	1,7877	0,6559	0,6700	0,7564	0,2506	0,2286
16:3n3	0,1616	0,0710	0,0710	0,1293	0,0524	0,0398
16:4n3	0,5476	0,2458	0,2514	0,0750	0,0910	0,0608
18	4,3379	4,3137	3,5237	5,4415	5,2015	3,4398
18:1n9	9,0950	14,1352	19,0946	13,4356	18,4337	23,7230
18:1n7	3,3721	1,7649	2,2466	5,8495	1,4773	1,9430
18:1n5	0,1314	0,0826	0,1154	0,3478	0,0762	0,1076
18:2n9	0,0417			1,4586		
18:2n6 LA	2,9523	11,7958	30,2211	4,5249	16,3180	40,7831
18:2n4	0,3882	0,1196	0,1242		0,0499	0,0362
18:3n6	0,3505	0,1379	0,1519	0,1734		0,0487
18:3n4	0,4476	0,1195	0,1764		0,0372	
18:3n3 LNA	1,3947	31,4852	4,4378	0,2988	34,5633	4,7885
18:3n 1				0,0801		
18:4n3	1,5063	0,6930	0,6869		0,2239	0,2346
18:4n1	0,3113		0,2979	0,2690	0,2317	0,3142
20	2,4190	1,9314	1,9761	2,0724	1,9560	1,6493
20:1n9	0,2717	0,1309	0,1349	0,5450	0,0981	0,0803
20:2n9	0,1281	0,0338	0,0350	0,3244	0,0802	
20:3n9					0,0496	
20:2n6	0,1835			0,1609		0,0716
20:3n-6 DHGLA	0,2231					
20:4n6 ARA	0,9353	0,3935	0,3952	0,9087	0,1458	0,1332
20:4n3	0,7824		0,3177	0,0710	0,0971	0,0890
20:5n3 EPA	7,5052	3,5175	3,4569	1,1254	0,9516	1,0674
22:1n11	1,5724	1,4657	1,5474	0,2874	1,6397	1,3341
22:4n6	0,2697				0,0450	0,0477
22:5n3	1,3239	0,5890	0,5732	0,4621	0,1369	0,1515
22:6n3 DHA	5,2781	3,4566	3,3501	0,5620	1,1617	1,4093
Total satuates	44,0829	23,8318	26,3308	43,2461	21,2775	21,3501
Total monoenes	27,1115	22,0364	27,6425	44,7056	23,9000	28,8787
n-3	18,4998	40,0582	13,1452	2,7236	37,2778	7,8408
n-6	5,3255	12,4796	30,9046	6,2081	16,5690	41,1328

n-9	9,5363	14,2998	19,2644	15,7636	18,6616	23,8032
n-3 HUFA	12,7832	6,9741	6,8070	1,6874	2,1133	2,4767
AA/EPA	0,1246	0,1119	0,1143	0,8075	0,1532	0,1248
OA/DHA	1,7232	4,0893	5,6997	23,9064	15,8678	16,8336
EPA/DHA	1,4220	1,0176	1,0319	2,0024	0,8191	0,7574
OA/n-3HUFA	0,7115	2,0268	2,8051	7,9625	8,7229	9,5785
n-3/n-6	3,4738	3,2099	0,4253	0,4387	2,2499	0,1906

100F=100% fish oil; 100L=100% Linseed oil; 100S=100% Soybean oil; 70L30F= 70% linseed oil and 30% fih oil; 70S30F= 70%soybean oil and 30%fish oil; 50S50L= 50% soybean oil and 50% linseed oil.

III.2 Second experiment

III.2.1 Primer design proof

III.2.1.1 Agarose gel proof

- Interleukin_1 beta

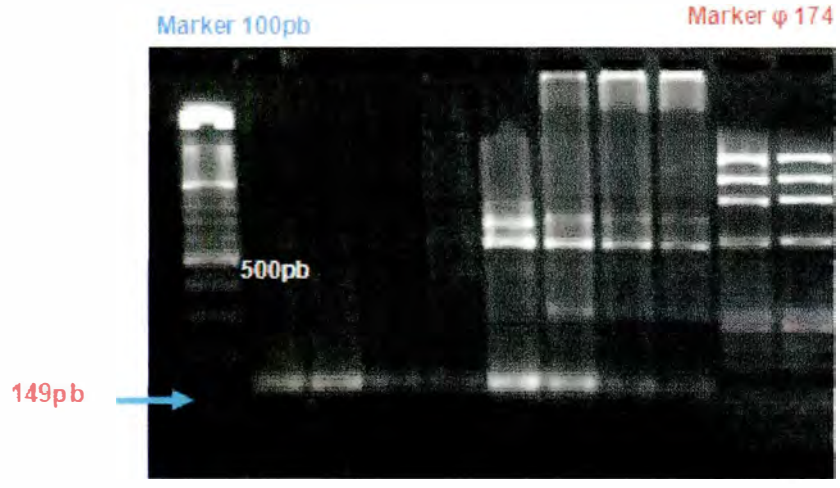


Figure 27: Agarose gel photo of Interleukin_1beta as proof to adequate primer design

- Beta-actina and Tumor Necrosis factor

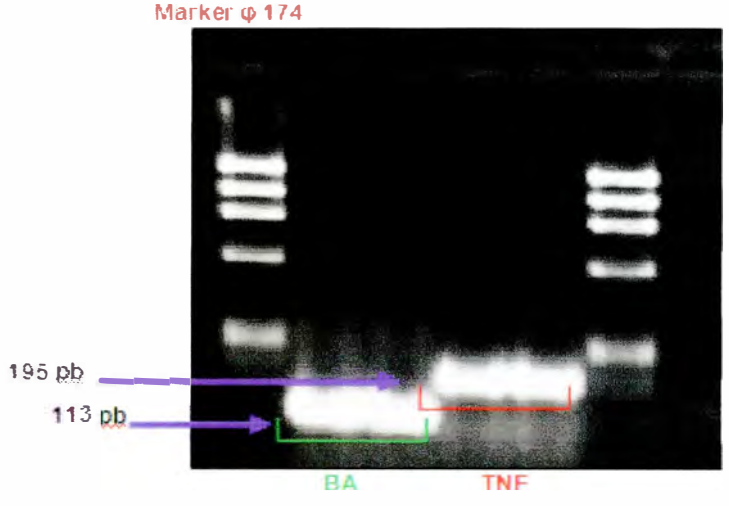


Figure 28: Agarose gel photo of Beta-actina and Tumor necrosis as proof of adequate primer design

The following ethitium bromide staining of cDNA separated in native agarose gel 2% visualized bands of amplified sequence respectively to IL_1B, TNF and B-actin. The marker φ 174 confirms the product weight of each searched band. Testing several amplification temperature per above and below the annealing temperature of each target gene allowed us to avoid favorable cases of non specific product amplification (Figure 27, 28).

Sequencing and alignment proof

- Interleukin_1 beta

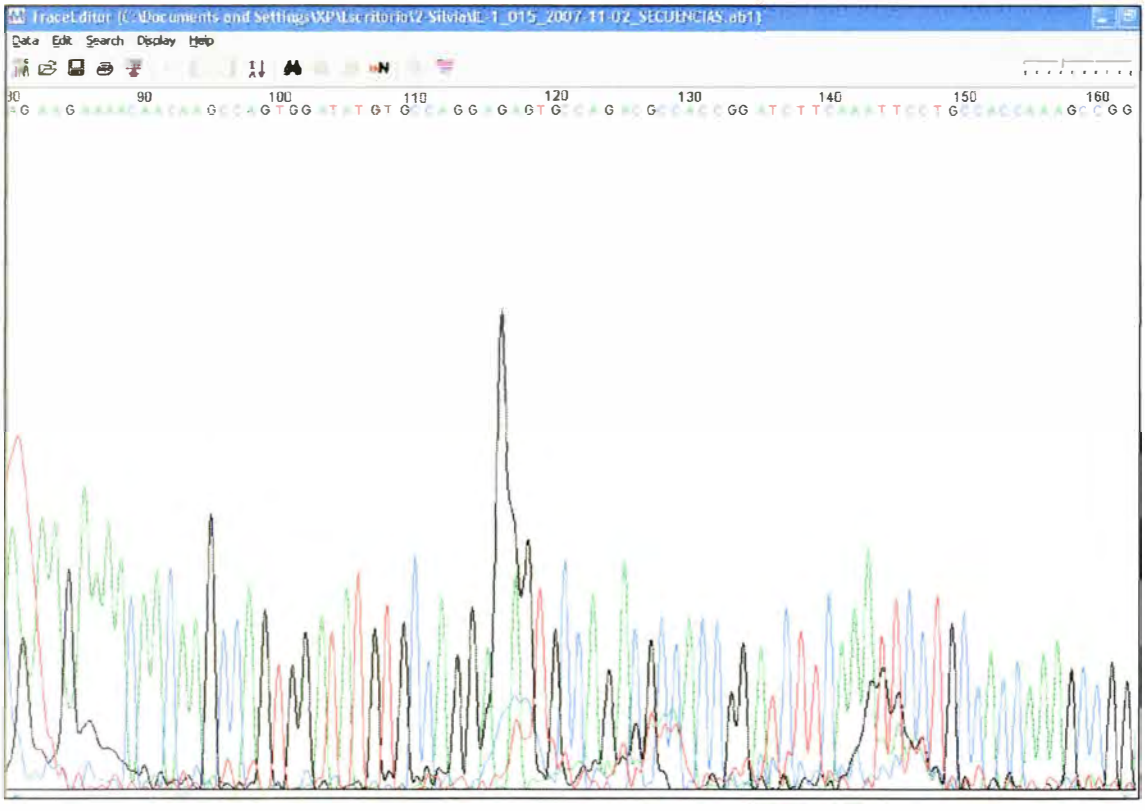


Figure 29: Sequencing result of Interleukin-1beta gene

Primer specificity was been tested through sequencing technique of the amplified fragment. Alignment of obtained sequence paste successfully with the interleukin_1 beta sequence of gilthead seabream published in the Gen bank

(Figure 29) and (Figure 30).

