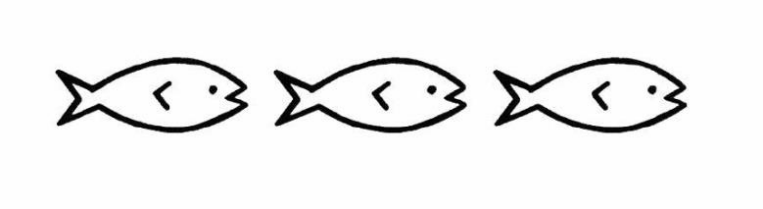
UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Study of novel carbohydrate sources on rainbow trout "*Oncorhynchus mykiss*" diets



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Valencia, Junio 2018

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

INSTITUTE OF ANIMAL SCIENCE AND TECHNOLOGY

RESEARCH GROUP OF AQUACULTURE AND BIODIVERSITY

Study of novel carbohydrate sources on rainbow trout "*Oncorhynchus mykiss*" diets

A thesis submitted to the Universitat Politècnica de València in partial fulfilment of the requirement of the requirements for the degree of doctor by

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"¿Qué sabe el pez del agua dónde nada toda su vida?". **Albert Einstein.**

"Todo somos genios. Pero si juzgas a un pez por su habilidad de escalar un árbol, vivirá su vida entera creyendo que es un estúpido". **Albert Einstein.**

"La vida no debería ser un viaje hacia la tumba con la intención de llegar a salvo con un cuerpo bonito y bien conservado, sino más bien, llegar derrapando de lado, entre una nube de humo completamente desgastado y destrozado y proclamar en voz alta ¡Uf! ¡vaya viajecito!". **Hunter S. Thompson.**

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RESUMEN

En la presente Tesis Doctoral se utilizaron cebada y remolacha como fuentes alternativas de carbohidratos en dietas para trucha arcoíris con el objetivo de evaluar su efecto sobre parámetros productivos, histología hepática e intestinal, estrés y calidad de la carne.

Los marcadores estudiados fueron los mismo tanto para los experimentos de cebada como de remolacha. En las pruebas de la cebada se utilizaron concentraciones crecientes de este ingrediente (0-32%) en la dieta, se inició con un peso medio de 127.72 ± 5.65 g y se finalizó cuando alcanzaron el peso comercial al cabo de 84 días. En las pruebas de remolacha se utilizaron dos niveles de remolacha (14% y 28%) y dos de betaína (0.9% y 1.63%), se inició con un peso medio de 69 ± 2.2 g y se finalizó cuando los peces alcanzaron el peso comercial al cabo de 105 días.

La inclusión de cebada en dietas de trucha arcoíris no mostró diferencias significativas en los parámetros de crecimiento ni biométricos. La inclusión de 14% remolacha y 0.9% betaína no afectó al crecimiento, parámetros nutritivos, biométricos y retenciones de nutrientes en comparación con el control, concentraciones mayores de remolacha y betaína tuvieron un efecto negativo.

Cuando se estudiaron los parámetros histológicos y morfométricos en hígado e intestino, los resultados mostraron que los peces alimentados con concentraciones crecientes de cebada mostraron hepatocitos más pequeños, mientras que los peces alimentados con remolacha y betaína presentaban hepatocitos más grandes, en ambos casos el hígado mostró un menor nivel de vacuolización. La concentración de cebada de un 8% produjo un efecto negativo a nivel morfológico del intestino, sin embargo, la inclusión de remolacha no produjo ningún efecto.

RESUMEN

Los peces fueron sometidos a estrés por ausencia de oxígeno y aumento de densidad durante 10 minutos. Cuando el estrés fue analizado a nivel bioquímico los resultados mostraron que a niveles basales, la inclusión de cebada y remolacha no causó ningún cambio en los niveles de cortisol, glucosa y MDA, aunque los valores basales de lactato fueron significativamente más bajos en los peces alimentados con remolacha y betaína. Treinta minutos después del estrés la concentración de cortisol, glucosa y lactato aumentó significativamente en los peces de todos los grupos y la concentración de MDA disminuyó. La inclusión de cebada mostró valores más bajos de cortisol, glucosa y lactato que la dieta control.

Cuando los peces alcanzaron el peso comercial se analizó la calidad de los filetes y las propiedades antioxidantes. En ambas pruebas los peces alimentados con los ingredientes objeto de estudio mostraron valores de actividad de agua más bajos y una mejora en la textura y el color de los filetes comparado con los peces alimentados con la dieta control. La inclusión de remolacha y betaína no tuvo ningún efecto sobre los parámetros oxidativos del filete, mientras que la inclusión de cebada inhibió su oxidación lipídica. Concentraciones de cebada por encima del 8% mejoraron la actividad antioxidante de los filetes y aumentaron el contenido en alpha-tocoferol, sin embargo, los peces alimentados con remolacha y betaína mostraron un mayor contenido en flavonoides y fenólico pero no se observó ningún efecto sobre las propiedades antioxidantes del filete.

Por último, cuando se llevó a cabo el análisis sensorial se observó que los peces alimentados con dietas con más de un 8% de cebada mostraron unas agallas más rojas y mejor textura, además de un color rojo del filete más intenso, parámetros que se correlacionan con una mejora de la frescura del pescado. Sin embargo, la inclusión de cebada o remolacha en dietas de trucha arco iris no tuvo ningún efecto sobre la

aceptabilidad de los filetes.

ABSTRACT

The current Doctoral Thesis used barley and red beet as alternative carbohydrate sources in rainbow trout diets. The aim was to evaluate their effect on productive, histological and morphometric parameters, their effect on biochemical indexes after an acute stress and their effect on the quality of rainbow trout (*Oncorhynchus mykiss*).

The different markers studied were the same in the barley and red beet experiments. In the barley experiment, increasing concentrations of barley (0-32%) were used in the diet, starting with an average weight of 127.72 ± 5.65 g and ending when they reached commercial weight after 84 days. In the red beet experiment, two red beet levels (14% and 28%) and two betaine levels (0.9% and 1.63%) were used, starting with an average weight of 69 ± 2.2 g and finishing when they reached commercial weight at the end of 105 days.

The inclusion of barley in rainbow trout diets did not show significant differences in growth and biometric parameters. While the inclusion of 14% of red beet and 0.9% betaine did not affect growth, nutritive or biometric parameters and nutrient retentions compared to control, higher red beet and betaine concentrations showed a negative effect.

When the histological and morphometric parameters were studied in liver and intestine, the results showed that fish fed at high barley concentrations showed smaller hepatocytes than control, while hepatocytes were larger in fish fed with red beet and betaine than control, however, in both cases, the liver had a lower level of vacuolization. Barley inclusion at 8% produced a negative effect at intestine morphological level; however, no effects were observed with the inclusion of red beet.

Fish were submitted to stress, a lack of oxygen and increased of fish density,

during 10 minutes. The results showed that at basal levels, the inclusion of barley and red beet did not cause any change in cortisol, glucose and MDA levels, although basal lactate values were significantly lower in the fish fed with red beet and betaine. Thirty minutes after stress the concentration of cortisol, glucose and lactate increased significantly in the fish of all groups and the concentration of MDA decreased. The inclusion of barley showed lower cortisol, glucose and lactate values than control.

When fish reached commercial weight, the quality of the fillets and antioxidant properties were analysed. In both trials, fish fed with the experimental ingredients showed lower water activity values and textural and colour properties were enhanced. Red beet and betaine inclusion did not show any effect on the oxidative parameters of the fillets, while the inclusion of barley showed an inhibitory effect on fillets lipid oxidation. Concentrations of barley above 8% improved the antioxidant activity of the fillets and increased the content of alpha-tocopherol, however, fish fed with red beet and betaine showed a higher content of flavonoids and phenolics but no effect on the antioxidant properties of the fillet.

Finally, when the sensory analysis was carried out, it was observed that fish fed diets with more than 8% barley showed redder gills and better texture than control, in addition to a more intense red colour of the fillet, these characteristics are correlated with an improvement of fish freshness. However, the inclusion of barley or red beet in rainbow trout diets had no effect on the acceptability of fillets.

RESUM

En la present tesi doctoral es van utilitzar ordi i remolatxa com a fonts alternatives de carbohidrats en dietes per a truita amb l'objectiu d'avaluar el seu efecte sobre paràmetres productius, histologia hepàtica i intestinal, estrès i qualitat de la carn.

Els marcadors estudiants fòren els mateixos en els experiments d`ordi I remolatxa. En les proves de l'ordi es van utilitzar concentracions creixents d'aquest ingredient (0-32%), es va iniciar amb un pes mitjà de 127.72 ± 5.65 g i es va finalitzar quan van arribar al pes comercial al cap de 84 dies. En les proves de remolatxa es van utilitzar dos nivells de remolatxa (14% i 28%) i dos de betaïna (0.9% i 1.63%), es va iniciar amb un pes mitjà de 69 ± 2.2 g i es va finalitzar quan els peixos van aconseguir el pes comercial al cap de 105 dies.

La inclusió d'ordi en dietes de truita no va mostrar diferències significatives en els paràmetres de creixement i biomètrics. La inclusió de 14% remolatxa i 0,9% betaïna no va afectar el creixement, paràmetres nutritius, biomètrics i retencions de nutrients en comparació amb el control, concentracions majors de remolatxa i betaïna van tenir un efecte negatiu.

Quan es van estudiar els paràmetres histològics i morfomètrics en fetge i intestí, els resultats van mostrar que els peixos alimentats amb concentracions creixents d'ordi tenien hepatòcits més petits, mentres que els peixos alimentats amb remolatxa i betaïna presentaven hepatòcits més grans, i en ambdós casos el fetge va mostrar un menor nivell de vacuolització. La concentracion d'ordi d'un 8% va produir un efecte negatiu a nivell morfològic de l'intestí, mentres que la inclusió de remolatxa no va produir cap efecte a nivell d'intestí.

Els peixos van ser sotmesos a estrès per absència d'oxigen i augment de densitat

durant 10 minuts. Quan l'estrès va ser analitzat a nivell bioquímic els resultats van mostrar que a nivells basals, la inclusió d'ordi i remolatxa no va causar cap canvi en els nivells de cortisol, glucosa i MDA, encara que els valors basals de lactat van ser significativament més baixos en els peixos alimentats amb remolatxa i betaïna. 30 minuts després de l'estrès la concentració de cortisol, glucosa i lactat va augmentar significativament en els peixos de tots els grups i la concentració de MDA va disminuir. La inclusió d`ordi va mostrar valors més baixos de cortisol, glucosa i lactat que la dieta control.

Quan els peixos van aconseguir el pes comercial es va analitzar la qualitat dels filets i propietats antioxidants. En ambdues proves els peixos alimentats amb els ingredients objecte d'estudi van mostrar valors d'activitat d'aigua més baixos i una millora en la textura i el color dels filets comparat amb els peixos alimentats amb la dieta control. La inclusió de remolatxa i betaïna no va tenir cap efecte sobre els paràmetres oxidatius del filet, mentre que la inclusió d'ordi va inhibir l'oxidació lipídica dels filets. Concentracions d'ordi per sobre del 8% van millorar l'activitat antioxidant dels filets i van augmentar el contingut en alpha-tocoferol, però, els peixos alimentats amb remolatxa i betaïna van mostrar un major contingut en flavonoides i fenòlic però no es va observar cap efecte sobre les propietats antioxidants del filet.

Finalment, quan es va dur a terme l'anàlisi sensorial es va observar que els peixos alimentats amb dietes amb més d'un 8% d'ordi van mostrar unes ganyes més vermelles i millor textura, a més d'un color vermell del filet més intens, paràmetres que es correlacionen amb una millora de la frescor del peix. No obstant això, la inclusió d'ordi o remolatxa en dietes de truita no va tenir cap efecte sobre l'acceptabilitat dels filets.

ABBREVIATIONS

AA	Amino acid
AD	Apparent Digestibility
ADC	Apparent Digestibility Coefficient
AIA	Acid-insoluble Ashes
ARA	Arachidonic acid
A_w	Water activity
B&D	Blight and Dyer
BCA	Bicinchonininc acid
BI	Biomass Increment
CF	Condition Factor
CHO	Carbohydrate
CF	Crude Fat
CP	Crude Protein
DHA	Docosahexaenoic acid
DP/DE	Digestible protein/Digestible energy
DPPH	1,1-diphenyl-2-picrylhydrazyl
dw	Dry weight
ECR	Economic Conversion Ratio
EDTA	Ethylenediaminetetracetic acid
EE	Ether Extract
EPA	Eicosapentaenoic acid
EPI	Economic Profit Index
EPI_{st}	Standarized Economic Profit Index
FA	Fatty Acid
FAME	Fatty Acid Methyl Esther
FCR	Feed Conversion Ratio
FI	Feed Intake
HPLC	High-Performance Liquid Chromatography
HSI	Hepatosomatic index
LC/MS	Liquid Chromatography/Mass Spectrometry
L_{max}	Maximum legth
L_{min}	Minimum legth
MDA	Malondialdehyde

ABBREVIATIONS

MUFA	Monounsaturated fatty acids
n-3	omega 3 fatty acid
n-6	omega 6 fatty acid
n-9	omega 9 fatty acid
ORAC	Oxygen Radical Absorbance Capacity
PUFA	Polyunsaturated fatty acids
PV	Peroxide value
QDM	Quality Descriptive Method
QIM	Quality Index Method
RACI	Relative Antioxidant Capacity Index
RAS	Recirculation Aquaculture System
RB	Red beet
RIA	Radioimmunoassay
ROS	Reactive Oxygen Species
SFA	Saturated fatty acids
SGR	Specific Growth Rate
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric acid Reactive Substances
TEAC	Trolox Equivalent Antioxidant Activity
TFC	Total Flavonoid content
TGC	Thermal Growth Coefficient
TP	Total Phenols
VSI	Viscerosomatic Index
Wi	Initial weight
Wf	Final Weight
WR	Working Reagent

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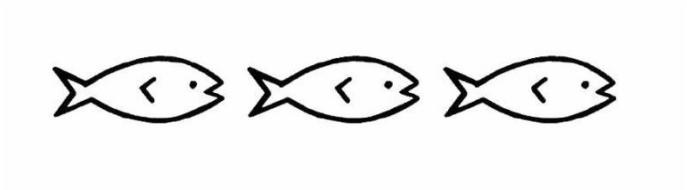
CHAPTER 1. GENERAL INTRODUCTION



1. Global aquaculture sector

Aquatic food production has undergone a shift from being mainly based on capture of wild fish to produce of increasing number of farmed species. Capture of wild fish has maintained stable since 1980s, however, aquaculture has significantly increased supplying most of the fish for human consumption (Figure 1), moreover it is an important source of employment that contributes to the sustainable rural livelihoods and indirectly improves human nutrition.

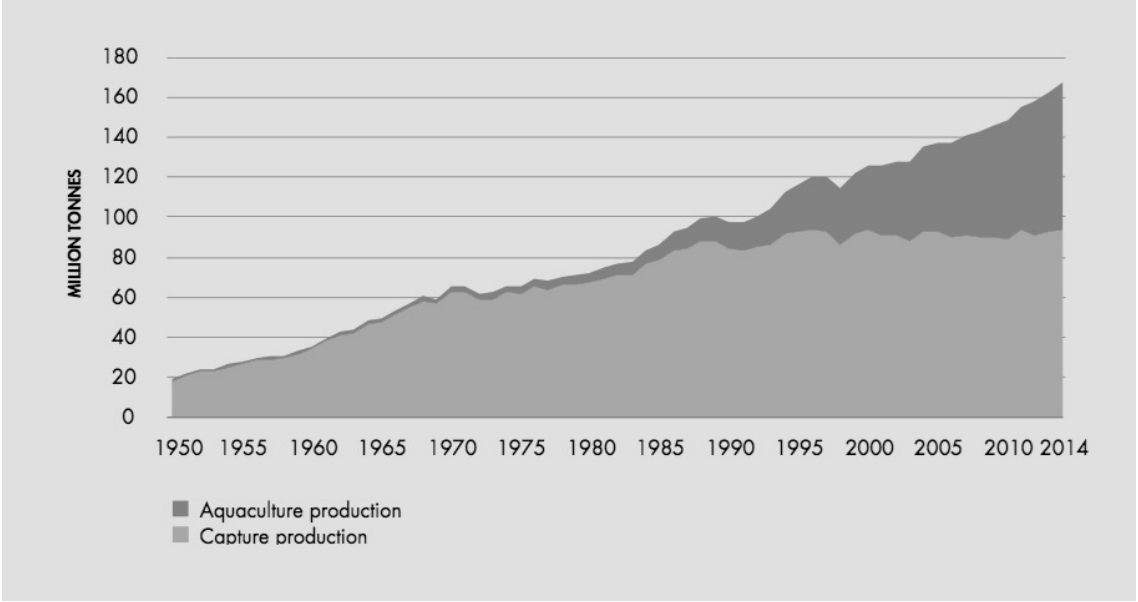


Figure 1. World capture and aquaculture production (FAO 2016. The state of world fisheries and aquaculture)

World per capita fish consumption (aquaculture and fisheries) has increased from 9.9 kg in the 1960s to 19.7 Kg in 2013 and has grown to 20 kg in 2014 and 2015. Fish consumption corresponded in 2014 to a 86.9% (146.3 million Tm) of the world total production. In addition to the production growth, other factors that have promoted fish consumption have been the reduction in wastage, better utilization of resources, improved of distribution channels and growing demand linked to population growth and

the rising incomes and urbanization. Also, international trade provides wider choices to consumers.

In 2013, fish supply about 17% of global population`s intake of animal protein and 6.7% of all protein consumed. Fish and fish products, in addition offers easily digested, high-quality proteins containing essential amino acids and provide essential fatty acids such as n-3 (eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA), vitamins (A, B, D) and minerals (calcium, iodine, zinc, iron and selenium).

The remaining 13.1% (20.9 million Tm) of the world fish production is not used for human consumption and is used for non-food products, of which 76% was reduced mainly for the manufacture of fish meal and fish oil, the rest it has been used as raw materials for direct use in aquaculture. Fish by-products are becoming an important industry with a focus on reducing waste. Fish meal and oil are still considered the most nutritious and digestible ingredients for farmed-fish feeds. However, as aquaculture increases, feed demand increases and fish meal and oil prices increase as well. In this sense to control their high prices, the volume of fish meal and fish oil used in aquaculture feed has shown a clear downward trend, using them as strategic ingredients at lower concentrations and for specific stages of production, such as hatchery, broodstock and finishing diets.

Sustainability of aquaculture is the main goal of its management. Today, aquaculture success is based on the adequate management of the specie biology, on the introduction of technologic innovations, on the development of specific-specie fish feed and on a good business organization (FAO, Declaración y Estrategia de Bangok, 2000). It is crucial to the livelihood, food security and nutrition of billions of people.

Global annual aquaculture production has been increasing at an average rate of

3.2% during the last five decades (APROMAR, 2017), being the global aquatic production in 2015 of 199.7 million Tm. All continents have shown a general trend of increasing share of aquaculture production, except Oceania where it has declined in the last three years. In the case of Europe, aquaculture is an important sector, being the total production in 2015 of 1.3 million Tm (Figure 2).

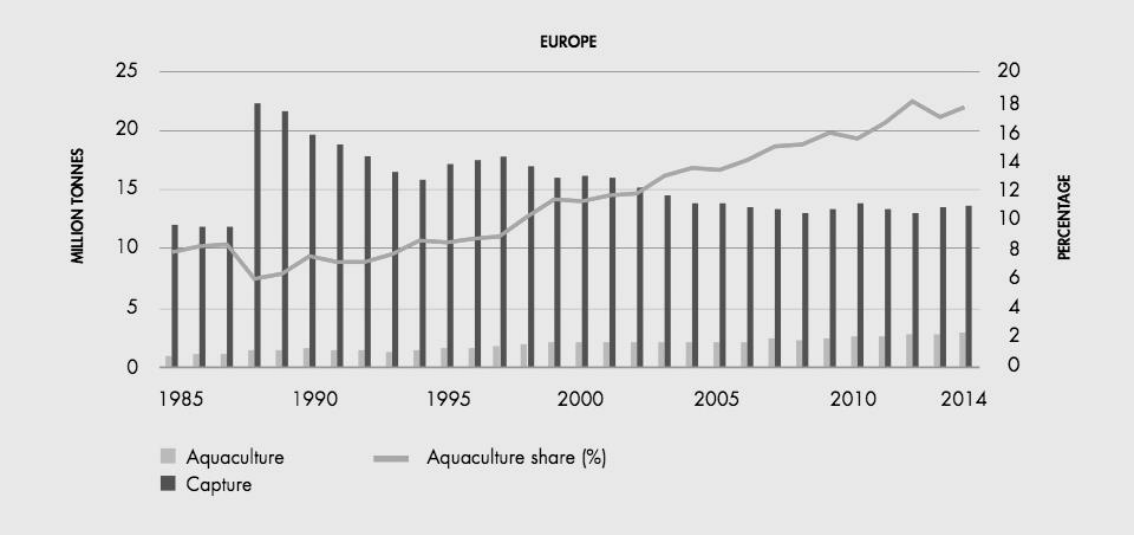


Figure 2. Share of aquaculture in total European production of aquatic animals (FAO 2016. The state of world fisheries and aquaculture).

The main groups of species produced from inland aquaculture and marine aquaculture differ among continents. The main fish specie produced in the European Union in 2015 is the Atlantic salmon (185995 Tm), followed by rainbow trout (185889 Tm) and seabream (82526 Tm). United Kingdom is the member state of the European Union with the highest aquaculture fish production (185189 Tm), the second is Greece (87290 Tm) and the third Spain (61810 Tm) in 2015 (APROMAR 2017). European aquaculture growth since 2000 has been very low. During the last decade its average growth has been 1.2% annually compared with the 6.4% of the rest of the world. In the specific case of Spain, annual increases were only 2% (aquaculture and fishery),

however, it is expected that aquaculture statistics will increase in the following years. In 2015 Spain sums a total of 289821 Tm of the total aquaculture production. The principal species produced were mussel (225300 Tm), seabass (21324 Tm), seabream (16231 Tm) and rainbow trout (16.179 Tm). In 2016, rainbow trout production increased 113% (17.732 Tm) and between the principal regions that produced rainbow trout is Castilla y León. Most of the rainbow trout are produced on freshwater (70%), however, some countries, such as Norway or Chile, its production finished on marine water.

2. Feed and Feeding

With the increase in intensive aquaculture, the demand for more efficient dry diets for fish is rising. Feed is the principal operating cost in the production of fish. When formulating aquafeeds, price and availability of ingredients are the most relevant factors. Therefore, the aquaculture industry is searching for feed ingredients for the sustainable development of the industry (Lupatsch et al. 1997, Stone 2003, Abro 2014, Couto et al. 2017). Incorporation of novel ingredients needs to be balanced of economic and product quality aspects in order to be successful (Pratoomyot et al. 2010, Valente et al. 2015).

2.1. Requirements

Fish must be fed adequately by using their nutrient requirements (Trichet 2010). Feeding animals with diets that do not contain nutrient requirements affects directly in the feed efficiency and therefore, in the growth, disease susceptibility, produces the appearance of deficiency signs such as altered behaviour and pathological changes and at the end leads on a loss quality of the final product (Oliva-Teles 2012). However, information about specific nutrient requirements of different aquaculture species is still

limited (Webster and Lim 2002) and only basic nutritional data are available to reassure minimum requirements in their diets.

Rainbow trout require all the nutrients found to be essential in the diets of other monogastric animals.

2.1.1. Protein requirements

There are several factors that make difficult to estimate dietary protein needs, such as fish size, water temperature, activity level, reproductive status, dietary energy level, feed ingredient costs and other variables. In practice, rainbow trout feed formulations generally contain between 35-50% protein depending on their production stage (Hardy 2002, Lovell 2002).

For an optimum physiological status, protein and energy levels should be coordinated (Oliva-Teles 2012). When the ratio DP/DE (Digestible protein/Digestible energy) is high in diets, fish will eat more protein than required for growth and the excess of protein will have negative economic and environmental impacts. On the contrary, when the ratio DP/DE in diets is low, fish will not eat the required protein for the maximum growth and increase fish fat content.

Fish meal is still the principal protein source in carnivorous fish feeds (Tacon and Metian 2008, Oliva-Teles 2012, 2015) due to its high protein content, adequate amino acidic (aa) profile, high palatability, high protein digestibility and its lack of antinutrients (Gatlin et al. 2007, Hardy 2010). However, the limited availability of fish meal in the world market urgently requires to reduce its use and search alternative protein sources in aquafeed (Watanabe 2002, Hardy 2008, Oliva-Teles 2012, 2015). Partial replacement of fish meal by alternative ingredients such as animal by-products and plant protein concentrates has been achieved successfully in rainbow trout (Slawski

et al. 2012, 2013); however, the total replacement of fish meal by an alternative protein source has been rarely achieved in carnivorous fish species. This option has to be considered by food industry because it is not always the most effective economical choice (Martínez-Llorens et al. 2012, Oliva-Teles 2015).

2.1.2. Lipid requirements

Lipids are the main energy source in carnivorous fish diets (Lovell 2002, Oliva-Teles 2012). Nowadays the trend in carnivorous fish production is to use energy-dense diets that are formulated with high lipid levels and the ability to use high dietary lipid levels on the diet is different between species (Oliva-Teles 2012, 2015). For this reason, there are limits on maximum lipid incorporation in diets without affecting growth performance and body composition.

Hardy (2002) and Lovell (2002) reported lipid levels in rainbow trout diets between 14-25% depending on their production stage. Rainbow trout, as other fish species and vertebrates, have n-3 polyunsaturated fatty acid requirements. Trout require between 0.5% and 2% n-3 fatty acids in their diet to prevent essential fatty acid deficiency signs (Hardy 2002, Lovell 2002). Trout can desaturate and elongate linolenic acid (C18:3) and produce EPA and DHA from this shorter-chain precursor, unlike marine species. Trout may also require small amounts of n-6 fatty acids, particularly arachidonic acid (C20:4). A level of 4-5% of marine fish oil in the diets provides a sufficient quantity level of n-3 polyunsaturated fatty acids (PUFA) for rainbow trout.

Fish oil is the main lipid source in aquafeed for most fish species, due to its n-3 PUFA composition it does not alter lipid composition and organoleptic properties of fish (Oliva-Teles 2012). However, as occurred with fish meal, fish oil availability it is limited worldwide, thus, at the expected rates of aquaculture increase, current fish oil

incorporation in fish feed will not be economically sustainable and should be partially replaced by vegetable oils or animal fats (Turchini et al. 2009, Oliva-Teles et al. 2012, 2015). Those alternative lipid sources are cheaper than fish oil and may provide all the essential fatty acids required by rainbow trout (Naylor et al. 2009, Turchini et al. 2009, Glencross and Turchini 2010, Tocher et al. 2010, Oliva-Teles et al. 2015).

2.1.3. Carbohydrates requirements

Carbohydrates are not essential nutrients in fish diets. They are mainly used as energy source and for their binding properties (Aderolu et al. 2009, Abro et al. 2014). They are important in formulated diets because they are cheaper than lipids and proteins and knowledge of the optimal level of protein and protein sparing effect on dietary carbohydrate may be useful in reducing the cost of fish diets (Abro et al. 2014). Dietary carbohydrate inclusion in several fish species diets produces positive effects on growth and digestibility (Watanabe 2002, Hung et al. 2003, Li et al. 2013, Abro et al. 2014). However, using the appropriate level of carbohydrates in feed is important to ensure the correct performance. High carbohydrates levels may have negative effects on nutrient utilisation, growth, metabolism and health (Li et al. 2012, Couto et al. 2017).

Carbohydrates fish utilization depends on the fish specie (carnivorous species tolerate lower levels of dietary carbohydrates than omnivorous or herbivorous species) and also on the carbohydrate source, molecular complexity of the molecule, processing treatments and dietary inclusion level (Krogdahl et al. 2005, Enes et al. 2009, Oliva-Teles 2012). Generally, it is recommended for carnivorous fish between 15-25% carbohydrates and for herbivorous fish between 25-40%.

2.1.3.1. Carbohydrate sources

Dietary carbohydrates have been widely used in fish diets to improve the feed

quality and to provide a source of energy (Vielma et al. 2003, Kamalam et al. 2017). Although grains and grain products are the main carbohydrate (CHO) sources in the diets of farmed fish and other livestock (Darunma et al. 2000, Sealey et al. 2008, Gaylord et al. 2009, Aderolu et al. 2009), an attempt at fulfilling the energy requirement of livestock through the use of root and tubers could ameliorate the stiff competition with cereals and grains (Agbede et al. 2002). Furthermore, to meet the annual increase of fish production, research should be targeted towards the use of alternative or unconventional feed ingredients such as root and tubers which could probably improve the food water stability and nutrient retention, increase efficiency of digestibility and reduce cost of fish feed production (Falayi et al. 2003 and 2004, Aderolu et al. 2009).

Wheat is the cereal traditionally used as carbohydrate source in commercial trout diets (Sealey et al. 2008, Gaylord et al. 2009). More cereal grains such as barley, oat and corn have been also traditionally utilized as carbohydrate sources in commercial salmonid diets (Sealey et al. 2008, Gaylord et al. 2009). Sealey et al. (2008) reported that the substitution of barley meal for wheat meal in a fish meal based diet did not alter growth performance or proximate composition of rainbow trout. Suárez et al. (2002) showed that the used of gelatinized maize starch in a level of 20% in rainbow trout diets did not modify weight gain, feed efficiency and protein body content. Arnesen et al. (1995) fed Atlantic salmon with increasing levels of oat meals and showed that fish fed with oat diets improved lipid digestibility, whereas no changes were observed in protein digestibility, however, a significant growth reduction was observed at high oat inclusion on diets.

Yamamoto et al. (2001) fed rainbow trout with different levels of gelatinized potato and showed that did not alter feed efficiency, protein efficiency ratio and starch digestibility of rainbow trout.

All those ingredients generally contain high fibre content and starch content and these, together with the presence of some antinutritional components, produce limitations to the inclusion of plant ingredients on carnivorous fish diets (Oliva-Teles et al. 2015). Also, some CHO sources produce a reduction of feed palatability, which leads to reduce fish intake and growth (Lim et al., 2016) and the presence of antinutritional components such as tannins, oxalates, phytates can produce a reduction on rearing parameters (Francis et al. 2001). On the other hand, plant ingredients can be an important source of antioxidant and other bioactive components (Ganessan et al. 2011). There are few studies that use alternative ingredients with bioactive compounds in order to promote fish quality and enhance oxidative stability.

2.1.3.1.1. Barley

Barley (*Hordeum vulgare*) is one of the most spread crops worldwide (Ferreres et al. 2009, Ghafoor 2014, Benito-Román et al. 2015). It is used for livestock feeding, however, its use in aquaculture is still scarce, although a few studies showed that its incorporation into fish feed did not have any detrimental effect on growth parameters (Sealey et al. 2008). Barley is rich in a wide range of antioxidant compounds such as phenolic acids, proanthocyanidins, quinones and flavonoids (Bonoli et al. 2004, Ferreres et al. 2009). The presence of phenolic compounds with antitioxidat activity and β-glucan, with prebiotic properties has renewed the interest in barley (Ghafoor 2014, Benito-Román et al. 2015). Phenolics are also present in barley, benozoic and cinnamic acid derivatives are present in the grain (Benito-Román et al. 2015).

Barley β-glucans in nature are in the cell wall; depending on the variety of barley, β-glucan content can range from 4 to 11% (Gatlin et al. 2007). The acceptance of β-glucans as a functional, bioactive ingredient has increased their popularity (Lazaridou and Biliaderis 2007) and potential due to their immunostimulant effect.

Different studies have been carried out to evaluate the beneficial effects of β-glucans on the growth and survival rates (Hai and Fotedar 2009; Lin et al. 2011), disease resistance and protection against pathogens (Dalmo and Bøgwald 2008; Lokesh et al. 2012), and immune system enhancement (Gu et al. 2011) in a wide range of aquaculture species (Sealey et al. 2008; Meena et al. 2013). In particular, several studies on trout have reported the growth enhancement when β-glucans were added on fish feed (Heidarieh et al. 2012; Ghaedi et al. 2015). Jeney et al. (1997) observed that low doses of β-glucans (0.1%) in the feed may prevent stress caused by transport.

Probably one of the reasons of the scarce use of barley in aquaculture is due to the presence of anti-nutritive components in its composition, such as phytic acid (Cheng and Hardy 2003). The presence of phytic acid limits the absorption of some minerals in diets such as phosphorus, zinc, and calcium caused by the formation of insoluble salts (Cheng and Hardy 2003; Overturf et al. 2003; Gaylord et al. 2009; Kumar et al. 2012). However, in order to decrease the presence of phytates, new varieties low in phytic acid levels have been developed (Overturf et al. 2003; Gaylord et al. 2009). Another limiting factor for the use of barley is the low protein content compared to that found in other different sources (soy, corn, etc).

2.1.3.1.2. Red beet

Red beet (*Beta vulgaris* L.) is a traditional vegetable all over the world and in recent years has attracted special attention as a health-promoting functional food product (Clifford et al. 2015, Nistor et al. 2017). It is a source rich in important nutrients including magnesium, sodium, potassium, vitamin C and betalains (Han et al. 2014). But also it is rich in valuable active compounds such as carotenoids, polyphenols, flavonoids, betalains and betaines. Red beet betaines and betalains have been widely studied for their nutritional and health benefits; since present a high radical scavenging

antioxidant activity (Paciulli et al. 2016). Betalains represents the principal pigment in red beet. Betaine has been reported widely to have antioxidant, antimicrobial and antiviral activities (Pedreno and Escribano 2001, Attia et al. 2013). In aquaculture, betaine is widely used as a common additive due to its bioactive properties as osmoprotector and enhancing feed palatability that promotes feed intake in some fish species (Tiril et al. 2008, Lim et al. 2015). Betaine has been extensively used in salmon feeding to protect them from the stress related to salinity variations (Virtanen 1995). And also, betaine plays an important role in cell metabolism substituting the aminoacid choline (Guérin 2000). Its incorporation could also enhance the quality of the final product, especially on the colour of fish flesh.

2.1.4. Vitamins and minerals

Vitamins are organic compounds required in trace amounts but essential in rainbow trout diets (Hardy 2002) for normal growth, reproduction and fish health (NRC 1993). Vitamin requirements are very similar between species (Oliva-Teles 2012). Halver et al. (2002) reported the vitamin requirements of some fish between of them there is rainbow trout (Table 1).

Table 1. Vitamin requirements of rainbow trout (mg/kg dry diet)

B1	B2	B6	B5	Niacin	Folacin	B12	myo-Inositol	Choline	Biotin	Ascorbate	A	D	E	K
10-12	20-30	10-15	40-50	120-150	6-10	R	200-300	2000-4000	1-1.2	100-150	2000-2500 IU	2400 IU	30	10

R means that it is a required mineral but level not known.

Fish mineral requirements are poorly studied. Most of the required minerals are obtained from the surrounding water and from the diet (Hardy 2002, Oliva-Teles 2012). The exchange of ions from the aquatic environment across gills and skin of fish complicates the determination of the quantitative dietary requirements (Lall 2002). Lall et al. (2002) reported the mineral requirements of some fish between of them there is

rainbow trout (Table 2).

Table 2. Mineral requirements of rainbow trout[a]

Species	Calcium (%)	Phosphorous (%)[c]	Potassium (%)	Magnessium (%)	Iron (mg)	Copper (%)	Manganese (%)	Zinc (mg)	Iodine (µg)[d]	Selenium (mg)
Rainbow trout	-[b]	0.6	R[e]	0.05	R	3	13	15-30	1.1	0.15-0.3

R means that it is a required mineral but level not known.

[a] Percentage or amount per Kg feed.

[b] Requirement below the level of detection under normal rearing conditions. When reared in low-calcium freshwater calcium requirements ranges from 0.03 to 0.65% of the diet.

[c] Inorganic phosphorus.

[d] The estimated iodine requirement of salmonids is 1.1 mg/kg (NRC, 1993).

[e] Essential in the diet but the quantitative requirement not reported.

2.2. Effect of feed on rainbow trout histology

Histological analysis of the digestive system is considered an important indicator of the nutritional status of fish (Caballero et al. 2003, Escaffre et al. 2007). The intestine and liver are the most important organs in digestion and absorption of nutrients from food (Rašković et al. 2011).

Several histological studies on diets with a higher proportion of vegetable protein and oil for salmonid have been described. However, the effect of the carbohydrate fraction on histological tissues has not been enough studied. The negative effects of plant ingredients are mainly due to the presence of non-starch polysaccharides and antinutritional factors (Mosberian-Tanha et al. 2017). The inclusion of soybean meal has shown to develop histological alterations in the intestine of Atlantic salmon and rainbow trout such as a shortening of mucosal folds, a loss of the normal supranuclear vacuolation of the absorptive cells in the intestinal epithelium, a widening of the central stroma within the mucosal folding, infiltration of inflammatory cells in the lamina propria or enteritis (Krogdahl et al. 2000, Refstie et al. 2000, Krogdahl et al.

2003). Feeding rainbow trout with lupin produce also intestine morphological changes such as a decrease in the number of basophil granulocytes, distal displacement of enterocyte nucleus, increment in lipid drops or a shortening of the villous height (Farhangi and Carter 2001, Borquez et al. 2011).