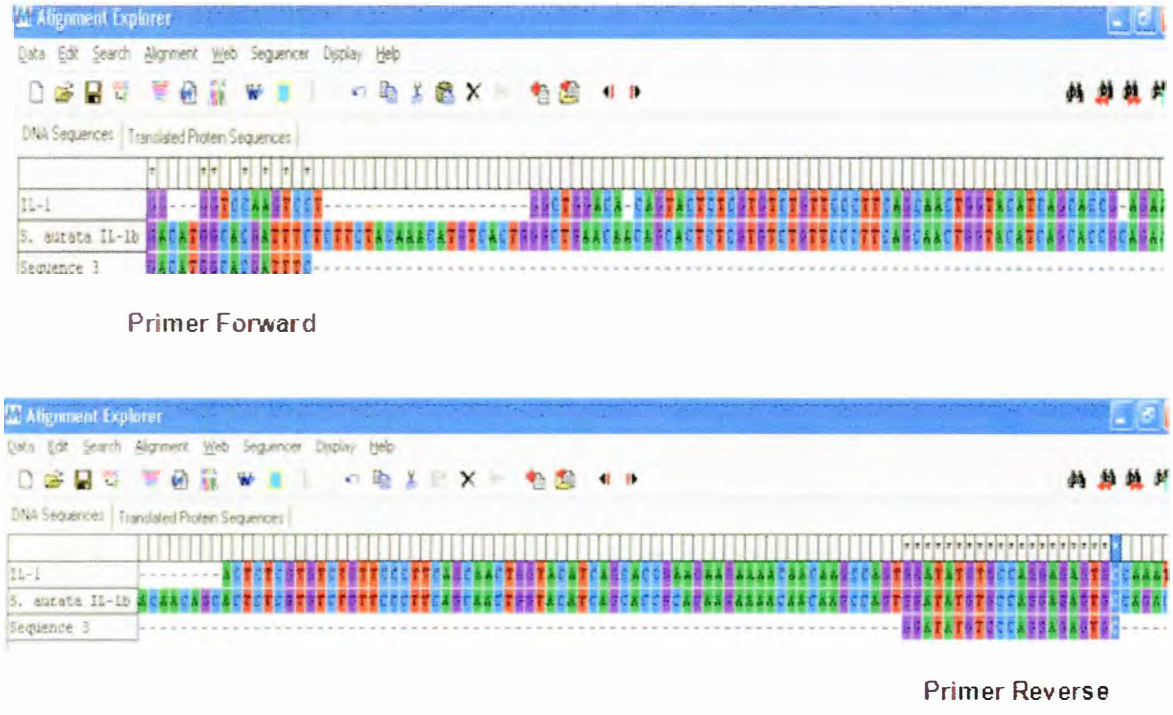


Figure 30: Alignment of primers and sequence of interleukin-1 beta of seabream

Designed primers are able to amplify an amplicon of 140 pair of bases from the base 2354 to 2493 base of the sequence of interleukin-1 beta of gilthead seabream.

- Tumor Necrosis factor α

Primer specificity was been tested through sequencing technique of the amplified fragment. Alignment of obtained sequence paste successfully with the Tumor Necrosis factor α sequence of gilthead seabream published in the Gen bank (Figure 31) and (Figure 32).

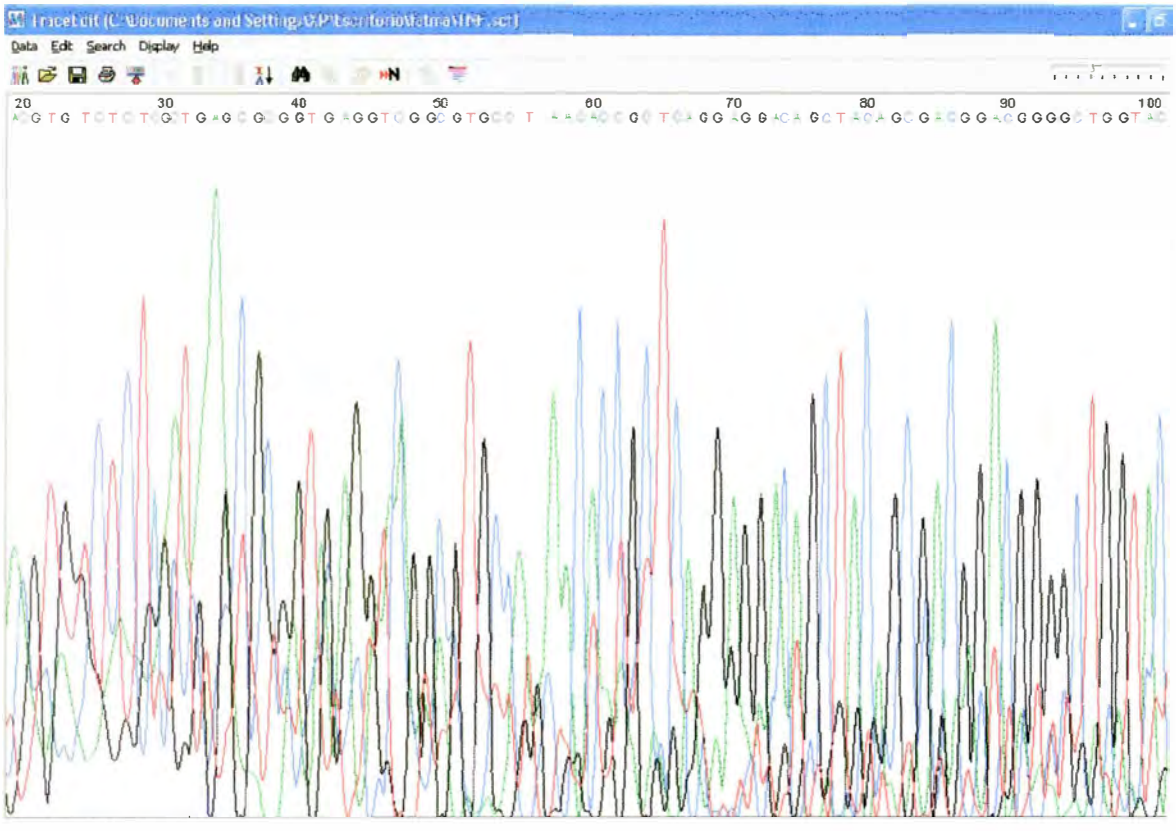


Figure 31: Sequencing result of Tumor Necrosis Factor α

Figure 32: Alignment of primers and sequence of Tumor necrosis factorα of seabream

Designed primers are able to amplify an amplicon of 195 pair of bases from the base 1126 to 1320 base of the sequence of Tumor Necrosis Factor α of gilthead seabream.

III.3II.2. Gene Expression

III.3.1.1 RNA Integrity

The (Annex II) resumed the different concentration and absorbance ratio of 126 templates (each one represented a pool of four fish). Each diet is defined by four point of sampling for control subject as well as infected fish. All samples shown a good quality, almost all presented a ratio of A260/280 above 1.7 and several reach the value of 2.

III.3.1.2 Specificity and Efficiency of cDNA amplification

- Specificity of qPCR for different genes

Because the Syber Green I binds to all dsDNA, it was been necessary checking the specificity of our qPCR assay for each target gene by analyzing the reaction products. The melting curve function of our reaction enables us to ensure the optimal annealing temperature for IL-1B, TNF and B-actin avoiding amplification of non specific product such as dimers. (Figure 33), (Figure 34) and (Figure 35) All charts shown a unique tip well defined coupled with good efficiency amplification Table XIV.

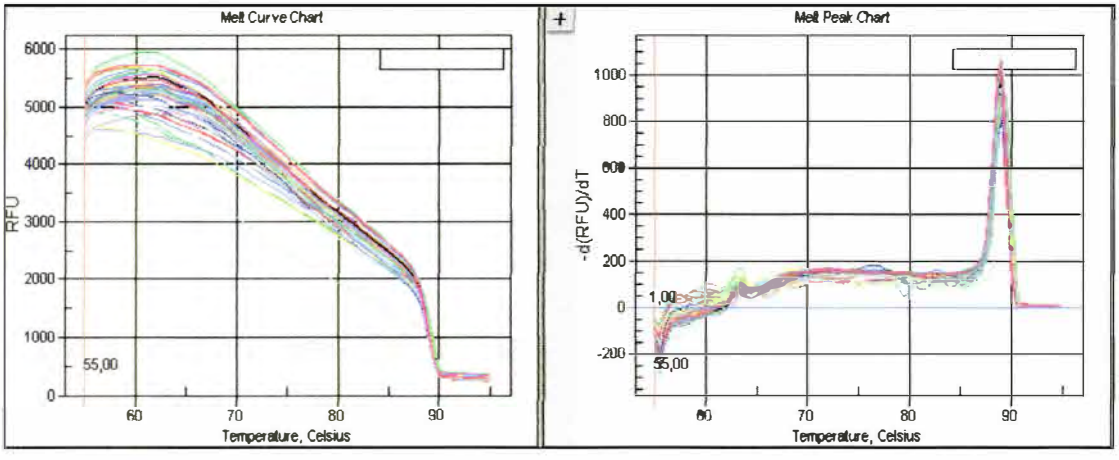


Figure 33: Melting point curve of TNF gene

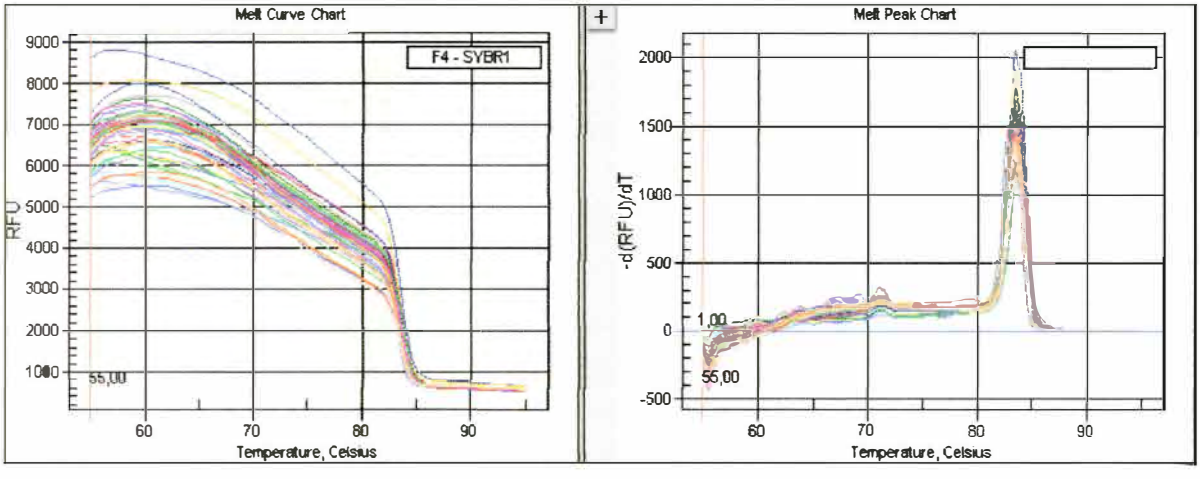


Figure 34: Melting point curve of IL_1B gene

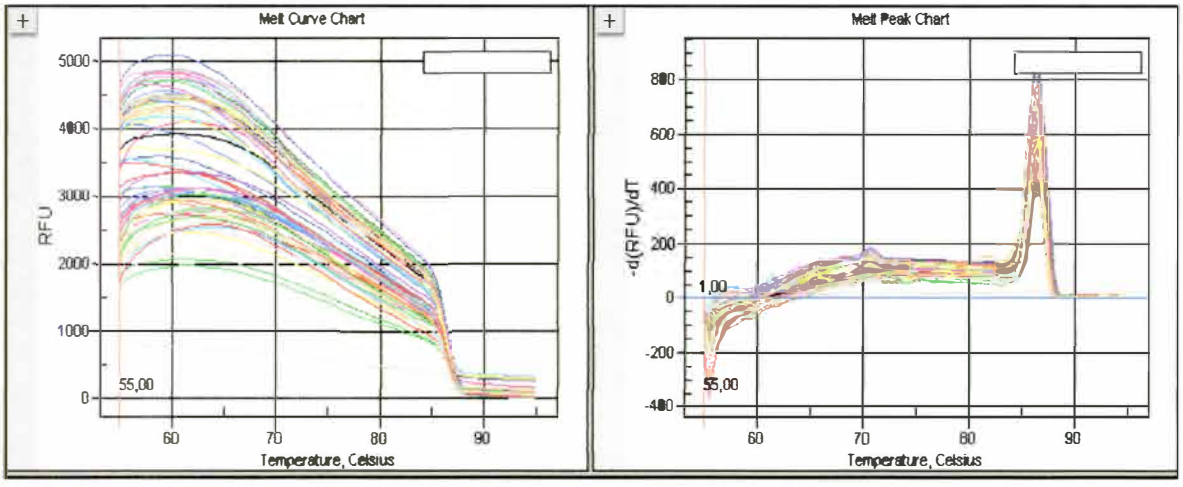


Figure 35: Melting point curve of B-actin gene

Efficiency of qPCR

Table XIV: Efficiency of TNF and IL-1B amplification

Genes	qPCR EFFICIENCY	
	Oh+ 3D	**1D+7D**
B- actin	107	124.9
TNF	101.8	107.5
IL-1B	98.3	114.5

The table above sum up efficiency of amplification of each gene for different carried plaque. Efficiency close to 100 % is the best indicator of robust, reproducible assay.

III.3.1.3 Data expression

The following table XV resumes C_T values of different samples.

Table XV: C_T values of different target genes for both Infected and control sample

Condition	C_T Interleukin	C_T TNF	C_T B-actina
50 D1 C1	23,83	25,41	17,35
50 D1 C2	21,82	24,47	21,30
50 D1 C3	20,77	22,08	20,55
50 D1 I1	22,13	24,71	19,03
50 D1 I2	23,68	25,29	22,18
50 D1 I3	23,14	24,96	17,30
50 D7 C1	23,47	25,93	22,48
50 D7 C2	22,92	24,98	22,00
50 D7 C3	23,74	25,20	23,56
50 D7 I1	26,28	26,47	23,51
50 D7 I2	22,91	23,59	20,36
50 D7 I3	22,46	20,36	19,89
100F D1 C1	26,87	28,97	15,97
100F D1 C2	28,14	28,90	18,83
100F D1 C3	26,72	25,94	15,91
100F D1 I1	24,97	27,78	15,43
100F D1 I2	26,49	28,97	17,14
100F D1 I3	26,19	28,17	18,10
100F D7 C1	24,06	29,11	20,20
100F D7 C2	21,71	24,89	21,05
100F D7 C3	23,46	28,02	22,43
100F D7 I2	24,50	29,29	21,22
100F D7 I3	24,22	28,57	20,14
100L D1 C1	27,91	29,16	17,05
100L D1 C2	26,33	27,19	17,41
100L D1 C3	23,87	26,07	17,93
100L D1 I1	25,78	27,04	15,71
100L D1 I2	27,24	29,05	19,33

100L D1 I3	26,70	28,56	20,52
100L D7 C1	24,02	27,52	17,76
100L D7 C2	22,57	25,48	19,07
100L D7 C3	24,70	28,28	18,14
100L D7 I1	22,83	26,37	20,17
100L D7 I2	26,60	29,12	19,78
100L D7 I3	25,77	30,98	18,16
100S D1 C1	25,99	27,69	18,15
100S D1 C2	26,55	27,20	16,61
100S D1 C3	25,96	27,16	19,10
100S D1 I1	27,38	28,73	16,22
100S D1 I2	24,49	27,01	18,74
100S D1 I3	23,41	26,93	22,41
100S D7 C1	24,02	25,09	15,65
100S D7 C2	24,82	25,95	16,73
100S D7 C3	25,34	28,16	18,67
100S D7 I1	24,19	26,39	20,92
100S D7 I2	26,80	26,21	17,02
100S D7 I3	24,33	26,32	16,14
70L D1 C1	27,58	28,10	21,23
70L D1 C2	24,52	27,97	21,50
70L D1 C3	23,71	26,30	20,31
70L D1 I1	24,84	25,60	20,06
70L D1 I2	25,02	26,01	19,35
70L D1 I3	22,94	25,74	23,10
70L D7 C1	24,20	25,47	16,25
70L D7 C2	21,34	22,01	17,78
70L D7 C3	28,05	29,44	20,93
70L D7 I1	23,44	27,56	21,13
70L D7 I2	25,49	27,88	17,95

70L D7 I3	22,60	25,63	18,13
70S D1 C1	30,71	29,19	17,06
70S D1 C2	24,18	26,89	17,68
70S D1 C3	25,30	26,33	17,68
70S D1 I1	28,95	28,79	16,55
70S D1 I2	29,20	28,99	16,98
70S D1 I3	26,52	27,90	19,14
70S D7 C1	24,77	25,89	15,41
70S D7 C2	24,25	26,04	17,59
70S D7 C3	26,53	28,60	18,64
70S D7 I1	26,42	27,77	20,75
70S D7 I2	27,01	27,27	18,01
70S D7 I3	27,77	26,55	17,57

III.3.1.4 Assessment of profile gene expression of TNF and IL_1B in the proximal intestine

III.3.1.4.1 TNF α gene

- **Basal level of TNF α expression in the different diets**

The chart bellow (Figure 36) shows TNF gene basal level expression for each type of diet. Statistical test (One-Way ANOVA) not proved significant expression difference between different diets except between 100L and 70S (Post Hoc Multiple comparisons: Duncan test). Except the 100 L diet, TNFα of fish fed 100% or 70% soybean oil and the 70% linseed oil expressed less than the fish fed control diet. Estimated means are presented in the following table Table XVI.

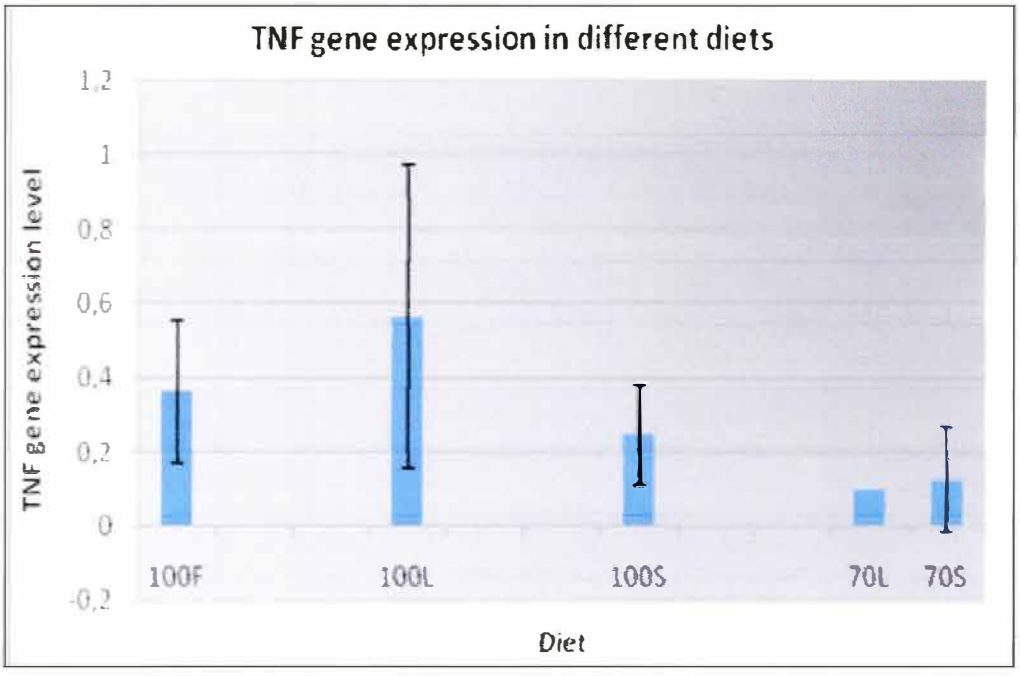


Figure 36: Baseline expression level of TNFα of different diets

Table XVI: Estimated means value of basal level TNFα for different diets

Diet	Mean TNF expression level in the proximal intestine
100F	0,361 ± 0,19
100L	0,563 ± 0,40
100S	0,245 ± 0,134
70L	0,0005
70S	0,124 ± 0,142

- **Post infection expression of TNF α in the control diet (100% fish oil)**

An over expression is observed at third day post infection that decrease in the followings days (Figure 37).It is about 3 times up the basal level. In spite of this important difference of average expression, this do not seems to be statistically significant due to the great variance of values. Nevertheless, excluding one value

that seems to be clearly out of rank put in evidence the existence of significant but transitory up regulation of TNFα expression in the control diet (Table XVII).