High levels of vegetable oil inclusion in fish diets have been showed to favour degenerations on histological structures of fish (Figueiredo-Silva et al. 2005, Yildiz et al. 2013), mainly associated to the accumulation of large lipid vacuoles in enterocytes and hepatocytes (Tucker et al. 1997, Olsen et al. 1999, 2000, Caballero et al. 2002, Figueiredo-Silva et al. 2005, Wassef et al. 2009, Yildiz et al. 2013). However, in rainbow trout the inclusion of different vegetable oils in the diet did not significantly change the liver histology. For example, Figueiredo-Silva et al. (2005) and Caballero et al. (2002) reported that soybean oil replacing fish oil up to 50% had no significant effect on liver histology of rainbow trout. Yildiz et al. (2013) also showed that liver and intestinal histology of rainbow trout fed with fish oil, cottonseed oil and canola oil were similar. Total replacement of fish oil by soybean oil resulted in a high hepatocytes vacuolation in livers of red drum (Tucker et al. 1997) and turbot (Bell et al. 1995). Feeding Arctic charr with linseed oil showed an accumulation of lipid droplets on the enterocytes from pyloric caeca and midgut (Olsen et al. 1999, 2000).

2.3. Effect of feed on stress

Under intensive culture conditions, fish are subjected to many factors such as stocking densities, handling, weighing, feeding, water quality, temperature, dissolved oxygen among other factors (Bertotto et al. 2010, 2011, Oliva-Teles 2012), which can induce a stress state and negatively affect the fish welfare. Also these factors can affect the performance and productive parameters, producing an important economic impact (Oliva-Teles 2012). For this reason it is clearly a decisive goal in aquaculture to find

different alternatives to manage stress. Pottinger (2003) showed the importance to investigate the feasibility of selectively farmed fish such as rainbow trout, in order to minimise their responsiveness to a common aquaculture stressor with better aquaculture practices and selecting fish genetics more resistant to stress (Øverli et al. 2006). The search of methods to reduce stress responses and/or strengthen immunity is an important area of study, specially the use of nutrients and other compounds such as ascorbic acid in gilthead seabream (Ortuño et al. 2003), in rainbow trout (Dabrowski et al. 2004), or in fish tambaqui (Chagas and Val 2006), vitamin E in gilthead seabream (Montero et al. 2001, Ortuño et al. 2003), fatty acids in gilthead seabream (Van Anholt et al. 2004), β-glucans in rainbow trout (Jeney et al. 1997) or in yellow croaker (Zeng et al. 2016), or betaine in Labeo rohita (Virtanen 1995, Kumar et al. 2012).

2.4. Effect of feed on rainbow trout quality

Fish quality includes a variety of aspects (Figure 3). Fish nutrition has an important impact on several parameters that affect directly to fish quality, such as nutritional parameters, colour, texture, sensory properties, lipid oxidation and shelf life. Consumers are becoming more concerned about how the fish are produced, which feed ingredients are used, etc. As fish quality, up to a point, remains in the hand of the aquaculture industry, it is necessary to increase knowledge regarding specific characteristics of farmed fish and how to standardize and/or manipulate them to contribute to the production of high quality fish (Rasmussen 2001).

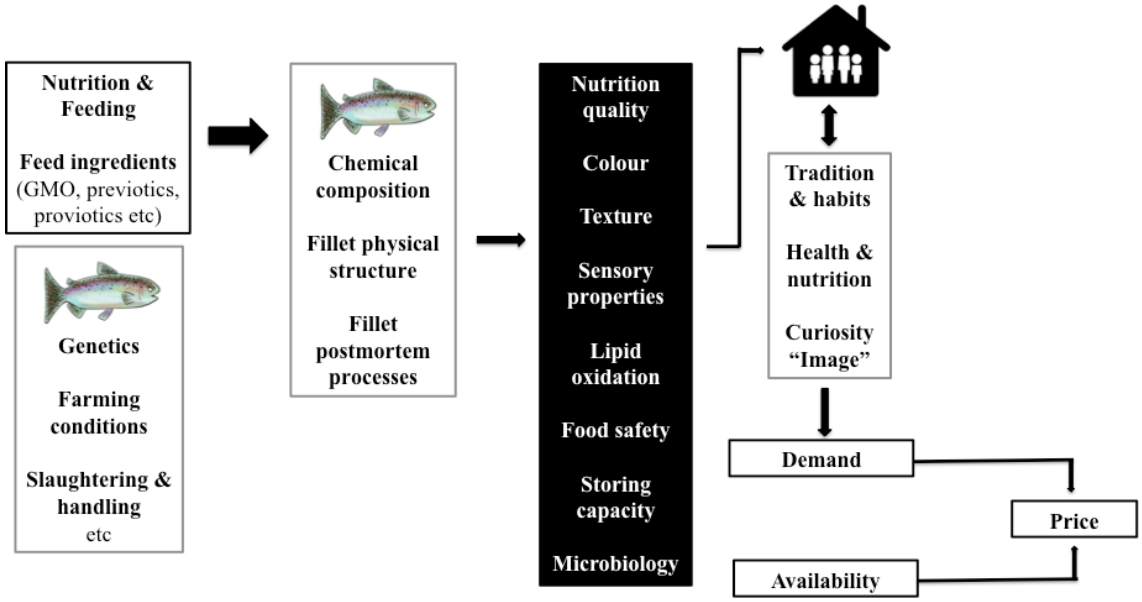


Figure 3. Overview of factor affecting fish quality parameters (Source: Lie (2001), Flesh quality - the role of nutrition. Aquaculture Research 32, 341-348, with some modifications)

2.4.1. Effect of feed on nutritional parameters

Nutritional quality of fish is related to the nutrient content and bioavailability of aquafeeds. Consumers have increased the interest on healthy food and nutritional quality of food and fish has gained special attention because it is an excellent source of proteins and fish lipid, especially of n-3 polyunsaturated fatty acids. High-lipid fish, such as rainbow trout, are the only natural source of vitamin D and fish in general provide B vitamins and minerals.

The evaluation of fish composition is generally related to lipid and protein content (Rasmussen 2001). Fish protein content or amino acid profile are not greatly modified by diets (Kim et al. 1992, Rønsholdt 1995, Rasmussen et al. 2000, Hardy and Lee 2010). This could be explained by the limited amount of protein that can be produced into the fish body upon feeding high protein rations (Rasmussen et al. 2000). In contrast, lipid content and fatty acid profile are directly related to dietary input. Over

the years, there is an interest on the production of feeds of high nutritive value that contain high energy levels by increasing lipid levels, and the use of these feeds, has been associated with a loss in fish quality (Rasmussen 2001). For example, lipid-rich diets increase both visceral and fillet fat content in rainbow trout and other fish species (Rasmussen et al. 2000, Regost et al. 2001, Rosenlund et al. 2010, Hung et al. 2017).

Fatty acid composition of fish fillets can be influenced by dietary lipid sources when rainbow trout are fed with different plant oils (Lie 2001, Sargent et al. 2002, Rinchard et al. 2007, Hardy and Lee 2010, Yildiz et al. 2013). Fish tend to accumulate high dietary levels of fatty acids such as linoleic (18:2 n-6), α-linoleic (18:3 n-3) or oleic (18:1 n-9) acid, reduced levels of total n-3 polyunsaturated fatty acids (PUFA) and increase levels of n-6 PUFA, however, saturated fatty acids are minimally affected by the inclusion of plant oils (Caballero et al. 2002, Sener and Yildiz 2003, Yildiz et al. 2013).

In the literature, not much attention has been paid to carbohydrates and their effect on fish fillet quality, and different studies have reported that dietary carbohydrates have no effect on the body composition of rainbow trout, although there is little information studied (Ki and Kaushik 1992, Sealey et al. 2008).

2.4.2. Effect of feed on rainbow trout colour

Colour in rainbow trout fillets is an important property. Spanish consumers expect trout fillet to be reddish-orange in colour, however, in other countries, such as Poland, they do not consider this characteristic as important as in Spain. Colour in fish skin and fillets results from the deposition of carotenoids. To obtain this pigmented skin or fillet it is necessary to supplement diets with carotenoids. Carotenoids sources used in fish feed can be obtained from natural sources, such as shrimp or krill meal or from

industrial production (Torrissen et al. 1995, Lie et al. 2001, Hardy and Lee 2010).

2.4.3. Effect of feed on rainbow trout fillet texture

Texture is one of the main quality parameters. Texture of fish is defined by its dryness, chewiness and juiciness (Lie 2001). In industry, it is commonly tested by the "finger method", which depends on a subjective evaluation of the person who does the test. However, instruments for the determination of texture are becoming more commonly used and different methods and analysis have recently been evaluated (Sigurgisladottir et al. 1999). Lipid content and distribution have effect on textural properties. Faergemand et al. (1995) reported that rainbow trout fillets with more than 20% lipids did not affect texture measurements. Specie, storage time, nutritional state and other factors, contribute on fish meat firmness. Compared with other animal meat, which contains up to 23% collagen, the collagen in fish is lower than 3%, which results in a tender product (Rasmussen 2001). The influence of diet on fillet texture has been evaluated in several studies. Andersen et al. (1997) reported that rainbow trout fed with high-lipid diets had softer fillets than those fed with low-lipid diets. However, other authors reported no differences on firmness, fattiness or hardness regarding the lipid content on diets (Bjerkeng et al. 1997, Rørå et al. 1998, Rasmussen et al. 2000, Regost et al. 2001).

2.4.4. Effect of feed on rainbow trout fillet lipid oxidation

Oxidation of fish lipids is the major quality problem leading to off-flavour compounds. Oxidation can also lower nutritional quality and modify texture and colour. There are several studies that investigate the use of different compounds to increase fillet oxidative stability. The higher level of antioxidants in fish tissue, the longer lipid oxidation is prevented. Different strategies have been proposed to prevent oxidative deterioration. Some of these strategies are focused on processing process such as

packaging and/or the use of antioxidants incorporated in food products. However, special attention during these years has been taking to the use of antioxidant ingredients on diet. These ingredients have been reported as a strategy to maintain fish good quality and control oxidative processes (Baron et al. 2009, García-Romero et al. 2014, Secci and Parisi 2016). Supplementing the diet with antioxidants enable these substances to be incorporated into the phospholipid membrane, which can effectively inhibit oxidation reactions (Lauridsen et al. 1997). Previous studies have reported the use of different antioxidants such as α-tocopherol, astaxanthin or canthaxanthin enhance the quality of different fish species protecting fish muscle against oxidative degradation. Jensen et al. (1998) showed that the addition of astaxanthin on rainbow trout diets protects against lipid oxidation during the early stages of oxidative deterioration. Choubert et al. (2011) reported the same effect of astaxanthin on rainbow trout diets during long-term frozen storage. Other authors have confirmed the effect of α-tocopherol or ascorbic acid as antioxidants in feed of different fish species: turbot (Scophthahus maximus) (Stéphan et al. 1995), rainbow trout (Oncorhynchus mykiss) (Chaiyapechara et al. 2003), hybrid tilapia (Oreochromis niloticus x O. aureus) (Huang et al. 2003), red seabream (Pagrus major) (Gao and Koshio 2015). New natural antioxidants have been utilised as feed additives such as thymol (Giannenas et al. 2012) or rosemary extracts (Álvarez et al. 2012, Hernández et al. 2014).

2.4.5. Effect of feed on rainbow trout sensorial properties

Sensory characteristics, as perceived by consumers, have important effects on the acceptance and market value of the product and they are very useful to be compared with the results of instrumental methods. The most common sensory attributes evaluated in fish quality are skin or fillet colour, texture, flavour and odour. Colour, flavour and odour can be altered by fish diet and its formulation. However, texture is

not easily modified, although there are several studies where fish fillet texture (not determined instrumentally) is modified by adding algae to fish feeds (Nakagawa and Montgomery 2007). Colour is an indicator of freshness and it is expected to be reddish-orange in rainbow trout. Flavour and odour attributes are related and it has been well studied that certain feed ingredients can alter them when use them in rainbow trout diets: rainbow trout fed with sunflower oil was less fishy than fish fed with fish oil (Skonberg et al. 1993). Rainbow trout were described as less sweet and with less odour intensity when a plant protein mixture replaced fish meal in the diet (De Francesco et al. 2004). Efforts to modify sensory attributes of farmed fish have to take into account consumers preferences and acceptability.

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CHAPTER 2. OBJECTIVES

The general objective of the current Doctoral Thesis is to develop a sustainable use of novel ingredients that allow improving aquaculture productivity and fish products quality. And within this objective, the development of a product with a differentiate quality, including ingredients, such as barley and red beet, with immunostimulatory activity and bioactivity which are both important at the production level and at the product level.

The specific objectives of this thesis are:

* Evaluate the effect of the inclusion of barley on growth performance, apparent digestibility, stress, histology and bioactivity on rainbow trout.
* Evaluate the effect of the inclusion of red beet on growth performance, apparent digestibility, stress, histology and bioactivity on rainbow trout.

CHAPTER 3. RESUME OF THE EXPERIMENTS

The current Doctoral Thesis consists on several experimental proofs (Table 3) in which barley and red beet were used as alternative ingredients as carbohydrate source. Barley diets contained increasing barley concentrations (0 – 32%) till the complete substitution of wheat. And on red beet experiments, diets contained two red beet (14% and 28%) and two betaine (0.9% and 1.63%) concentrations. 28% of red beet substitute the whole wheat fraction and the reason for using betaine was that betaine concentration on the red beet was very low (0.6%).

Table 3. Different experiments carried out on the current Doctoral Thesis

		Time (days)	Initial weight	Fish number	Diets*
Barley Experiment	Growth	84	127	500	CONTROL 40B 80B 160B 319B
	Stress	45	248	400	
	Histology	45	248	400	
	Quality	84	127	500	
	Bioactivity	84	127	500	
Red beet experiment	Growth	105	69	900	CONTROL A B C D
	Stress	45	250	400	
	Histology	45	250	400	
	Quality	105	69	900	
	Bioactivity	105	69	900	

* CONTROL (0% barley, 0% red beet); 40B (4% barley); 80B (8% barley); 160B (16% barley); 319B (31.92% barley); A (14% red beet, 0.9% betaine), B (14% red beet, 1.63% betaine), C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine).

CHAPTER 4. ENHANCEMENT OF QUALITY OF RAINBOW TROUT (*Oncorhynchus mykiss*) FLESH INCORPORATING BARLEY ON DIET WITHOUT NEGATIVE EFFECT ON REARING PARAMETERS.

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ABSTRACT

Barley concentrations ranging from 0% to 32% (0B, 40B, 80B, 160B and 319B) were incorporated into rainbow trout, *Oncorhynchus mykiss* (Walbaum) diets. The experiment started with an initial average fish weight of 127.72 ± 5.65 g and finished when they reached commercial weight (final weight between 312-330 g) after 84 days. The inclusion of barley in the diets did not show a significant effect on growth and biometric parameters, fat and carbohydrate digestibilities, however, protein digestibility decreased significantly with the incorporation of barley on diets. Glucose levels increased significantly with barley concentration in the diet and lactate and cortisol levels were also significantly affected after a stress period regardless of the diet. Meat quality was influenced as well by barley concentration. Lower water activity values and an enhancement in textural and colour properties, were observed in fish fed with the diet containing the highest barley concentration. Trout fed feed with higher concentrations of barley (160B) showed lower lipid oxidation levels than those fed with lower concentrations (control and 40B). The sensory panel found that fish fed with diets higher than 8% in barley content (80B), exhibited a brighter red colour in gills and a better texture, also meat colour became redder with a higher barley inclusion (160B and 319B), being all these sensory parameters correlated with fish freshness. Thus, results indicate that barley can be substituted for wheat fraction without any detrimental effect on production efficiency and enhancing fish quality.

***Keywords**: Barley, β-glucans, growth parameters, meat quality, trout diet*.

1. INTRODUCTION

In the course of just a few decades fish farming has evolved into a highly productive and efficient industry in animal protein production for human consumption (Caballero et al., 2002). Rainbow trout, *Oncorhynchus mykiss* (Walbaum) is one of the most important freshwater cultured fish worldwide. European rainbow trout production represents 21% (176.983 metric Tons in 2012; APROMAR, 2014) of the world production and Spain holds 10% of this production (14.009 metric Tons in 2015, MAGRAMA). Aquaculture requires nutrition optimization in order to raise fish with food production purposes efficiently (Hixson 2014).

Incorporation of novel ingredients need to balance economic and product quality aspects (Pratoomyot et al., 2010; Valente et al., 2015) without compromising sensory attributes and consumer acceptance. Cereals are usually incorporated in extruded diets of rainbow trout as a carbohydrate and starch source. Wheat is the cereal traditionally used as a carbohydrate source in commercial trout diet (Sealey et al., 2008, Gaylord et al., 2009), however, barley has not been used widely as an ingredient in aquaculture feed, although a few studies showed that its incorporation into fish feed did not have any detrimental effect on growth parameters (Sealey et al., 2008). Probably one of the reasons of the scarce use of barley is due to the presence of anti-nutritive components in its composition, such as phytic acid (Cheng and Hardy 2003). The presence of phytic acid limits the absorption of some minerals in diets such as phosphorus, zinc and calcium caused by the formation of insoluble salts (Cheng and Hardy 2003, Overturf et al., 2003, Gaylord et al., 2009, Kumar et al., 2012). However, in order to decrease the presence of phytates, new varieties low in phytic acid levels have been developed (Overturf et al., 2003, Gaylord et al., 2009). Another limiting factor for the use of barley is the low protein content compared to that found in other different sources (wheat, soy,

corn, etc).

However, barley presents many advantages due to its β-glucan content (Sealey et al., 2008; Meena et al., 2013). β-glucans in nature are in the cell walls of several plants such as barley, oats, rye and wheat at concentrations ranging from 2 to 7% and < 1% respectively. However depending the variety of barley β-glucan content can range from 4 to 11% (Gatlin et al. 2007). The acceptance of β-glucans as a functional, bioactive ingredient has increased their popularity (Lazaridou and Biliaderis 2007) and potential due to their immunostimulant effect. Different studies have been carried out to evaluate the beneficial effects of β-glucans on the growth and survival rates (Hai and Fotedar 2009; Lin et al., 2011), disease resistance and protection against pathogens (Dalmo and Bøgwald 2008; Lokesh et al., 2012), and immune system enhancement (Gu et al., 2011) in a wide range of aquaculture species (Sealey et al., 2008; Meena et al., 2013). In particular, several studies on trout have reported the growth enhancement when β-glucans were added on fish feed (Heidarieh et al., 2012; Ghaedi et al., 2015). Jeney et al. (1997) observed that low doses of β-glucans (0.1%) in the feed may prevent stress caused by transport.

The objective of the present work was to study the effect of the inclusion of barley, as an alternative ingredient in rainbow trout, *Oncorhynchus mykiss* (Walbaum) diets and evaluate the impact on growth performance, apparent digestibility, response to stress and final fish meat quality parameters.

2. MATERIAL AND METHODS

2.1. Production system

The trial was conducted in 20 cylindrical fiberglass tanks (500 L) within a freshwater recirculation system (RAS). Throughout the experiment temperature

remained constant at 13.58 ± 1.06 ºC and so were dissolved oxygen levels, kept between values of 9.18 ± 1.35 mg L^{-1}. All tanks were equipped with aeration and an oxygen probe. Water pH was 8.03 ± 0.07 and ammonia and nitrites concentration in water were 0.16 ± 0,23 and 0,15 ± 0.11 mg L^{-1} respectively. Water flow was 12.2 ± 0.5 L h^{-1}. The photoperiod consisted on 12 hours light and 12 hour dark intervals and all tanks had identical lightning conditions.

2.2. Fish and experimental design

A total of 500 rainbow trout from a commercial trout farm (IPEASA, Fuentidueña, Segovia, Spain) were used. Fish were randomly allocated in 20 tanks, 25 fish in each tank (initial stocking density 6.7 ± 0.4 kg m^{-3}). Prior to the feeding trial, all fish were acclimated to the indoor rearing conditions for 2 weeks and fish were fed once a day (8:00) to apparent satiation using exclusively a control diet. The study lasted 84 days.

Rearing parameters (growth (final weight, biomass increment, survival and SGR), nutritional parameters (FI and FCR) and biometric indexes (CF, VSI and HSI) and meat quality (proximate composition, water activity, colour, texture and sensory analysis) were evaluated approximately every 28 days. All fish were starved for 24 h and anesthetized with (MS222®; 200 mg L^{-1}) prior to taking weight and length measurements. Fish were randomly sampled from each tank to determine rearing and meat quality parameters during the growth period (0, 28, 56 and 84 days). At day 44, fish were controlled stressed by decreasing the concentration of oxygen from 8 to 4 mg L^{-1}. The concentration of oxygen was decreased by lowering water level to a volume of 50 L and removing the aeration. When the levels of dissolved oxygen in water reached 4 mg L^{-1} it is started to count 10 minutes in these conditions, reaching levels of < 2 mg L^{-1}. Biochemical parameters in blood plasma (glucose, lactate and cortisol levels) were

determined.

2.3. Diets and feeding

Five isoproteic (40% crude protein) and isolipidic diets (18% crude lipid) were developed containing different barley levels (0B (0% barley, 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.22% β-glucans); 160B (16% barley, 0.53% β-glucans); 319B (31.92% barley, 1.5% β-glucans). Control diet (0B) has been prepared with the same ingredients as experimental diets but without barley on the formulation. This diet was not a commercial diet. There were five feeding treatment groups each in four replicates (n=4).

The barley which was used corresponded to an H13 and bred variety from plant breeding of merlin and volga varieties, harvested in the 2012/2013 period and commercially known as GALIS. This barley is bare with a β-glucan content of 5.2%.

The formulation and composition of the diets are shown in Table 4. Diets were prepared by an extrusion process using a semi-industrial twin-screw extruder (CLEXTRAL BC-45. St. Etienne, France). Raw material was processed at a speed of 100 rpm, at 110ºC and a pressure of 40-50 atmospheres.

Table 4. Formulation and proximate composition of the experimental diets

	Diets[1]				
	0B	**40B**	**80B**	**160B**	**319B**
Ingredients (gKg^{-1}) - international feed number in parentheses					
Fish meal (5-02-000)	220	220	220	220	220
Wheat (4-05-268)	318	278	238	159	0
Barley	0	40	80	160	319
Wheat gluten	192	186	182	177	160
Meat meal	103	111	116	120	140
Soybean oil (4-07-983)	91	89	88	88	85
Fish oil (4-08-048)	45	45	45	45	45
Maltodextrin (4-08-023)	11	11	11	11	11
Multivitamin and minerals mix[2]	20	20	20	20	20
Analyzed composition (% dry matter)					
Dry matter	90.30	90.90	90.40	90.90	90.40
Crude Protein (% CP)	38.70	39.30	39.80	40.20	39.40
Crude Fat (% CF)	17.60	17.10	17.10	16.90	15.90
Ash (%)	7.50	7.70	7.90	8.00	8.40
Carbohydrate (% CHO)[3]	36.20	35.90	35.20	34.90	36.30
β-glucans (%)	0.00	0.14	0.22	0.53	1.50

[1] Different experimental diets: 0B (0% barley, 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.22% β-glucans); 160B (16% barley, 0.53% β-glucans); 319B (31.92% barley, 1.5% β-glucans).

[2] Vitamin and mineral mix (values are g kg^{-1} except those in parenthesis): Premix: 25; Choline, 10; DL-a-tocopherol, 5; ascorbic acid, 5; $(PO_4)_2Ca_3$, 5. Premix composition: retinol acetate, 1000000 IU kg^{-1};

calciferol, 500 IU kg^{-1}; DL-a-tocopherol, 10; menadione sodium bisulfite, 0.8; thiamine hydrochloride, 2.3; riboflavin, 2.3; pyridoxine hydrochloride, 15; cyanocobalamin, 25; nicotinamide, 15; pantothenic acid, 6; folic acid, 0.65; biotin, 0.07;ascorbic acid, 75; inositol, 15; betaine, 100; polypeptides 12.

[3] Carbohydrate (% CHO) were calculated by difference for all the nutrients: %CHO = 100 – (% CP + % CF + % Ash)

Fish were fed twice a day (8:00 am and 15:00), 6 days per week to apparent satiation level during the whole experimental period. Pellets were distributed manually to allow all fish to eat. The amount of pellets not consumed for fish were collected to determine feed intake (FI).

2.4. Apparent digestibility coefficients

Simultaneously to the feeding trial, digestibility studies were conducted. After fish were fed for a second time, tanks were completely cleaned and faeces were collected in a settling column (Cho et al. 1982), which was emptied in the following morning at 8:00 hours. Wet fecal content was then collected and dried at 60ºC for 48 hours prior to analysis (crude protein (CP), crude fat (CF), carbohydrates (CHO) and acid-insoluble ashes (AIA)). Over the whole experimental period, samples of feces were collected from each tank (n=4).

The ***apparent digestibility coefficients (ADCs)*** of protein, fat and carbohydrates in the diets tested were calculated according to the following formula:

$$ADC(\%) = 100 \times \left[100 - \left(\frac{marker\ in\ diet}{marker\ in\ faeces} \times \frac{PN\ in\ faeces}{PN\ in\ diet}\right)\right]$$

Where PN is the percentage of nutrient.

2.5. Biochemical parameters in blood plasma

To determine the stress response (hypoxia conditions, < 4 mg L^{-1} per 10 min) 3 fish per tank (n=3) were alternatively captured before stress conditions (basal levels),

during stress condition and after one and two weeks, to measure their ability to recover basal levels.

Blood samples were withdrawn from the caudal vein using 1 ml syringes (BD Plastipak) with ethylenediaminetetracetic acid (EDTA) as anticoagulant, 0.5 ml were centrifuged (Hettich Zentrifugen, Universal 320 R, Germany) at 5000 rpm for 20 min at 4ºC and the plasma was extracted to measure cortisol, glucose and lactate levels. Samples were stored at -80ºC till analysis.

Concentration of plasma cortisol was determined using the method described by Thomas (1992), using a Enzyme-Linked Immunosorbent Assay (DEMEDITEC CORTISOL ELISA® Ref. DE1887). Briefly, aliquots (20 µl) from plasma that were dispensed into appropriate wells and incubated with 200 µl of enzyme conjugate solution for 60 min at room temperature. After incubation the wells were rinsed 3 times with wash solution (400 µl per well) and incubate with 100 µl substrate solution for 15 min at room temperature. The enzymatic reaction was stopped by adding 100 µl of stop solution and the absorbance was measured at 450 nm with an absorbance microplate reader (Bibby Scientific Limited, Jenway 7315, United Kindom).

Concentration of glucose and lactate were measured by an enzymatic colorimetric assay, in particular by GOD-POD (SPINREACT® Ref. 1001191) and LOD-POD (SPINREACT® Ref. 1001330) method respectively (Kaplan and Pesce 1984). Briefly, aliquots (5 µl) from plasma samples were mixed with 500 µl of reactive and incubated for 10 min for glucose determination and 5 min for lactate determination at 37ºC in dark. The absorbance was determined at 490 nm in a 96-well microplate reader (Bibby Scientific Limited, Jenway 7315, United Kindom).

2.6. Quality markers of fish meat

2.6.1. Proximate composition analysis

Proximate analyses (moisture, crude protein, crude fat and ash, % of dry weight) were evaluated from ingredients, diets and feces obtained from the digestibility trial and from fish flesh (n=4 for flesh, one fish per tank). Analyses were determined according to AOAC (1990) procedures: Dry matter (60ºC to constant weight), ash (incinerated at 550ºC to constant weight), crude protein (N × 6.25 and nitrogen was analyzed by Dumas principle, TruSpec CN; Leco Corporation, St. Joseph, MI, USA) and crude lipid content using the Soxhlet extraction method. AIA was used as an indicator for the ADC, and was analyzed according to the method described by Atkinson et al. (1984) with some modifications. Briefly, 5 g of sample were ashed for 5 hours at 550ºC to ensure complete combustion of the organic material in the sample. The resulting ash was boiled till dryness in 75 mL of HCl (2N) and boiled in other 75 mL HCl during 15 min. Samples were filtered hot through ashless filter paper and washed in boiling destilled water till neutralized the samples. Finally as Atkinson et al. (1984) method, samples were ashed for 5 hours at 550ºC.

β-glucan content was measured in barley, control and all experimental diets. β-glucan content on barley and different diets were evaluated using McCleary method (Megazyme mixed-linkage beta-glucan assay procedure K.BGLU04/06). Briefly, 0.5 g of sample were mixed with 1 mL ethanol (50% v/v) and 5 mL of sodium phosphate buffer (20 mM, pH 6.5). It was incubated in a water bath during 5 minutes. It was cooled at 40ºC and mixed with 0.2 mL of liquenase (10 U) during 1 h at 40ºC. After this time, the mixture was centrifuged at 100 xg during 10 minutes. 0.1 mL of supernatant is transferred and mixed with 0.1 mL of sodium acetate buffer (50 mM pH 4) and 0.1 mL of β-glucosidase (0.2 U). The mixture was incubated during 15 minutes at 40ºC for the

determination of β-glucan. The absorbance was determined at 510 nm in a 96-well microplate reader (Bibby Scientific Limited, Jenway 7315, United Kindom).

2.6.2. Water activity (a_w)

Water activity (aw) was instrumentally measured using an Aqualab 4TE (Decagon Devices inc., Pullman, WA, USA). Measurements were taken directly from the muscle. Six measurements were made in each flesh at three different locations (front, central and tail). The study was evaluated in four independent fish flesh (n=4).

2.6.3. Colour

CIELAB parameters (lightness (L*), redness (A*), yellowness (B*)) were evaluated using a portable colorimeter (Minolta CM-2002, Osaka, Japan). Hue and Chroma were calculated using the formulas.

$$Chroma = (a^{*2} + b^{*2})^{1/2}$$

$$Hue = arctan\left(\frac{b^*}{a^*}\right)$$

Measurements were taken directly over the muscle. Six measurements were evaluated randomly over skinless fish meat. The study was evaluated in four independent fish flesh (n=4).

2.6.4. Texture analysis

Texture was determined using a texture analyzer TA-XT2i (ANAME, Stable Micro System, Vienna Court, Lammas Road, Godalming, Surrey, UK). A texture profile analysis (TPA) was carried out using a penetration probe of 4 mm diameter at speed of 1 mm s-1 with a 5 mm distance and the instrument was equipped with a 25 kg load cell. The time delay between cycles was 5 seconds. Previous to analysis, samples were peeled manually and texture was analyzed in the front, middle and tail parts. Fish

flesh were evaluated in the same position (with the muscle fibers perpendicular) to the test probe. The study was evaluated in four independent fish flesh per treatment (n=4).

Curves were evaluated and the following parameters were determined: ***hardness*** (g) (maximum force required to compress the sample), ***cohesiveness*** (capacity of the sample to deform before rupture (A2 / A1, where A1 is the total energy required for the first compression and A2 is the total energy required for the second compression)), ***elasticity*** (mm) (capacity of the sample to recover its original shape after the deformation force ends) and ***gumminess*** (g) (strength to disintegrate a sample to a constant state of swallowing (hardness × cohesiveness)).

2.6.5. Thiobarbituric Acid Reactive Substances (TBARS)

TBA as an indicator of lipid oxidation was evaluated using the methodology described by Vyncke (1975). Briefly, 10 g of samples were mixed with 30 mL of 7.5% TCA. The mix was homogenized and centrifuged for 5 min at 4 ºC and 4000 rpm, then filtered with Whatman nº 1 filters (Prat Dumas, France). 5 mL of the filtrate were mixed with 5 mL 0.02 M TBA, incubated at 90ºC in a water bath during 40 min and then read in spectrophotometer (Fluostar® Omega, BMG labtech, The microplate reader company, Germany) at 530 nm. One fish per tank was analysed during the entire experiment (n=4) and results were expressed as µmol malondialdehyde (MDA) per kilogram of fresh muscle.

2.7. Sensory analysis

All sensory analysis were performed according to ISO standards (ISO 2001, 2008) in a sensory room compliant with ISO 2007 by a panel of 8 people (4 male and 4 female aged between 25 and 50) with previous experience in sensory analysis of food products. Nonetheless, in order to familiarise the panel with the sensory assessment of

fish products and optimise the tables used for sensory evaluation, the panel were trained in the main characteristics we wished to study.

Sensory analysis comprised fresh whole fish and fish meat samples (n=4). Whole fish were evaluated using the quality index method (QIM) and fish flesh were analysed using a quality descriptive method (QDM). Panellists were trained to perform both analysis. QIM was assessed following the guideline of QIM Eurofish (Martinsdóttir et al., 2001). Freshness was evaluated by giving demerit points according to certain aspects associated with general appearance such as skin, stiffness, odour, gills pots colour and odour, belly, and eyes brightness and shape. The trained judges scored ranked from 0-3 for each attribute. The maximum score of 3 corresponded to the fish with the worst quality parameters.

For the QDM, panellists were trained to discriminate colour, texture, odour and acceptability of fish meat. A continuous non-structured scale (1-10) was used for evaluation. The left side of the scale corresponded to the lowest intensity (value 1: white, soft, fresh odour and acceptable sample) whereas the right side corresponded to the highest intensity (value 10: dark, hard, rancid odour and non-acceptable sample).

Panellists evaluated one fish per treatment every 28 days during the whole experiment. 5 samples, in pairs of whole fish and flesh of each treatment, were individually presented in porcelain dishes to each panellist. Samples were coded with random numbers and maintained at room temperature during evaluation.

2.8. Statistical analyses

Statistical analysis was performed using SAS version 9 (SAS Institute Inc., Cary, North Carolina, USA) by a GLM procedure for the variance analysis (ANOVA). In the rearing parameters, the initial weight (Wi) was included as covariable.

For the rearing parameters and digestibility diet was included as the only fixed factor. For biochemical parameters, in order to evaluate the differences between diets at a certain moment and at different moments within a same diet, the fixed factors of diet and time and their interaction were included in the model. For proximal composition, colour, sensorial analysis, TBARs, water activity (aw) and texture in the GLM model only the diet was included as fixed effect. In addition, for aw and texture the section where these parameters were measured (front, middle or tail of the fish flesh) was also included as a fixed effect.

When the ANOVA revealed a significant effect, values were compared using the t-Student test and were considered to be significant at $p<0.05$. When the interaction was proven to be significant the data correspond to the double interaction and are presented as least-squares means (LSM) ± the standard error of the mean (SEM).

2.9. Ethical statement

The rainbow trout study complied with the European Union Council Directive 2010/63/UE, which provides the minimum standards for animal protection, and was also in accordance with the Spanish national legislation (Spanish Royal Decree 53/2013) based on animal protection in experimentation and other scientific practices and approved by the Animal Ethics Committee of Agro-Technological Institute of Castilla y León (Spain).

Fish in tanks were checked on a daily basis. Every four weeks, fish were weighed individually and their health status was assessed by observation, after sedation with MS222 dissolved in water (MS222®; 200 mg L^{-1}) to minimize animal suffering.

Animals were euthanized by excess of MS222 (300 mg L^{-1}) or with ice (when quality samples were taken) and then fish were dissected.

3. RESULTS

3.1. Rearing parameters: growth and biometric analysis

The experiment started with an initial average fish weight of 127.72 ± 5.65 g and finished when fish reached commercial weight (range 312-330 g). Every 28-days fish were weighed and length measured to determine the growth and biometric indexes (Table 5). The study did not show significant differences in any of the parameters studied.

Table 5. Growth and biometric indexes of rainbow trout fed with different experimental diet for 84 days (n=4).

	DIETS[1]					
	0B	**40B**	**80B**	**160B**	**319B**	**SEM**
Initial weight (g)	125	125	127	131	131	2.62
Final weight (g)	328	312	330	328	330	11.64
Biomass increment (g)	4354	4215	4444	4415	4387	233.85
Survival (%)	91	99	96	97	95	2.16
SGR (%day^{-1}) [2]	1.26	1.19	1.24	1.19	1.20	0.04
FI (g 100 g fish^{-1} day^{-1}) [3]	1.23	1.15	1.16	1.14	1.16	0.03
FCR [4]	1.17	1.13	1.11	1.11	1.14	0.02
CF (g cm^{-3}) [5]	0.93	0.93	0.93	0.96	0.94	0.01
VSI (%) [6]	10.75	9.94	10.36	10.71	10.80	0.35
HSI (%) [7]	1.63	1.60	1.68	1.72	1.58	0.11

[1] Diets explanation as in Table 1.

Absence of superscript letters indicate no significant differences between treatments ($p>0.05$).

[2] Specific growth rate (%day^{-1}) SGR = 100 x ln(final weight/initial weight)/days.

[3] Feed Intake ratio (g 100 g fish^{-1} day^{-1}). FI = 100 x feed consumption (g)/average biomass (g) x days.

[4] Feed Conversion Ratio FCR = feed intake (g)/weight gain (g).

[5] Condition factor (g cm^{-3}) CF = 100 x final weight/length^3

[6] Viscerosomatic Index (%) VSI = 100 x visceral weight/final weight.

[7] Hepatosomatic Index (%) HSI = 100 x liver weight/final weight

3.2. Apparent digestibility coefficients (ADC)

The results showed that ***protein digestibility*** of fish fed with the control and 40B diets were significantly higher (98.28%) ($p<0.05$) than that of fish fed with higher

barley concentrations. The ***fat*** and ***carbohydrate ADC*** on experimental diets was not significantly affected by diet (Table 6).

Table 6. Apparent digestibility coefficients (ADC) of protein, fat and carbohydrate in rainbow trout fed with five experimental diets differing on the source of carbohydrate (wheat and barley) (n=3).

	DIETS[1]					
	0B	**40B**	**80B**	**160B**	**319B**	**SEM**
Apparent Digestibility Coefficient (ADC)						
Protein ADC	98.28[b]	98.28[b]	96.61[a]	96.41[a]	96.49[a]	0.45
Fat ADC	96.88	97.93	96.17	95.90	97.20	1.20
Carbohydrate ADC	84.76	88.89	79.23	80.67	83.48	3.04

[1] Diets explanation as in Table 1.

Data in the same row with different superscripts letter indicate significant differences between treatments ($p<0.05$). Absence of superscript letters indicate no significant differences between treatments ($p>0.05$).

3.3. Biochemical parameters

Higher concentrations of barley on the diet showed higher concentration of glucose in blood plasma in the moment of stress. But not significant effects were observed in lactate and cortisol levels (Figure 4). When the stress response results were analyzed a significant increase ($p<0.05$) of glucose, lactate and cortisol were observed under stress, recovering basal levels of cortisol and lactate after 7 days, while hyperglycemia persisted 7 days more (Figure 4). An interactive effect was only observed in glucose levels on the different experimental diets. Changes in glucose levels have been significantly ($p<0.05$) affected by the inclusion of barley and the effect of stress.

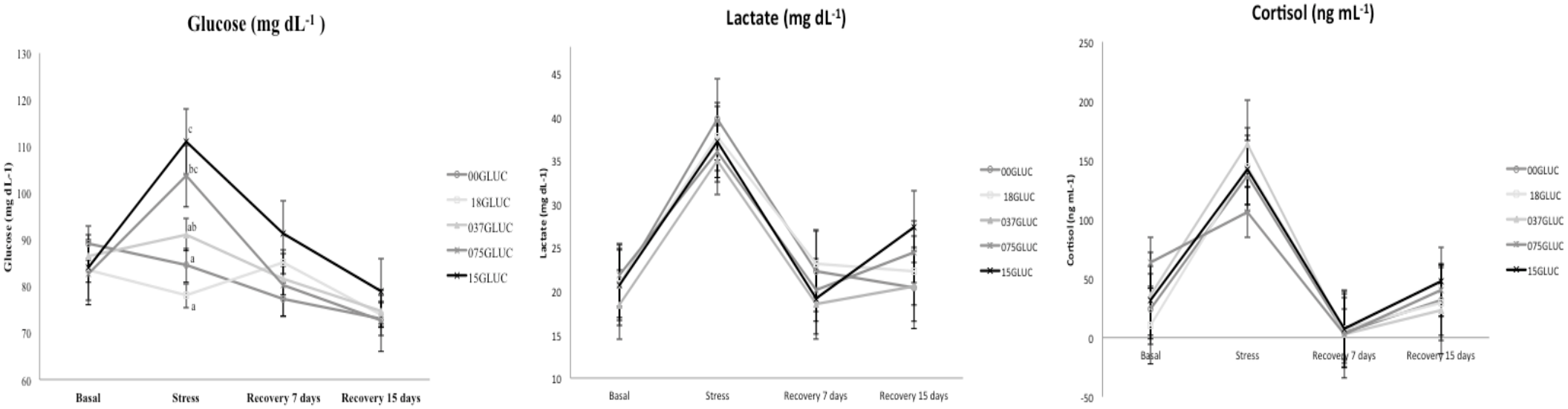

Figure 4. Effect of hypoxia challenge on the biochemical parameters (glucose, lactate and cortisol) of trout fed different experimental diets measured along the time (basal levels, just after the stress, one week of recovery and two weeks recovery) (n=3).

Small letters indicate significant differences between diets ($p<0.05$). 0B (0% barley, 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.22% β-glucans); 160B (16% barley, 0.53% β-glucans); 319B (31.92% barley, 1.5% β-glucans) are the different experimental diets.

3.4. Quality markers of fish meat

3.4.1. Proximate composition

Results showed that barley increased significantly ($p<0.05$) crude fat and ash content on meat proximate composition (Table 7) while moisture and crude protein were not affected. At the end of the experimental growth, crude fat content of fish fed 160B diets increased significantly ($p<0.05$), while ash content decreased significantly with the concentration of barley.

Table 7. Proximate composition of rainbow torut meat fed with increasing levels of barley at the end of the experimental period (data are expressed as % of dry matter) (n=4)

		DIETS[1]					
	Initial	**0B**	**40B**	**80B**	**160B**	**319B**	**SEM**
Analyzed composition (% dry matter)							
Moisture	75.80	73.60	75.50	75.50	74.30	76.00	1.30
Crude Protein (CP)	17.22	17.30	16.30	16.60	17.00	17.00	0.68
Crude Fat (CF)	5.74	7.84^{c}	6.81^{b}	6.39^{ab}	7.78^{c}	5.71^{a}	0.28
Ash	1.08	2.85^{c}	2.57^{b}	2.57^{b}	2.69^{b}	2.30^{a}	0.05

[1] Diets explanation as in Table 1.

Data in the same row with different superscripts (small letters) indicate significant differences between treatments ($p<0.05$). Absence of superscript letters indicate no significant differences between treatments ($p>0.05$).

3.4.2. Water activity (a_w)

Aw was significantly ($p<0.05$) affected by the different diets (Figure 5). Lower aw values were observed in fish fed with diets high in barley (319B) at the end of the experimental growth period and in the front and middle parts of the fillet (results not shown).

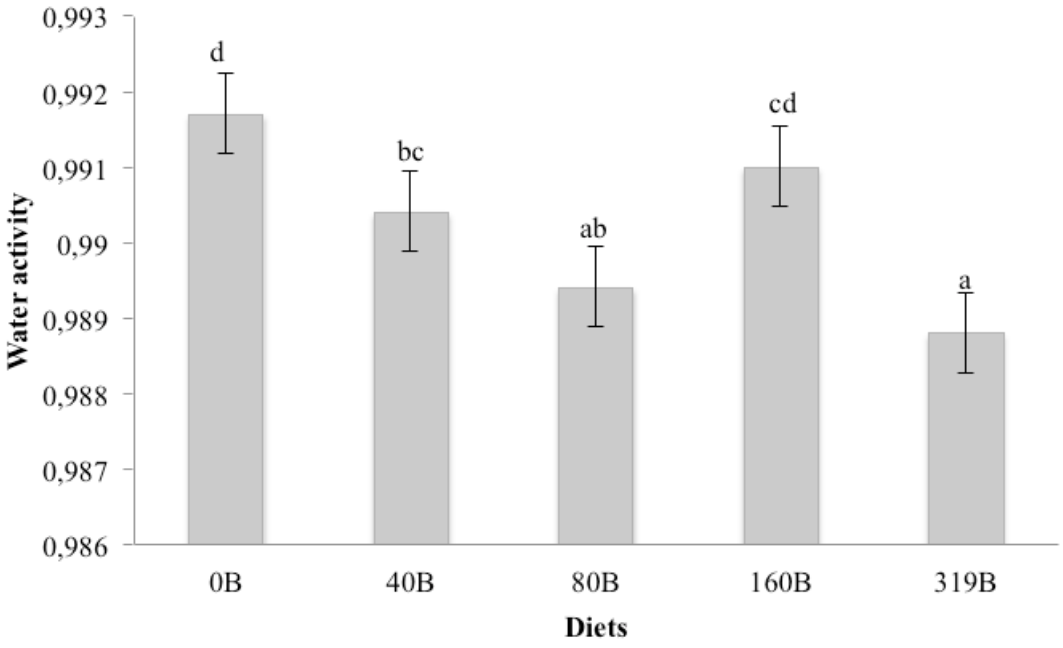

Figure 5. Effect of barley concentration on water activity (aw) of fish at the end of the experimental growth period.

Data are presented as least-squares means ± standard error of the mean (n=4); significant differences ($P<0.05$) are indicated with different letters above the column. 0B (0% barley, 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.22% β-glucans); 160B (16% barley, 0.53% β-glucans); 319B (31.92% barley, 1.5% β-glucans) are the different experimental diets.

3.4.3. Colour

No significant differences were observed despite barley concentration in meat of fish fed the different diets (Table 8).

Table 8. Effect of barley concentration on the CIELAB parameters of fish meat at the end of the experimental growth period (n=4).

		DIETS[1]					
	Initial	**0B**	**40B**	**80B**	**160B**	**319B**	**SEM**
L*	53.86	57.83	57.54	57.16	57.32	55.13	0.76
A*	3.01	-0.84	- 0.24	-0.52	-0.75	-0.46	0.30
B*	12.50	7.82	7.46	7.57	7.02	6.41	0.52
Hue	0.96	-0.65	-0.16	-0.33	-0.48	-0.28	0.27
Chroma	13.05	7.98	7.66	7.77	7.34	6.55	0.49

[1] Diets explanation as in Table 1.

Absence of superscript letters indicate no significant differences between treatments ($p>0.05$).

3.4.4. Texture

The results obtained in the present study showed that barley concentration on the diet had a significant effect on meat gumminess (Table 9 and Figure 6). Compared to control diet (0B) the increase of barley on diet produced a significant decrease of meat gumminess. Hardness, cohesiveness and elasticity were not significantly affected by the diet. Different sections of the fish flesh were also studied (front, middle and tail). Results showed the tail region was the stiffest part of the flesh (results not shown).

Table 9. Effect of barley concentration on hardness, cohesiveness, elasticity and gumminess of fish meat fed with diets with increasing concentrations of barley at the end of the experimental growth period (n=4).

		DIETS[1]					
	Initial	**0B**	**40B**	**80B**	**160B**	**319B**	**SEM**
Hardness	198.18	36.78	55.16	42.53	44.81	45.53	7.80
Cohesiveness	0.12	0.20	0.21	0.21	0.22	0.21	0.01
Elasticity	4.63	4.82	4.83	4.71	4.78	4.68	0.09
Gumminess	20.35	7.46^{a}	12.01^{b}	8.74^{ab}	9.75^{ab}	6.26^{a}	1.27

[1] Diets explanation as in Table 1.

Data in the same row with different superscripts (small letters) indicate significant differences between treatments ($p<0.05$). Absence of superscript letters indicate no significant differences between treatments ($p>0.05$).

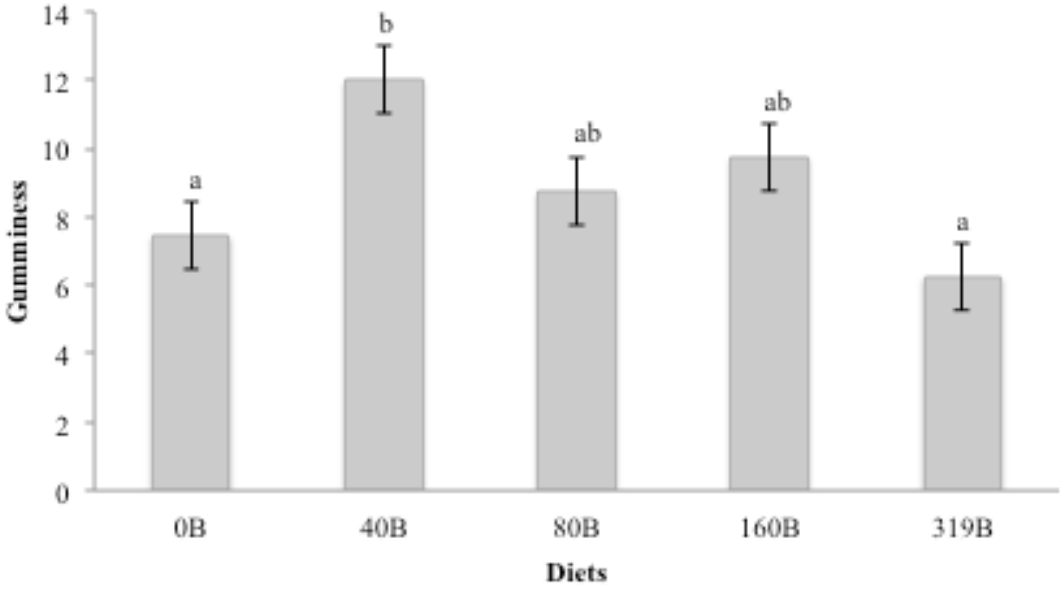

Figure 6. Effect of barley on meat gumminess of fish fed different experimental diets.

Data are presented as least-squares means ± standard error of the mean (n=4); significant differences ($P<0.05$) are indicated with different letters above the column. 0B (0% barley, 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.22% β-glucans); 160B (16% barley, 0.53% β-glucans); 319B (31.92% barley, 1.5% β-glucans) are the different experimental diets.

3.4.5. Thiobarbituric Acid Reactive Substances (TBARS)

The inclusion of barley in the diet had an inhibitory effect. Trout fed with diets higher in barley concentration had a lower level of TBARS in meat than those obtained from trouts fed with lower barley concentrations diets (Figure 7).

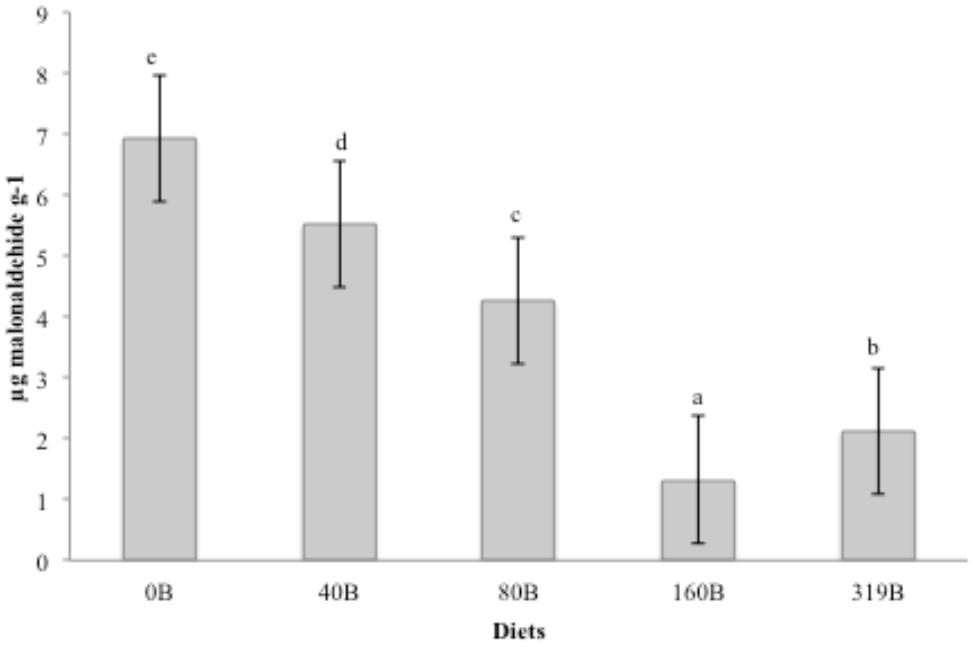

Figure 7. Effect of barley concentration on lipid oxidation (TBARS) of fish meat at the end of experimental growth period.

Data are presented as least-squares means ± standard error of the mean (n=12); significant differences (P<0.05) are indicated with different letters above the column. 0B (0% barley, 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.22% β-glucans); 160B (16% barley, 0.53% β-glucans); 319B (31.92% barley, 1.5% β-glucans) are the different experimental diets.

3.5. Sensory analysis

Results from QIM showed that barley concentration significantly affected ($p<0.05$) gill colour (Figure 8). Gills became pale on fish fed with 40B diets but diets with higher barley concentrations enhanced the redness, so barley with a β-glucan content of 0.22% (80B) or higher, enhanced fish freshness by making gills appear redder.

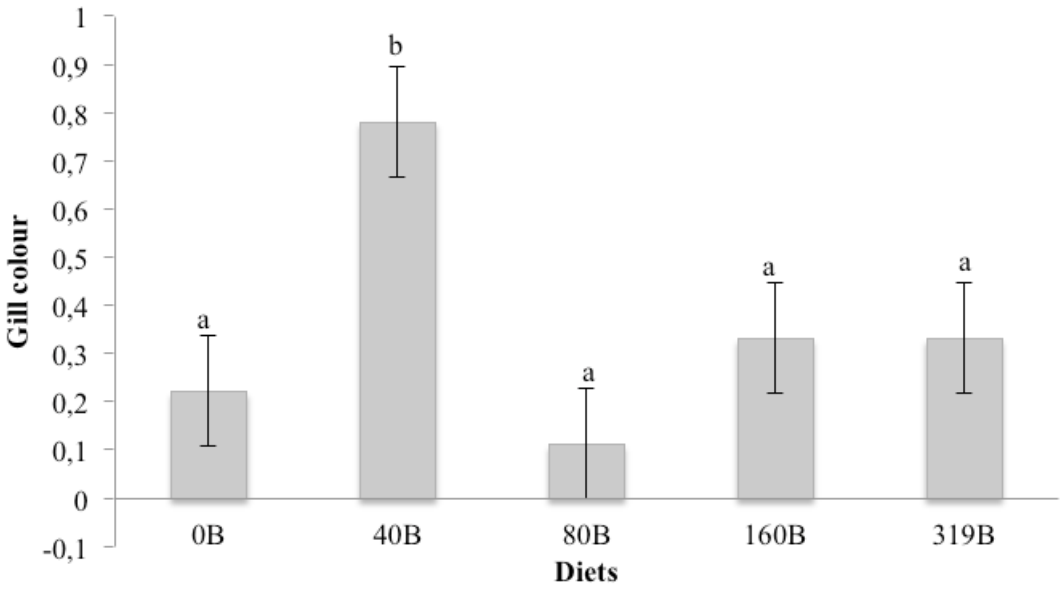

Figure 8. Effect of barley concentration on the gill colour of fish from sensory analysis (QIM) at the end of the experimental growth period.

Data are presented as least-squares means ± standard error of the mean (n=4); significant differences (P<0.05) are indicated with different letters above the column. 0B (0% barley, 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.22% β-glucans); 160B (16% barley, 0.53% β-glucans); 319B (31.92% barley, 1.5% β-glucans) are the different experimental diets.

On the other hand, QDM was evaluated on fish flesh. Experimental diets showed a significant ($p<0.05$) effect on meat colour (Figure 9). Fish colour was redder in those fish fed with diets at higher barley concentrations (0.53%, 160B). Texture was also affected by diets; fish fed with 80B showed a higher hardness than those fish fed with diets higher in barley concentrations (Figure 10). When acceptability was analyzed, no significant differences were observed regardless barley concentration, so fish samples were considered to be acceptable compare to control.

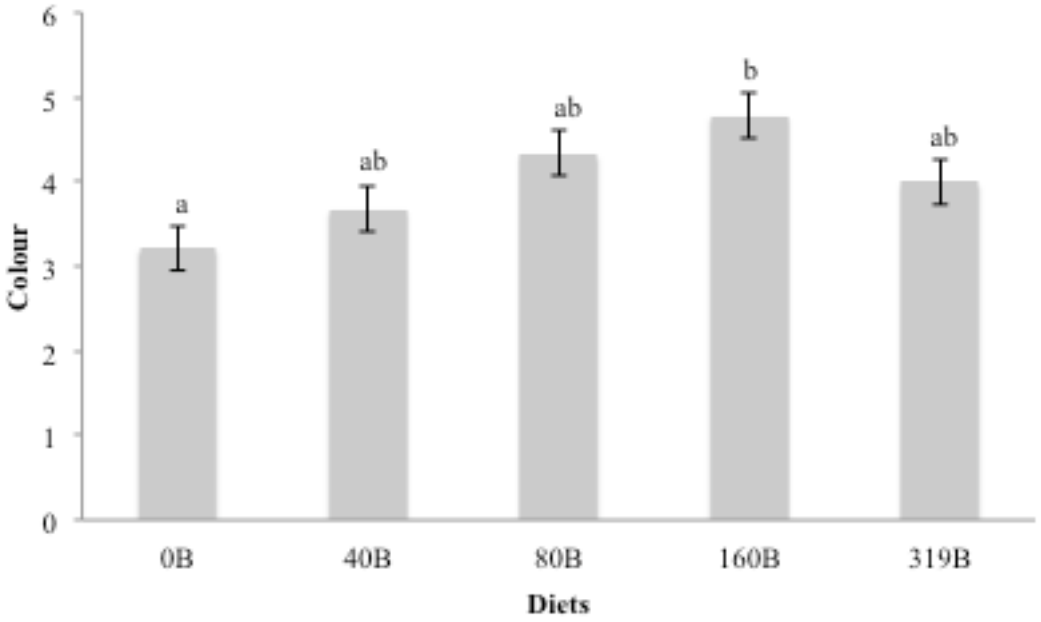

Figure 9. Effect of barley concentration on the colour of fish meat from sensory analysis (QDM) at the end of the experimental growth period.

Data are presented as least-squares means ± standard error of the mean (n=4); significant differences (P<0.05) are indicated with different letters above the column. 0B (0% barley, 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.22% β-glucans); 160B (16% barley, 0.53% β-glucans); 319B (31.92% barley, 1.5% β-glucans) are the different experimental diets.

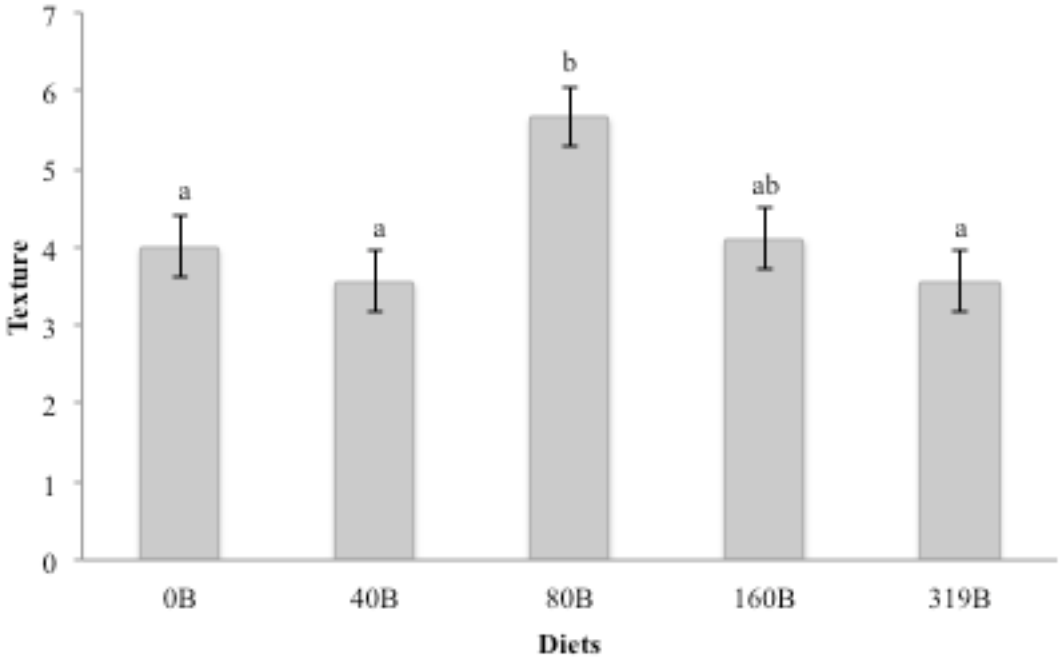

Figure 10. Effect of barley concentration on the texture of fish meat from sensory analysis (QDM) at the end of the experimental growth period.

Data are presented as least-squares means ± standard error of the mean (n=4); significant differences ($P<0.05$) are indicated with different letters above the column. 0B (0% barley, 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.22% β-glucans); 160B (16% barley, 0.53% β-glucans); 319B (31.92% barley, 1.5% β-glucans) are the different experimental diets.

4. DISCUSSION

The present study was mainly focused on evaluating the effect of increasing levels of barley, as an ingredient rich in β-glucans, on rainbow trout diets. The findings concerning growth performance and digestibility obtained on the present study have showed the potential use of barley on commercial diets for rainbow trout. In the actual study, substituting wheat for barley did not substantially altered growth performance of rainbow trout suggesting that both cereals could be equally used even though barley contains more dietary fiber than wheat. However, barley, in contrast to wheat, enhanced fish meat quality. Similar results were obtained by Sealey et al. (2008), who studied the effect of 3 barley genotypes on growth performance of rainbow trout and did not observed significant differences on final weight regardless barley concentration. The fact that growth has not been disadvantaged could also been explained that the phytic acid content of this barley variety had not a negative effect on the growth of rainbow trout, as it has also been reported by other authors (Overturf et al., 2003, Gaylord et al., 2009). Despite barley has more dietary fibre than wheat it was not observed a greater feed intake (FI). It is common that when fibre levels are very high, digestive transit is faster and FI increase, possibly as a result that this higher fibre level is not harmful for rainbow trout. Results did not show significant differences on survival rate. Probably one of the reasons of this could be that experimental diets studied in the present study were very well balanced nutritionally. However, it has been reported a significant increase on survival rates with the incorporation of β-glucan in other fish species:

croaker (*Pseodosciena crocea*) (Ai et al., 2007), Pacific white prawns (*Penaeus monodon*) (Chang et al., 2003) and juvenile western king prawns (*Penaeus latisulcatus kishinouye*) (Hai and Fotedar 2009). In the case of Chang et al. and Ai et al. they tested immunity, so the survival rate is related to resistance of fish to a disease factor.

In the present work has been observed that diets with barley and wheat were properly digested by rainbow trout, since all apparent digestibility coefficients were high. It is true that protein digestibility coefficient was slightly lower in trouts fed with diets containing higher barley levels, but considering the high percentage of this coefficient it is not possible to conclude a negative effect of this ingredient in the overall digestibility of diets. The ability of salmonids to digest fiber is rather limited due to the low α-amylase activity and the large amounts of undigested starch in the intestinal content which it would reduce digestibility of other macronutrients (Skrede et al. 2002, Stone 2003, Krogdahl et al. 2005, Couto et al. 2016). The concentration of undigested carbohydrate in the gut has been related to reduction in fat digestibility in rainbow trout (Storebakken et al. 1998, Morken et al. 2011). In the present study the ADC of fat on experimental diets was not significantly affected by diet, fat digestibility was higher than values reported in other carnivorous fish species studies: rainbow trout (*Oncorhynchus mykiss*) (Storebakken et al, 1998), atlantic salmon (*Salmo salar*) (Skrede et al., 2002), gilthead seabream (*Sparus aurata*) (Couto et al., 2016). The ADC for protein and fat were higher than 80%, values in agreement with the results reported by Cheng and Hardy (2002, 2003) who reported ADC for protein and fat in barley were also higher than 80% for rainbow trout. Starch digestibility decreased with the increase of wheat and barley levels in the diets, in accordance with previously reported data (Grisdale-Helland and Helland 1997, Skrede et al. 2002). Skrede et al. (2002) performed a study with lactic acid fermentation of both barley and wheat, reporting a

higher starch digestibility in the case of barley. Results which were similar to those obtained in the present study, indicating that barley would be an interesting ingredient in extruded diets for rainbow trout.

For rainbow trout, it was observed high glucose values following feeding with high levels of available carbohydrates (Walton 1986, Krogdahl et al. 2004). When the stress response results were analyzed a significant increase of all parameters (glucose, lactate and cortisol) were observed under stress, recovering basal levels of cortisol and lactate in 7 days, while hyperglycemia persisted 7 days more. Rainbow trout, as a carnivorous fish, has limited capability to digest fibre (Skrede et al. 2002, Stone 2003, Krogdahl et al. 2005, Couto et al. 2016), which will explain why plasma glucose levels increased significantly with the inclusion of barley in the diet. During any type of stress, cortisol levels can reach up to more than 100 ng ml^{-1} and later drop to 10-20 ng ml^{-1}, their basal level (Flores-Quintana 2002). Changes in cortisol levels during hypoxia produced a hyperglycemia due to glucogenolysis and gluconeogenesis pathways (Hemre et al. 2002). Changes in cortisol and glucose plasma levels occurred at different kinetics (Mommsen et al. 1999), that is why the hyperglycemia persisted for 14 days while basal cortisol levels were reached in 7 days. Lactate is produced by glucose from anaerobic glycolysis, and as glucose, it incremented significantly at the time stress occurred, but recovered basal levels in 7 days. Hemre (1992) reported in the case of Atlantic cod, that even 96 h after transport stress, sustained hyperglycemia was detected only in fish adapted to high dietary starch levels, while adaptation to a low starch diet resulted in a lower glucose peak coupled with a shorter recovery period to establish basal levels. This adaptation also influenced muscle and liver ability to regulate plasma glucose levels after peaking, assuming that the space for glycogen storage can be modified by an adaptation diet, in agreement with studies on glucose space in halibut

(*Hippoglosus hippoglossus*) (García-Riera and Hemre, 1996) and Atlantic salmon (*Salmo salar*) (Hemre and Krogdahl, 1996).

Proximate composition values were similar to those reported by other authors (Yildiz et al. 2004, Popelka et al. 2014). Substituting wheat for barley did not significantly affect proximate composition of rainbow trout flesh, results that were in accordance with those reported by Sealey et al. (2008). Lower aw values were observed on fish fed with diets at high barley concentration (319B) at the end of the experimental growth period. The reduction of aw would help to reduce lipid oxidation process and microbial growth. For this reason the incorporation of barley seems to have a positive effect on shelflife.

The appearance of food products is of major importance to consumers, both from the acceptability and preference point of view. The colour of rainbow trout is generally considered as one of the most relevant quality parameters. Therefore, colour plays a decisive characteristic during quality evaluation of the product at the point of sale (Ortiz et al., 2013). No significant differences were observed despite barley concentration at different diets. These results differed from the studies obtained from sensory analysis where in the QDM analysis it was observed that when fish reached commercial weight, fish fed with 319B diets were significantly redder than fish fed with control diets.

Fish muscle texture is based on many intrinsic biological factors such as collagen or fat content. Some autolytic enzymes and microbiological effects could be induced to degradation, which made muscles less elastic and softer (Asghari et al. 2014, Xu et al. 2015). Casas et al. (2006) reported cohesiveness as a parameter to measure muscle elasticity since it describes the ability of the muscle to recover from deformation

and its resistance to subsequent deformation. If cohesiveness is < 1, the deformation suffered by the first compression is partly irrecoverable. In the present samples, the deformation along the experimental growth period was < 1 for every experimental diet. Different sections of the fish flesh were also studied (front, middle and tail). Results showed the tail region to be the stiffest part of the flesh also in accordance with the results obtained by Casas et al. (2006).

Lipid oxidation of fish meat was measured through TBARs indicators. Lakshmanan (2000) proposed a range of 1-2 mg malonealdehyde per kg of sample as the limit of acceptability, when TBAR index is above this value it affects to the fish. At the end of the experimental period TBARs index was between the range proposed by Lankshmanan (2000) and fish fed with 80B and diets higher in barley concentration reached those TBAR index levels. Trout fed with diets higher in barley concentration had a lower level of TBARS in meat than those obtained from trouts fed with lower barley concentrations diets. This decrease on the TBAR index was correlated with the lower water activity of fish fed with diets higher in barley concentrations at the end of the experimental growth period, which probably reduced microbial and enzymatic activity and probably with a positive effect of different compounds of barley which act as endogenous antioxidants.

Barley is a cereal with bioactive components, not only β-glucans, but also phenolic acids, polyphenols and non polar compounds such as tocols that can enhance growth and quality parameters, however, with the obtained data we cannot claim those improvements to be associated to the combined effect of all of these components or just to one of them, and so further studies should be done to evaluate the cause of those beneficial effects on rainbow trout. β-glucans are potential immunostimulant components, thus some immunological studies should be carried out to explain their

efficiency in growth and quality parameters.

5. CONCLUSIONS

Results indicated that wheat can be substituted by barley without any significant detrimental effect on rearing parameters and with a positive enhancing effect on fish quality, lower water activity values, as well as an enhancement in textural and colour properties, were observed in fish fed with the diet containing the highest barley concentration. Trout fed feed with higher concentrations of barley showed lower lipid oxidation levels than those fed with lower concentrations. The sensory panel found that fish fed with diets higher than 8% in barley content, exhibited a brighter red colour in gills and a better texture, also fillet colour became redder with a higher barley inclusion, being all these sensory parameters correlated with fish freshness. Considering the total of the results obtained and taking into account that the product quality (fish flesh) is a balance between rearing parameters (fish health) and quality of fish (fish flesh), is considered that barley concentrations of 31.9 g kg^{-1} is a suitable concentration to achieve this balance.

Acknowledgements

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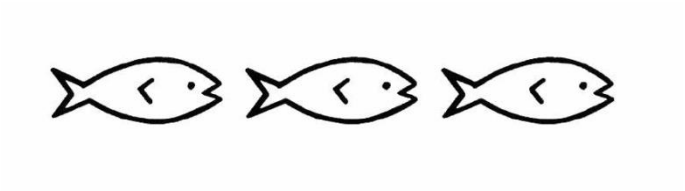
CHAPTER 5. EFFECTS OF DIETARY BARLEY ON RAINBOW TROUT EXPOSED TO AN ACUTE STRESS CHALLENGE



Submitted to Aquaculture. Ready for decision.

ABSTRACT

The present study evaluates the effect of dietary barley in rainbow trout under acute stress challenge (hipoxia and crowding) and their recovery. Diets were formulated containing increasing barley concentrations (0, 4, 8, 16, 32%). Cortisol on plasma and fin, glucose and lactate plasma levels and malondialdehyde (MDA) in muscle were determined under normoxia before the stress test (basal levels), 30 minutes after the acute stress challenge and also during normoxia recovery (6 and 12 hours after the stress). Results showed that at basal levels the inclusion of barley had no influence on cortisol, glucose nor on lactate levels. After 30 minutes from the stress challenge, there was a significant increase in cortisol, glucose and lactate concentration in fish of all groups. Plasma cortisol showed the lowest levels in fish fed with diets at a medium (8%) of barley concentration and returrned to basal levels 6 hours after the stress stimulus with no differences between diets. Glucose values showed a less clear tendency 30 minutes after the stress challenge with lower levels in the control group, fish fed with 8% and 32% of barley in the diets and returned to basal levels in almost all the groups only 12 hours after the stress challenge. Lactate showed the glucose trend after the stress challenge but it returned to basal levels in 6 hours. Interestingly, there was a significant decrease on lipid oxidation (MDA) in muscle of fish fed with the máximum barley inclusión on the diet son after the stress test. The present results suggest a potential positive effect of dietary barley on trout stress response.

***Keywords**: Barley, β-glucans, rainbow trout diets, stress challenge*

1. INTRODUCTION

During the last years, the growth of the aquaculture industry has focused on fish welfare as a relevant aspect (Conte 2004, Bertotto et al. 2010, Oliva-Teles 2012, Naderi et al. 2017). Under intensive aquaculture conditions, fish are subjected to many factors such as stocking densities, handling, weighing, feeding and water quality among others (Conte 2004, Bertotto et al. 2010, 2011, Oliva-Teles 2012), which can induce to a stress state and negatively affect the fish welfare. These factors can also affect the performance and productive parameters, having an important economic impact (Oliva-Teles 2012). A lack of oxygen can regularly occur on fish in their natural environment (Omlin & Weber 2010, Poulsen et al. 2011, Pascoli 2012, Gesto et al. 2015), however, in this situation fish are able to escape from it (Vianen et al. 2001), while in aquaculture closed systems fish are forced to put up with it (Poulsen et al. 2011). A correct aquaculture management tries to provide the right dissolved oxygen concentration so as to guarantee fish welfare and production efficiency although it is not possible to have a complete control of it. This leads to an occasional oscillation and depletion of oxygen up to values close to hypoxia (Pérez-Jiménez et al. 2012) and cause stress. Under hypoxia, fish exhibit behavioural, anatomical and physiological responses. Although those responses are protective responses, stress intensity and exposure to time can determine, at medium and long term, immunosuppression producing a higher susceptibility to diseases and growth depletion (Ming et al. 2012). Antibiotics and hormones have been used to control fish diseases, however, these do not contribute to the sustainability of the aquaculture. For this reason, during the last years alternative dietary ingredients have gained more attention than conventional methods to mitigate stress response. According to our knowledge, few studies have explored the effects of diet on acute hypoxia. Chagas and Val (2006) described the effect of hypoxia stress on

the Amazon fish tambaqui (*Colossoma macropomum*); McKenzie et al. (2008) reported an increase of tolerance under hypoxia in sole larvae and juveniles fed with diets enriched with essential fatty acids; Pérez-Jiménez et al. (2011) also found that sea bream fed with diets with white tea showed similar behaviour as fish fed with control diets after an hypoxia challenge.

The use of β-glucans have been widely studied in aquaculture (Jeney et al. 1997, Meena et al. 2013, Al-Faragi 2014, Pinedo-Gil et al. 2017). β-glucan is one of the most important immunostimulants (Meena et al. 2013) and has been widely studied because it plays an important role in immune system (Zeng et al. 2016, Miest et al. 2016) protecting fish against stress factors (Dawood et al. 2015, Zeng et al. 2016). Barley is rich in β-glucans and, for this reason, it has been widely used for livestock feeding, but on the other hand, it is scarcely used in aquaculture. In this sense, the incorporation in aquafeed of ingredients with specific components that can prepare the animal to overcome eventual adverse situations, such as oxygen depletion, it is a challenge for the aquaculture research (Pérez-Jiménez et al. 2011).

To study the stress response in fish, different parameters can be used (Bertotto et al. 2010, 2011). Plasma cortisol is one of the most commonly used indicator of stress in fish (Barton and Iwama 1991, Weendelar-Bonga 1997, Bertotto et al. 2010) but this parameter is not always an ideal marker of stress due to its rapid increase after the exposure to stressors and variability of these values (Bertotto et al. 2010, Gesto et al. 2015). For this reason, the study of cortisol levels in alternative matrices such as in fin and in muscle can provide a more real and stable measurement of the cortisol level. Along with the increase in plasma cortisol, stress also causes an increase on plasma glucose and lactate levels due to the stress hormone mediated by glycogenolysis and gluconeogenesis and muscle lactate formation during anaerobiosis (Pankhurst 2011).

At cellular level, the production of reactive oxygen species (ROS) due to a stressful factor is normal (Pascoli et al. 2011, Rahal et al. 2014). These ROS can determine oxidative damage to proteins, lipids and nucleic acids (Pascoli et al. 2011, Lushchak 2011). During oxidative stress conditions, ROS increase till levels that cells cannot remove causing lipid peroxidation, protein carbonyl formation and cell death. Lipid peroxides are unstable indicators of oxidative stress (Aldini et al. 2007, Pascoli et al. 2011, Lushchak 2011). Other byproducts of lipid oxidation such as malondialdehyde (MDA) have also been shown to be produced by oxidative stress, that presents a good correlation with stress (Aldini et al. 2007, Pascoli et al. 2011, Lushchak 2011).