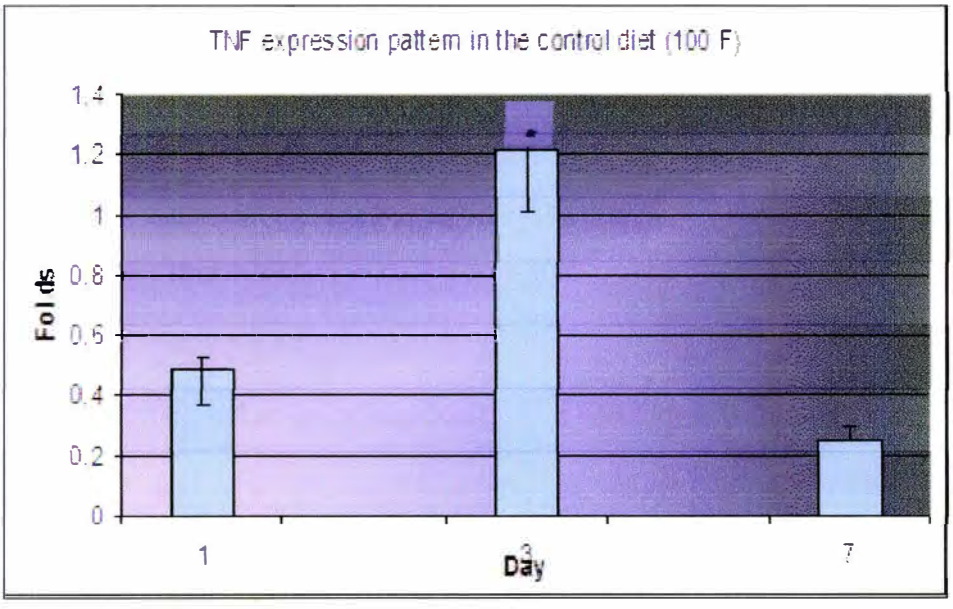


Figure 37: TNFα gene expression in the 100 F diet during the time course of post infection

Table XVII: Significant mean difference during the time course of TNF expression in the control diet

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1,043	2	,521	27,268	,012
Within Groups	,057	3	,019		
Total	1,100	5			

The new means values respective to such modification are showed in the following table (XVIII). They are presented as means ±Sdt. Deviation.

Table XVIII: The new mean values of TNFα expression during the time course post infection of control diet

Day	Folds expression of TNFα in the proximal intestine
D1	$0,48856959^{a} \pm 0,12318953$
D3	$1,21418889^{b} \pm 0,20140584$
D7	$0,22917686^{a} \pm 0,04022892$

Means with a letter in different superscript differ significantly ($P < 0.05$)

- **Pattern expression for 100 S diet**

Picture shows (Figure 38) that TNFα of fish fed the 100% soybean oil diet, presents a gradual increase of expression during the course time post infection that reaches a tip of over expression at third day when infected subjects are expressing at 5.5 folds higher level than control fish and about 23 times above the basal level. At seventh day, mRNA decreases but not reaches basal value. This expression pattern is not demonstrated significant due to the high variance existing between samples. Means values are presented in the following table (Table XIX).

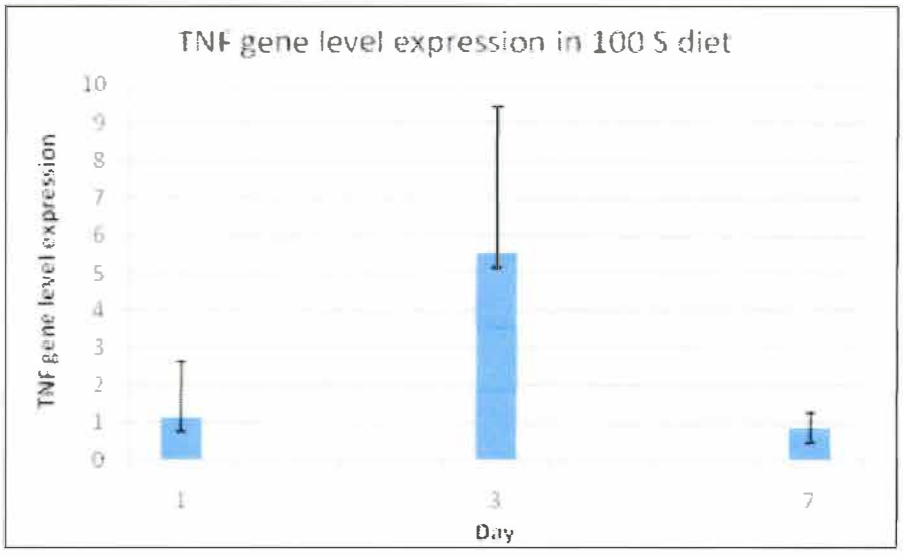


Figure 38: TNFα gene expression in the 100S diet during the time course of post infection

Table XIX: Mean value of TNFα expression for 100S diet during course time

Day	Mean
1	1,14 ± 1,46
3	5,53 ± 3,88
7	0,85 ± 0,40

- **Pattern expression for 100 L diet**

The bacterial infection appears inducing up regulation of TNFα gene expression especially at third day post inoculation in samples fed the 100L diet ((Figure 39). Such fish expressed the target gene 3 times more compared with control animals and at 6 folds higher level than the basal level. This expression pattern is demonstrated significant (p<0.05) (Table XX). After, gene expression level falls to reaches newly basal level. Means values are presented in the table below (Table XXI).

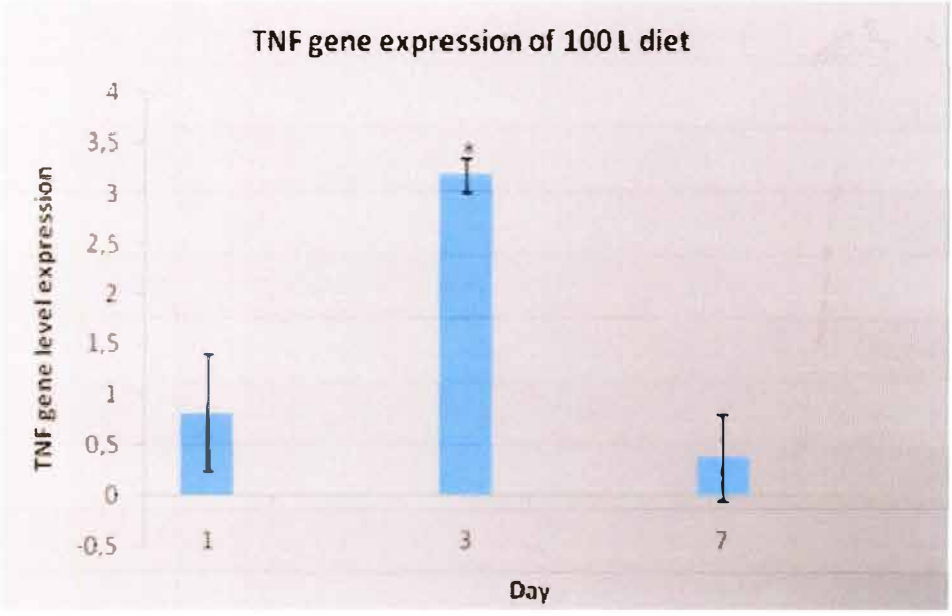


Figure 39: TNFα gene expression in the 100 L diet during the time course of post infection

Table XX: Significant mean difference of TNFα expression in the 100L diet

TNF_EXP	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9,101	2	4,551	24,589	,014
Within Groups	,555	3	,185		
Total	9,657	5			

Table XXI: Mean value of TNFα expression for 100L diet during time course post infection

Day	Mean
1	$0,81^{a}$ ± 0,57
3	$3,17^{b}$ ± 0,16
7	$0,365^{a}$ ± 0,43

Pattern expression for 50S:50L diet

Up regulation at the 3 day post infection was observed in samples fed mixed vegetable oil diet 50S50L (Figure 40). Over mRNA TNFα transcription reaches to expresse 10 times more than control fish and subsequently decreases in the followings days. This oscillation not appears significant except if (p<0, 06). Nevertheless, excluding one value that seems to be out of rank transforms data to be significant (p< 0, 05) Table **XXII, XXIII**.

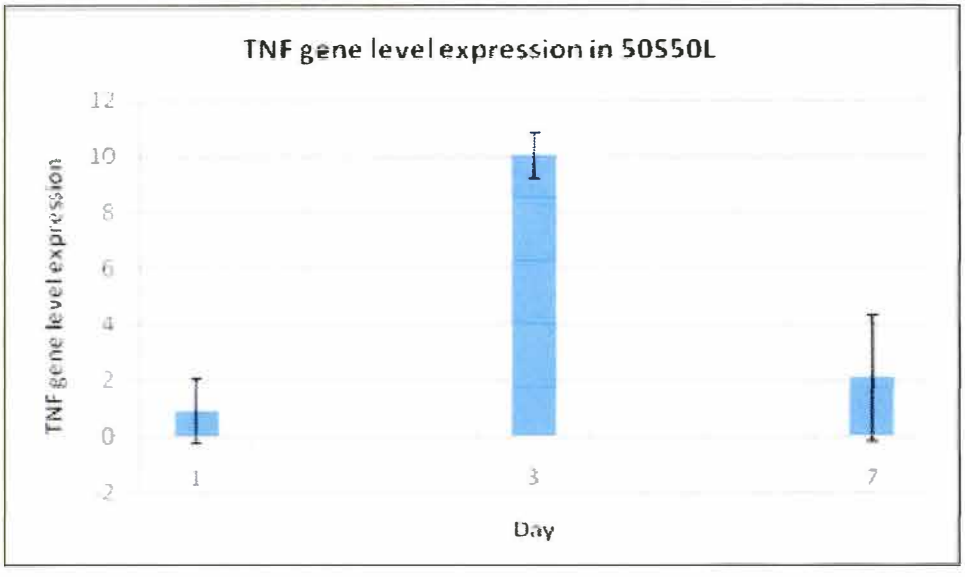


Figure 40: TNFα gene expression in the 50S50 L diet during the time course of post infection

Table XXII: Significant mean difference of TNFα expression in the 50S50L diet

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	112,043	2	56,022	20,762	,004
Within Groups	13,491	5	2,698		
Total	125,534	7			

Table XXIII: Mean value of TNFα expression for 50S:50L diet during course time

Day	Mean ± Sdt
1	$0,89^{a}$ ± 1,14
3	$10,03^{b}$ ± 0,83
7	$2,05^{a}$ ± 2,25

- **Pattern expression of 70S diet**

Unlike to other pattern expression, samples fed diet with partial vegetable oil substitution at 70% soybean oil shown a later up regulation expression at the seventh day post inoculation (Figure 41). Furthermore, expression magnitude seems to be lower than what we have observed with other diets. Although, this low level expression, induction of TNF gene expression after bacterial infection is clearly marked comparing with the basal level. Statistical test not proved a significant difference between means due to the high variance aspect of data. Excluding same ones from the data base change ANOVA- one way analysis results to be significant ($p<0,05$) Table XXIV, XXV.