The present study was conducted to evaluate, base don the antioxidant properties of barley, the antioxidant ability as well as the potential stress-relieving properties of dietary administration of barley in rainbow trout (*Oncorhynchus mykiss*).

2. MATERIAL AND METHODS

2.1. Diets

Five isoproteic (40% crude protein) and isolipidic diets (18% crude lipid) were formulated using barley as experimental ingredient (0B: 0% barley; 4B: 4% barley; 8B: 8% barley; 16B: 16% barley; 32B: 32% barley). The composition and proximate analysis of barley diets are described in Pinedo-Gil et al. 2017.

The control diet was prepared with the same ingredients as experimental diets but without barley on the formulation. A semi-industrial twin-screw extruder was used (CLEXTRAL BC-45. St. Etienne, France). Raw material was processed at a speed of 100 rpm, at 110ºC and a pressure of 40-50 atm. The experimental diets were assayed in duplicate. The fish were fed by hand twice a day (8:00 and 15:00), 6 days per week to apparent satiation level during the whole experimental period. The pellets were

distributed slowly to allow all fish to eat.

2.2. Production system

Rainbow trout (*Oncorhynchus mykiss*) were obtained from a local fish farm (Cien Fuentes fishfarm, 19420 Cifuentes, Guadalajara, Spain) and transported alive to the Research Aquaculture Centre of the Agro-Technological Institute of Castilla y León, Segovia, Spain. A total of 400 rainbow trout were used. Fish were randomly allocated in 10 cylindrical fiberglass tanks (500 L) and 40 fish (initial stocking density 19.9 ± 0.1 Kg $(m^3)^{-1}$) were randomly allocated in each tank. Prior to the feeding trial, all fish were acclimated to the indoor rearing conditions for 1 week and fed once a day (8:00) to apparent satiation using exclusively the control diet. The study lasted 45 days.

The trial was conducted in a freshwater recirculation system (RAS). During the experimental period, water temperature was mantained at 15.06 ± 0.30 ºC (mean ± SD). The level of dissolved oxygen was 6.20 ± 0.61 mg L^{-1} (61% saturation). All tanks were equipped with aeration and an oxygen probe. Water pH was 7.96 ± 0.22 and ammonia and nitrites concentration in water were 0.93 ± 0.46 and 0.97 ± 0.74 mg L^{-1} respectively. Water flow was 10.35 ± 0.80 L h^{-1}. The photoperiod consisted on 12 hours light and 12 hours dark intervals. All tanks had identical light conditions.

2.3. Stress challenge: Acute stress (hypoxia)

At the end of the experimental period (after 45 days of feeding), fish were exposed to a controlled stress test by decreasing oxygen concentration from 6.20 to 4 mg L^{-1} (acute stress, hypoxia). The concentration of oxygen was decreased by lowering the water in the tanks to a volume of 50 L and removing the aeration (crowding and hypoxia). Once that water dissolved oxygen reached 4 mg L^{-1} (oxygen-saturated value of 39.7%) (approximately 15-20 minutes), fish were kept in these conditions for 10

minutes and after this time, tanks were fulfilled again with water and in these aerated. During the acute stress challenge (hipoxia and crowding), oxygen decreased to levels lower than mg L^{-1} (oxygen-saturated values below 19.8%). Before applying the stress all fish were starved for 2 days.

2.3.1. Sampling

Samples were taken before the stress (basal levels), 30 minutes, 6 hours and 12 hours after the stress. For each sampling time, 6 fish per tank (n=12) were rapidly sacrificed with and overdose of anaethetic (300 mg L^{-1} MS222, 100% w/w; PHARMAQ®). Fish were bled from the caudal vein using 1 mL syringer (BD Plastipak) and blood was transferred to lithium heparin tubes. Soon after collection, blood was centrifuged at 1200 xg for 10 min at 4 ºC and plasma was mantained at -80 ºC until its analysis. Small portions of muscle (about 1x1x1 cm from the caudal peduncle and without skin) and caudal fin (about 1x1 cm from the upper lobe) were collected and stored at -80 ºC until analysis.

2.3.2. Cortisol analysis

Cortisol was determined in plasma and caudal fin by a specific radioimmunoassay (RIA) as described Bertotto et al. (2010) after extraction in diethyl ether. The sensitivity of the assay was 3.125 pg $well^{-1}$ and was defined as the dose of hormone at 90% binding (B/B0).

2.3.3. Glucose and lactate

Glucose and lactate concentrations were determined in plasma by enzymatic colorimetric assay, in particular by GOD-POD (SPINREACT® Ref. 1001191) and LOD-POD (SPINREACT® Ref. 1001330) methods respectively (Kaplan and Pesce 1984). Briefly, aliquots (5 µL) from plasma samples were mixed with 500 µL of

reactive and they were incubated for 10 min for glucose determination and 5 min for lactate determination at 37 ºC in dark. The absorbance was determined at 490 nm in a 96-well microplate reader (Bibby Scientific Limited, Jenway 7315, UK). Values were expressed as mg dL^{-1}.

2.3.4. MDA

The amount of lipid oxidation was determined in muscle. 100 mg of tissue was mixed with Tris HCL 0.125 M pH 6.9, centrifuged at 13000 xg at 4ºC for 15 min. Supernatant used for the assays.

Lipid oxidation was determined by measuring thiobarbituric acid-reactive substances (TBARS) according to Yoshida et al. (2005) as detailed in Pascoli et al. (2011). Tetramethosypropane was used as standard to estimate TBARS formation.

Total proteins in muscle were determined using the Piece BCA Protein Assay Kit (bicinchoninic acid assay; Thermo Fisher Scientific) following manufacturer´s instructions. Results were expressed as μg mL^{-1}.

MDA values were expressed as nM of malondialdehyde (MDA) per mg of protein.

2.4. Statistical analyses

Data were analysed by ANOVA using the PROC MIXED (SAS, 2013) with dietary treatment and time after stress as factors of variability and the tanks were considered as a random effect. The probability of the linear, cubic and quadratic components of variance was calculated by contrast statement to test differences among dietary treatments and sampling time after stress. Differences among means with $P < 0.05$ were accepted as representing statistically significant differences.

2.5. Ethical statement

The present study complied with European Union Council Directive 2010/63/UE, and is in accordance with Spanish national legislation (Spanish Royal Decree 53/2013) protecting animals used in experimentation and for other scientific purposes. Moreover, the experimental plan has been approved by Animal Ethics Committee of Agro-Technological Institute of Castilla y León (Spain).

Fish in the tanks were checked on a daily basis. At the end of the trials, fish were weighed individually and their health status was assessed by observation, after sedation with MS222 dissolved in water (MS222®; 200 mg L^{-1}). Animals were euthanized by excess of MS222 (300 mg L^{-1}) and then dissected.

3. RESULTS

3.1. Cortisol

Basal plasma levels ranged from 2.5 to 5 ng mL^{-1} and these values did not change with the diet ($P > 0.05$). Thirty minutes after the stress test a significant increase ($P < 0.05$) of plasma cortisol was observed with average values 30 times higher than basal values. The diet showed a significant effect ($P < 0.05$), the values reached by fish fed with 8B were significantly lower than the control ones. It was observed that cortisol values in plasma decreased rapidly recovering basal levels after 6 hours and were kept the same after 12 hours except for fish fed with 32B diet (Figure 11A).

Result of cortisol in the caudal fin are shown in Figure 11B. Basal values did not show significant differences between diets ($P > 0.05$) and significantly increased ($P < 0.05$) after the stress test reaching values 6 times higher than basal values. Fish groups fed with 4B, 8B and 32B diets recovered basal values after 6 hours, meanwhile fish fed with control diets and 16B did not recover these values even after 12 hours. Twelve

hours after the stress the inclusion of barley had a significant effect on fin cortisol values. Fish fed with 16B diet showed the highest value, which was significantly higher than the fish fed with the control diet or the diet 8B.

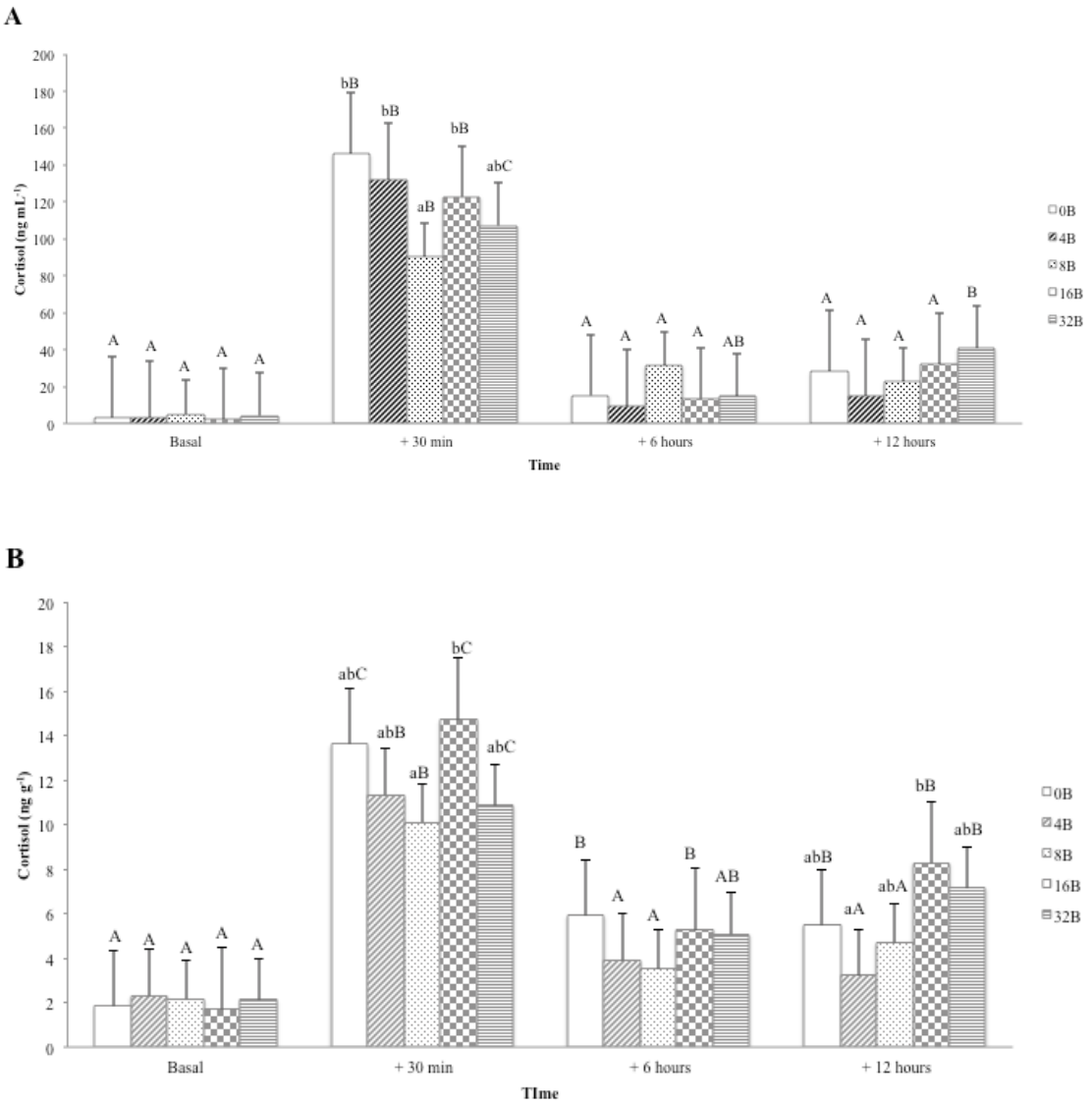


Figure 11. Effect of barley on plasma cortisol (A) and fish cortisol (B) content of rainbow trout under normoxia (basal), 30 minutes, 6 and 12 hours after stress.

Data are expressed as least-square means ± SEM, n=6. Capital letters indicate significant differences ($p<0.05$) at different time points and small letters above the bars indicate significant differences ($p<0.05$) between experimental diets in the same time point. Different experimental diets: 0B (0% barley); 4B (4% barley); 8B (8% barley); 16B (16% barley); 32B (32% barley).

3.2. Plasma glucose and lactate concentrations

Basal glucose plasma levels ranged from 41.70 to 51.06 mg dL^{-1} (without significant differences between groups) and significantly increased ($P < 0.05$) 30 minutes after the stress. Fish fed with 4B and 16B diets showed significantly higher glucose values than fish fed with the diet 32B, but there were no significant differences with fish fed with the control and 8B diets. Six hours after the stress, fish fed with the 8B diet were the only group that recovered basal values and showed significantly lower values than the control group. Glucose recovered in most of the groups after 12 hours, except in fish fed with 16B diet (Figure 12A).

Plasma lactate increased in fish of all experimental groups after the acute stress and, after 6 hours, it decreased with a trend similar to that of the cortisol. Basal lactate plasma levels ranged from 18.60 to 23.20 mg dL^{-1} and after 30 minutes from the stress challenge, lactate levels significantly increased ($P < 0.05$) to values 3 times higher than basal values. The lowest value was observed on fish fed with the 8B diet (44.89 mg dL^{-1}) and was significantly lower than fish fed with 4B (60.86 mg dL^{-1}) and 16B (58.93 mg dL^{-1}) diets. After 6 hours fish of all groups recovered lactate basal levels, not showing significant differences ($P > 0.05$) with the control regardless of the diet and maintained constant values in most of the groups after 12 hours (except with the 32B diet) (Figure 12B).

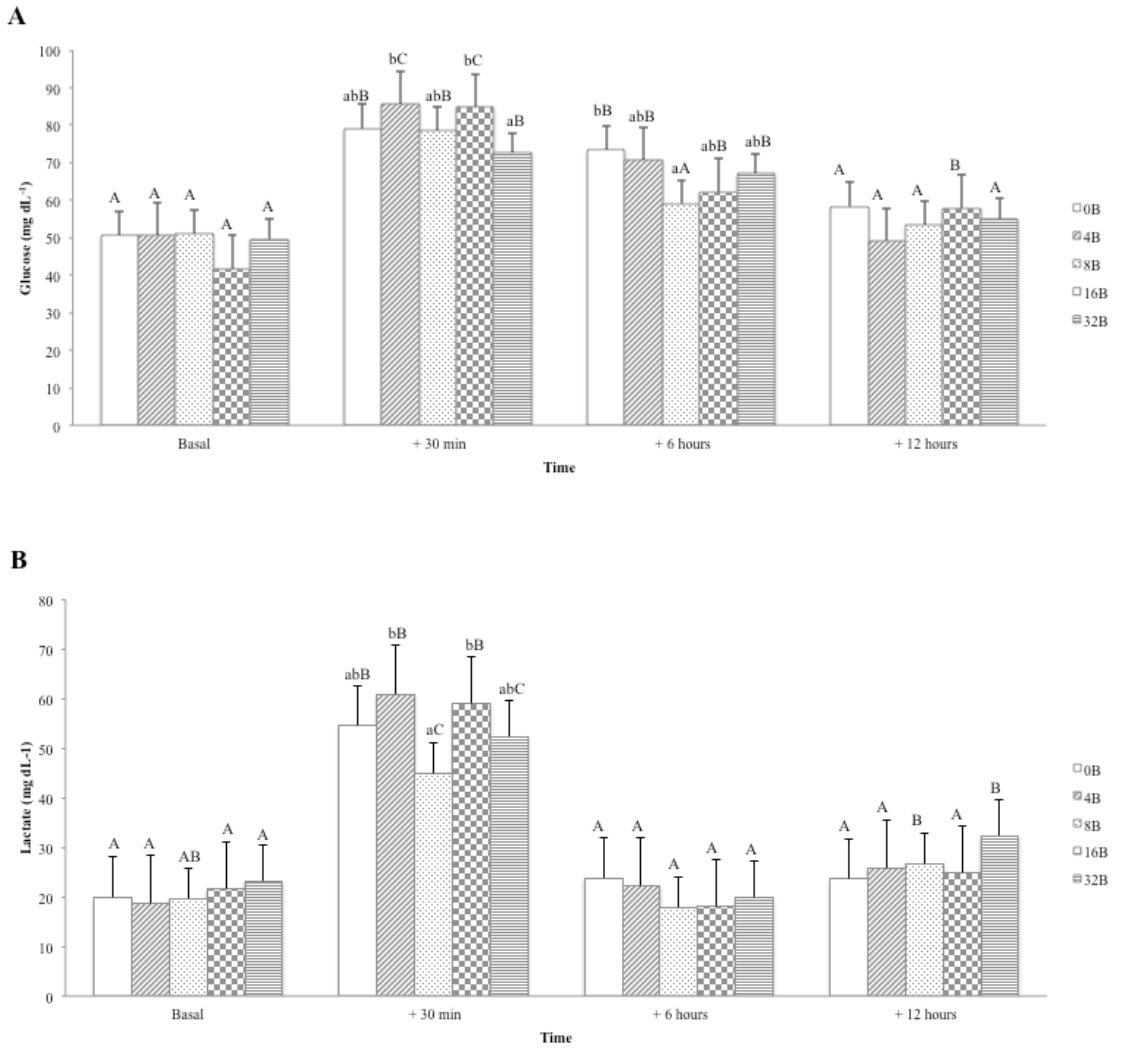


Figure 12. Effect of barley on plasma glucose (A) and lactate (B) content of rainbow torut under normoxia (basal), 30 minutes, 6 and 12 hours after stress.

Data are expressed as least-square means ± SEM, n=6. Capital letters indicate significant differences ($p<0.05$) at different time points and small letters above the bars indicate significant differences ($p<0.05$) between experimental diets in the same time point. Different experimental diets: 0B (0% barley); 4B (4% barley); 8B (8% barley); 16B (16% barley); 32B (32% barley).

3.3. MDA

MDA levels are shown in Figure 13. MDA basal values showed significant differences between groups fed with different barley concentrations. Fish fed with the highest barley concentration diet (32B) showed significantly higher MDA value than fish fed 16B diet but without significant differences with the rest of the diets. Thirty

minutes after the stress, fish fed the highest barley concentration showed significantly lower MDA concentration than control. Between 6 and 12 hours after the stress fish of all groups showed a similar trend. After stress challenge, values observed at the end of the recovery period for fish fed with barley (except for fish fed with 4B diet) were even lower than basal values.

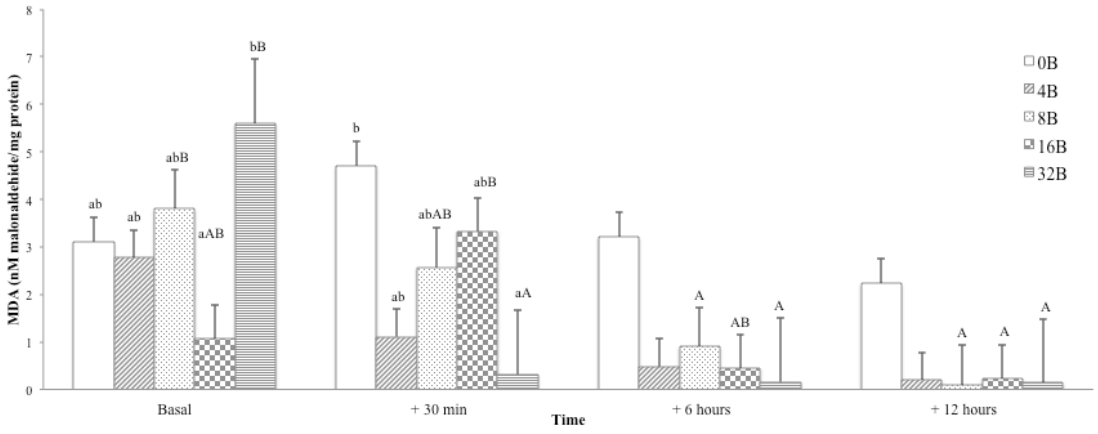


Figure 13. Effect of barley on MDA values (nM malonaldehide per mg of protein) of rainbow trout under normoxia (basal), 30 minutes, 6 and 12 hours after stress.

Data are expressed as least-square means ± SEM, n=6. Capital letters indicate significant differences ($p<0.05$) at different time points and small letters above the bars indicate significant differences ($p<0.05$) between experimental diets in the same time point. Different experimental diets: 0B (0% barley); 4B (4% barley); 8B (8% barley); 16B (16% barley); 32B (32% barley).

4. DISCUSSION

Results of the present study suggest a potential positive effect on fish under acute stress challenge and their recovery when barley is included in the diet.

Basal plasma and fin cortisol values obtained in the results, in general are similar to those obtained in similar conditions and in the same specie by Ellis et al. (2004) and Bertotto et al. (2010). The effect of hipoxia and crowding produced a significant

increase in plasma cortisol but returned to basal levels soon after 6 hours as it was observed also in previous studies (Raaij et al. 1996). The initial cortisol levels increased significantly after the stress, about 30 times in plasma and 6 times in fin. This shows that the depletion of oxygen levels to values of 4 mg L^{-1} and exposure to low oxygen levels for 10 minutes was an stressful factor for this specie and all matrices were valuable to asses this stress. Cortisol increases in fin may be explained by the lipophilic nature of cortisol, which can diffuse through cell membranes into several tissues such as fins, mucus or muscle (Bertotto et al. 2010). The increase on plasma cortisol levels was expected and has been described in several studies after various stress conditions in teleost species (Weendelar-Bonga 1997, Mommsen et al. 1999, Pichavant et al. 2002, Bertotto et al. 2010, Pinedo-Gil et al. 2017). The lowest values observed in fish fed with 8B diets probably may be explained by a possible immonumodulatory effect of barley. Barley had a positive effect only when 8% barley is included on diets. The stress-reducing effect of barley could be due to its content on β-glucans (Meena et al. 2013), although no mechanisms have been proposed to explain this effect. Dawood et al. 2015, Zeng et al. 2016 and Miest et al. 2016 reported that β-glucan produces an activation of the immune system and protects fish against various stress factors. Cain et al. (2003) also observed a decrease on cortisol plasma response when handling stress in tilapia fed a 0.2% β-glucan diet or Jeney et al. (1997) observed a significant reduction in cortisol 2 hours and 1 week after a transportation stress in rainbow trout fed with a 0.1% β-glucan diet. Some authors (Aluru and Vijayan 2006, Ings et al. 2012, Gesto et al. 2013, 2015) observed that rainbow trout needs between 4-8 hours to recover basal cortisol plasma levels. Results of the present study showed that 6 and 12 hours after stress test, plasma cortisol levels significantly decreased to basal levels, which was in accordance with the studies reported by other authors (Ings et al. 2012, Gesto et al. 2013, 2015).

Plasma glucose and lactate increased during stress challenge in the present study as reported in fish by several other authors (Raaij et al. 1996, Pichavant et al. 2002, Pinedo-Gil et al. 2017). These results were expected because in stressful conditions, the chromaffin cells reléase catecholamine hormones, adrenaline and noradrenaline towards blood circulation that in conjuction with cortisol mobilize and elevate the glucose production in fish through gluconeogenesis and glycogenolysis pathways (Iwama et al. 1999). All this makes it possible to cope with the energy demand produced by the stressor for the "fight of flight" reaction and to increase lactate due to the muscle anaerobiosis (Mommsen et al. 1999, Chagas and Val 2006, Ming et al. 2012, Pérez-Jiménez et al. 2012). In the present study, plasma glucose and lactate increased in all experimental groups after the stress challenge and then decreased during the recovery period. Before the stress (basal levels) there were no significant differences on glucose and lactate levels of fish of all the groups but 30 minutes after the stress significant differences were observed among fish fed with barley and no differences were observed with the control group. Fish fed with the highest barley concentration showed the lowest glucose plasma values. Six and twelve hours after the stress period, glucose and lactate plasma levels recovered to basal values in almost all the groups. These results point at a possible effect of barley (their β-glucans or other compounds) in glucose metabolism. Fish facing up a stressor mobilize energy reserves as an adaptive response. Barley provided an extra resistance reducing the stress associated with the depletion of oxygen, and modifying the plasma glucose metabolic pathway. Another explanation of the barley effect on cortisol, glucose and lactate levels may be related to the cortisol synthesis. Barley can control lipid peroxidation and that prevented from the production of cholesterol (Ming et al. 2012), thereby avoiding the cortisol production (Kitabchi 1967).

Lipid oxidation results showed a less clear effect of the stress challenge on trouts. MDA values resulted higher in fish fed with the highest barley concentration (32B) than those fed with 16B diets before the stress challenge (basal levels). Interestingly, a significant decrease of MDA was observed after the stress challenge in fish fed with the highest barley level, with lowest recorded MDA value if compared with the control group. The use of different tissues and techniques in lipid oxidation evaluation makes difficult to compare the results of the present study with those of other studies but the low levels of MDA in fish fed with barley diet after the stress test and during their recovery suggest a potential antioxidant effect of this dietary ingredient. Some studies have shown an increase on lipid oxidation under hypoxia (Lushchak et al. 2005, Pérez-Jiménez et al. 2011); other studies such as the described by Chagas and Val (2006) showed that lipid oxidation is prevented by antioxidants. Interestingly and in accordance with the present results, Leveelahti et al. (2014) in a study of three species (the epaulette shark, threespine stickleback and rainbow trout) exposed to hypoxia, reported that in general, fish do not show an increase in redox-active antioxidant defence in response to oxidative stress associated with hypoxia. Rather, the changes in antioxidant defences during hypoxia are very much species- and tissue-specific and are not linked to the levels of hypoxia tolerance. It is well known that the response of MDA is tissue-specific (Lushchak and Bagnyukova 2006) and depends on the type of stress. In the present study, the lowest MDA level observed in fish fed with the highest barley level suggests a protective effect from oxidative stress. Lushchak et al. (2005) observed in carp that TBARS levels in liver increased due to the hypoxia, however in brain and liver lipid peroxides decreased, maybe due to low oxygen availability which could reduce the reactive oxygen species (ROS) levels and thus reducing lipid oxidation.

5. CONCLUSIONS

The inclusion of barley on rainbow trout diets controlled stress markers which were determined in plasma, fin and muscle. Cortisol levels significantly decreased when fish were fed at medium (8%) and maximum barley concentrations (32%). Glucose values significantly decreased when fish were fed at maximum barley concentrations (32%) and lactate at medium barley concentrations (8%). It was also observed a significant decrease on lipid oxidation (MDA) in muscle. All these indicate a better control of acute hypoxia and crowding when fish were fed with barley.

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CHAPTER 6. EFFECTS ON HISTOLOGICAL PARAMETERS OF RAINBOW TROUT FED WITH DIFFERENT BARLEY CONCENTRATIONS BEFORE AND AFTER AN HYPOXIA CHALLENGE

One related publication:

Pinedo-Gil J., Martín-Diana A.B., Caballero-Cansino M.J., Sanz-Calvo M.A., Jover-Cerdá M., Tomás-Vidal A. (2017). Effect of barley on liver histology of rainbow trout, *Oncorhynchus mykiss*. Journal of Aquaculture & Marine Biology 5(3): 00123 DOI:10.15406/jamb.2017.0500123

ABSTRACT

This study investigates the effects of the incorporation of barley on rainbow trout diets on histological parameters of the liver and gut before and after an acute hypoxia stress challenge. Experimental diets were used containing different barley concentrations (0-32%) randomly assigned to two groups per treatment. Results showed that liver histological studies indicated that the increase of barley concentrations produced significant modifications, reducing hepatocytes size, vacuolation processes and increasing the incidence of appearance of lymphocytic foci. Barley in a concentration of 8% caused significant negative changes in the intestinal morphology of rainbow trout especially in the proximal intestine. After the acute hypoxia stress challenge it was not observed any change on histological and morphological on liver and gut. However, 30 minutes after the stress, hepatocytes size significantly decreased and this decrease was more evident in those fish fed at higher barley concentration. Liver cell vacuolation was also lower, similar to what was observed before the stress. It seems a positive effect of barley on liver inflammatory cells when fish were fed with 4% of barley. Barley did not have any effect on gut histology and morphology after the stress. Only gut vacuolation was lower at higher barley concentration. Probably, 30 minutes is not enough time to detect stress changes on histological parameters, however, it seems a positive effect on liver and gut health.

***Key words**: Barley, rainbow trout, hypoxia, liver and gut histology and morphology*

1. INTRODUCTION

To improve the profitability of fish production and to reduce the cost of feeds, a great variety of alternative and sustainable ingredients are under evaluation. These new ingredients must ensure the welfare of the fish because a healthy digestive system is fundamental for ideal performance. Vegetable crops are promising alternative feed ingredients, particularly if they are produced by terrestrial agriculture in the region and could contribute directly to the sustainability and cost effectiveness of the aquaculture industry (Adamidou et al. 2011; Martínez-Llorens et al. 2012). In this respect, the use of cereal grains such as wheat, barley, oats and corn in carnivorous fish diets is a common practice around the world (Gatlin et al. 2007; Glencross et al. 2012), as they are highly available in the world market at low prices and have a relatively stable composition (Couto et al. 2016). Wheat is a cereal traditionally used in rainbow trout diets, however, barley has not been used widely as an ingredient in aquaculture feed (Pinedo-Gil et al. 2017). The scarce use of barley in aquaculture might be responded by the presence of some antinutritional factors, such as phytic acid (Cheng & Hardy 2003) that reduce the absorption of some minerals in diets, and has a low protein content compare to other cereal grains such as wheat, soy, corn, etc. (Pinedo-Gil et al. 2017). However, barley also presents advantages due to its β-glucan content. β-glucans have been reported to have important health benefits on different fish species (Lazaridou et al. 2007; Gatlin et al. 2007; Dalmo et al. 2008; Sealey et al. 2008; Lin et al. 2011; Lokkesh et al. 2012; Meena et al. 2013). β-glucans are routinely used as a vaccine adjuvant (Rørstad et al. 1993; Jørgensen et al. 2003) and immunostimulant feed ingredient (Robertsen et al. 1990; Burrells et al. 2001). Many studies have been carried out with Atlantic salmon (*Salmo salar*) (Paulsen et al. 2001; Refstie et al. 2010), rainbow trout (*Oncorhynchus mykiss*) (Sealey et al. 2008; Meena et al. 2013), gilthead seabream (*Sparus aurata*)

(Castro et al. 1999; Spain-Ortuño et al. 2002). There is strong experimental evidence that the administration of β-glucans through immersion, dietary inclusion or injection has been found to enhance many types of immune responses, resistance to bacterial and viral infections and to environmental stress in many fish species (Vetvicka et al. 2013).

During the last years, one of the main concerns of aquaculture is to guarantee animal welfare and production efficiency (Bertotto et al. 2010). However, sometimes can occur oscillations on water parameters that can be a stress factor to fish (Pérez-Jiménez et al. 2012). One of these oscillations could be a depletion of the water-dissolved oxygen to levels close to hypoxia. When this occurs, fish react with protective responses (behavioural, anatomical and physiological) depending on the stress intensity and the time of exposure, which may lead to immunosuppression, higher susceptibility to disease and growth depletion (Ming et al. 2012). The decrease in oxygen availability to tissues can produce necrotic or apoptotic lesions in different organs (Harper and Wolf 2009). Liver and gut are organs that indicate the nutritional and physiological status of the fish. Stress responses may be evident in liver because its important role on energy storage and metabolism (Harper and Wolf 2009). Liver histological alterations can be visible as differences on hepatocellular vacuolation and tinctorial staining characteristics (Wolf and Wolfe 2005). Also, fish exposure to low levels of oxygen may result in alterations of the gut barrier function and induced morphological changes in the distal intestine as it was reported for Atlantic salmon by Sundh et al. 2010.

There is scarce information about how the combination of a stress factor, such as an acute hypoxia, and a new cereal ingredients rich in a bioactive compound, such as barley and its β-glucan content may affect liver and intestinal health in rainbow trout. Tran-Ngoc et al. (2016) reported that negative changes in the intestinal morphology induced by a soybean based diet to Nile tilapia were aggravated with a low water

dissolved oxygen level. Mosberian-Tanha et al. (2017) reported that rainbow trout fed with a soybean based diet combinated with an hypoxia challenge presented a more aggravated enteritis than fish exposed to the dietary challenge alone. Barley as a good source of bioactive compounds can provide physiological benefits to fish acting as dietary immunostimulant. For this reason, the aim of the present work was to evaluate the effect of different barley concentrations on liver and gut histological parameters before and after an acute hypoxia.

2. MATERIAL AND METHODS

The current study was complied with European Union Council Directive 2010/63/UE, which lays down minimum standards for the protection of animals, was also in accordance with Spanish national legislation (Spanish Royal Decree 53/2013) protecting animals used in experimentation and for other scientific purposes and approved by Animal Ethics Committee of Agro-Technological Institute of Castilla y León (Spain).

2.1. Fish and rearing conditions

A total of 400 rainbow trout were used. Fish were randomly allocated in 10 cylindrical fiberglass tanks (500 L) and 40 fish (initial stocking density 19.9 ± 0.1) were randomly allocated in each tank. The tanks were all connected to a recirculation system which allowed online measurement of actual and cumulative water flow, oxygen concentration, temperature, pH and conductivity. During the experiments water temperature remained constant at 15.06 ± 0.30 ºC (mean ± SD). The level of dissolved oxygen was 6.20 ± 0.61 mg L^{-1}. All tanks were equipped with aeration and an oxygen probe. Water pH was 7.96 ± 0.22 and ammonia and nitrites concentration in water were 0.93 ± 0.46 and 0.97 ± 0.74 mg L^{-1} respectively. Water flow was 10.35 ± 0.80 L h^{-1}. The photoperiod consisted on 12 hours light and 12 hour dark intervals. All tanks had

identical light conditions.

Five isoproteic and isolipidic diets were formulated using barley as experimental ingredient. Barley was incorporated on the different diets at increasing levels (0B = 0% barley; 40B = 4% barley; 80B = 8% barley; 160B = 16% barley; and 319B = 31.9% barley). Control diet (0B) was prepared with the same ingredients as experimental diets but without barley on the formulation. This diet was not a commercial diet. The composition and proximate analysis of the diets are described in Pinedo-Gil et al. 2017.

The extrusion process in which diets were prepared is described in Pinedo-Gil et al. 2017. The experimental diets were assayed in duplicate. The fish were fed by hand twice a day (8:00 and 15:00), 6 days per week to apparent satiation level during the whole experimental period. The pellets were distributed slowly to allow all fish to eat. Prior to the feeding trial, all fish were acclimated to the indoor rearing conditions for 1 week and fish were fed once a day (8:00) to apparent satiation using exclusively the control diet. The study lasted 45 days.

2.2. Stress challenge: acute stress (hypoxia)

At the end of the experimental period (after 45 days of feeding), fish were controlled stressed by decreasing oxygen concentration from 6.34 to 4 mg L^{-1} (acute stress, hypoxia). The concentration of oxygen was decreased by lowering water level to a volume of 50 L and removing the aeration. When the levels of dissolved oxygen in water reached 4 mg L^{-1}, it is started to count 10 min in these conditions, reaching levels of < 2mg L^{-1}. After 10 min of hypoxia, tanks were fulfilled with water and aerated. Before applying the stress all fish were starved for 2 days.

2.3. Sampling procedure

Samples were taken before the stress (basal levels) and 30 minutes after the

stress (starting when oxygen level in water reached 4 mg L^{-1}). For each sampling time 6 fish per tank (n=6) were sacrificed with 300 mg L^{-1} MS222 (100% w/w; PHARMAQ®) (in order fish died immediately). Once fish were dead, the liver and gut of fish were taken for histological analysis. They were preserved in phosphate buffered formalin (4% pH 7.4). Three sections were obtained from each liver (two transversal and one longitudinal) and from the posterior intestine; these were embedded in paraffin. For histological analysis, 4 μm tissue slices were stained with hematoxylin-eosin alcian-blue. Liver slices were evaluated for the infiltration of perpancreatic fat, hepatocytes area and length and presence of lymphocytes foci. Gut slices were evaluated for vacuolation, incidence of mucus cells and presence of cell infiltrations. This evaluation was carried out individually. Microphotographs were taken with Nikon Microphot-FXA microscope and Olympus DP50 camera. The liver photographs (100X) were used to measure the area and maximum (Lmax) and minimum (Lmin) lengths of randomly selected hepatocytes passing through nucleus. For each fish, 15 hepatocytes were measured. All measurements were made with the aid of Image Pro® Plus software (media Cybernetics, Inc.; MD, USA).

2.4. Statistical Analysis

Statistical analysis was performed using SAS version 9 (SAS Institute Inc., Cary, North Carolina, USA). All data were tested for normality and homogeneity of variance. Least-squares means (LSM) ± the standard error of the mean (SEM) were calculated for the hepatocytes areas and lengths using one-way analysis of variance (ANOVA). When the ANOVA revealed a significant effect, values were compared using the t-Student test and were considered to be significant at $P < 0.05$.

The infiltration of perpancreatic fat and the presence of lymphocytes foci on liver, gut vacuolation, incidence of mucus cells and cells infiltration were analysed

using the CATMOD procedure, and the significance was determined using a chi-square test at $P < 0.05$.

3. RESULTS

3.1. Effect of barley on liver and gut histology and morphology

Liver morphological studies showed that the inclusion of barley affect significantly ($P < 0.05$) to the hepatocyte size. Fish fed at higher barley concentrations showed lower hepatocyte areas and lengths (Table 10) than fish fed the control and 40B diets, showing the smallest hepatocytes fish fed with the diet 320B.