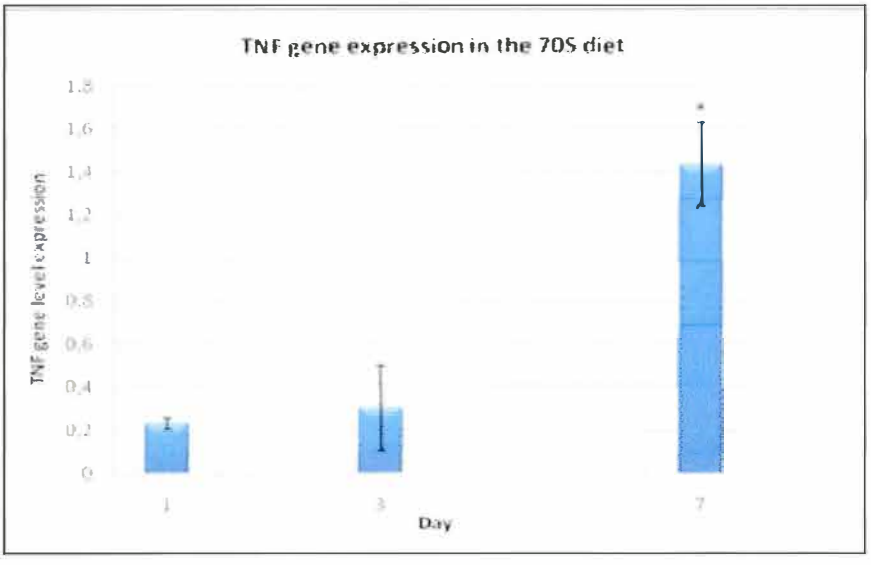


Figure 41: TNFα gene expression in the 70 S diet during the time course of post infection

Table XXIV :Significant mean difference of TNFα expression in the 70S diet

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	,851	2	,426	7,390	,045
Within Groups	,230	4	,058		
Total	1,081	6			

Table XXV: Mean value of TNFα expression for 70S diet during course time

Day	Mean
1	0,229 [a] ± 0,025
3	0,298 [a] ± 0,196
7	1,43 [b] ± 0,195

- **Pattern TNF expression in 70 L diet**

Unlike to TNF gene expression of infected fish fed with 70% soybean oil, infected fish fed a diet with a linseed oil partial inclusion expressed early front of pasterellosis infection (Figure 42). A marked up regulation was detected since the first day post inoculation, and TNF mRNA transcripted was 6 times higher than control fish and over expressed comparing with the basal level. After, gene expression was down regulated and almost reached the basal level at the third day post infection.

This expression deference was not demonstrated statistically significant. Means values are presented in the table below (Table XXVI).

Figure 42: TNF gene expression in the 70 L diet during the time course of post infection

Table XXVI: Mean value of TNFα expression for 70L diet during time course post infection

Day	Mean
1	5,56 ±7,28
3	0,9± 0,79
7	0,966± 0,84

Post infection TNF gene expression pattern of in all diets

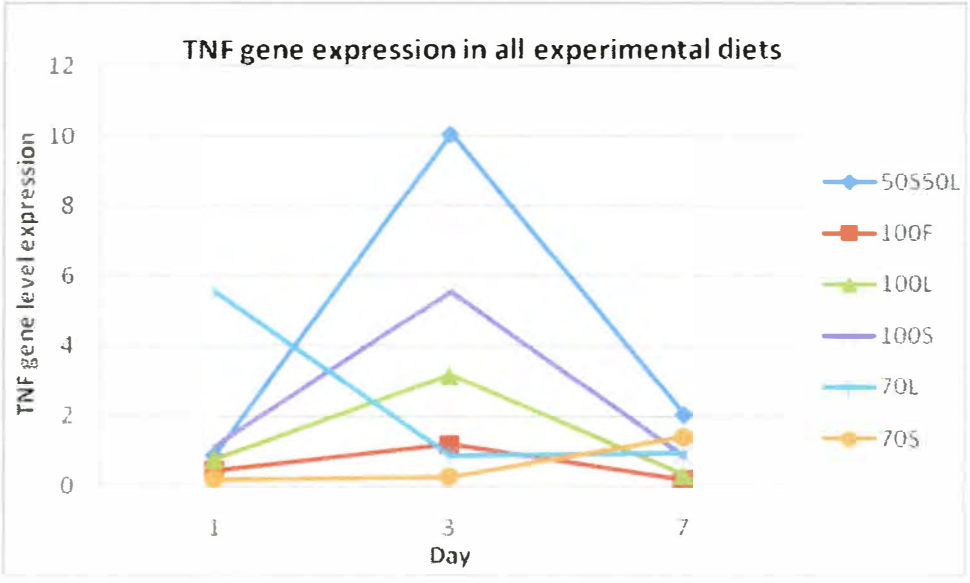


Figure 43: TNF gene expression level of different diets

The following figure (Figure 43) shows the estimated marginal mean value of TNF expression for each type of diet, and considering the three points of sampling 1, 3 and 7 days post infection. As it is observed, third day post infection is a crucial point in which an over expression but transitory was observed for 100% vegetable oil substitution diet (100S and 100L), same behavior gene expression was attributed also to the mixed diet 50S:50L (50%soybean oil, 50% linseed oil).

Unlike to anterior diets, fish fed partial substitution soybean oil witnessed a marked over expression at seventh day post inoculation.

Divergence en time TNF gene expression take more evidence with the 70L diet for which infected fish expressed more early in response to the bacterial invasion.

With the regard to the Control diet 100 % fish oil, infected fish expressed more at third day post infection but at lower magnitude compared with other diets.

Comparing different magnitude expression between all diets and during the time course of pasteurellosis post infection, ANOVA test reveals significant expression difference at third day post inoculation ($p<0.05$). First, difference appears clearly between soybean diets with its different percentage of substitution: 50%, 70% and 100%. The partial inclusion of linseed oil seems to have significant difference with all other diets. Finally the control diet and the 100% linseed oil not manifest significant difference between each other.

The following table translates such significant difference at third day post infection (Table XXVII).

Table XXVII: Different mean values of TNF expression for all diet at third day post infection

Diet	Mean value
100F	$1.21^{(abc)} \pm 0.20$
100L	$3.17^{(abc)} \pm 0.16$
100S	$5.53^{(c)} \pm 3.88$
70L	$0.9^{(ab)} \pm 0.79$
70S	$0.22^{(a)} \pm 0.025$
50S50L	$10.03^{(bc)} \pm 0.83$

Means with a letter in different superscript differ significantly ($P < 0.05$)

➢ Interleukin_1B gene

Baseline IL_1B gene expression

The following picture (Figure 44) shows IL-1β gene basal level expression for each type of diet. Statistical test (One-Way ANOVA) not proved significant expression difference between different diets. (Post Hoc test are not performed because existed diet with fewer than three groups). Similarly to basal TNF gene expression of samples fed the 70L diet, IL-1β expression was very low or almost zero. Means values were presented in the following table XXVIII.

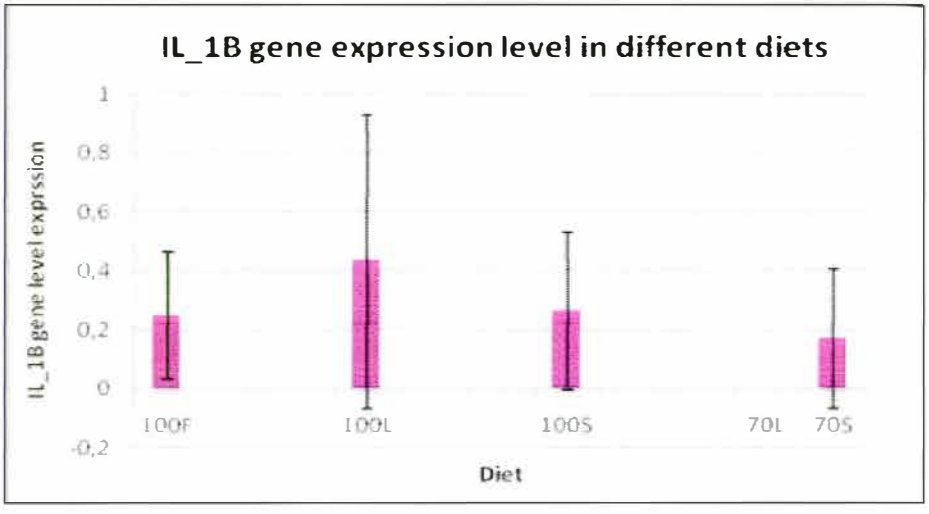


Figure 44: Basal IL-1β gene expression in different diets

Table XXVIII: Means values of basal IL-1β expression in different diets

Diet	Mean± SD
100F	0,24 ±0,21
100L	0,429 ± 0,49
100S	0,26 ± 0,26
70L	0,00052
70S	0,166 ± 0,23

- **IL-1β gene expression pattern in the 100F diet**

Figure 45: IL-1β gene expression in the 100F diet during the experimental phase

The chart above reveals an over expression of IL-1β expression during the three first days post infection with a magnitude of about 3 times more than control expression (Figure 45). After, a significant down regulation occurs at seventh day post inoculation. It is a marked fall that represents almost 10% of the initial expression value. ANOVA result analysis and mean expression value are resumed in the following tables XXIX.

Table XXIX: Significant ANOVA test and different means values of IL-1β expression in the 100F diet

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1,271	2	,635	5,953	,048
Within Groups	,534	5	,107		
Total	1,805	7			

Day	Mean ± SD
1	2,828[a] ± 1,67
3	3,049[a] ± 3,16
7	0,292[b] ± 0,11

- **IL-1β gene expression pattern in the 50S50L diet**

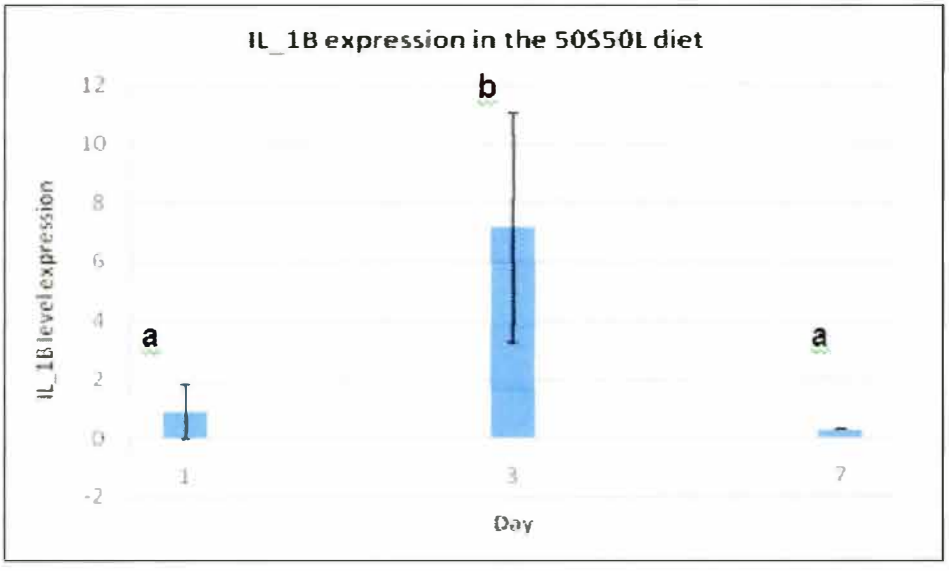


Figure 46: IL-1β gene expression in the 50S50 L diet during the time course of post infection

The figure above (Figure 46) showed a significant up regulation at third day post inoculation ($P<0.05$) of the IL-1β gene expression of subject reared with mixed diet of 50 % soybean and 50% linseed. Such expression decrease after to reach almost the same initial value. Significant difference proof and means values expression are mentioned in tables bellow Table XXX.

Table XXX: Significant ANOVA test and different means values of IL-1β expression in the 50S50L diet

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3,217	2	1,608	9,217	,015
Within Groups	1,047	6	,175		
Total	4,264	8			

Day	Mean ± SD
1	$0,861^{a}$ ± 0,91
3	$7,131^{b}$ ± 3,89
7	$0,262^{a}$ ± 0,02

- **IL-1β gene expression pattern in the 100L diet**

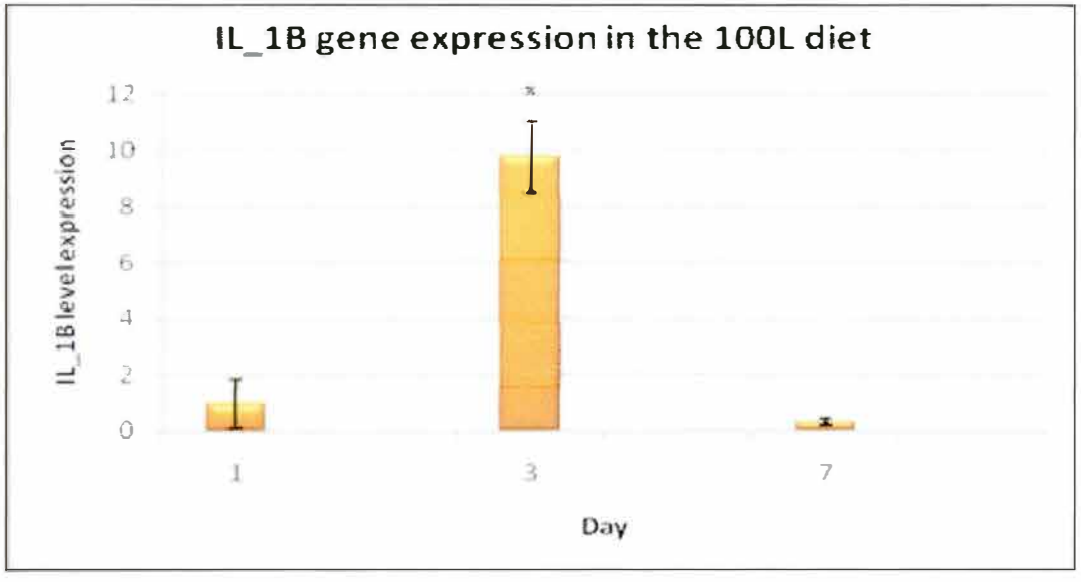


Figure 47: IL-1β gene expression in the 100L diet during the experimental phase

The bacterial infection appears inducing up regulation of IL-1β gene expression especially at third day post inoculation in samples fed the 100L diet (Figure 47). Such fish expressed the target gene 10 times more compared with control animals

and at 25 folds higher level than the basal level. This expression pattern is demonstrated significant (p<0.05). After, gene expression level falls to reache newly basal level. Means values are presented in the table below Table XXXI.

Table XXXI: Significant ANOVA test and different means values of IL-1β expression in the 100L diet

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	111,068	2	55,534	68,783	,003
Within Groups	2,422	3	,807		
Total	113,491	5			

Day	Mean ± SD
1	$0{,}965^{a}$ ± 0,86
3	$9{,}745^{b}$ ± 1,27
7	$0{,}3^{a}$ ± 0,11

- **IL-1β gene expression pattern in the 100S diet**

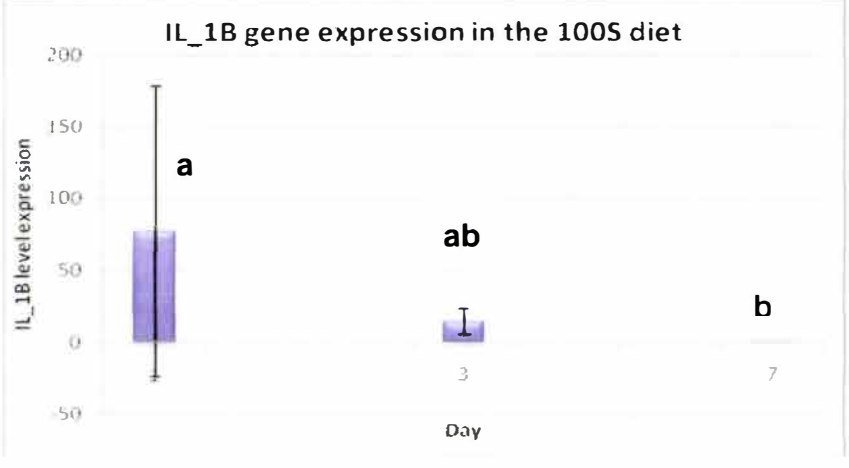


Figure 48: IL-1β gene expression in the 100S diet during the experimental phase

Unlike to other pattern expression, samples fed diet with total vegetable oil substitution at 100% soybean oil shown an early up regulation expression at the first day post inoculation (Figure 48). Furthermore, expression magnitude seems to be higher than what we have expected. At third day, IL-1β expression decreased reaching the half level comparing with anterior days.

Statistical test not proved a significant difference between means due to the high variance aspect of data. Excluding same ones from data base changed ANOVA- one way analysis results to be significant ($p<0.05$). Means values of IL-1β expression are summed up in the following table XXXII.

Table XXXII: Different means values of IL-1β expression in the 100S diet

Day	Mean ± SD
1	$76,95^{a}$ ± 101,038
3	$13,82a^{b}$ ± 8,762
7	$0,48^{b}$ ± 0,339

IL-1β gene expression pattern in the 70L diet

IL-1β gene of infected fish fed 70L diet proved an over expression since the first day post inoculation. A down regulation is observed at third day post infection, after that IL-1β expression newly increase to reach almost initial level expression (Figure 49). This statement not demonstrated being significant and different means values are shown in the following table XXXIII.

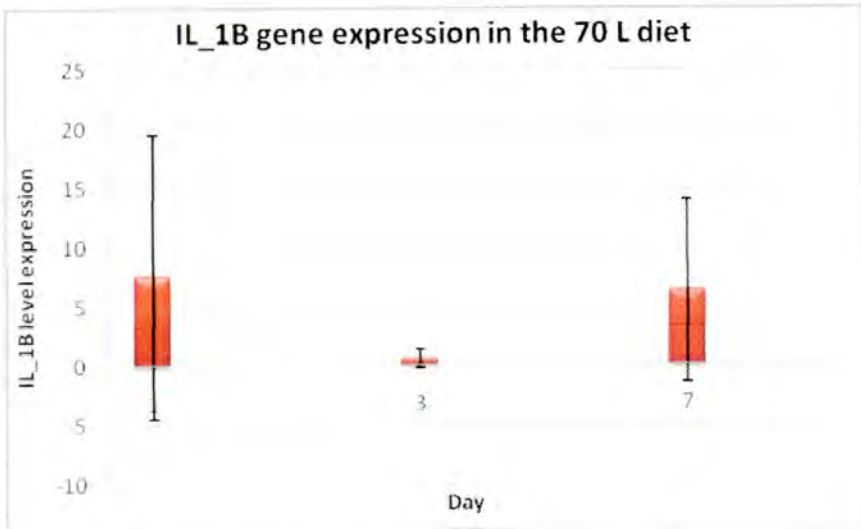


Figure 49: IL-1β gene expression in the 70L diet during the experimental phase

Table XXXIII: Different means values of IL-1β expression in the 70L diet

Day	Mean
1	7,47 ±12,02
3	0,56 ± 0,75
7	6,22 ± 7,68

IL-1β gene expression pattern in the 70S diet

IL-1β gene expression pattern of infected fish fed the 70S diet appeared have same behavior that was been mentioned in infected fish fed 70L diet. Target gene manifested an over expression since first post infection days which decreased at

the third day to newly increased in the seventh day. Level expression appeared lower than those detected with 70L diet (Figure 50).Respectives means values are advanced in the table below Table XXXIV.