Table 10. Areas, maximum (Lmax) and minimum (Lmin) lengths of hepatocytes of fish fed different experimental diets (n=4).

	DIETS[1]						
	0B	**40B**	**80B**	**160B**	**320B**	**SEM**	**P-value**
Area	1.269^{b}	1.200^{b}	0.997^{a}	0.996^{a}	0.946^{a}	0.067	0.0013
Lmax	2.113^{b}	2.051^{b}	1.812^{ab}	1.883^{ab}	1.764^{a}	0.088	0.0207
Lmin	0.925^{b}	0.934^{b}	0.793^{a}	0.756^{a}	0.815^{ab}	0.046	0.0167

[1] 0B (0% barley. 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.223% β-glucans); 160B (16% barley, 0.53% β-glucans) and 320B ((31.9% barley, 1.5% β-glucans) are the different experimental diets.

Data in the same row not sharing a common superscripts letter are significantly different ($p < 0.05$). SEM: standard error of the mean

Liver cell vacuolation was significantly lower on fish fed with diets 80B and 160B than in the rest of the groups and higher barley level did not show significant differences with control (Figure 14).

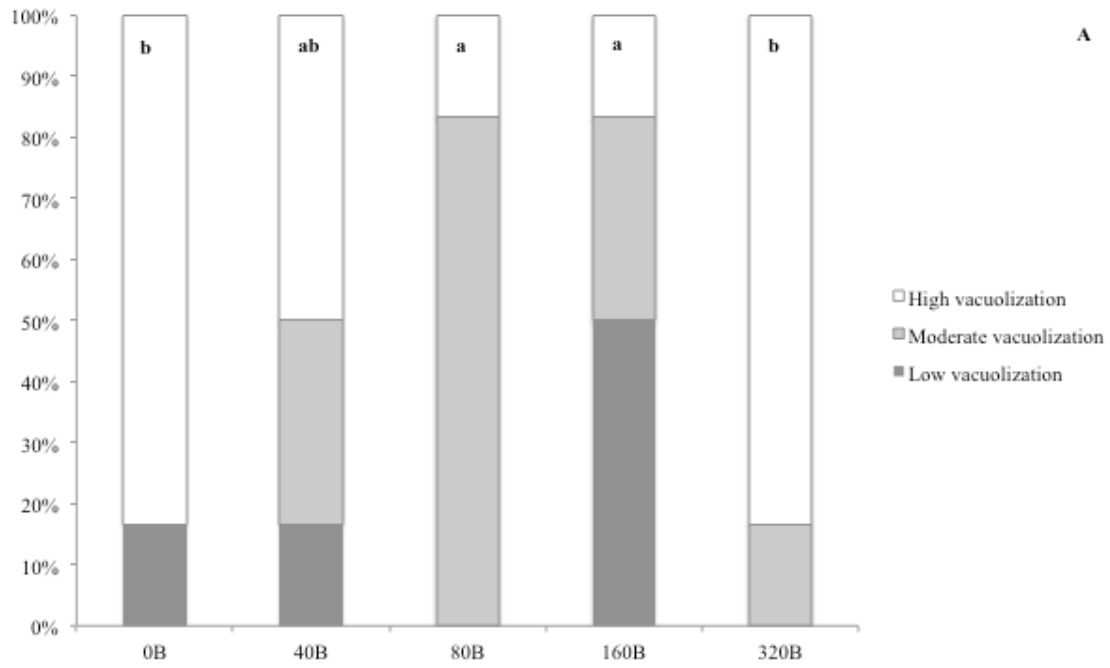

Figure 14. Hepatocytes vacuolation of fish fed different experimental diets.

0B (0% barley. 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.223% β-glucans); 160B (16% barley, 0.53% β-glucans) and 320B (31.9% barley, 1.5% β-glucans) are the different experimental diets. Different letters indicate significant differences ($P < 0.05$) between different experimental diets.

In the present work the liver tissue examination revealed inflammation by the presence of lymphocytic cells foci in fish of all groups (Figure 15). Fish fed with diets 80B and 160B showed the highest degeneration of liver and fish fed with diets 40B and 320B did not show significant differences with control.

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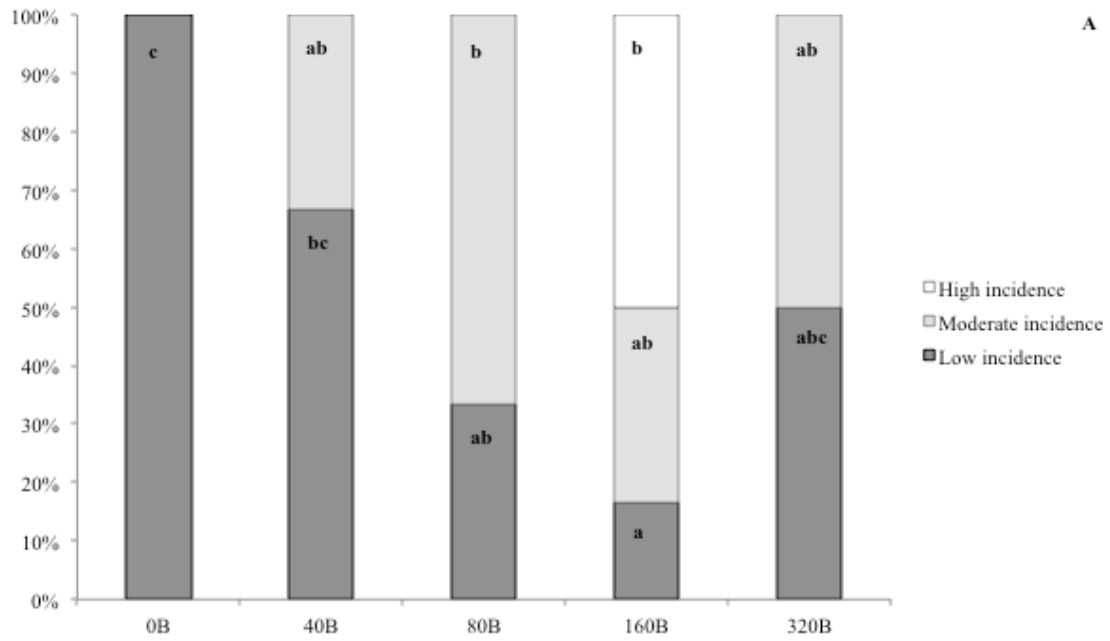

Figure 15. Incidence of lymphocytic foci appearance in hepatocytes of fish fed different experimental diets.

0B (0% barley. 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.223% β-glucans); 160B (16% barley, 0.53% β-glucans) and 320B (31.9% barley, 1.5% β-glucans) are the different experimental diets. Different letters indicate significant differences ($P < 0.05$) between different experimental diets.

When the posterior intestine was examined it was observed cell infiltration in all groups (Figure 16). Fish fed with diet 80B showed significantly ($P < 0.05$) the highest gut degeneration.

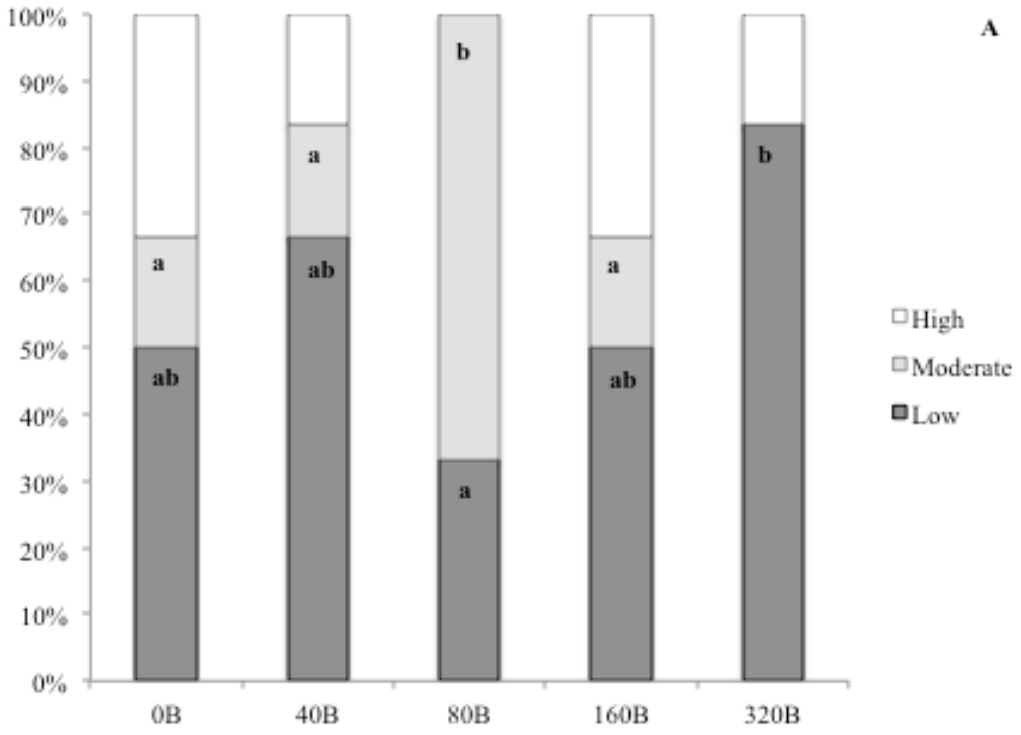

Figure 16. Cell infiltration on the posterior intestine of fish fed different experimental diets.

0B (0% barley. 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.223% β-glucans); 160B (16% barley, 0.53% β-glucans) and 320B (31.9% barley, 1.5% β-glucans) are the different experimental diets. Different letters indicate significant differences ($P < 0.05$) between different experimental diets.

Fish fed with control, 80B and 160B diets showed significantly ($P < 0.05$) higher number of mucus cells then fish fed with 40B and 320B diets (Figure 17).

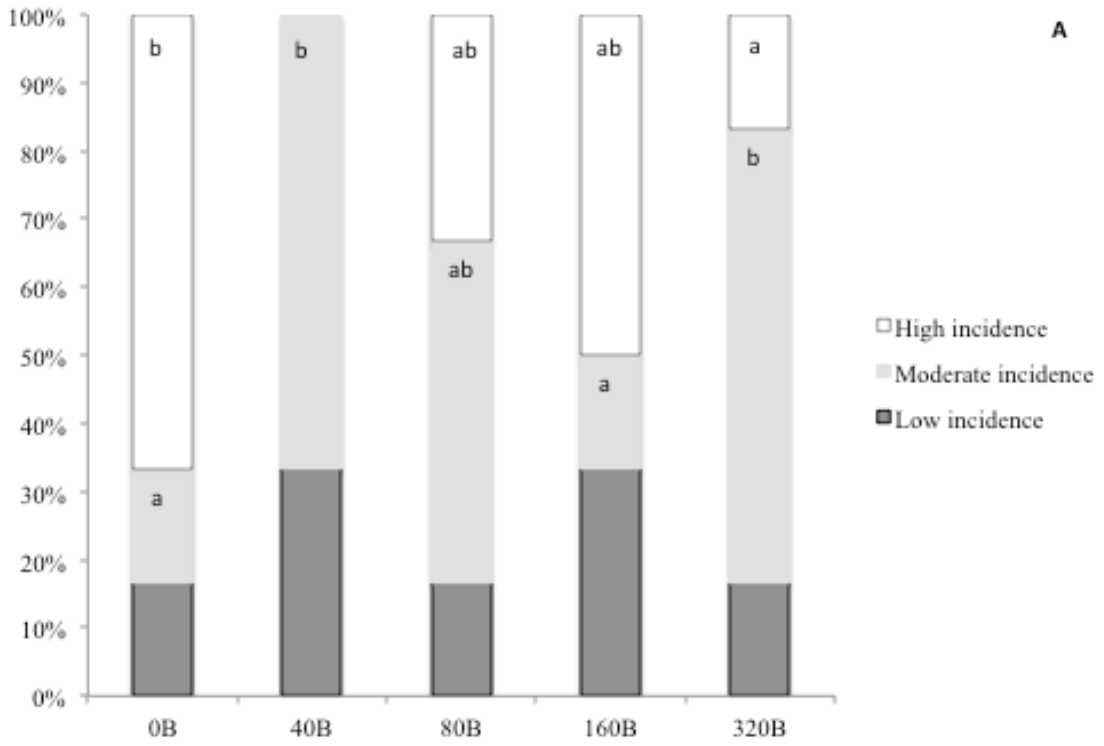

Figure 17. Mucus cells on the posterior intestine of fish fed different experimental diets.

0B (0% barley. 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.223% β-glucans); 160B (16% barley, 0.53% β-glucans) and 320B (31.9% barley, 1.5% β-glucans) are the different experimental diets. Different letters indicate significant differences ($P < 0.05$) between different experimental diets.

And gut vacuolation was significantly lower on fish fed with 40B and 160B diets than fish fed with the control diet (Figure 18).

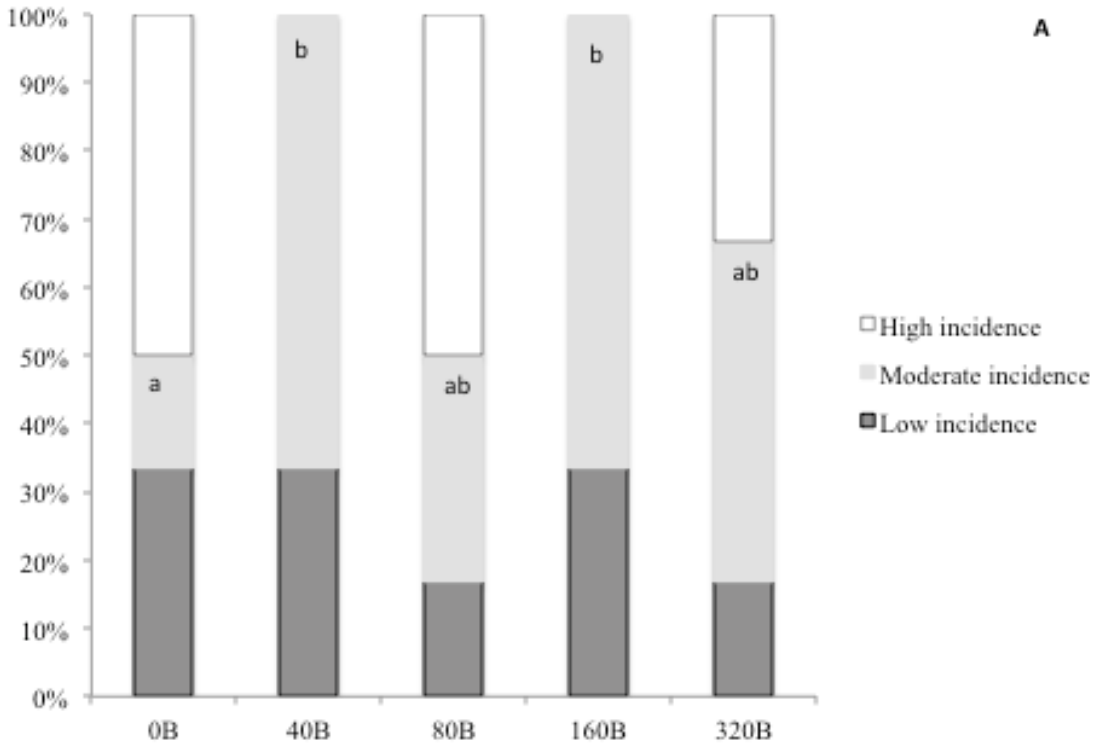

Figure 18. Gut vacuolation (posterior intestine) of fish fed different experimental diets.

0B (0% barley. 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.223% β-glucans); 160B (16% barley, 0.53% β-glucans) and 320B (31.9% barley, 1.5% β-glucans) are the different experimental diets. Different letters indicate significant differences ($P < 0.05$) between different experimental diets.

3.2. Effect of stress on liver and gut histology and morphology

Thirty minutes after the stress, hepatocytes size (areas and lengths) significantly ($P < 0.05$) decreased (Figure 19). Liver cell vacuolation (Fig. 1 – supplementary data) and the incidence of appearance of lymphocytic foci (Fig. 2 – supplementary data) did not change regardless the stress (data not shown).

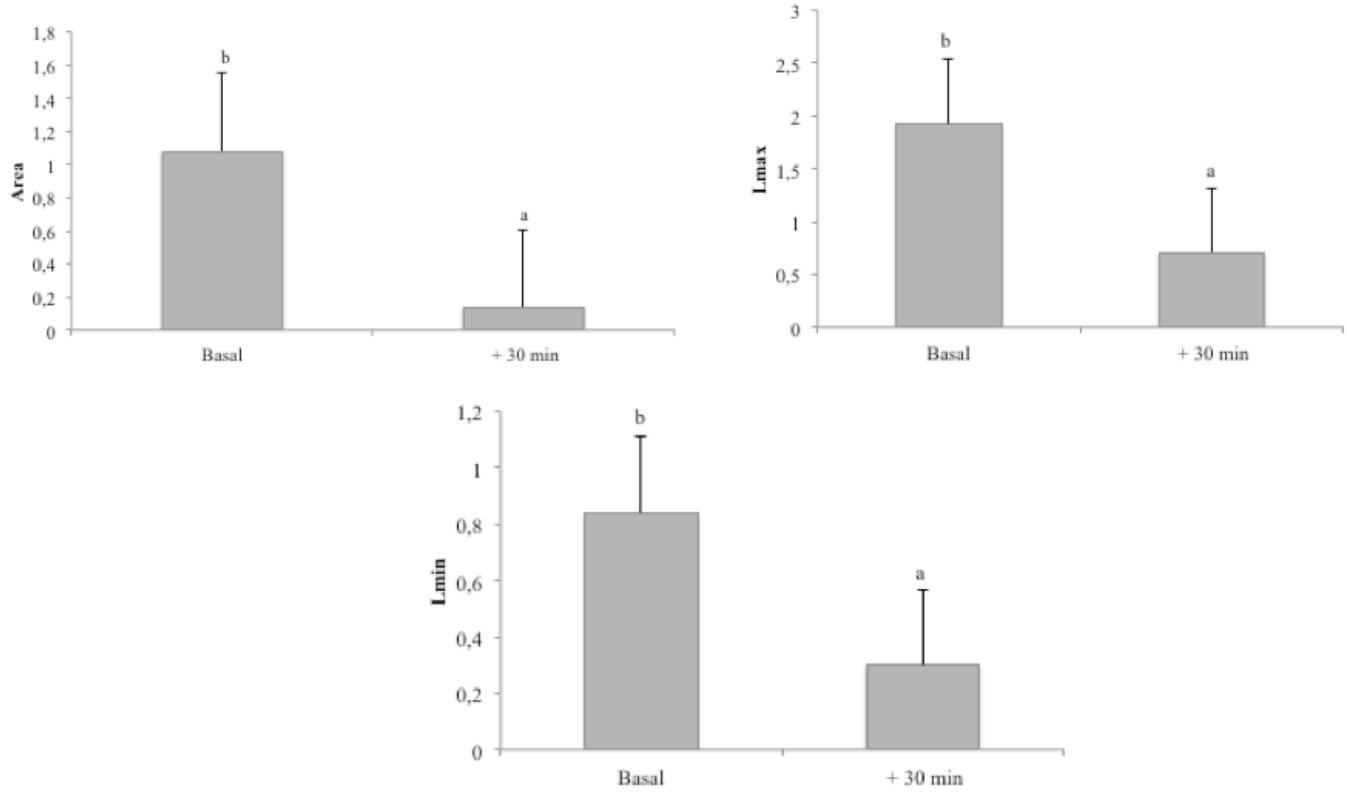

Figure 19. Effect of stress on hepatocytes size (areas and lengths).

0B (0% barley. 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.223% β-glucans); 160B (16% barley, 0.53% β-glucans) and 320B (31.9% barley, 1.5% β-glucans) are the different experimental diets. Different letters indicate significant differences ($P < 0.05$) between different experimental diets.

Results showed that the stress did not have a significant effect on the incidence of appearance of cell infiltration (Fig. 3 – supplementary data), on the vacuolation (Fig. 4 – supplementary data) and mucus cells (Fig. 5 – supplementary data) of the posterior intestine (data not shown).

3.3. Interaction between diets and hypoxia on liver and gut histology and morphology

Thirty minutes after the stress higher barley concentrations showed lower hepatocyte areas and lengths (Table 11) as it was observed before the stress, showing

the smallest hepatocytes sizes on fish fed with the diet 320B (areas of 0.125 μm^2 on 320B diets compared to 0.157 μm^2 on control diet).

Table 11. Areas, maximum (Lmax) and minimum (Lmin) lengths of hepatocytes of fish fed different experimental diets after the stress challenge (n=4).

	DIETS[1]						
	0B	**40B**	**80B**	**160B**	**320B**	**SEM**	**P-value**
Area	0.157^{b}	0.156^{b}	0.137^{ab}	0.129^{a}	0.125^{a}	0.008	0.0159
Lmax	0.741^{b}	0.775^{c}	0.694^{abc}	0.687^{ab}	0.644^{a}	0.031	0.0362
Lmin	0.313	0.316	0.286	0.273	0.287	0.015	0.2251

[1] Experimental diets described in Table 1.

Data in the same row not sharing a common superscripts letter are significantly different ($p < 0.05$). SEM: standard error of the mean.

According to these results, liver cell vacuolation was also lower on fish fed with diets at higher barley concentration (Figure 20), except on fish fed with diet 80B that did not show significant differences with fish fed with the control diet.

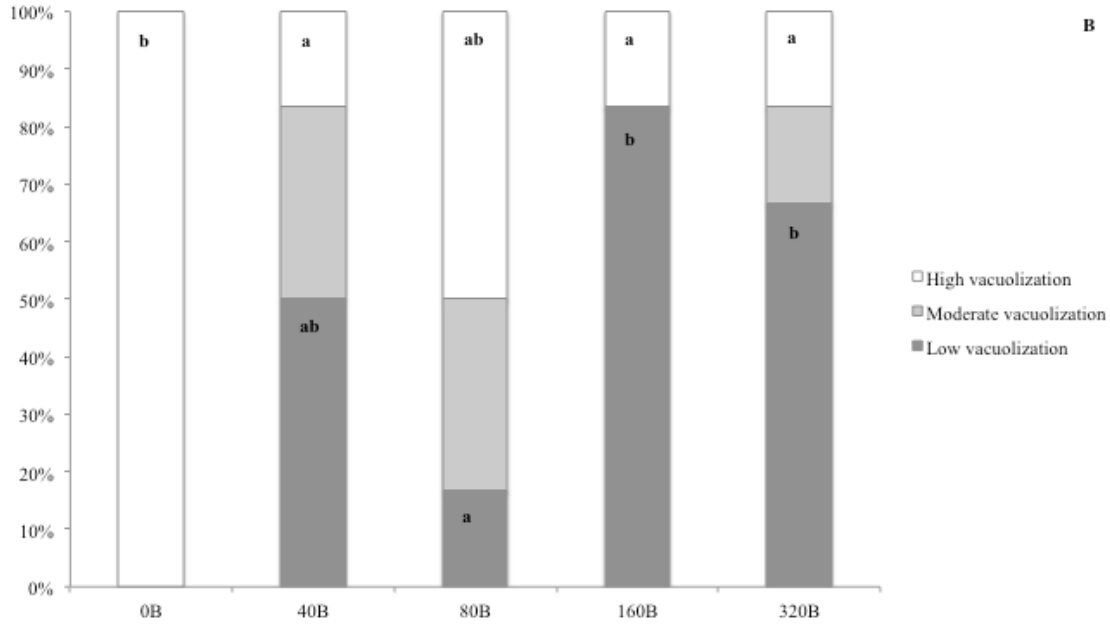

Figure 20. Hepatocytes vacuolation of fish fed different experimental diets after the stress challenge.

0B (0% barley. 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.223% β-glucans); 160B (16% barley, 0.53% β-glucans) and 320B (31.9% barley, 1.5% β-glucans) are the different experimental diets. Different letters indicate significant differences ($P < 0.05$) between different experimental diets.

The incidence of appearance of lymphocytic foci of fish fed with diets 40B was significantly lower than fish fed at higher barley concentrations, but did not show significant differences with control group (Figure 21).

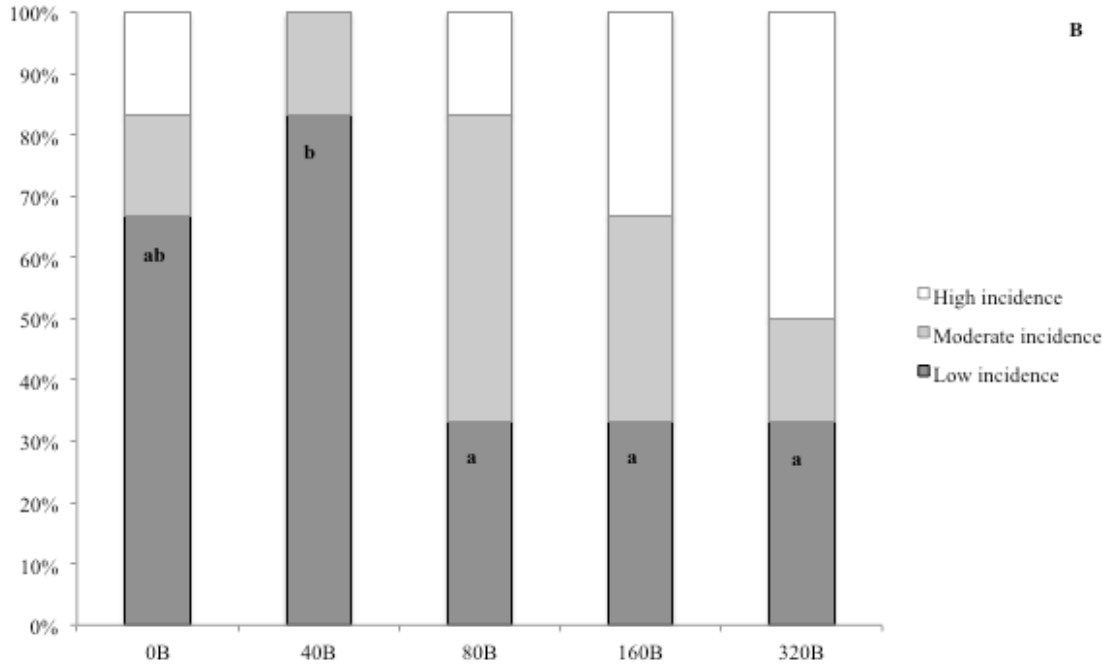

Figure 21. Incidence of lymphocytic foci appearance in hepatocytes of fish fed different experimental diets after the stress challenge.

0B (0% barley. 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.223% β-glucans); 160B (16% barley, 0.53% β-glucans) and 320B (31.9% barley, 1.5% β-glucans) are the different experimental diets. Different letters indicate significant differences ($P < 0.05$) between different experimental diets.

When the posterior intestine was examined after the stress the incidence of appearance of cell infiltration (Fig. 6 – supplementary data) and mucus cells (Fig. 7 – supplementary data) did not show significant differences regardless of the diet. Fish fed with diets 80B, 160B and 320B showed lower gut vacuolation than fish fed with control

and 40B diets (Figure 22).

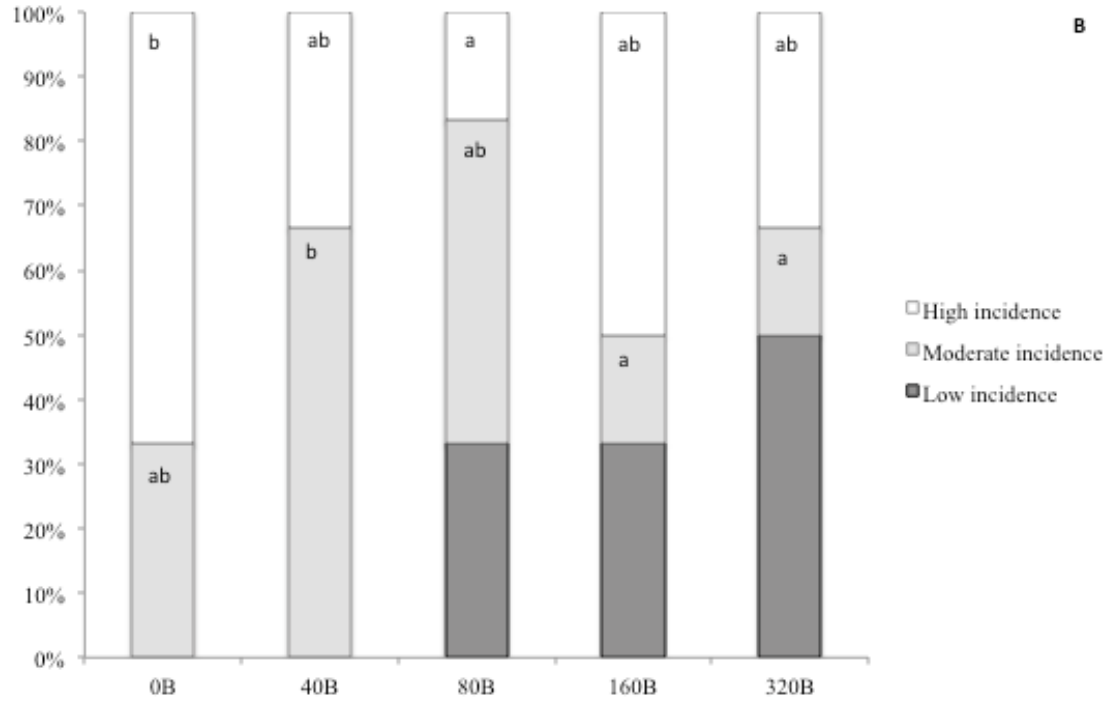

Figure 22. Gut vacuolation (posterior intestine) of fish fed different experimental diets after the stress challenge.

0B (0% barley. 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.223% β-glucans); 160B (16% barley, 0.53% β-glucans) and 320B (31.9% barley, 1.5% β-glucans) are the different experimental diets. Different letters indicate significant differences ($P < 0.05$) between different experimental diets.

4. DISCUSSION

Histological analysis of fish fed diets containing different barley concentrations exhibited morphological changes before and after an acute stress, unlike results obtained on rearing and quality parameters (Pinedo-Gil et al. 2017), where it was not observed any detrimental effect on rearing parameters and a positive enhancing effect on fish quality. Before the stress, it was observed the effect of barley on liver and gut histology and morphology. Fish fed at higher barley concentrations showed lower sized hepatocytes than fish fed with the control and 40B diets, showing the smallest

hepatocytes fish fed with the diet 320B. Liver cell vacuolation was significantly lower on fish fed with diets 80B and 160B than in the rest of the groups and higher barley level did not show significant differences with control. In general, bigger hepatocytes are more vacuolized than smaller hepatocytes as it was observed by other authors (Rusell et al. 2001, Pereira et al. 2002, Figueiredo-Silva et al. 2005). Similar results were observed by Rusell et al. (2001) when substituting cornstarch by pea seed meal on European sea bass diets. This substitution produced a lower vacuolation on liver of fish fed pea seed meal than the control diet, concluding that more lipids in the livers could be associated with a slower rate of glucose uptake and hence glucose is transformed into lipids. This effect was also observed by Pereira et al. (2002) feeding rainbow trout with brassica byproducts. Glencross et al. (2004) fed rainbow trout with yellow lupin as a source of protein and also observed that fish fed with diets with 50% of this ingredient had a significant decreased on hepatocytes lipid vacuolation. However, under rearing conditions has been reported by other authors a frequent liver vacuolation when diets are modified (Caballero et al. 2002, Figueiredo-Silva et al. 2005). Figueiredo-Silva et al. (2005) reported for juvenile sea bass that the accumulation of lipid in the liver could be due to a hepatocyte reaction to a new metabolic state when fish were fed with soybean in the diets, corresponding to a reactive adaptation to a new ingredient in short-term, and could lead into liver necrosis if the diet was not corrected. In the present work the liver tissue examination revealed inflammation by the presence of lymphocytic cells foci in fish of all groups. Fish fed with diets 80B and 160B showed the highest degeneration of liver and fish fed with diets 40B and 320B did not show significant differences with control. This was in accordance with what was reported by Figuereido-Silva et al. (2005), liver alteration appeared in those livers less vacuolated.

Results showed that barley significantly affect the histology of posterior gut. It

was observed cell infiltration in all groups, however, fish fed with diet 80B showed significantly the highest gut degeneration. Borquez et al. (2011) reported that the appearance of inflammatory cells infiltration in the lamina propria occur when carnivorous fish are fed with plant ingredients. The presence of inflammatory cells may be due to the presence of some antinutrients on barley. Many plant sources contain antinutrients (Francis et al. 2001) that may produce intestinal inflammation in some species. Barley can contain phytic acid that limit the absorption of some minerals and can produce hypertrophy and vacuolation of the intestinal epithelium (Francis et al. 2001). Mucus cells, which are mainly distributed along the villi, play an important role in synthesising and secreting mucin into the mucus layer to destroy pathogens (Heidarieh et al. 2013). In the present work, fish fed with control, 80B and 160B diets showed significantly higher number of mucus cells than fish fed with 40B and 320B diets. This is in accordance with the appearance of inflammatory cells. Fish fed with 80B diets showed the highest incidence of inflammation and mucus cells. The increase in the number of mucus cells is a good indicator to detect enteritis in carnivorous fish as it is supported by Urán (2008) and Tran-Ngoc et al. (2016). And gut vacuolation was significantly lower on fish fed with 40B and 160B diets than fish fed with the control diet. Probably, as it was said before, the presence of some antinutritional factor produce the vacuolation of the intestinal epithelium (Francis et al. 2001).

Results showed that the stress did not have a significant effect on liver and gut cell vacuolation and the incidence of appearance of lymphocytic foci and mucus cells in gut. Probably 30 minutes was not enough time to detect an histological or morphological change. However, 30 minutes after the stress, hepatocytes sizes (areas and lengths) significantly decreased and this decrease was more evident in those fish fed at higher barley concentration. Liver cell vacuolation was also lower in fish fed with

diets at higher barley concentration, similar to what was observed before the stress, except on fish fed with diet 80B that did not show significant differences with fish fed with the control diet. Surprisingly, after the stress the incidence of appearance of lymphocytic foci of fish fed with diets 40B was significantly lower than fish fed at higher barley concentrations but did not show significant differences with control group. And when the posterior intestine was examined after the stress the incidence of appearance of cell infiltration and mucus cells did not show significant differences regardless of the diet. After the stress, the differences observed before it disappeared and fish fed with diets 80B, 160B and 320B showed lower gut vacuolation than fish fed with control and 40B diets. In some way barley is playing a protective role. It seems that fish fed with barley showed smaller hepatocytes, less vacuolized and less inflammatory indicators. Barley β-glucan content or other components might have different immunostimulative effects. However, it should be study what happen in more than 30 minutes after the stress to observed if the stress has an effect or not on the different histological parameters.

5. CONCLUSION

In conclusion, although previous studies have demonstrated that wheat can be substituted by barley without any significant detrimental effect on rearing parameters and with a positive enhancing effect on fish quality (Pinedo-Gil et al. 2017), liver histological studies indicated that the increase of barley concentrations produced significant modifications, reducing hepatocytes size, vacuolation processes and increasing the incidence of appearance of lymphocytic foci. Barley in a concentration of 8% caused significant negative changes in the intestinal morphology of rainbow trout especially in the proximal intestine. After the acute hypoxia stress challenge it was not observed any change on histological and morphological on liver and gut. However, 30

minutes after the stress, hepatocytes size significantly decreased and this decrease was more evident in those fish fed at higher barley concentration. Liver cell vacuolation was also lower, similar to what was observed before the stress. It seems a positive effect of barley on liver inflammatory cells when fish were fed with 4% of barley.

Acknowledgements

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Supplementary data:

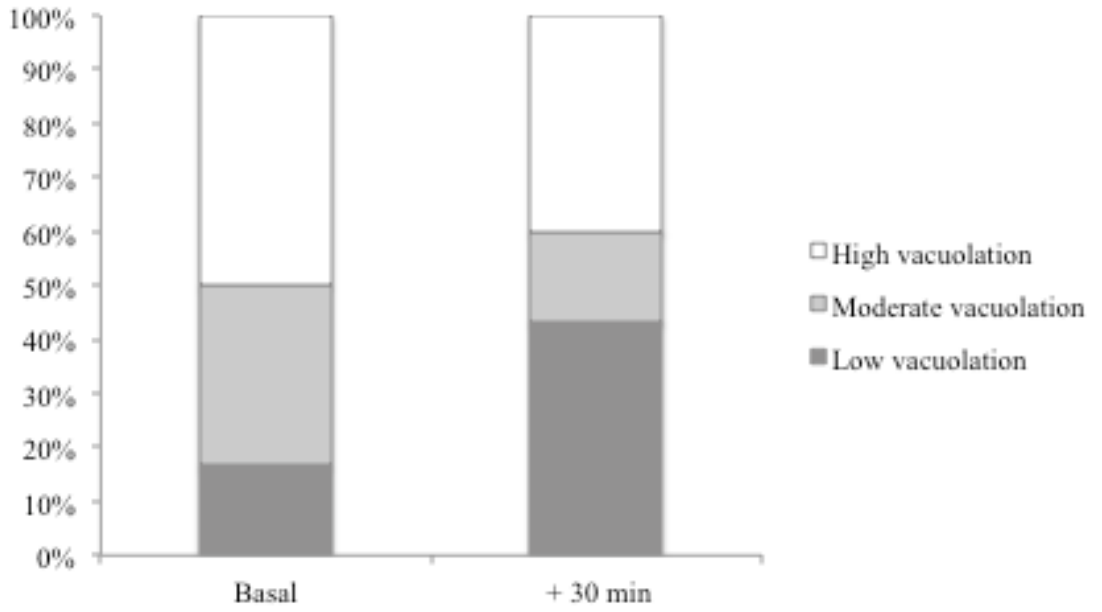


Fig. 1. Effect of stress on liver cell vacuolation. 0B (0% barley); 40B (4% barley); 80B (8% barley); 160B (16% barley) and 320B (31.9% barley) are the different experimental diets. Absence of different letters indicate no significant differences ($P < 0.05$).