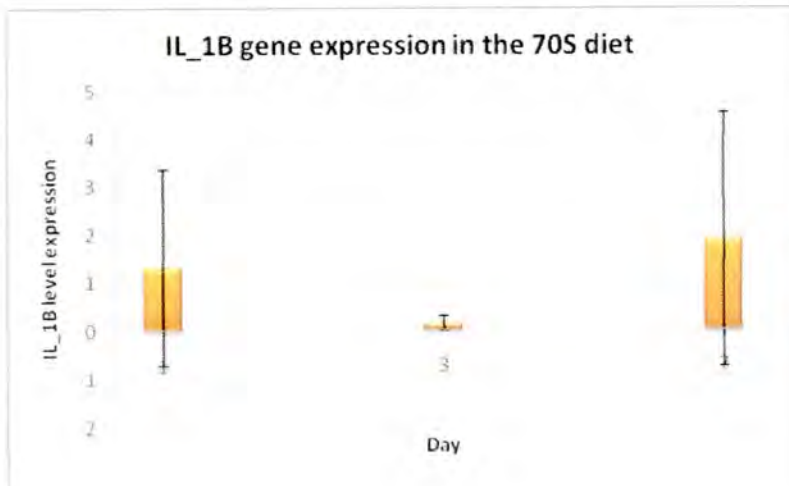


Figure 50: IL-1β gene expression in the 70S diet during the experimental phase

Table XXXIV: Different means values of IL-1β expression in the 70S diet

Day	Mean± SD
1	1,3 ± 2,05
3	0,14 ± 0,14
7	1,87 ± 2,64

- **IL-1β gene expression in different diets**

The following figure 51, shows the estimated marginal mean value of IL-1β expression for each type of diet, and considering the three points of sampling 1, 3 and 7 days post infection. As it is observed the third day post infection is a special point in which an over but transitory expression was observed for 100L diet (100% linseed oil), 50S:50L diet (50%soybean oil, 50% linseed oil), a down

regulation is detected for 70S diet (70%soybean oil) and 70 L (70% linseed oil). These later have singular behavior in which IL-1β expression increased early, one day after infection may be before, decreased at three days post inoculation and returned to over expresse at the seventh day. The case of 100% soybean oil presents a singular expression behavior.

Broadly, infected subject expressed IL-1β after bacterial challenge differently according to its specific diet. Those fed diet with total vegetable oil substitution or a mixed vegetable oil diet expressed above the control diet level expression at third day post inoculation whereas target gene expression of fish fed partial vegetable oil inclusion appeared down regulated with comparison to control diet.

Statistical analysis confirmed such statement and the scheffe test gave us the following significant difference between diets (Table XXXV).

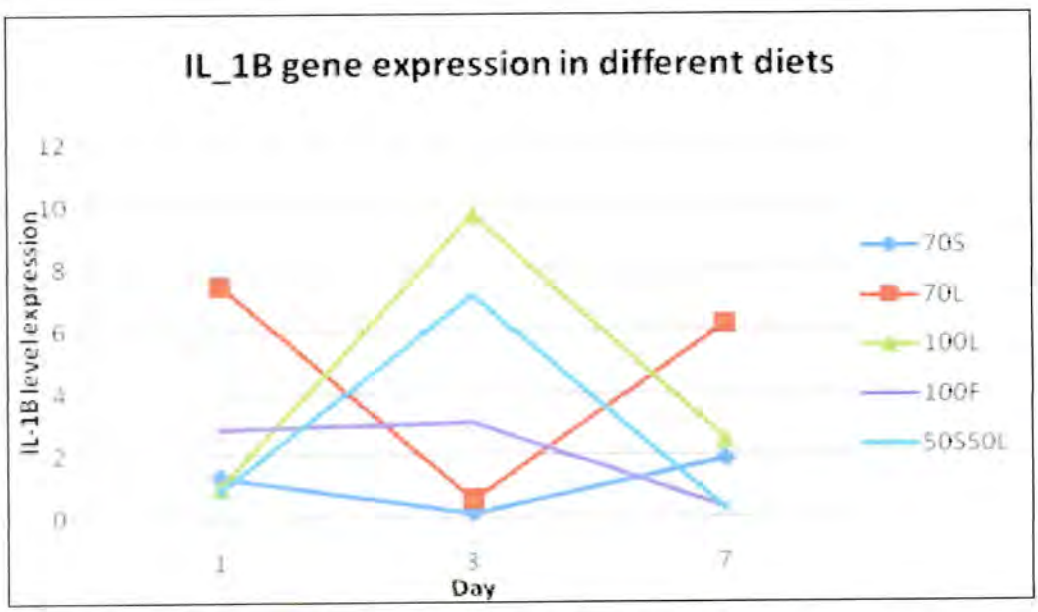


Figure 51: IL-1β gene expression in all diet during the experimental phase

Table XXXV: Different means values of IL-1β expression in all diets at third day post infection

Diet	Mean value
100F	3.04 $^{(bc)}$± 3.16
100L	9.74 $^{(c)}$ ± 1.27
100S	13.82 $^{(c)}$ ± 8.7
70L	0.56 $^{(ab)}$± 0.75
70S	0.14 $^{(a)}$± 0.14
50S50L	7.13 $^{(c)}$ ± 3.16

IV **Discussion**

As shown in previous studies (Izquierdo *et al.* 2003; Izquierdo *et al.*, 2005; Ganga, 2005; Montero *et al.*, 2008) the substitution of 60% and 70% dietary fish oil by vegetable oils did not affect growth of juveniles of gilthead sea bream. However, complete substitution (100S and 100L) affect negatively the growth and template fed such diet demonstrated lower body weight reached during the experimental period.

This statement appeared en agreement with many studies carried out in the same purpose but with other species such as yellowtail (*Seriola sp.*) (Watanabe, 2002), turbot (*Psetta maxima*) (Regost *et al.*, 2003), European sea bass (*Dicentrarchus labrax*) (Mourente *et al.*, 2005; Yildiz *et al.*, 2004) and sharp snout sea bream (*Diplodus puntazzo*) (Piedecausa *et al.*, 2007). The amount of vegetable oils included in diets for marine fish is limited by the quantity of fish oil incorporated to cope with the requirement of essential fatty acids for the species studied and the total amount of dietary fat.

Concerning the conversion index and specific growth rate, no significant difference was found among fish fed different experimental diets during the 70 experimental days (Ganga, 2004). Nevertheless a bit difference started to manifest between subjects fed diet with partial o total substitution of fish oil. In fact, our results agreed with what has been mentioned in bibliography about the increase of conversion index and the decrease of specific growth rate with inclusion of 80% of vegetable oil for the case of sea bream (Grasso, 2006; Montero et *al.*, 2003) as well as other species such as sea bass (Montero et *al.*, 2005).

Photobacteriosis, described also as pasteurellosis, is caused by the halophilic bacterium *Photobacterium damselae* subsp. *Piscicida*. It is an increasingly important pathogen for several marine fish species, causing mortalities mainly in larvae and juveniles less than 50g (Toranzo *et al.*, 2005). Innate immunity is the first line of defense in fish and other invertebrates and therefore has a

relevant role after body injuries and infection. Inflammatory cytokines such as interleukins and tumor necrosis factors that participate in acute phase response are one of the most studied.

In this study we observed a basal expression for both target genes TNFα and IL_1β in the proximal intestine. The magnitude of expression obtained for the later was a bite less marked than TNFα. Such expression was more induced after infection (Pelegrin *et al.*, 2001). Castillo *et al.*, (2002) confirmed the constitutive expression of TNFα in all seabream tissues. They found that whatever that is about a systemic immune tissue or not, samples from fish challenged with not virulent strain of *V.anguillarum,* expressed at the same way of healthy fish. Furthermore, in vitro assays failed to enhance macrophage gene expression up the baseline level. However, the same authors suggested that contradictory results were obtained when working with virus sub-lethal dose.

Muelder *et al.*, (2007) reported through their experiment with rainbow trout the non constitutive expression of TNFα in head kidney, proximal and distal intestine whereas trout gene was mentioned constitutively expressed in the gill of unstimulated fish in previous studies (Laing *et al.*, 2001). Moreover, no one of examined tissues from healthy Japanese flounder demonstrated accumulating TNFα mRNA (Hirono *et al.*, 2000).

In the second part of our study we focused inters in gene profile expression during the time course of post infection with *Photobacterium damselae* subsp. *Piscicida*. Real time PCR data shown a significant over expression of TNF as well as IL_1β at third day post inoculation. Nevertheless, the former was 3 times higher. This over expression had the tendency to decrease or reaching basal level at the seventh day. Such deduction agreed with recent study carried out by (Poisa-Beiro *et al.*, 2008). They demonstrated through

intramuscular viral infection of both seabream and sea bass fish an over expression of the same genes in the brain, at 3 days post infection. Authors suggested that up regulation of the pro- inflammatory cytokine in immune tissue sample seems to be earlier than in another such the brain. In fact a strong up regulation of TNF expression was detected 1 day post infection for both species in head kidney. This up regulation was no longer obvious 3 and 7 days after infection.

Rojo et al., 2007 reported through intraperitoneal infection of zebrafish by a LD_{50} of *listonella anguillarum*, a progressive increase in the TNF and IL_1 β spleen expression. Such expressions reach their maximum levels at 24 hours post infection. Same statement was deduced for turbot infected by *L. anguillarum*. Specifically, IL_1 β mRNA levels increased progressively reaching a maximum at 24 h and then declined at 72 h (Chai *et al.*, 2006).

This pattern of induction for the IL_1 β gene through zebrafish infection is also in accordance with results from other inoculation experiments using Gram-negative and -positive bacteria (Lin *et al.*, 2007).

Gonzalez *et al.*, 2007 shared the same point of view with later authors and précised that such up regulation occurred at third day post ectoparasite infection of the carp (*Cyprinus carpio* L.) challenged with *Ichthyophthirius multifiliis*, and decrease subsequently.

All previous studies converge at the same conclusion that pro-inflammatory cytokines reach their over expression in systemic immune tissues before their over expression in local level were the pathogen proliferates. This statement was in accordance with studies carried out with rainbow trout (Sigh *et al.*, 2004; Lindenstrom *et al.*, 2004).

The effects of *n*-3 PUFAs on immune system have been reviewed several times in recent years. All studies were been in accordance that vegetable oil inclusion in fish diet alters lipid membrane composition and affect negatively the macrophage phagocytic as well as serum alternative complement pathway activity (Montero *et al* ,1998; 2003; 2008). This drastic affect was been detected for many species either of warm or cold water but with modulated effect due the different physiology of both, especially in which is related with desaturation ability of LA and LNA.

Little are studies that focused on the n-3 PUFA effect on cytokines production in fish. All studies dealing with this topic are been carried on mammals and agreed with the anti-inflammatory effect of such lipids through in vitro macrophages culture as well as in vivo feeding subjects. (Meydani *et al.*, 1991; 1992, 1993)(Endres *et al.*, 1993). In a recent study carried out by Kesavalu *et al.* (2007),testing the hypothesis of possible anti-inflammatory effect of omega 3 fatty acids in the gingival tissues of porphyromonas gingivalis-infected rats, researchers postulated that supplementing the diet with n-3 PUFA decreased pro-inflammatory gene expression (IL-1β and TNFα) and enhanced IFNδ messenger RNA. Nevertheless, in 2003, Sijben *et al.*, assumed that dietary n-3 polyunsaturated fatty acid administration (corn oil, linseed oil, menhaden oil and beef tallow) to chicken challenged or not with *Salmonella typhimurium* lipopolysaccahride not modulate several splenic cytokines genes expression such as IL-6, IL-8, IL-15, IL-18 whereas only IFN-δ mRNA was significantly higher in the chickens fed the fish oil enriched diet compared with the LO, CO and BT.

As a sum up of all reviewed studies, we may suggest that probably n-3PUFA affect differently innate immune system comparing between mammals, birds and why not fish template. This depends not only to the genetic background of

samples but also to the experimental design. Front of this variety of experimental protocol, it wasn't able to generalize such effect.

Following the concept of the eicosanoides regulator effect of cytokines production it is possible suggesting that since dietary lipids affect eicosanoid production, it might be expected that dietary lipids, especially those containing *n*-3 PUFAs, will affect cytokine production. It was been proved that high contain of dietary linolenic acid leads to the suppression of ARA-derived eicosanoids in Atlantic salmon (Bell et al., 1993). Ganga *et al.* (2005), through their study of the effect of different levels of substitution of fish oil by until 60% of vegetable oil rich en oleic, linoleic and and linolenic acids on juveniles gilthead seabream not proved significant alteration of ARA-derived eicosanoids PG_2 production in plasma among different diets, whereas the PG_3 in plasma was reduced due to the decrease of plasma EPA. This unbalanced production of PG_3 and alteration in DHGLA/ARA ratio in vegetable oil inclusion diets could be one of the responsible factors of changes in cytokine production. It´s important to mention that basic lipid amount derived from fish meal in diet affect the possible alteration that could cause vegetable oil inclusion in diet. Thus such effects not are similar among diets with 22% of constant lipid contain and those with only 15%. Many reviews reported the antagonist effect of PG_1 in cytokines synthesis as well as high levels of DHGLA acids (Utsunomiya *et al.*, 2000; James *et al.*, 2000).

In this study, we have tried to elucidate the modulator dietary n-3 PUFA effect on cytokines gene expression. First, it was difficult to suggest any thing on the basis of basal level expression for both target genes (TNFα and IL-1β) since control fish seem to be expressing in the same way in all diets. Although, a little difference was been observed among expression of templates fed partial o total vegetable oil substitution. Such difference was more induced after bacterial challenge with *Photobacterium damselae*. The profile gene expression for both

target genes revealed almost the same behavior what make suggesting the synergetic effect of TNFα and IL-1β (secombes *et al.*1996). Expression gene pattern shown up regulation at third day post infection for subject fed diet very rich en LA and LNA whereas early expression was detected with individual fed partial vegetable oil inclusion since first day post infection. Obtained pattern at third day post inoculation seemed to be similar to what was advanced in the study of Mx gene expression of gilthead seabream down total substitution of fish oil by vegetable oil in (Montero *et al.* 2008).

It´s difficult to give explication to this modulator effect of vegetable oil inclusion, may be the distinct ratio of OA/DHA that appeared lower in partial diet vegetable oil inclusion compared to total substitution could direct investigation to more profound study.

In summary, the present study demonstrated partially that inclusion of high levels of vegetable oils in diets not effect only physiologic parameters of immune system as it was widely described in the last decade for many species en particularly gilthead seabream, but took also genetic expression of many genes related with innate immune system. Montero *et al.* (1998, 2003, 2008), proved that dietary inclusion of vegetable oils affected both humoral and cellular immune parameters in gilthead sea bream, reducing the alternative complement pathway activity in serum and the phagocytic activity of head kidney leucocytes. Ganga *et al.* (2005) mentioned that high vegetable substitution alter EPA concentration en plasma which affect negatively ARA-eicosanoides production. Such change may be explaining modulation of cytokines production.

Modulator effect of vegetable oil substitution in gene expression in fish was mentioned newly by Montero *et al.* (2008) and our study is the second that deals with such topic. This is a new area where more research, in different immune tissues and more target genes, are required to investigate the possible

suppressive effect of dietary vegetable oils in seabream and its interaction with gene expression.

V Conclusion

- ✓ We confirm that vegetable oils can be use in diets for seabream at percentage of substitution up to 70% without affecting its growth, but extreme substitution may reduce the growth.
- ✓ Vegetable oil substitution not affect hardly basal level expression of TNF α and IL-1β
- ✓ TNF α and IL-1β expression are up regulated three days post infection after *Photobacterium damselae* challenge in the control diet. This gene pattern decrease in the following days
- ✓ Vegetable oil inclusion modulates TNF α and IL-1β gene expression. Fish fed diet with total substitution of fish oil expressed differently to those fed diet with partial substitution.

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Annex II

Table : Data integrity and concentration of RNA of all control and infected samples during different sampling point for all diets

Name	Final conc. (µg/µl)	A260/A280
100F 0h1	0,213	1.99
100F 0h2	1,737	1.96
100F 0h3	0,504	1.86
100F 1d1c	1,249	1.77
100F 1d2c	0,583	2.02
100F 1d3c	1,461	2.01
100F 1d1i	1,383	1.96
100F 1d2i	1,743	1.90
100F 1d3i	1,259	1.95
100F 3d1c	1,441	1.71
100F 3d2c	2,068	1.70
100F 3d3c	1,560	1.87
100F 3d1i	2,050	1.82
100F 3d2i	1,967	1.86
100F 3d3i	2,747	1.84
100F 7d1c	1,104	1.90
100F 7d2c	1,105	1.92
100F 7d3c	1,504	1.82
100F 7d1i	2,029	1.95
100F 7d2i	1,728	1.80
100F 7d3i	1,274	1.81
100L 0h1	1,013	1.88
100L 0h2	0,388	1.94
100L 0h3	2,038	1.92
100L 1d1c	0,424	1.92
100L 1d2c	2,670	1.89
100L 1d3c	1,243	2.01
100L 1d1i	1,425	1.96
100L 1d2i	3,165	1.91
100L 1d3i	1,688	1.95
100L 3d1c	1,615	1.50
100L 3d2c	1,380	1.89
100L 3d3c	1,484	1.80
100L 3d1i	2,043	1.75
100L 3d2i	1,708	1.76
100L 3d3i	1,771	1.70
100L 7d1c	1,412	1.92
100L 7d2c	1,165	1.94
100L 7d3c	1,881	1.79
100L 7d1i	1,151	1.94
100L 7d2i	2,220	1.82

100L 7d3i	0,953	1.78
100S 0h1	1,287	1.92
100S 0h2	1,733	1.95
100S 0h3	0,895	1.98
100S 1d1c	0,762	1.86
100S 1d2c	2,665	1.90
100S 1d3c	2,685	1.87
100S 1d1i	1,860	1.93
100S 1d2i	2,205	1.93
100S 1d3i	1,855	1.92
100S 3d1c	2,241	1.64
100S 3d2c	1,639	1.83
100S 3d3c	2,461	1.85
100S 3d1i	2,593	1.70
100S 3d2i	2,304	1.83
100S 3d3i	3,172	1.73
100S 7d1c	1,530	1.92
100S 7d2c	1,514	1.95
100S 7d3c	0,748	2.00
100S 7d1i	2,238	1.83
100S 7d2i	1,152	1.86
100S 7d3i	1,017	1.86
70L 0h1	0,621	1.83
70L 0h2	1,357	1.96
70L 0h3	0,000	0.00
70L 1d1c	0,602	1.92
70L 1d2c	1,670	1.88
70L 1d3c	1,928	1.88
70L 1d1i	1,860	1.88
70L 1d2i	1,936	1.93
70L 1d3i	1,654	1.91
70L 3d1c	1,828	1.61
70L 3d2c	1,346	1.87
70L 3d3c	0,930	1.80
70L 3d1i	1,461	1.72
70L 3d2i	0,762	1.83
70L 3d3i	2,980	1.83
70L 7d1c	1,490	1.91
70L 7d2c	1,062	1.91
70L 7d3c	0,775	2.00
70L 7d1i	2,160	1.85
70L 7d2i	1,352	1.86
70L 7d3i	1,342	1.85
70S 0h1	0,545	1.90
70S 0h2	0,467	2.04
70S 0h3	0,790	1.94
70S 1d1c	0,337	2.02
70S 1d2c	2,490	1.89
70S 1d3c	1,468	1.91
70S 1d1i	0,988	1.99
70S 1d2i	2,550	1.8
70S 1d3i	1,640	1.98

70S 3d1c	2,685	1.54
70S 3d2c	1,338	1.86
70S 3d3c	2,556	1.8
70S 3d1i	4,258	1.74
70S 3d2i	1,649	1.82
70S 3d3i	3,330	1.72
70S 7d1c	1,150	1.90
70S 7d2c	0,786	1.95
70S 7d3c	0,855	1.97
70S 7d1i	0,783	1.90
70S 7d2i	1,309	1.75
70S 7d3i	1,316	1.83
50S 0h1	1,525	1.70
50S 0h2	1,170	1.63
50S 0h3	0,721	1.66
50S 1d1c	0,910	1.73
50S 1d2c	0,977	1.67
50S 1d3c	0,752	1.74
50S 1d1i	1,615	1.64
50S 1d2i	1,552	1.72
50S 1d3i	1,178	1.61
50S 3d1c	1,638	1.64
50S 3d2c	1,588	1.90
50S 3d3c	1,880	1.92
50S 3d1i	2,570	1.72
50S 3d2i	2,314	1.78
50S 3d3i	4,329	1.75
50S 7d1c	1,565	1.58
50S 7d2c	1,390	1.65
50S 7d3c	0,928	1.73
50S 7d1i	1,208	1.59
50S 7d2i	0,413	1.58
50S 7d3i	0,458	1.63