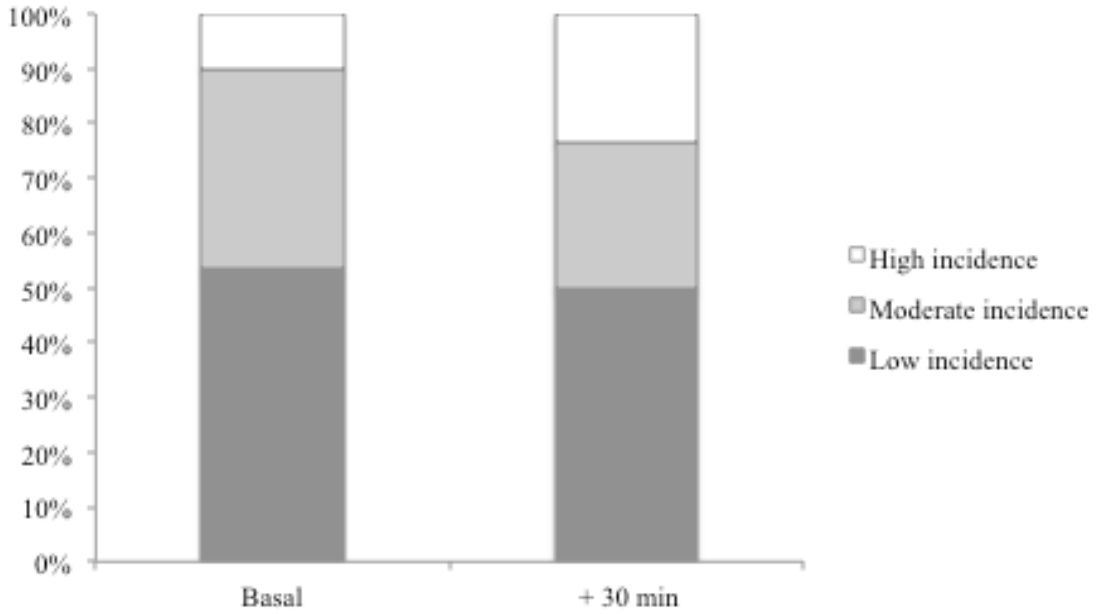


Fig. 2. Effect of stress on the incidence of appearance of lymphocytic foci. 0B (0% barley); 40B (4% barley); 80B (8% barley); 160B (16% barley) and 320B (31.9% barley) are the different experimental diets. Absence of different letters indicates no significant differences ($P < 0.05$).

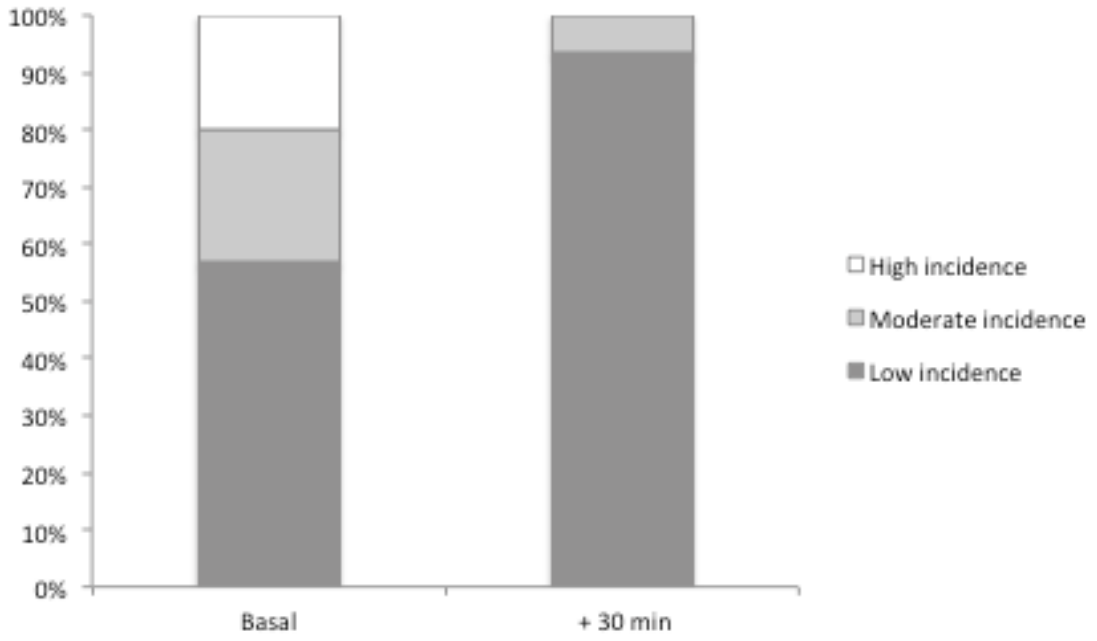


Fig. 3. Effect of stress on the incidence of appearance of cell infiltration on the posterior intestine. 0B (0% barley); 40B (4% barley); 80B (8% barley); 160B (16% barley) and 320B (31.9% barley) are the different experimental diets. Absence of different letters indicates no significant differences ($P < 0.05$).

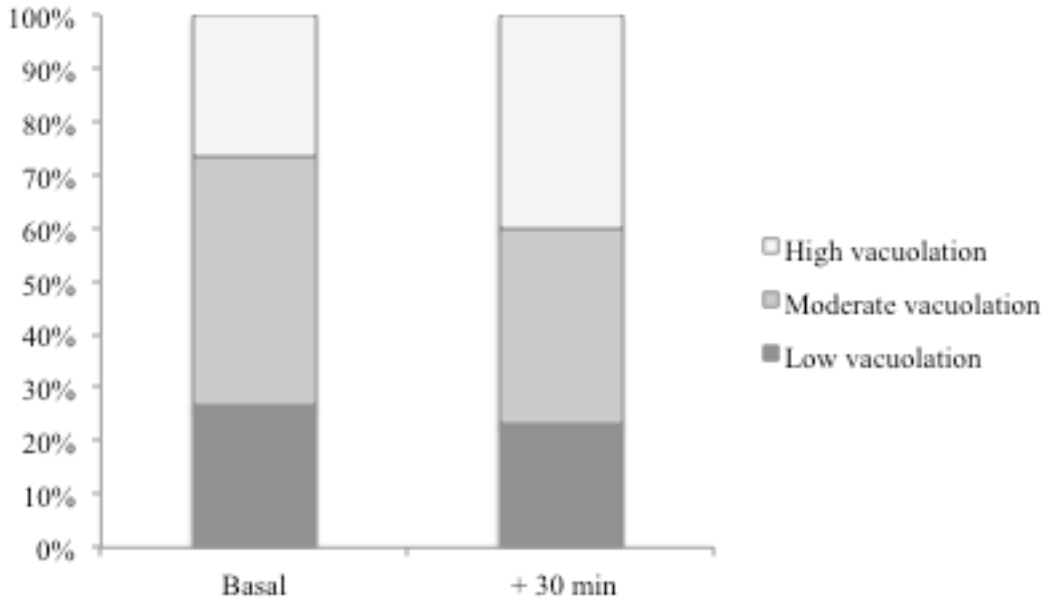


Fig. 4. Effect of stress on the incidence of vacuolation of the posterior intestine. 0B (0% barley); 40B (4% barley); 80B (8% barley); 160B (16% barley) and 320B (31.9% barley) are the different experimental diets. Absence of different letters indicates no significant differences ($P < 0.05$).

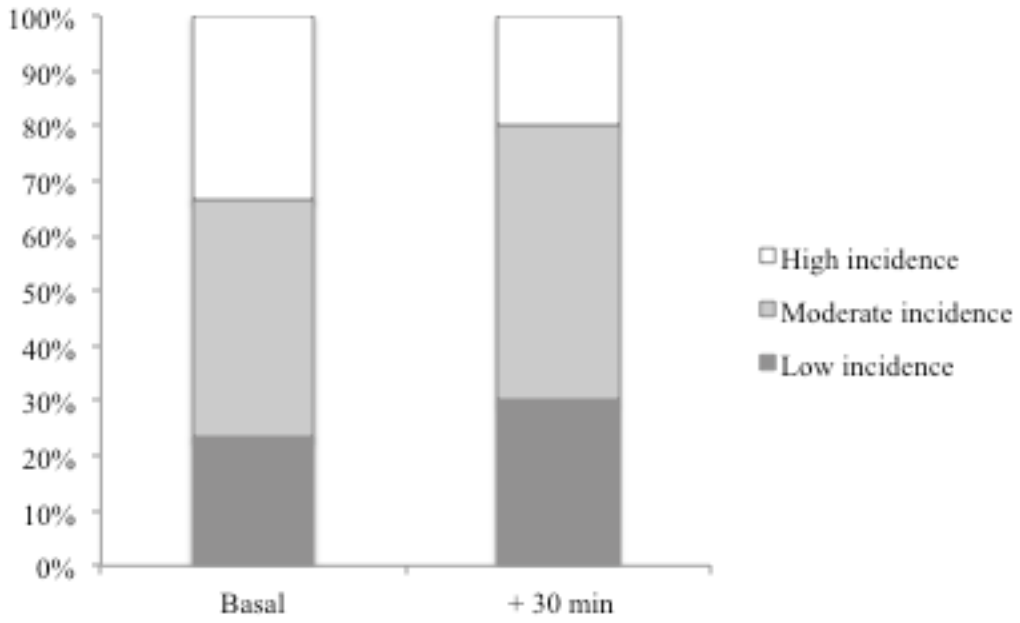


Fig. 5. Effect of stress on the incidence of appearance of mucus cells on the posterior intestine. 0B (0% barley); 40B (4% barley); 80B (8% barley); 160B (16% barley) and 320B (31.9% barley) are the different experimental diets. Absence of different letters indicates no significant differences ($P < 0.05$).

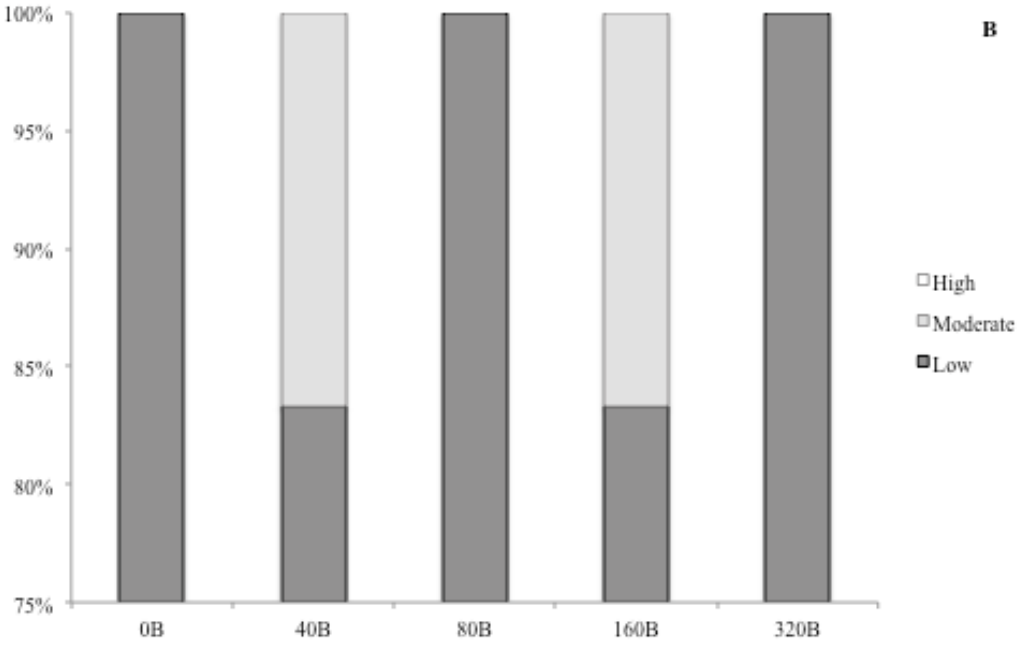


Fig. 6. Cell infiltration on the posterior intestine of fish fed different experimental diets after the stress challenge. 0B (0% barley. 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B

(8% barley, 0.223% β-glucans); 160B (16% barley, 0.53% β-glucans) and 320B (31.9% barley, 1.5% β-glucans) are the different experimental diets. Different letters indicate significant differences ($P < 0.05$) between different experimental diets

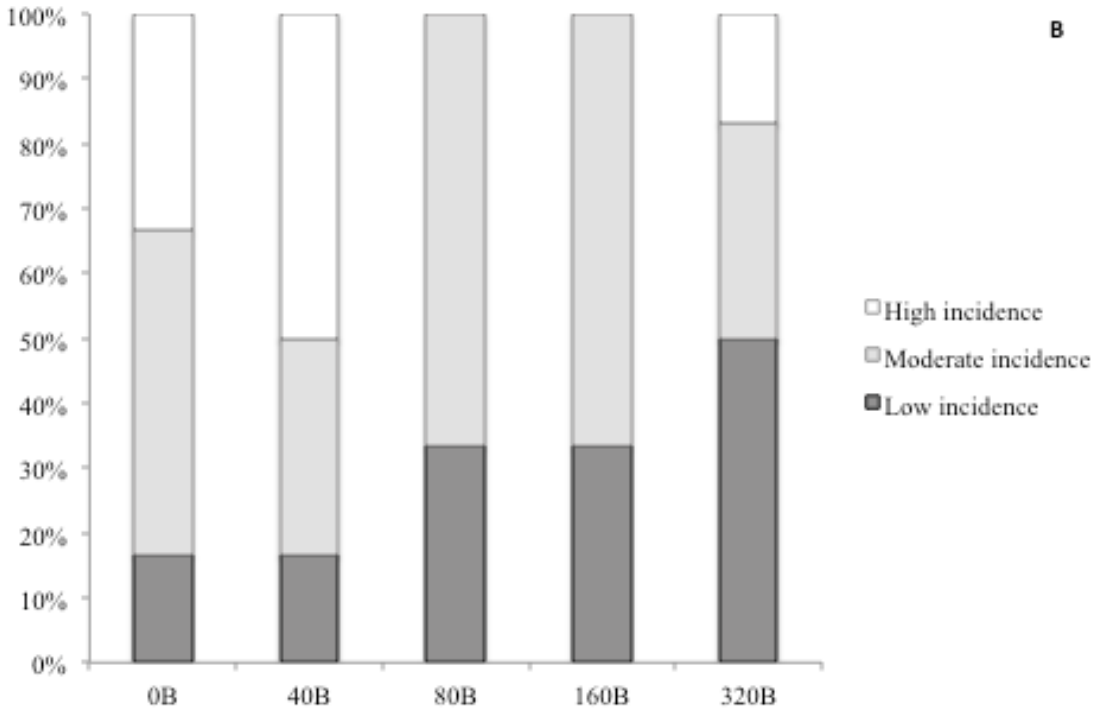
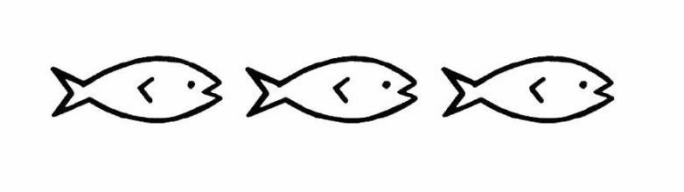


Fig. 7. Mucus cells on the posterior intestine of fish fed different experimental diets after the hypoxia challenge. 0B (0% barley. 0% β-glucans); 40B (4% barley, 0.14% β-glucans); 80B (8% barley, 0.223% β-glucans); 160B (16% barley, 0.53% β-glucans) and 320B (31.9% barley, 1.5% β-glucans) are the different experimental diets. Different letters indicate significant differences ($P < 0.05$) between different experimental diets

CHAPTER 7. EFFECTS ON LIPID OXIDATION AND BIOACTIVE PROPERTIES OF RAINBOW TROUT FILLETS FED WITH BARLEY.



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ABSTRACT

Barley concentrations ranging from 0% to 32% were incorporated into rainbow trout, *Oncorhynchus mykiss*, diets. The effect of barley concentration on lipid peroxidation and antioxidant activity were analysed on fish fillets. Results showed that the inclusion of barley on rainbow trout diets had an inhibitory effect on lipid oxidation probably associated with certain bioactive compounds reported on barley, which could interact scavenging and reducing metabolites involved in lipid oxidation. Concentrations up to 8% of barley produced an enhanced of fish fillets showing high antioxidant activity and higher levels of alpha-tocopherol.

Keywords: *Barley, lipid oxidation, antioxidant activity*.

Practical Applications: Barley is a cereal not frequently used in aquaculture. The present study demonstrate that its use would be interesting due to its capacity to enhance quality, oxidative stability and the antioxidant activity of fish fillet. That makes rainbow trout fillet healthier and could promote its sale.

1. INTRODUCTION

Rainbow trout (*Oncorhynchus mykiss*) is one of the major aquaculture fish species worldwide and is the second most consumed fish in Europe (FAO, 2013). During the last decade, the demand of rainbow trout has increased significantly for its high nutritional value, taste and aroma (Volpe et al. 2015, Shadman et al. 2017, Erbay et al. 2017). Trout is an important source of high-quality proteins, polyunsaturated fatty acids (PUFA´s), lipid soluble vitamins and micronutrients (Alparslan et al. 2014, Volpe et al. 2015, Erbay et al. 2017), although can be rapidly oxidised leading to important sensory and nutritional quality losses (Pereira de Abreu et al. 2010, Yildiz et al. 2016, Erbay et al. 2017).

Incorporation of novel ingredients to subside the aquafeed cost is essential to balance product quality in order to control some negative aspects such as lipid oxidation (Pratoomyot et al. 2010, Valente et al. 2015, García-Romero et al. 2014, Pinedo-Gil et al. 2017). The use of natural antioxidants or ingredients preserve and reduce oxidation during and after fish processing. Therefore, organoleptic properties can be maintained, since lipid oxidation (hydroperoxide, peroxide value (PV) and their break down into other secondary compounds, most of them volatile products), is involved in the production of off-flavours (Razaei and Hosseini 2008).

Barley is an important source of β-glucans and other bioactive components such as phenolic compounds, which can control oxidative processes (Sandhu and Punia 2017). The use of barley on rainbow trout diets is not currently implemented at industrial scale, although few studies have investigated their incorporation on diet. It was observed that the incorporation of barley did not produce any negative effect on productive parameters (Sealey et al. 2008, Pinedo-Gil et al. 2017), however, more studies are required. For this reason, the objective of this study was the evaluation of

oxidative parameters and bioactive properties of rainbow trout fed at different barley concentrations.

2. MATERIAL AND METHODS

2.1. Experimental design

Rainbow trout were provided by a commercial trout farm (IPEASA, Fuentidueña, Segovia, Spain). The average weight for each fish was 127 ± 2.62 g (least-square mean ± SEM). Fish were fed with five isoproteic (40% crude protein) and isolipidic diets (18% crude fat), which contained different barley levels (0-31.9%, named 0B, 40B, 80B, 160B and 319B) (Pinedo-Gil et al. 2017). There were five feeding treatment groups each in four replicates (n=4). Fish were randomly sampled every 28 days (0, 28, 56 and 84 days) and skinless fish fillets were prepared for the evaluation. Three fish per replicate were evaluated for the different analysis (n=12). The skin was removed and fillets were kept frozen until analysis. Prior to analyses, all fish were starved for 24 h and anesthetized with MS222®; 200 mg L^{-1}. The duration of the trial was 84 days.

2.2. Fatty acid profile (FAME)

Fatty acid profile (FA) was determined in barley, diets and fish fillets by triplicate. Blight and Dyer (B&D) method (Blight & Dyer 1959) was used for lipid extraction. Lipid-containing chloroform phase was separated and after evaporated. The remaining phase was dissolved in 1 mL of hexane and a methylated procedure carried out by adding 100 µL of 0.5 M methanolic KOH and leaving the reaction for 10 min at room temperature (RT). The upper layer was transferred to a 2 mL vial. Analysis of FA methyl esters (FAME) were carried out on a gas chromatograph Agilent 7890A (Agilent Technologies, PA, California, USA) and a flame ionization detector. For the analysis

the method was run on 50 ºC to 200 ºC during the first 7 min at a rate of 3 ºC min^{-1} and held for 26 min. Injector and detector temperature were 250 ºC and 280 ºC, respectively. After, 1 µL of the hexane extract was injected in split mode (ratio 25:1), and FAMEs were identified by comparison of retention times with those of 37 FAMEs standard mix (Supelco, Sigma-Aldrich, CO).

2.3. Alpha-tocopherol content

Tocopherol content was determined according to the AOCS official method (1992) for fish fillets samples, using Agilent 1200 series HPLC equipped with a diode array detector. Two grams of the B&D extract was evaporated and resuspended in 2 mL of hexane with 20 µL of tocopherol acetate as internal standard. An aliquot (10 µL) was injected and a column (250 mm x 4.6mm 5 µm) (Teknokroma Anlítica S.A., Barcelona, Spain) was used. Elution was performed using an isocratic mixture of hexane:2-propanol (99.6:0.4; v:v) at a flow rate of 1.3 mL min^{-1}. Detection was set at 295 nm and 284 nm for tocopherol acetate. Results were expressed in µg tocopherol per gram of fillet.

2.4. Oxidative parameters

2.4.1 Peroxide value (PV)

PV was measured on the fish fillet using B&D extract according to the International IDF Standards method (1991). Results were expressed in meq of active oxygen per kg of lipids.

2.4.2. Conjugated hydroperoxides (dienes and trienes)

Conjugated hydroperoxides (fish fillet B&D extract) were measured as described by Undeland et al. (1998). Results were calculated as mmol of hydroperoxides per kg lipid.

2.5. Antioxidant markers

2.5.1. Total Flavonoid determination (TFC)

TFC was determined using the method described by Lin and Tang (2007) for barley and diets. Aliquots of 0.1 g of sample were dissolved in 1 mL of 10% aluminium chloride hexahydrate ($AlCl_3$), 0.1 mL of 1 M potassium acetate (CH_3COOK) and 2.8 mL of deionized water. After incubation at room temperature (RT) for 40 minutes the reaction was measured at 415 nm (Shimadzu PharmaSpec UV-1700. Milton Keynes, UK). The data were expressed as quercetin equivalent (QE) per 100 g of sample based on the moisture content of lyophilized powder and "fresh sample".

2.5.2. Extract preparation

Barley, diets and fish fillet were used for extracts preparation to measure antioxidant activity. One gram of blended sample was dissolved in 10 mL of 90% methanol. The extraction was accelerated using a ceramic homogenizer in the test tubes and stirring for 30 s. Following samples were centrifuged at 1.635 x g for 10 min at 4 ºC and the supernatants were collected, filtered and stored at -80 ºC. All the extracts were used for the determination of total phenols and oxygen radical absorbance capacity (ORAC).

2.5.3.Total phenols (TP)

TP were measured using the Folin-Ciocalteu method (Slinkard and Singleton 1977) on barley, diets and fish fillets. Results were expressed as mg of gallic acid per gram of dried weight (dw) sample.

2.5.4. Phenolic characterization using high-performance liquid chromatography (HPLC)

Phenolic characterization was determined on barley and diets. Five gramms of

sample were mixed with 45 mL of 80% ethanol (v/v) and after it was sonicated in a water bath for 1 h. After centrifugation (5000 x g, 20 min., 10 ºC), the supernatant was removed and the extraction was repeated twice. Supernatants were mixed and after evaporated at 40 ºC with nitrogen until complete dryness, reconstituted in 2 mL of 40% acetonitrile and then it was filtered through 0.45 µm membrane for HPLC analysis (Bonoli et al. 2004, Zhao et al. 2006).

The phenolic compounds were separated and quantified using the method described by Schieber et al. (2001) with modifications, briefly as follows. Water Alliance 2795 Chromatography Separations Module (Waters Corp., Milford, USA) coupled to a Waters 2996 PDA detector fixed at 280 nm of wavelength. Column employed was Zorbax sb-c18 Agilent (4.6 x 150 nm) 5 microns. The mobile phases consisted in 0.5% acetic acid (buffer A) and 20% (0.5% acetic acid):80% acetonitrile (buffer B). Initial gradient started with 5% of buffer B for 1 minute, and then was increased up to a 55% for 50 minutes; the column was cleaned for 5 minutes by pumping 95% of buffer B and finally it was re-equilibrated for another 10 minutes. Calibration curves were constructed using the following standards: gallic acid, chlorogenic acid, ferulic acid, p-cumaric acids, synaptic acid, 3-coumaric acid, 4-coumaric acid, syringic acid, maleic acid, transcinamic acid, vanilic acid, caffeic acid and 4-hydroxibenzoic acid at concentration of 5, 10, 20, 40 and 80 µg mL^{-1}.

2.5.5. Determination of the oxygen radical absorbance capacity (ORAC)

The procedure was based on the method described by Ou et al. 2001. The determination was measured on fish fillets. Results were expressed as µmol of Trolox Equivalent (TE) per g of sample (dw).

2.6. Statistical analysis

Statistical analysis was performed using SAS version 9 (SAS Institute Inc., Cary, North Caroline, USA) by a GLM procedure for the variance analysis (ANOVA) followed by a t-Student test and considering significant differences between values with a P-value < 0.05.

3. RESULTS AND DISCUSSION

Proximate composition and β-glucan in barley, experimental diets and fillets were determined in Pinedo-Gil et al. 2017. It was observed that all diets were isoproteic (40% protein) and isolipidic (18% lipid), barley contained 5.2% β-glucan and its inclusion on experimental diets introduce this component to the diets (0 to 1.5% β-glucan). In fillet, the inclusion of barley increased significantly crude fat while protein was not affected.

3.1. Fatty acid profile

The replacement of wheat with barley in rainbow trout diets resulted in a marked decrease of linoleic acid (C18:2 n-6), linolenic acid (C18:3 n-3) and docosahexanoic acid (DHA, C22:6 n-3) in the experimental diets compared with the control (Table 12).

Table 12. Fatty acid profiles (FA) of barley and expeirmental diets.

	Barley	DIETS[1]				
		0B	**40B**	**80B**	**160B**	**319B**
SFA						
C14:0	0.00	0.205	0.125	0.200	0.195	0.200
C16:0	0.36	2.22	2.28	2.29	2.30	2.38
C18:0	0.36	3.81	2.125	0.50	0.49	0.59
MUFA						
C16:1	0.00	0.23	0.24	0.23	0.24	0.22
C18:1 (n-9)	0.00	0.00	1.89	3.57	3.57	3.30
PUFA						
C18:2n6	0.84	5.61	5.49	5.03	5.01	3.82
C18:3n3	0.03	0.42	0.42	0.39	0.39	0.3
C20:5n3 (EPA)	0.00	0.27	0.27	0.11	0.21	0.09
C22:6n3 (DHA)	0.02	0.46	0.44	0.40	0.38	0.27

[1] Experimental diets: 0B (0% barley); 40B (4% barley); 80B (8% barley); 160B (16% barley); 319B (31.92% barley).

SFA (saturated fatty acid); MUFA (monounsaturated fatty acid); PUFA (polyunsaturated fatty acid); DHA (docosahexaenoic acid, 22:6 n-3); EPA (Eicosapentaenoic acid, 20:5 n-3).

However, results showed that although the fatty acid profile change with the concentration of barley, it had not a significant effect on fillets total SFA´s, MUFA´s or PUFA´s (Table 13). Significant differences ($P < 0.05$) were observed when individual FA´s were analysed: an increase on myristic acid (C14:0) and palmitic acid (C16:1) levels on fish fed with 319B diets. The fatty acid composition was in agreement with values for fresh rainbow trout fillets as reported by other authors (Ozden 2005, Volpe et al. 2015). It is well known that the fatty acid composition of fish fillets reflects the fatty acid composition of the diet (Turchini et al. 2009, Volpe et al. 2015) but is also modified by metabolic processes (Drew et al. 2007). Trout can elongate and desaturate C18:3 n-3 into the longer chain n-3 fatty acids (Tocher et al. 2001). Probably, for this reason, although fish fed with barley showed less PUFA content these differences were not significant.

Table 13. Fatty acid profiles (FA) of rainbow trout fillets when fish were fed with increasing barley levels. (data are expressed as % of dry matter) **(n=12).**

	DIETS[1]					SEM	Sign.
	0B	**40B**	**80B**	**160B**	**319B**		
SFA	20.24	20.74	21.27	17.29	19.94	1.47	N.S.
C14:0	1.57^{ab}	1.66^{abc}	1.70^{bc}	1.40^{a}	1.89^{c}	0.10	**
MUFA	33.22	33.95	35.52	43.49	37.70	3.58	N.S.
C16:1	2.37^{ab}	2.75^{b}	2.67^{b}	2.05^{a}	3.36^{c}	0.16	**
C18:1 (n-9)	27.27	27.61	29.08	38.05	30.40	3.86	N.S.
PUFA	46.54	45.31	43.21	39.22	42.36	2.56	N.S.
n-3	14.60	13.98	13.69	12.52	12.70	0.94	N.S.
n-6	31.39	30.97	29.09	24.79	29.18	1.63	N.S.
n-9	30.86	31.20	32.86	41.44	34.34	3.70	N.S.
n-3 / n-6	0.47	0.45	0.47	0.50	0.44	0.03	N.S.
ARA	0.75	0.68	0.68	0.61	1.43	0.36	N.S.
EPA	2.07	2.02	2.06	1.99	178	0.17	N.S.
DHA	8.88	8.28	7.95	7.25	7.37	0.60	N.S.
ARA / EPA	0.37	0.33	0.33	0.32	0.85	0.22	N.S.
EPA / DHA	0.24	0.24	0.26	0.27	0.24	0.01	N.S.

[1] Experimental diets as shown in table 1.

SFA (saturated fatty acids); MUFA (monounsaturated fatty acids); PUFA (polyunsaturated fatty acids); ARA (arachidonic acid, 20:4 n-6); DHA (docosahexaenoic acid, 22:6 n-3); EPA (Eicosapentaenoic acid, 20:5 n-3).

Different superscript letters indicate significant differences ($P < 0.05$) between the experimental diets. ** indicates P-values < 0.0001.

3.2. Alpha-tocopherol content

Alpha-tocopherol content was measured on fillets from fish fed at different barley concentrations. Fish fed with diets 80B showed significantly higher α-tocopherol content than control and fish from the rest of experimental diets (Figure 23). Alpha-tocopherol has an important antioxidant activity and is well absorbed by rainbow trout when included on their diets (Timm-Heinrich et al. 2013, Valente et al. 2015). In spite of α-tocopherol was not determined on barley, the inclusion of 8% of barley on rainbow trout diets improved fillet α-tocopherol content, and probably enhanced the antioxidant properties of fillets of fish fed with 80B diets. Several studies have reported that some

vegetable ingredients contain some endogenous antioxidants, which are present in small amounts and can enhance tocopherol antioxidant activity and this might explain the stability of fish fed with 80B diets (Lauritzsen et al. 1999, Thiyam et al. 2006).

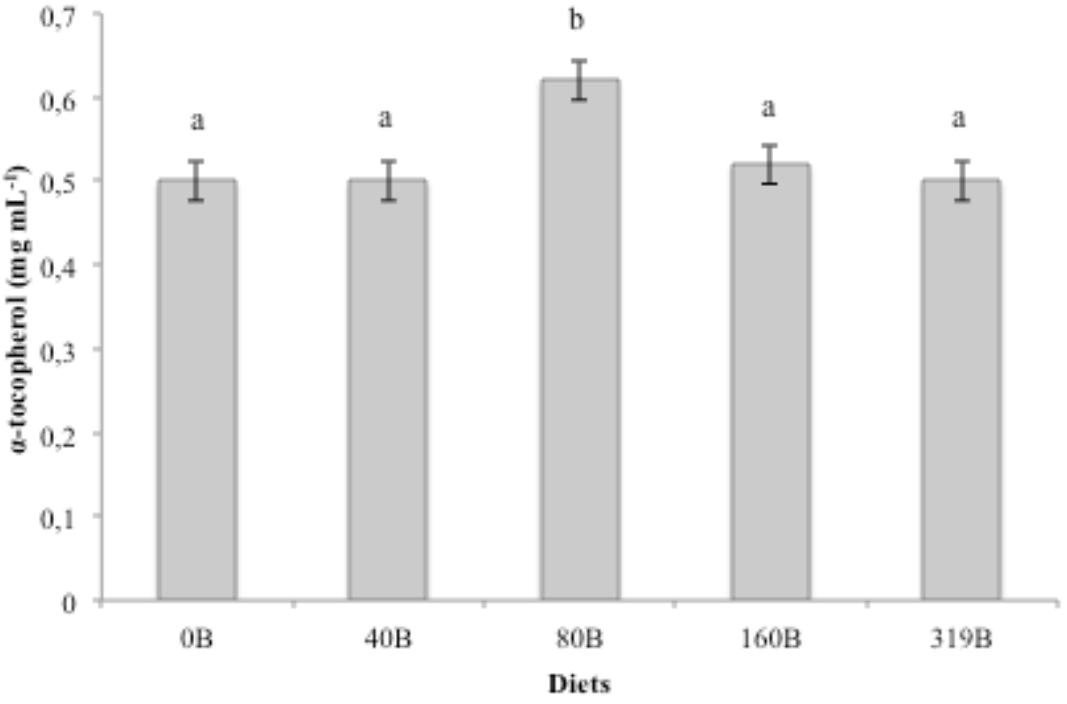

Figure 23. Fillet alpha-tocopherol content of fish fed different experimental diets (n=12). Different experimental diets

0B (0% barley); 40B (4% barley); 80B (8% barley); 160B (16% barley); 319B (31.92% barley). Different small letters (a, b) correspond to significant differences ($P < 0.05$) between different samples.

3.3. Oxidative parameters

3.3.1. Peroxide value (PV) and conjugated hydroperoxides (dienes and trienes)

No differences were observed regardless of the diet on the peroxide value (PV) (Table 14). However, when conjugated hydroperoxides (dienes and trienes) were evaluated, it was observed that barley concentration only showed a significant effect ($P < 0.05$) on trienes (Table 14). The highest value of trienes was observed in control fillets (7.61 mmol of hydroperoxides kg lipid^{-1}) and the lowest value in fish fillets at the

highest barley concentration (319B) (2.66 mmol of hydroperoxides Kg lipid^{-1}).

These results suggested that barley had a positive effect on the control of oxidative process, since oxidative markers were lower in fillets came from fish fed with barley. This could be related to the higher antioxidant activity of fish fed at higher barley concentration and with the lower TBARS values as shown in Pinedo-Gil et al. 2017.

Table 14. Effect of barley on the peroxide value (PV) and conjugated hydroperoxides (dienes and trienes) of rainbow torut fillet of fish fed at different barley concentrations (data are expressed as % of dry matter) (n=12).

	DIETS[1]						
	0B	**40B**	**80B**	**160B**	**319B**	**SEM**	**Sign.**
PV	13.26	12.87	11.83	11.77	7.62	3.40	N.S.
Dienes	18.65	15.70	18.00	17.46	13.71	1.35	N.S.
Trienes	7.61^{b}	4.02^{a}	4.46^{a}	4.18^{a}	2.66^{a}	1.07	*

[1] Experimental diets as shown in table 1.

Different superscript letters indicate significant differences ($P < 0.05$) between the experimental diets. * indicates P-values >0.0001.

3.4. Antioxidant markers

3.4.1. Total flavonoid content (TFC) and total phenolic content (TP)

Barley TFC values were of 0.02 µg QE g^{-1} (Figure 24). Differences in barley flavonoid content may be influenced by genotype, agronomic practices, climatic conditions, maturity at harvest, postharvest and handling and storage conditions (Erdman et al. 2007). Total flavonoid content was determined in the different experimental diets it was observed that the substitution of wheat for barley significantly decreased the TFC (Figure 24). Flavonoid content in diets comes from other compounds different from barley.

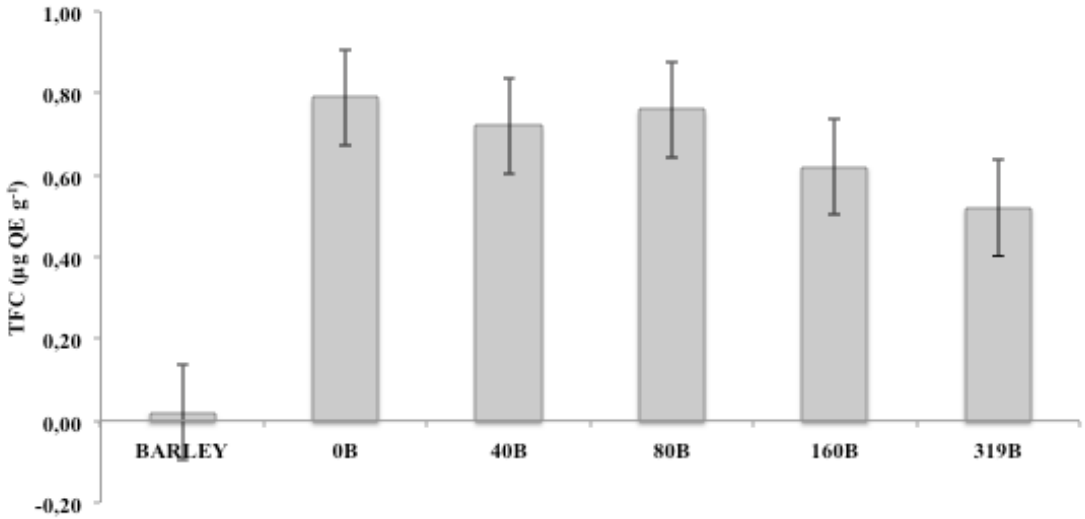

Figure 24. Barley and experimental diets total flavonoid contents (TFC). Different experimental diets

0B (0% barley); 40B (4% barley); 80B (8% barley); 160B (16% barley); 319B (31.92% barley).

Phenolic acids are present in cereal grains and their content in cereals is usually lower than 1% of dry matter (Abidi et al. 2015). It has been reported that barley contains more total phenols than wheat (Ragaee et al. 2006, Fogarasi et al. 2015). The value obtained in the study was 1.17 mg of GAE g^{-1} (Figure 25). This result was in accordance with the values obtained by Zhao et al. (2006), who reported that the values of TP varied from 1.03 to 1.87 mg of GAE g^{-1}. However, TP can vary significantly among barley varieties (Abidi et al. 2015). Surprisingly, it was not expected that diets containing higher barley concentration showed significantly lower TP than control or diets with 4% and 8% of barley (Figure 25), since, as it was said before barley contains more total phenols than wheat. The reason of this result could be that phenolic compounds are heat labile (Sharma & Gujral 2011) and less resistant to the heat that can alter their nature (Sharma et al. 2012). The reduction in TP may be due to the decomposition of phenolic compounds due to the high extrusion temperature during the feed elaboration process.

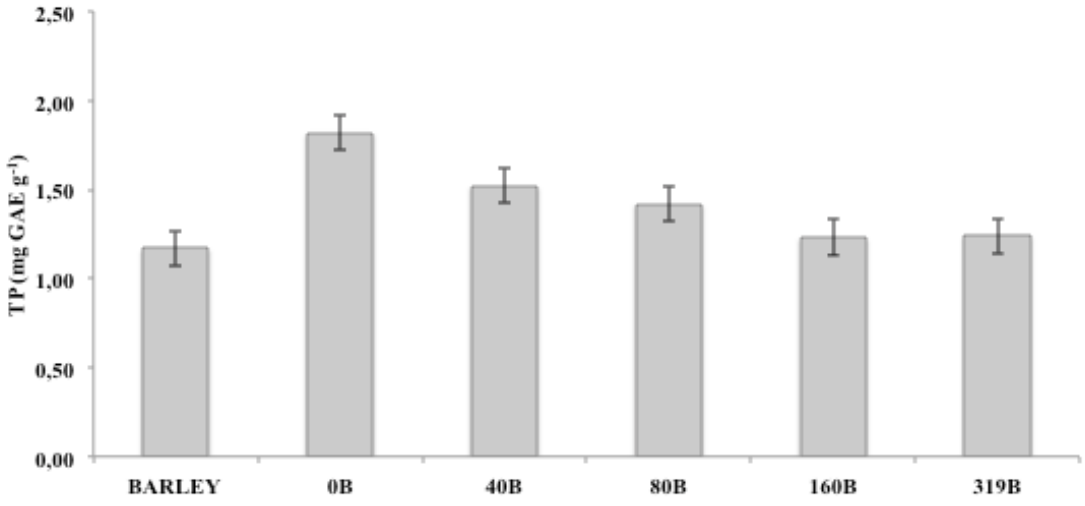

Figure 25. Barley and experimental diets total phenolic contents (TP). Different experimental diets

0B (0% barley); 40B (4% barley); 80B (8% barley); 160B (16% barley); 319B (31.92% barley).

According to these results when TP was determined on fish fillets, TP was higher in fillets of fish fed without barley than those fed with barley, regardless the concentration used (Table 16).

3.4.2. Individual phenolic compounds

Individual phenolic compounds were determined in barley and experimental diets. HPLC barley profile appears in Figure 26, and the contents of individual phenolic compounds are summarized in Table 15. Eleven phenolic compounds were identified and quantified in barley and the experimental diets. The results showed that maleic acid (14.76 μg mL^{-1}) was the most abundant phenolic compound in barley, followed by 4-hydroxybenzoic (5.34 μg mL^{-1}), 3-coumaric acid (2.87 μg mL^{-1}), caffeic acid (2.58 μg mL^{-1}), vanillic acid (1.72 μg mL^{-1}), gallic acid (1.45 μg mL^{-1}), ferulic acid (1.43 μg mL^{-1}), 4-coumaric acid (1.40 μg mL^{-1}), syringic acid (0.97 μg mL^{-1}), chlorogenic acid (0.65 μg mL^{-1}) and transcinnamic acid (0.50 μg mL^{-1}). These results were not in agreement to results reported by Naczk & Shahidi (2006) where ferulic acid and hydroxybenzoic acid

were the main phenolics. Probably differences can be associated to the variety of barley. In this study naked barley was used and the lack of cover or peel can be the responsible on the differences on the profile.

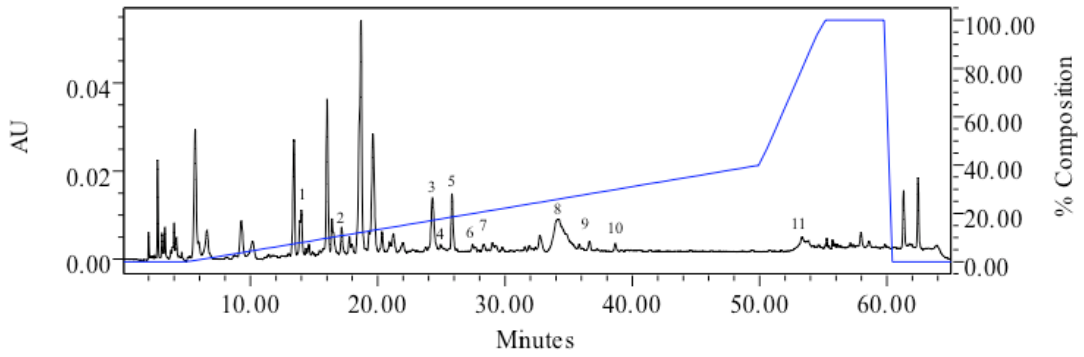

Figure 26. HPLC chromatogram of phenolic compounds profile in barley extracts

1. Gallic acid; 2. Maleic acid; 3. 4-hydroxybenzoic acid; 4. Chlorogenic acid; 5. Vanillic acid; 6. Caffeic acid; 7. Syringic acid; 8. 4-coumaric acid; 9. Ferulic acid; 10. 3-coumaric acid; 11. Transcinamic acid.

Table 15. Content in individual phenolic compounds in barley and experimental diets (μg mL^{-1})**.**

	Barley	DIETS				
		0B	40B	80B	160B	319B
1 Gallic acid	1.45	69.97	18.65	89.28	40.78	59.06
2 Maleic acid	14.76	94.52	79.17	53.59	45.30	56.73
3 4-hydroxybenzoic acid	5.34	4.14	4.10	2.20	3.35	3.91
4 Chlorogenic acid	0.65	0.11	1.61	5.03	11.78	17.60
5 Vanillic acid	1.72	4.30	6.52	3.53	2.01	3.60
6 Caffeic acid	2.58	4.45	2.63	4.42	2.52	2.52
7 Syringic acid	0.97	0.23	0.89	0.41	2.65	7.68
8 4-coumaric acid	1.40	0.06	0.11	0.10	0.44	0.70
9 Ferulic acid	1.43	1.02	0.68	0.65	0.07	0.01
10 3-coumaric acid	2.87	3.66	6.89	1.95	1.95	1.95
11 Transcinamic acid	0.50	5.78	8.11	1.04	11.58	12.93

• Experimental diets as shown in table 1.

When the different phenolic compounds were determined in the experimental diets results showed that the inclusion of barley increased 4-coumaric acid content and

decreased maleic acid content. This study showed no differences on the rest of the phenolic compounds.

3.4.3. Fillets antioxidant activity

The antioxidant activity of fish fillets from fish fed with the different experimental diets was evaluated through total phenol content (TP) and ORAC activity. Table 16 shows that TP was higher in fillets of fish fed without barley than those fed with barley, regardless the concentration used. However, antioxidant capacity measured using ORAC method, showed that fillets from fish fed 8% barley concentration had the highest antioxidant activity. Probably, the absence of correlation between ORAC and TP could was due to the presence of non-phenolics compounds with high antioxidant activity. It is important to remember that barley is rich in β-glucans.

Table 16. Effect of barley on antioxidant properties of rainbow trout fillets of fish fed at different barley concentrations (data are expressed as % of dry matter) (n=12).

	DIETS[1]						
	0B	**40B**	**80B**	**160B**	**319B**	**SEM**	**Sign.**
TP (µmol Trolox Eq. g-1)	224.39	194.20	214.91	216.54	188.63	15.45	N.S.
ORAC (µmol Trolox Eq. g^{-1})	18.43	18.93	20.07	16.70	17.04	1.39	N.S.

[1] Experimental diets as shown in table 1.

Total phenols (TP), ORAC (oxygen radical absorbance capacity).

Different superscript letters indicate significant differences ($P < 0.05$) between the experimental diets.

4. CONCLUSION

Results indicated that the inclusion of barley had an inhibitory effect on fish fillets lipid oxidation probably associated with certain compounds present on barley, which could act by scavenging and reducing lipid oxidation. Concentrations of 8% barley enhance antioxidant properties, improving α-tocopherol content and ORAC values in fish fillets. However, concentrations higher than 8% produced a negative

effect on fish fillets.

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CHAPTER 8. RED BEET AND BETAINE AS INGREDIENTS IN DIETS OF RAINBOW TROUT (*Oncorhynchus mykiss*): EFFECTS ON GROWTH PERFORMANCE, NUTRIENT RETENTION AND FLESH QUALITY.

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ABSTRACT

A control diet was compared to 4 experimental diets in which two red beet (14 and 28%) and betaine (0.9 and 1.63%) levels were incorporated on rainbow trout diets. The study was set up with an average weight of 69 ± 2.21 g and finished when fish reached commercial weight (175 to 250 g) after 105 days. The impact of the diets was studied based on the growth performance, biometric indexes, proximal composition, protein and fat retention efficiencies and apparent digestibility of fish reared on a recirculation system. Moreover, it was studied the effect of red beet and betaine on the flesh proximate composition and quality (water activity, colour, texture, TBARS and sensory characteristics) of the final product. Results showed that inclusions of 14% of red beet and 0.9% of betaine did not produce an effect on growth, nutritive or biometric parameters, nor nutrient retentions compared to control diet, however, higher concentrations had a negative effect on growth and nutritive parameters. These ingredients enhanced quality parameters regardless of the concentration used. Fish flesh enriched with the new ingredients showed lower water activity and better textural and colour properties than control diet and also had a dose-dependent effect on lipid oxidation.

***Keywords**: red beet; betaine; growth; rainbow trout; diet; fish product; quality; sensory scores*.

1. INTRODUCTION

Carnivorous fish species, including salmonids, the incorporation of digestible carbohydrates (CHO) should not exceed 20% of the diet. Cereals (wheat, barley, oat, corn) have been traditionally the most utilized CHO sources in commercial salmonid diets (Sealey et al. 2008, Gaylord et al. 2009, Pinedo-Gil et al. 2016). However, those ingredients generally contain high fibre and starch content and these, together with the presence of some antinutritional components, produce limitations to the inclusion of plant ingredients on carnivorous fish diets (Oliva-Teles et al. 2015). Also, some CHO sources produce a reduction of feed palatability, which leads to reduce fish intake and growth (Lim et al., 2016). On the other hand, plant ingredients can be an important source of antioxidant and other bioactive components (Ganessan et al. 2011). Red beet (*Beta vulgaris* L.) is a source rich in natural betaine and also rich in important nutrients including magnesium, sodium, potassium, vitamin C and betalains (Han et al. 2014). In aquaculture, betaine is widely used as a common additive due to its bioactive properties as osmoprotector and enhancing feed palatability. Its incorporation could also enhance the quality of the final product, especially on the colour of fish flesh. However, to the best of our knowledge, the use of red beet as a source of betaine in fish nutrition has been scarce studied. For this reasons, natural sources, such as red beet, as an alternative CHO ingredient in fish diets should be taken into account from a health concern point of view and its effect on the quality parameters of rainbow trout flesh. The objective of this work was to evaluate the impact of red beet and betaine incorporation at different concentrations on a controlled population of rainbow trout diets on their growth performance and final fish flesh quality parameters.

2. MATERIAL AND METHODS

2.1. Diets

Five extruded isoproteic (40% crude protein (CP) and isolipidic diets (18% crude lipid (CL) diets were formulated. A control diet was compared to four experimental diets using two red beet (14 and 28%) and betaine (0.9 and 1.63%) levels. Betaine was of natural origin obtained from red beet betaine. Both ingredients were combined in a factorial design. The five diets were identified as: Control diet (0% red beet; 0% betaine), diet A (14% red beet; 0.9% betaine), diet B (14% red beet; 1.63% betaine), diet C (28% red beet; 0.9% betaine) and diet D (28% red beet; 1.63% betaine). The formulation and the composition of the diets are given in Table 17. Control diet was prepared using same ingredients as experimental diets but without red beet and betaine on the formulation. The control diet was not a commercial diet, was produced in the same conditions than modified diets. There were five feeding treatment groups, each in three replicates (n=3).

Diets were prepared using the cooking extrusion process with a semi-industrial twin-screw extruder (CLEXTRAL BC-45. St. Etienne, France). Processing conditions were the following: a screw speed at 0.63 x g, a temperature of 110 ºC and a pressure of 40-50 atm. Experimental diets were assayed in triplicate groups (n=3). Fish were fed by hand twice a day (8:00 am and 15:30 pm) until apparent satiation, six days per week during the whole experimental period. Pellets were distributed slowly to allow all fish to eat. The uneaten diet was collected and dried to determine feed intake (FI).

Table 17. Formulation and proximate composition of the experimental diets.

	Diets*				
	CONTROL	**A**	**B**	**C**	**D**
Ingredients [g/kg] - international feed number					
Fish meal	222	222	222	222	222
Wheat	338	168	160	0	0
Red Beet	0	140	140	280	280
Natural betain	0	23	48	20	45
Wheat gluten	170	175	160	201	189
Meat meal	103	103	101	105	92
Soybean oil	91	93	93	96	96
Fish oil	45	45	45	45	45
Maltodextrin	11	11	11	11	11
Multivitamin and mineral mix¶	20	20	20	20	20
Analyzed composition [% dry matter]					
Dry matter	95.00	96.50	96.10	94.70	94.40
Crude Protein (% CP)	38.30	40.60	41.10	39.90	41.20
Crude Fat (% CF)	17.60	17.40	19.50	17.30	16.80
Ash (%)	8.20	8.60	8.20	7.90	8.20
Betain (%)	0.00	0.90	1.63	0.90	1.63

- Different experimental diets: CONTROL (0% red beet, 0% betaine), A (14% red beet, 0.9% betaine), B (14% red beet, 1.63% betaine), C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine); ¶Contains: Choline, 10 g; DL-α-tocopherol, 5 g; ascorbic acid, 5 g; $Ca_3(PO_4)_2$, 5 g and a premix: 25 g. This premix contains per kg: retinol acetate, 20000 IU; calciferol, 10 IU; DL-α-tocopherol, 0.2 g; menadione sodium bisulfite, 0.016 g; thiamine hydrochloride, 0.05 g; riboflavin, 0.05 g; pyridoxine hydrochloride, 0.3 g; cyanocobalamine, 0.5 mg; nicotinamide, 0.3 g; pantothenic acid, 0.12 g; folic acid, 13 mg; biotin, 1.4 mg; ascorbic acid, 1.5 g; inositol, 0.3 g; betaine, 2 g; polypeptides, 0.24 g; Zn, 0.1 g; Se, 0.4 mg; I, 10 mg; Fe, 4 mg; CuO, 0.3 g; Mg 0.115 g; Co, 0.4 mg; methionine, 0.024 g; cysteine, 0.016 g; lysine, 0.026 g; arginine, 0.012 g; phenylalanine, 8 mg; tryptophan, 0.014 g (Dibaq Diproted S.A., Spain).

2.2. Rearing markers

2.2.1. Growth trial and fish sampling

A total of 900 rainbow trouts were provided by a local fish farm (Cien Fuentes Fishfarm, 19420 Cifuentes, Guadalajara, Spain) and transported alive to the Aquaculture Research Centre of the Agro-Technological Institute of Castilla y León, Spain. Prior to the feeding trial, all fish were acclimated to the indoor rearing conditions for two weeks and fish were fed once a day (8:00 am) up to apparent satiation using exclusively the control diet. Groups of 60 fish (average initial weight of 69 ± 2.21 g (mean ± SD)) were housed in 15 cylindrical fiberglass tanks (three per treatment, n=3). The capacity of each tank was 500 L (initial stocking density 8.4 ± 0.5 kg m^{-3}).

The trial was conducted in a recirculating freshwater system (RAS). Water temperature was 14.67 ± 0.57 ºC (mean ± SD). Level of dissolved oxygen in water was 7.97 ± 0.87 mg L^{-1}. All tanks were equipped with aeration and an oxygen probe. Water pH was 7.93 ± 0.12 and ammonia and nitrites concentration in water were 0.16 ± 0.14 and 0.19 ± 0.17 mg L^{-1} respectively. Water flow was 10.30 ± 0.98 L h^{-1}. The photoperiod consisted on 12 h of light and 12 h of dark intervals, having all tanks identical lightning conditions.

Fish were weighed and length measured at approximately 35-day intervals to study all rearing parameters (growth, final weight, biomass increment (BI), survival, thermal growth coefficient (TGC), specific growth rate (SGR) and nutritional parameters, FI and feed conversion ratio (FCR). Prior to weighing, all fish were starved for 24 h and anesthetized with MS222®; 200 mg L^{-1}. At the end of the growth trial, all fish were individually weighed and measured. Three fish were randomly sampled from each tank (n=3) and used for the determination of biometric indexes (condition factor

(CF), viscerosomatic index (VSI) and heptosomatic index (HIS) and final whole fish proximate composition. The duration of the trial was 105 days.

2.2.1.1. Calculations of rearing markers.

Different indexes were evaluated in order to assess rearing parameters.

BI was evaluated as an indicator of fish biomass increment from day one to day 105 (1).

$$BI\ [g] = Bf - Bi \quad (1)$$

Where Bi and Bf are the initial and final biomasses of fish at the beginning and end of the feeding trial, respectively [g].

To determine the impact of stress response to the fish survival, mortality was registered during the whole experimental period. Knowing the initial number of fish and dead fish allowed calculating **mortality** (2) and once determined, **survival** was calculated as follows (3):

$$\text{Mortality [\%]} = (\text{Number of fish died} / \text{Initial fish number}) \cdot 100 \quad (2)$$

$$\text{Survival [\%]} = 100 - \text{Mortality} \quad (3)$$

An accurate prediction of growth potential for fish under husbandry conditions is a prerequisite to estimate energy or feed requirements. The most commonly used formula is the **SGR**, which is based on the natural logarithm of body weight (4), but also **TGC** was calculated (5)

$$SGR = 100 \cdot ((\ln Wf - \ln Wi) / t) \quad (4)$$

$$TGC = (Wf^{(1/3)} - Wi^{(1/3)}) / [\text{days} \cdot \Sigma (T - 4)] \quad (5)$$

Where Wi and Wf are the initial and final body weights of fish at the beginning and end of the feeding trial, respectively [g], t is the experimental duration [d] and T is

the temperature in ºC.

FCR measures animal efficiency in converting nutriment into muscle or weight gained overtime (6).

$$FCR = (F / (Bf - Bi)) \quad (6)$$

Where Bi and Bf are the initial and final biomasses of fish at the beginning and end of the feeding trial, respectively [g] and F is the weight of feed supplied to fish in the feeding trial.

In order to avoid an excessive amount of feed given, **FI** [g per 100 g fish and day] was calculated (7). Protein is the main nutrient in fish diets and to evaluate the weight gained per unit of protein fed protein efficiency ration (**PER**) was determined as shown in (8).

$$FI = 100 \cdot (\text{Feed consumption [g]} / (\text{average biomass} \cdot t)) \quad (7)$$

Where t is the experimental duration [d].

$$PER = \text{wet weight gain} / \text{protein intake} \quad (8)$$

Calculated biometric indexes were the **CF** based on the weight-length data to evaluate fish population fitness (9); and **HSI** (10) and **VSI** (11) were used to evaluate the nutritional status.

$$CF = 100 \cdot (Wf / L^3) \quad (9)$$

Where Wf is the final body weight of fish at the end of the feeding trial [g] and L is the average body length of fish [cm].

$$HSI = 100 \cdot (\text{wet weight of the liver} / Wf) \quad (10)$$

Where Wf is the final body weight of fish at the end of the feeding trial [g].

$$VSI = 100 \cdot (\text{wet visceral weight} / Wf) \quad (11)$$

Where Wf is the final body weight of fish at the end of the feeding trial [g].

2.2.2. Apparent digestibility coefficients (ADCs)

Digestibility studies were conducted simultaneously to the feeding trial. After fish were fed for a second time, tanks were completely cleaned and faeces were collected in a settling column (Cho et al. 1982), which was emptied in the following morning at 8:00 h. Wet faecal content was then collected and dried at 60 ºC for 48 h prior to analysis (CP, CL, and ash-insoluble ashes (AIA). Over the whole experimental period, samples of faeces were collected from each tank (n=3).

The ADCs of protein, fat and carbohydrates in the diets tested were calculated according to the following formula (12):

$$ADC\ [\%] = 100 \cdot [100 - ((\text{marker in diet} / \text{marker in faeces}) \cdot (\text{PN in faeces} / \text{PN in diet})] \quad (12)$$

Where PN is the percentage of nutrient.

2.3. Proximate composition analysis

Compositional analyses were performed to the raw material (red beet), the ingredient (betaine), the diet, the fish and faeces obtained during the assay, and the final fish product (flesh). These analyses were performed in accordance with AOAC (1990) procedures: Dry matter (60 ºC to constant weight), ash (incinerated at 550 ºC to constant weight), crude protein (N · 6.25 and nitrogen was analysed by Dumas principle, TruSpec CN; Leco Corporation, St. Joseph, MI, USA) and crude lipid content using the Soxhlet extraction method. AIA was used as an indicator for the ADC, and was analyzed according to the method described by Atkinson et al. (1984) with some modifications. Briefly, 5 g of sample were ashed for 5 h at 550 ºC to ensure complete

combustion of the organic material in the sample. The resulting ash was boiled until dryness in 75 mL of HCl (2 N) and boiled in other 75 mL HCl for 15 min. Samples were filtered hot through ashless filter paper and washed in boiling distilled water until the samples were neutralized. Finally, following Atkinson et al. (1984) method, samples were ashed for 5 h at 550 ºC. Betaine content on diets, faeces and fish flesh were analysed. Briefly, betaine and esters were extracted from the sample in a mixture of methanol and water. For total betaine determination, a part of the extract was saponified with a 2 M KOH solution, hydrolysing the betaine ester to free the betaine, which is then quantified. The extract was further diluted and analysed on LC/MS ESI + ionization in which the quantification was based on the known isotopic marker internal standard. The betaine content was expressed as mg kg^{-1}.

2.4. Quality markers of fish flesh and fish sampling

Every 35-d intervals three fish per tank (n=3) were randomly taken for the determination of quality parameters (water activity, colour, texture and sensory analysis) until fish reached commercial weight (times of sampling: 0, 35, 70 and 105 d).

2.4.1. Water activity (a_w)

Water activity (a_w) was measured using an Aqualab 4TE (Decagon Devices inc., Pullman, WA, USA). Six measurements were carried out in each flesh at three different locations (front, central and tail). The study was evaluated in three independent fish flesh (n=3).

2.4.2. Colour

The colour was measured using a colorimeter (Minolta CM-2002, Osaka, Japan) for the evaluation of CIELAB parameters. The L* value represents lightness and + a*, - a* and b* values represent redness, greenness and yellowness, respectively. Six

measurements were taken directly over the muscle, randomly over skinless fish flesh. The study was evaluated in three independent fish flesh (n=3). Hue (13) and Chroma (14) were calculated using the following formulas for all experimental points:

$$Hue = \arctan (b^* / a^*) \quad (13)$$

$$Chroma = (a^{*2} + b^{*2})^{1/2} \quad (14)$$

2.4.3. Texture analysis

Texture was determined using a texture analyzer TA-XT2i (ANAME, Stable Micro System, Vienna Court, Lammas Road, Godalming, Surrey, UK). A texture profile analysis (TPA) was carried out using a penetration probe of 4 mm of diameter at speed of 1 mm/s with a 5 mm distance; the instrument was equipped with a 25 kg load cell. The time delay between cycles was 5 s. Previous to analysis, samples were peeled manually and texture was analysed in the front, middle and tail parts. Fish flesh was evaluated in the same position, with the muscle fibres perpendicular to the test probe. The study was evaluated by triplicate in three independent samples of fish flesh per treatment (n=3).

TPA curves were used to evaluate the ***hardness*** [g] (maximum force required to compress the sample), ***cohesiveness*** (capacity of the sample to deform before rupture (A2 / A1, where A1 is the total energy required for the first compression and A2 is the total energy required for the second compression)), ***elasticity*** [mm] (capacity of the sample to recover its original shape after deformation force ends) and ***gumminess*** [g] (strength to disintegrate a sample to a constant state of swallowing (hardness × cohesiveness)).

2.4.4. Thiobarbituric Acid Reactive Substances (TBARS)

TBARS as an indicator of lipid oxidation was evaluated using the methodology described by Vyncke (1975). Briefly, ten grams of samples were mixed with 30 mL of 7.5% TCA. The mixture was homogenized and centrifuged for 5 min at 4 ºC and 5570 x g, and then filtered with Whatman nº 1 filters (Prat Dumas, France). Five mL of the filtrate were mixed with 5 mL 0.02 M TBA, incubated at 90ºC in a water bath during 40 min; the reaction was measured at 530 nm (Fluostar® Omega, BMG labtech, Germany). Two fish were analysed per treatment during the entire experiment (n=6) and the results were expressed as μmol malonaldehide (MDA) per kg of fresh flesh produced.

2.4.5. Sensory analysis

All sensory analysis were performed according to ISO standards (ISO 2001, 2008) in a sensory room compliant with ISO 2007 by a panel of eight people (four male and four female aged between 25 and 50) with previous experience in sensory analysis of food products. Nonetheless, in order to train the panel with the sensory assessment of fish products and optimise the tables used for sensory evaluation, the panel were trained in the main characteristics necessary for the study.

Sensory analysis comprised fresh whole fish and fish meat samples. Whole fish was evaluated using the quality index method (QIM) and fish flesh was analysed using a quality descriptive method (QDM). Panellists were trained to perform both analyses. QIM was assessed following the guideline of QIM Eurofish (Martinsdóttir et al., 2001). Freshness was evaluated by giving demerit points according to certain aspects associated with general appearance such as skin, stiffness, odour, gill pots colour and odour, belly, and eyes brightness and shape. The trained judges scored ranked from 0-3 for each attribute. The maximum score of 3 corresponded to the fish with the worst quality parameters values.

For the QDM, panellists were trained to discriminate colour, texture, odour and acceptability of fish flesh. A continuous non-structured scale (1-10) was used for evaluation. The left side of the scale corresponded to the lowest intensity (value 1: white, soft, fresh odour and acceptable sample) whereas the right side corresponded to the highest intensity (value 10: dark, hard, rancid odour and non-acceptable sample).

Panellists evaluated one fish per treatment every 28 d during the experiment (n=2). Five samples, in pairs of whole fish and flesh of each treatment, were individually presented in porcelain dishes to each panellist. Samples were coded with random numbers and maintained at room temperature (RT) during evaluation.

2.5. Statistical analysis

The feeding trial was designed according to a factorial design with two red beet levels and two betaine levels. All data (rearing and quality parameters) were subjected to one-way ANOVA to determine the significance due to effects of dietary treatments, and two-way ANOVA to determine the significance due to levels of red beet, betaine or their interaction. Post Hoc was analysed by Tukey´s HSD test with statistical significance determined at $p > 0.05$. All statistical analysis were carried out using software SAS (SAS version 9, SAS Institute Inc., Cary, North Carolina, USA).

2.6. Ethical statement

The rainbow trout study complied with the European Union Council Directive 2010/63/UE, which provides the minimum standards for animal protection, and was also in accordance with the Spanish national legislation (Spanish Royal Decree 53/2013) based on animal protection in experimentation and other scientific practices and approved by the Animal Ethics Committee of Agro-Technological Institute of Castilla y León (Spain).

Fish in tanks were checked on a daily basis. Every four weeks, fish were weighed individually and their health status was assessed by observation, after sedation with MS222 dissolved in water (MS222®; 200 mg L^{-1}) to minimize animal suffering.

Animals were euthanized by excess of MS222 (300 mg L^{-1}) or with ice (when quality samples were taken) and then fish were dissected.

3. RESULTS

3.1. Diets

Table 17 shows the proximate composition of the different experimental diets. Diets were fish standard formulas in which the percentage of wheat was replaced by red beet. The whole-wheat portion substituted the highest red beet concentration; the other compounds were not modified.

3.2. Rearing markers

3.2.1. Growth performance, biometric parameters, body composition and nutrient retention efficiencies

The experiment started with an initial average fish weight of 69 ± 2.21 g (mean ± SD) and finished when fish reached commercial weight (175.27-250.72 g). Growth performance of rainbow trout fed with experimental diets is shown in Table 18. Results show that, at the end of 105 d, fish fed with red beet (RB1 and RB2) and betaine (B1 and B2) had a significant decrease ($p < 0.05$) on Wf and also on the SGR and TGC compared to control diets. When the interaction effect was studied, diet A did not show significant differences on growth performance in terms of Wf, SGR and TGC ($p > 0.05$) with the control group, whereas diet C significantly reduced ($p < 0.05$) those parameters compared to the other treatments. No significant differences were observed on FI.

Besides, compared to control diet showed a significant decrease on PER and changes in the FCR were only affected by the inclusion of red beet, following an opposite tendency from PER. Fish fed with diet A did not show significant differences with control diet, while diet C showed the worst values from a productive point of view for PER and FCR.

CF, VSI and HSI were significantly affected by red beet (RB1 and RB2) and betaine (B1 and B2) concentration (Table 19). CF decreased significantly ($p < 0.05$) with the inclusion of both ingredients. On the contrary, the interactive effect (experimental diets) showed that fish fed with control and D diets had significantly higher CF values than the other dietary treatments. VSI increased significantly ($p < 0.05$) with the inclusion of red beet (RB1 and RB2) and betaine (B1 and B2). Increasing levels of red beet and betaine on the diet increased significantly ($p < 0.05$) VSI. Fish fed with diet D did not show significant differences with control. On the other hand, HSI increased significantly with the inclusion of red beet, although this increase was only observed on fish fed with diet B1 and not in diets with higher betaine concentrations. The same effect was observed analysing the interactive effect (experimental diets).

In the present study, whole body composition was not significantly affected by the diet (Table 19).

Table 18. Effect of red beet and total betaine level on growth and nutritive parameters of rainbow trout (values are least-squares means ± SEM, n=3).

	Red beet Level [¶] [%]			Betaine Level [†] [%]			Interaction (Diets [*])					SEM	Two-way ANOVA (*p*-value)		
	CONTROL	RB1	RB2	CONTROL	B1	B2	CONTROL	A	B	C	D		Red beet	Betaine	Red beet •Betaine
Growth parameters															
Initial weight [g]	69.82	71.02	69.09	69.82	70.85	69.24	69.82	73,54	68.51	68.17	69.78	1.628	0.555	0.664	0.057
Final weight [g]	250.72^{b}	214.06^{ab}	198.27^{a}	250.72^{b}	208.37^{ab}	203.15^{a}	250.72^{d}	241.47^{cd}	186.66^{ab}	175.27^{a}	215.52^{bc}	7.481	0.019	0.033	< 0.05
SGR [% / day] [≠]	1.22^{b}	1.04^{a}	1.00^{a}	1.22^{b}	1.02^{a}	1.02^{a}	1.22^{d}	1.13^{cd}	0.95^{ab}	0.90^{a}	1.07^{bc}	0.031	0.012	0.016	< 0.05
TGC · 10^{-3} [§]	0.22^{b}	0.19^{a}	0.17^{a}	0.22^{b}	0.18^{a}	0.18^{a}	0.22^{d}	0.21^{cd}	0.17^{ab}	0.15^{a}	0.19^{bc}	0.007	0.013	0.019	< 0.05
FI [g / 100 g fish / day] [#]	1.05	1.03	1.02	1.05	1.04	1.01	1.05	1.06	1.00	1.01	1.02	0.035	0.617	0.528	0.224
FCR°	1.18^{a}	1.26^{ab}	1.37^{b}	1.18	1.34	1.30	1.18^{a}	1.19^{a}	1.32^{b}	1.50^{c}	1.28^{ab}	0.024	0.022	0.115	< 0.05
PER⊙	2.19^{b}	1.95^{a}	1.80^{a}	2.19^{b}	1.87^{a}	1.87^{a}	2.19^{d}	2.07^{cd}	1.84^{ab}	1.68^{a}	1.90^{bc}	0.037	0.001	0.007	< 0.05

[*] Diets explanation as in Table 1; [¶] Red beet concentration: RB1, diets with 14% of red beet and RB2, diets with 28% of red beet; [†] Betaine concentration: B1, diets with 0.9% of betaine and B2, diets with 1.64% of betaine; [a—c] Means with different superscripts in each row differ significantly ($p < 0.05$).

[≠] Specific growht rate [%/day] SGR = 100 · ln (final weight / initial weight) / days.

[§] Thermal growth coefficient TGC = (final weight $^{(1/3)}$ – initial weight $^{(1/3)}$) / [days · Σ (ºC – 4)]

[#] Feed Intake ratio [g/100 g fish/day]. FI = 100 · feed consumption [g] / biomass [g] · days.

° Feed Conversion Ratio FCR = feed intake [g] / weight gain [g].

⊙ Protein Efficiency Ratio PER =Weight gain [g] / Protein intake [g].

Table 19. Effects of red beet and total betaine level on biometric parameters, body composition and nutrient retention of rainbow trout (values are least-squares means ± SEM, n=3).

	Red beet Level ¶ [%]			Betaine Level ¶ [%]			Interaction (Diets*)					SEM	Two-way ANOVA (*p*-value)		
	CONTROL	RB1	RB2	CONTROL	B1	B2	CONTROL	A	B	C	D		Red beet	Betaine	Red beet •Betaine
Biometric indexes															
CF †	0.887^{b}	0.842^{a}	0.839^{a}	0.887^{b}	0.837^{a}	0.844^{a}	0.887^{b}	0.859^{ab}	0.823^{a}	0.803^{a}	0.861^{ab}	0.010	< 0.05	< 0.05	< 0.05
VSI ≠	8.64^{a}	9.40^{ab}	10.00^{b}	8.64^{a}	9.80^{b}	9.60^{ab}	8.64^{a}	9.04^{a}	9.75^{ab}	10.56^{b}	9.44^{ab}	0.299	< 0.05	< 0.05	0.032
HSI #	1.16^{a}	1.36^{b}	1.37^{b}	1.16^{a}	1.45^{b}	1.28^{a}	1.16^{a}	1.46^{b}	1.26^{a}	1.45^{b}	1.29^{ab}	0.053	< 0.05	< 0.05	0.040
Proximal composition [% dry matter]															
Moisture [%]	72.33	74.60	73.13	72.33	73.57	74.01	72.33	73.23	75.97	73.90	72.55	1.133	0.299	0.595	0.133
Crude Protein [%]	14.01	13.14	14.19	14.01	13.40	13.97	14.01	12.99	13.28	13.80	14.49	0.612	0.167	0.493	0.774
Crude Lipid [%]	11.27	9.18	9.70	11.27	9.95	9.03	11.27	10.53	7.82	9.36	9.95	0.882	0.636	0.302	0.119
Ash [%]	2.45	2.47	2.47	2.45	2.35	2.57	2.45	2.24	2.69	2.46	2.49	0.094	0.957	0.060	0.065
Feed Retention efficiency [%]															
Protein															
PIR (% intake) ■	35.21	26.16	27.68	35.21	25.50	28.24	35.21	27.47	24.85	23.53	30.79	3.393	0.768	0.494	0.159
PIR (% digested) °	41.06^{b}	29.88^{a}	31.34^{ab}	41.06^{b}	29.55^{a}	31.62^{ab}	41.06^{b}	32.41^{ab}	27.34^{ab}	26.69^{a}	34.83^{ab}	3.383	0.038	0.035	0.047
Fat															
FIR (% intake) ⊙	74.68	50.35	55.43	74.68	56.09	50.51	74.68^{b}	65.43^{ab}	35.27^{a}	46.74^{ab}	61.95^{ab}	8.252	0.627	0.368	0.015
FIR (% digested) §	80.92	54.51	63.91	80.92	63.04	56.60	80.92^{b}	71.51^{ab}	37.52^{a}	54.57^{ab}	70.91^{ab}	9.367	0.382	0.349	0.017

* Diets explanation as in Table 1; ¶ Red beet and betaine concentration on diets as explained in Table 2; [a—c] Means with different superscripts in each row differ significantly ($p < 0.05$).

† Condition factor [g/cm^3] CF = 100 · final weight / length^3 ; ≠ Viscerosomatic Index [%] VSI = 100 · wet visceral weight / final weight.

Hepatosomatic Index [%] HSI = 100 · wet liver weight / final weight ; ■ PIR (% intake) = 100 · (protein fish gain [g] / protein intake [g])

° PIR (% digested) = 100 · (protein fish gain [g] / protein digested [g]) ; ⊙ FIR (% intake) = 100 · (fat fish gain [g] / fat intake [g])

§ FIR (% digested) = 100 · (fat fish gain [g] / fat digested [g])

Feed retention efficiencies are shown in Table 19. A significant decrease ($p < 0.05$) on the protein retention efficiency (PIR , % digested) was observed with increasing levels of red beet (RB1 and RB2) and betaine (B1 and B2) on the diet. Fat retention efficiency (FIR, % intake and % digested) was not significantly affected by the inclusion of red beet and betaine individually, only an insignificant tendency of decreasing the values was observed. Compared to control diet, when the interaction was studied, it was observed a significant decrease ($p < 0.05$) on PIR and FIR (%intake and %digested) with increasing red beet and betaine concentrations on the diet.

3.2.2. Apparent digestibility coefficients (ADC)

The red beet and betaine concentration did not have any significant effect on the ADCprotein and ADCCHO. However, ADClipid was significantly affected by red beet concentration (RB1 and RB2). Increasing red beet levels on diets produced a decrease on ADClipid finding values ranging from 87.64% in RB2 diets to 92.36 % in control diets (Table 20).

Table 20. Apparent digestibility coefficients (ADCs) of protein (ADCprotein), lipid (ADClipid) and carbohydrates (ADCCHO) in rainbow trout fed the experimental diets differing on the source of carbohydrate (wheat and barley) (values are least-squares means ± SEM, n=3).

	Red beet Level ¶ [%]			Betaine Level ¶ [%]			Interaction (Diets *)					SEM	Two-way ANOVA (*p*-value)		
	CONTROL	RB1	RB2	CONTROL	B1	B2	CONTROL	A	B	C	D		Red beet	Betaine	Red beet •Betaine
Apparent digestibility Coefficient (ADCs)															
$ADC_{protein}$	85.56	86.62	88.78	85.56	86.81	88.60	85.56	84.42	88.83	89.21	88.36	1.948	0.285	0.374	0.197
ADC_{lipid}	92.36^{b}	91.51^{b}	87.64^{a}	92.36	89.02	90.12	92.36	90.52	92.49	87.53	87.74	1.818	0.050	0.558	0.637
ADC_{HCO}	42.82	41.98	42.48	42.82	47.82	38.83	42.82	40.00	42.48	49.77	35.19	7.876	0.907	0.574	0.432

* Diets explanation as in Table 1; ¶ Red beet and betaine concentration on diets as explained in Table 2; $^{a—c}$ Means with different superscripts in each row differ significantly ($p < 0.05$).

3.3. Fish flesh proximate composition

Results showed that red beet (RB1 and RB2) and betaine (B1 and B2) incorporated on diets did not affect water and protein content of fish flesh. However, fat and ash contents were significantly affected by the diets (Table 21). Fat content was significantly affected by red beet (RB1 and RB2) and by the experimental diets. The increase of red beet levels decreased significantly ($p < 0.05$) the content of fat in fish flesh, while, the incorporation of betaine produce a significant increment. The combination of both ingredients produced a decrease on fat content with increasing levels of red beet and betaine, showing the highest fat content in fish fed with diet A (6.36%). Ash content decreased significantly ($p < 0.05$) with increasing levels of red beet and betaine.

Regarding the betaine content in fish flesh, results showed that fish fed with diets containing higher betaine concentration (B and D) presented higher values of betaine on flesh than those with lower concentration or control (Figure 27).

Table 21. **Proximate composition of rainbow trout flesh fed with increasing red beet and betaine levels at the end of the experimental growth period** (data are expressed as % of dry matter) (values are least-squares means ± SEM, n=3).

	Initial	Red beet Level ¶ [%]			Betaine Level ¶ [%]			Interaction (Diet *)					SEM	Two-way ANOVA (*p*-value)		
		CONTROL	RB1	RB2	CONTROL	B1	B2	CONTROL	A	B	C	D		Red beet	Betaine	Red beet •Betaine
Proximate composition [% dry matter]																
Moisture	78.80	77.65	78.05	78.60	77.65	77.85	78.80	77.65	77.35	78.75	78.35	78.85	1.201	0.740	0.064	0.825
Protein	14.80	15.79	15.30	14.98	15.78	15.34	14.93	15.78	15.61	14.98	15.08	14.88	0.494	0.261	0.086	0.075
Fat	4.50	5.68^{ab}	5.84^{b}	4.80^{a}	5.69	5.56	5.08	5.69^{ab}	6.36^{b}	5.33^{ab}	4.77^{a}	4.83^{a}	0.303	0.004	0.258	0.041
Ash	1.80	2.09^{b}	1.87^{a}	1.97^{ab}	2.09^{b}	1.98^{ab}	1.87^{a}	2.09	1.86	1.89	2.09	1.85	0.054	0.021	< 0.05	0.134

* Diets explanation as in Table 1; ¶ Red beet and betaine concentration on diets as explained in Table 2; $^{a—c}$ Means with different superscripts in each row differ significantly ($p < 0.05$).

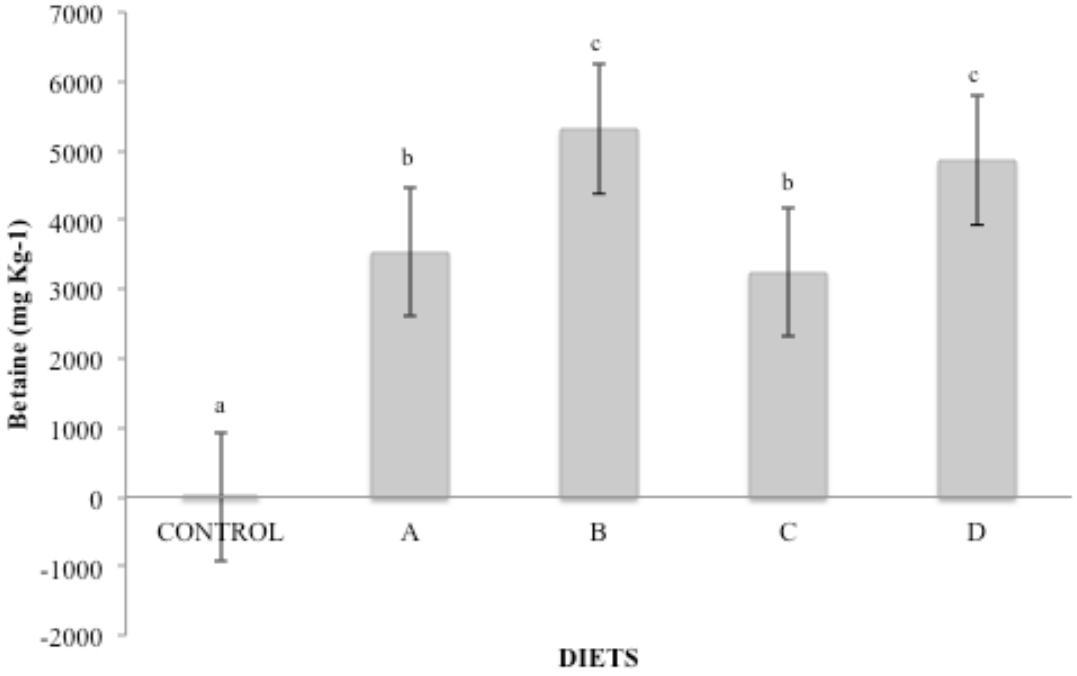

Figure 27. Effect of increasing levels of red beet and betaine on fish flesh betaine content.

Data are presented as least-squares means ± standard error of the mean (n=3); significant differences ($p < 0.05$) are indicated with different letters above the column. CONTROL (0% red beet, 0% betaine), A (14% red beet, 0.9% betaine), B (14% red beet, 1.63% betaine), C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets.

3.4. Fish flesh quality markers

3.4.1. Water activity (a_w)

Figure 28 shows the a_w of fish fed with different experimental diets. The inclusion of the ingredients individually and collectively produced a significant decrease on the aw of fish flesh compared to control diet.

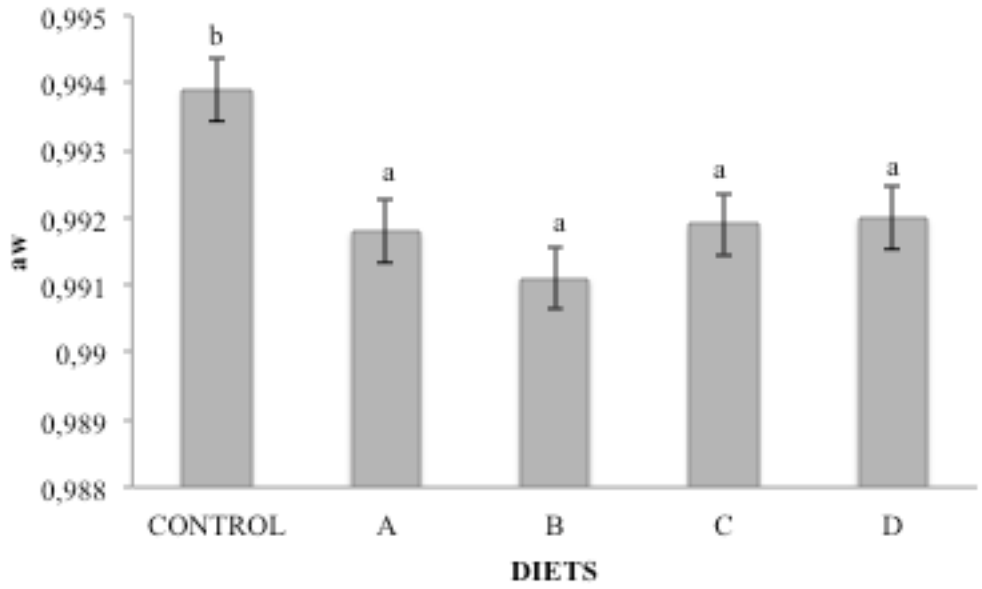

Figure 28. Effect of red beet and betaine concentration on water activity (aw) of fish meat at the end of the experimental growth period.

Data are presented as least-squares means ± standard error of the mean (n=3); significant differences ($p < 0.05$) are indicated with different letters above the column. CONTROL (0% red beet, 0% betaine), A (14% red beet, 0.9% betaine), B (14% red beet, 1.63% betaine), C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets.

3.4.2. Colour

The inclusion of red beet and betaine on diets was studied for CIELAB parameters. The study showed L* modification by the ingredients but those differences were attributed to fish variability of the product rather than a diet effect. As it was expected, fish flesh from fish fed with diets with the highest red beet and betaine concentration (D) showed higher redness values than samples from fish fed with lower red beet and betaine concentration and control (Figure 29). B*, hue and chroma values did not show significant effects between diets.

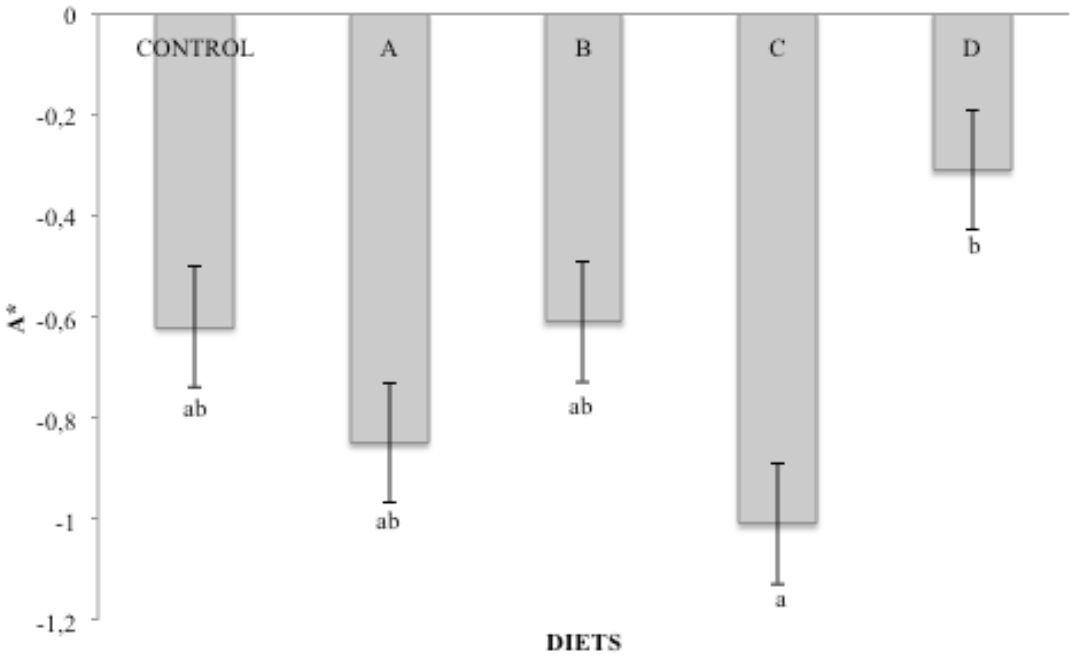

Figure 29. Effect of red beet and betaine on fish flesh redness (A* values) at the end of the experimental growth period.

Data are presented as least-squares means ± standard error of the mean (n=3); significant differences ($p < 0.05$) are indicated with different letters above the column. CONTROL (0% red beet, 0% betaine), A (14% red beet, 0.9% betaine), B (14% red beet, 1.63% betaine), C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets.

3.4.3. Texture

Red beet and betaine concentration did not have a significant effect on textural parameters. Elasticity was the only parameter affected by the diets (Figure 30). Compared to control diet, a significant lower elasticity was observed in flesh from the fish that were fed with lower betaine concentrations (diets A and C).

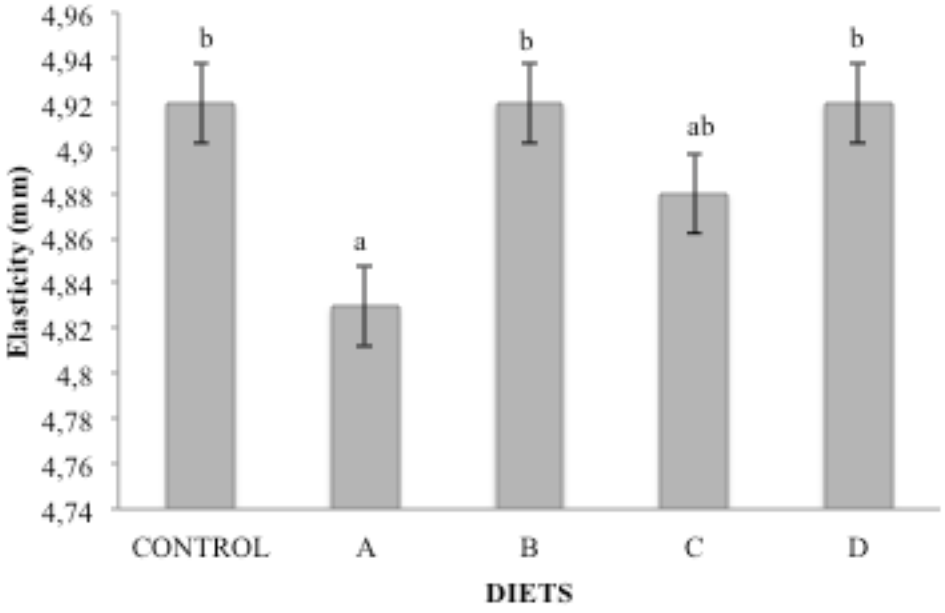

Figure 30. Effect of red beet and betaine on fish flesh elasticity at the end of the experimental growth period.

Data are presented as least-squares means ± standard error of the mean (n=3); significant differences ($p < 0.05$) are indicated with different letters above the column. CONTROL (0% red beet, 0% betaine), A (14% red beet, 0.9% betaine), B (14% red beet, 1.63% betaine), C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets.

3.4.4. Thiobarbituric Acid Reactive Substances (TBARS)

At the end of the experimental growth period, fish fed with control diets and the highest red beet and betaine concentrations (separately or together) had similar TBARS values (Figure 31), although the differences were not significant. It was observed a decrease when red beet and or betaine were included on the diet.

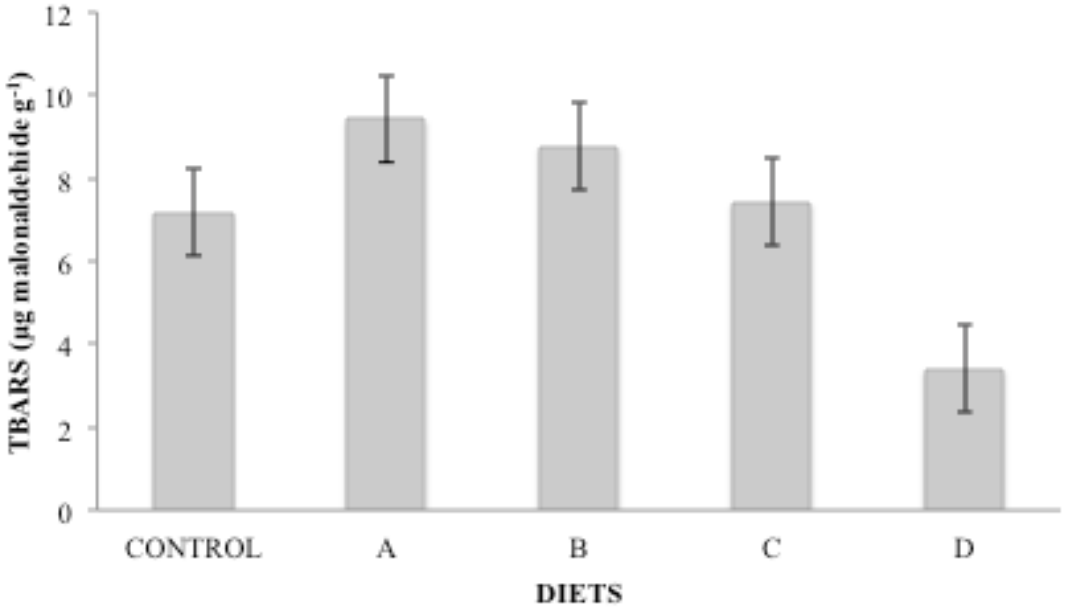

Figure 31. Effect of red beet and betaine concentration on lipid oxidation (TBARS) measured as µg malonaldehide g^{-1} of fish meat at the end of the experimental growth period.

Data are presented as least-squares means ± standard error of the mean (n=6); absence of different letters above the column indicates no significant differences ($p > 0.05$) between treatments. CONTROL (0% red beet, 0% betaine), A (14% red beet, 0.9% betaine), B (14% red beet, 1.63% betaine), C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets.

3.4.5. Sensory analysis

QIM was used for evaluating the sensory analysis of the whole fish. In all the parameters studied, at the end of the experimental growth period, only significant differences were found on odour and gills colour. Fish fed with the highest red beet and betaine concentration (D diets) showed higher rancid odour than the fish from the other experimental diets (data not shown). Fish fed with control and D diets had similar values on gills colour, with the characteristic red colour, while fish fed with B and C diets presented pale gills (data not shown).

On the other hand, QDM was evaluated in fish flesh. Only significant

differences were observed on meat colour. The study showed an effect on colour modification by the ingredients, but those differences found were rather due to fish variability than a diet effect (data not shown).

4. DISCUSSION

The inclusion of 14% of red beet and 0.9% of betaine did not affect growth, nutritive or biometric parameters, nor nutrient retentions compared to control, while higher red beet and betaine concentrations had a negative effect on growth and nutritive parameters. At the end of the experimental assay, the level of red beet and betaine separately, produced a significant decrease on Wf, SGR AND TGC, whereas fish fed with diet with 28% red beet and 0.9% betaine significantly reduced those parameters compared to the other dietary treatments. Betaine has been reported as a feeding stimulant to fish, inducing an increase of FI, and consequently, improving growth rate (Normandes et al. 2006, Tiril et al. 2008). However, in this study, the inclusion of betaine on rainbow trout diets did not show significant differences on FI and did not improve rainbow trout growth. Similar results were reported with other fish species such as Atlantic salmon (Duston 1993), sea bass and sea bream (García-Alcázar et al. 1994) or piauçu (Normandes et al. 2006) when they were fed with betaine on their diets. Additionally, compared to control diet, there was a significant decrease on PER and changes in FCR were only affected by the inclusion of red beet, following an opposite tendency from PER. These results may be due, in part, to the influence of some antinutritional components in red beet such as tannins or oxalates that reduced the growth and could lead to a poor FCR and PER (Shyamala and Jamuna 2010, Lawal et al. 2012, Focken et al. 2015, Nyonge 2015). However, for lower red beet and betaine concentrations, it seems to appear a positive interaction, presenting no differences with control diet.

ADCs obtained on the present study indicated an adequate quality and efficiency for the different experimental diets. Digestibility values in carnivorous fish normally range 75-95% for protein and 85-95% for lipid (NRC, 1993); the obtained values were between those ranges (84.42-89.21% for protein and 87.53-92.49% for lipid). Red beet and betaine concentrations (separately and together) did not have significant effects on ADCprotein and ADCCHO. However, ADClipid was significantly modified by the red beet concentration: the inclusion of red beet on rainbow trout diets significantly decreased ADClipid. This decrease might be associated to the modification on the lipid and/or carbohydrate metabolism pathways; also could be attributed to the presence of oxalate and its ability to bind minerals in the intestine, reducing the digestibility of fat (Francis et al. 2001). Also, this effect could be related to the higher VSI and HSI found on those diets higher in red beet. It seems that the inclusion of red beet and betaine on rainbow trout diets increase visceral adipose tissue mass and decrease growth, as it has been observed on the growth performance parameters. Similar results were reported in other studies with other carbohydrate sources and fish species (Tan et al. 2006, Wu et al. 2007, Cui et al. 2010). These authors indicated that CHO not absorbed, those not used as an energy source, can be accumulated in the liver and transformed into lipids and glycogen which lead on a higher HSI. More studies should be carried out to clarify if the negative effects on HSI and VSI are attributable to the synthesis of lipids from the structure of polysaccharides in red beet.

Whole body proximal composition was not significantly affected by the CHO source, which its in accordance with previous studies on sea bass (Enes et al. 2006), white sturgeon and hybrid tilapia (Lin et al. 1997) and for rainbow trout (Tekinay and Davies 2001). However, other authors have reported a significant effect of the CHO source on the whole body proximal composition (Tan et al. 2006, Wu et al. 2007).

The inclusion of red beet and betaine on rainbow trout diets produced a significant decrease on PIR (% digested). These results obtained were in agreement with PER values, but were not in accordance with ADCprotein, in which there were no significant differences between diets. Compared to control diet, PIR and FIR (% intake and digested) significantly decreased with higher red beet and betaine concentrations. A low PIR and PER are explained by an inappropriate protein metabolism into muscle. This effect can be associated to several reasons, one of them is because of an incorrect CHO and lipid metabolism, which produces an accumulation of lipids on visceral pack and liver, while the protein is used as an energy source (Hemre et al. 2002, Cui et al. 2010, Kamalan et al. 2012).

As it was expected, the inclusion of red beet and betaine in fish diets, increased betaine concentration in fish flesh compared to control diets. This is important from a bioactivity point of view of the product. Due to the high residual levels found on flesh from fish fed with red beet and betaine, the authors of the present study considered interesting to investigate the antioxidant properties that betaine can provide to the final product.

With regard to red beet and betaine effects on flesh quality, it has been observed that the inclusion of these ingredients produced a reduction of aw compared to control diet. aw plays an important role on spoilage of fish (Ježek and Buchtová 2014). This is in agreement to the observed with the inclusion of other CHO sources, such as barley (Pinedo et al. 2016). The reduction on aw values would help to reduce lipid oxidation and microbial growth, with advantages in shelflife.

When fish flesh colour was determined instrumentally, significant differences were observed on a* values, regarding the diet. As it was expected, redness (a* values)

of fish flesh, increased significantly with the inclusion of red beet and betaine, and fish fed with diets with 28% red beet and 1.63% betaine showed the reddest meat. The increase of redness at higher red beet and betaine concentrations can be associated to betaine pigment and betalains content (Stintzing et al. 2002, Zhong et al. 2005). Flesh from fish fed with this diet also presented the highest flesh betaine content, which could explain the increase of redness. These results were not consistent with the observations of panellist on the QDM analysis that were not able to perceive a flesh colour change.

Lipid oxidation was evaluated as one of the most important indicators of quality. TBARS values did not show significant differences between flesh from fish fed with control diet and fish fed with red beet and betaine. However, although no significant differences were observed, the inclusion of both ingredients seems to reduce TBARS values (dose-dependent effect).

Experimental diets did not have a significant effect on acceptability of fish flesh, but, surprisingly, during QIM analysis panellists detected that fish fed with diets with 28% red beet and 1.63% betaine presented a more rancid odour than fish fed with the other rest diets. These results were correlated with a loss of freshness in these fish.

5. CONCLUSIONS

The inclusion of 14% of red beet and 0.9% of betaine on rainbow trout diets had not a negative effect on rearing parameters compared to control diet, however, it enhanced the quality of the final product. In addition, it was expected a potential beneficial effect associated with betaine, which was present on red beet. Betaine content on flesh from fish fed control diet was < 2 mg kg^{-1} and it increased to values ranging from 3240 to 5310 mg kg^{-1} when red beet and betaine were present on the diet. For this reason, further studies would be necessary to verify if this ingredient enhances the

nutritional and healthy (antioxidant) value of rainbow trout flesh.

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CHAPTER 9. EFFECTS OF DIETARY INCLUSIONS OF RED BEET AND BETAINE ON THE ACUTE STRESS RESPONSE AND MUSCLE LIPID PEROXIDATION IN RAINBOW TROUT

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ABSTRACT

This study evaluates the effects of red beet (RB) and betaine on rainbow trout submitted to an acute stress challenge. A control diet was compared with four experimental diets in which red beet (14 and 28 %) and betaine (0.9 and 1.63 %) were incorporated in different concentrations according to a factorial design. Cortisol in plasma and fin, glucose and lactate plasma levels and malondialdehide (MDA) in muscle were all measured before the stress challenge, and 30 minutes, 6 and 12 hours after the stress challenge as parameters to determine the diet effects. RB and betaine had no effect on cortisol, glucose and MDA basal levels. However, lactate basal levels were significantly lower on fish fed with RB and betaine. Thirty minutes after the stress challenge, there was a significant increase in plasma and fin cortisol, glucose and lactate concentrations, although fish fed with diets containing RB and betaine showed significantly higher plasma cortisol values. MDA values of fish fed with 14 % RB and 0.9 % betaine were significantly higher than MDA values from fish fed with 28 % RB and 1.63 % betaine. After 6 and 12 hours, plasma and fin cortisol and lactate levels recovered in a similar trend. Glucose plasma levels recovered in almost all groups 12 hours after the stress. Also MDA values recovered basal levels after 6 and 12 hours. RB and betaine did not enhance the tolerance to the stress challenge compared to the control group, although the presence of these ingredients had no negative effect on any of the stress indicators.

Keywords: *Red beet, betaine, rainbow trout, acute stress challenge*.

1. INTRODUCTION

Fish welfare is crucial for the farming industry not only for public perception, marketing and product acceptance, but also in terms of efficiency, quality and quantity (Øverli et al. 2006, Ashley 2007). The welfare of farmed fish is influenced mainly by physical disturbances such as handling, weighing, crowding, grading, transport, temperature, dissolved oxygen which cause fish stress (Barton and Iwama 1991, Chagas and Val 2006, Bertotto et al. 2010, 2011, Madaro et al. 2015). The primary stress response in fish involves the release of catecholamines and activation of the hypothalamic-pituitary-interenal (HPI) axis, and the synthesis and release of cortisol. Both catecholamines and cortisol cause an energy source mobilisation, depletion of glycogen stores and increase of glucose and lactate plasma levels (Ashley 2007, Zolderdo et al. 2016). Therefore the levels of glucose and lactate in plasma are often used alongside cortisol to assess stress level in animals (Rollo et al. 2006, Ashley 2007).

Due to those stressful factors a decisive goal in aquaculture is to find different alternatives to manage stress. The development of novel methods to reduce stress responses and/or strengthen immunity is an important area of study, a good example being the use of nutrients and other compounds such as ascorbic acid in gilthead seabream (Ortuño et al. 2003), in rainbow trout (Dabrowski et al. 2004), or in fish tambaqui (Chagas and Val 2006), vitamin E in gilthead seabream (Montero et al. 2001, Ortuño et al. 2003), fatty acids in gilthead seabream (Van Anholt et al. 2004), β-glucans in rainbow trout (Jeney et al. 1997) or in yellow croaker (Zeng et al. 2016), or betaine in Labeo rohita (Virtanen 1995, Kumar et al. 2012). Over the last decade, there has been an increasing interest of the use of natural compounds present in fruits, vegetables and herbs as antioxidants and functional nutrients (Ganessan et al. 2011). Betaine is a natural compound that is widely found in animals, plants and microorganisms and has

been used as a dietary feed supplement in animal nutrition for more than 50 years due to its antioxidant and functional properties that protect against stressful factors (Kujala et al. 2002, Ganessan et al. 2011, Rabeh 2015). However, what about the activity of these functional compounds when they are not isolated but present in the whole raw material of the diet? Does it have the same effect? Few studies have been found about this topic. Red beet (Beta vulgaris L.) is a rich source in natural betaine but also in other important nutrients such as magnesium, sodium, vitamin C and betalains (Pinedo-Gil et al. 2017). To the best of our knowledge the use of red beet on aquaculture has been limited, possibly, due to their high fibre content that reduce fish digestibility (Hemre et al. 2002, Krogdahl et al. 2004, Tan et al. 2006, Enes et al. 2006, Wu et al. 2007, Cui et al. 2010); or due to a low palatability which would lead to a reduction of fish intake and growth; or due to probability of the presence of some antinutritional component such as tannins, oxalates or phytates which can also promote growth inhibition (Francis et al. 2001, Pinedo-Gil et al. 2017). However, Pinedo-Gil et al. (2017) showed a positive effect of red beet and betaine on quality parameters of rainbow trout. The main objectives of the study were to test, based on the antioxidant properties of betaine observed in other animals, the antioxidant ability as well as the potential stress-relieving properties of dietary administration of red beet and betaine in rainbow trout diet and the ability of these ingredients to control or reduce the stress.

2. MATERIAL AND METHODS

2.1. Production system

The trial was conducted at the Aquaculture Research Centre of Segovia, Spain, in 10 cylindrical fiberglass tanks (500 L) within a freshwater recirculation system (RAS). During the experiment water temperature remained constant at 15.04 ± 0.27 ºC (mean ± SD). The level of dissolved oxygen was 6.49 ± 0.37 mg L^{-1} (64 % saturation).

All tanks were equipped with aeration and an oxygen probe. Water pH was 7.91 ± 0.14 and ammonia and nitrites concentration in water were 0.65 ± 0.40 and 0.43 ± 0.30 mg L^{-1} respectively. Water flow was 10.29 ± 0.84 L h^{-1}. The photoperiod consisted on 12 hours of light and 12 hour of dark intervals and all tanks had identical light conditions.

2.2. Fish, diets and feeding

A total of 400 rainbow trout from a commercial fish farm (Piscifactoría Cien Fuentes, 19420 Cifuentes, Guadalajara, Spain) were used. Fish were randomly allocated in 10 tanks, 40 fish per tank (initial stocking density 20.0 ± 0.1 Kg m^{-3}, with and initial average weight of 250 ± 48.63 g). Prior to the feeding trial, all fish were acclimated to the indoor rearing conditions for 1 week and fish were fed once a day (8:00) to apparent satiation exclusively using the control diet. The study lasted 45 days.

Five isoproteic and isolipidic diets were formulated using red beet and betaine as experimental ingredients. Two red beet concentrations (14 and 28 %) and 2 betaine concentrations (0.9 and 1.63 %) were introduced: Control (0 % red beet, 0 % betaine), RB141 (14 % red beet, 0.9 % betaine), RB142 (14 % red beet, 1.63 % betaine), RB281 (28 % red beet, 0.9 % betaine) and RB282 (28 % red beet and 1.63 % betaine). The composition and proximate analysis of red beet diets are described in Pinedo-Gil et al. (2017). Control diet was prepared using the same ingredients than experimental diets but without red beet and betaine in the formulation. It was not a commercial diet. The diet extrusion process is described in Pinedo-Gil et al. 2017. The experimental diets were administered in replicate (two groups). The fish were fed by hand twice a day (8:00 and 15:00), 6 days per week to apparent satiation level during the whole experimental period. The pellets were distributed slowly to allow all fish to eat.

2.3. Stress trial

The stress trial was carried out after the feeding experimental period (45 days) by submitting the fish to a decrease of water oxygen concentration from 6.34 to 4 mg L^{-1}. The reduction of oxygen was obtained by lowering water level to a volume of 50 L and removing the aeration. Once the oxygen dissolved in water reached 4 mg L^{-1} (oxygen-saturated value of 39.7 %) (approximately 15-20 min), fish were kept in these conditions for 10 minutes, and after this time, tanks were filled again with water and aerated. During the acute stress challenge (hypoxia and crowding), oxygen decreased to less than 2 mg L^{-1} (oxygen-saturated value below 19.8 %). Before applying the stress all fish were starved for 2 days.

2.3.1. Sampling

Samples were taken before the stress test (basal levels) and 30 minutes, 6 hours and 12 hours after the stress. Fish were sacrificed with 300 mg L^{-1} MS222 (100% w/w; PHARMAQ®). Once fish were deeply anesthetized, they were blooded from the caudal vein with 1 mL syringers (BD Plastipak) and blood put in heparinized tubes on ice. When all the fish of every tank were bled (1.5 – 2 mL from each fish), the heads were removed and maintained in ice until all fish were blooded. Soon after collection, blood was centrifuged at 1200 xg for 10 min at 4 ºC and plasma (500 – 750 µL) and transferred to 1 mL eppendorfs and frozen at -80 ºC until analysis. Small portions of side muscle (about 1x1x1 cm of from the caudal peduncle and without skin) and caudal fin (about 1x1 cm from the upper lobe) were collected and conserved at -80 ºC until analysis.

An equal number of fish from each tank was subjected to the same sampling procedure at each time of sampling (6 fish per tank, n=6).

2.3.2. Cortisol analysis

Cortisol was measured in plasma and fin by a specific radioimmunoassay (RIA) as described by Bertotto et al. (2010) after extraction in diethyl ether. Briefly, a 96-well microtitre plate (Optiplate, Perkin Elmer Life Sciences, Waltham, MA, USA) was coated with anti-rabbit γ-globulin serum raised in goat, and the antiserum, diluted 1:1000 in 0.15 mM sodium acetate buffer, pH 9 at 4 ºC, was incubated overnight. The plate was washed twice with PBS and incubated again overnight at 4ºC with the specific antiserum solution. It was then carefully washed with PBS, standards, quality controls, unknown extracts and 3H tracer were added, and the plate was reincubated overnight at 4 ºC. Finally, the plate was washed with PBS, added with 200 µL scintillation cocktail (Microscint 20, Perkin Elmer Life Sciences) and counted on a β-counter (Top-Count, Perkin Elmer Life Sciences).

The sensitivity of the assay was 3.125 pg well^{-1} and was defined as the dose of hormone at 90 % binding (*B*/*B0*).

The anti-cortisol serum showed the following cross-reactions: cortisol 100 %, prednisolone 44.3 %, 11-deoxycortisol 13.9 %, cortisone 4.95 %, corticosterone 3.5 %, prednisone 2.7 %, 17-hydroxyprogesterone 1.0 %, 11-deoxycorticosterone 0.3 %, dexamethasone 0.1 %, progesterone < 0.01 %, 17-hydroxypregnenolone < 0.01 % and pregnenolone < 0.01 %.

2.3.3. Glucose and lactate

Glucose and lactate concentrations were determined only in plasma. They were measured by an enzymatic colorimetric assay, in particular by GOD-POD (SPINREACT® Ref. 1001191) and LOD-POD (SPINREACT® Ref. 1001330) method respectively (Kaplan and Pesce 1984). Briefly, aliquots (5 µL) from plasma samples

were mixed with 500 µL of reactive and incubated for 10 min for glucose determination and 5 min for lactate determination at 37 ºC in dark. The absorbance was determined at 490 nm in a 96-well microplate reader (Bibby Scientific Limited, Jenway 7315, UK). Values were expressed as mg dL^{-1}.

2.3.4. Lipid peroxidation

Muscle was used to determine the amount of lipid peroxidation. 100 mg of tissue was homogenized with Tris HCL 0.125 M pH 6.9 (500 µL), centrifuged at 13000 xg 4ºC for 15 min. and supernatant used for the assays.

The amount of lipid peroxidation was determined in muscle by measuring thiobarbituric acid-reactive substances (TBARS) according to Yoshida et al. (2005). Thiobarbituric acid reaction was carried out by mixing 0.2 mL sodium dodecyl sulphate solution (8.1 % w/v), 1.5 mL acetic acid buffer (20 % v/v, pH 3.5), 1.5 mL thiobarbituric acid (TBA 1 % v/v), 0.775 mL water and 0.05 mL ethanol containing butylated hydroxytoluene (0.8 wt % w/v) with 25 µL of supernatant. The reaction mixture was incubated at 100 ºC during 60 min and then cooled in ice followed by vigorous mixing with 1 mL water and 5 mL of n-butyl alcohol and pyridine (15/1, by volume). Afterwards, the mixture was centrifuged at 1400 xg at 0 ºC for 10 min and the supernatant was measured spectrophotometrically at 535 nm. Tetramethosypropane was used as standard to estimate TBARS formation as nmoles of malondialdehyde (MDA) equivalents per g of tissue.

2.4. Statistical analyses

All statistical analyses were carried out using software SAS (SAS version 9, SAS Institute Inc., Cary, North Carolina, USA). Data were analysed by ANOVA using the PROC MIXED with dietary treatment and time after stress as variable factors and

the tank as random effect. The probability of the linear, cubic and quadratic components of variance was calculated by contrast statement to test differences according to sampling time after stress. The contrast statements were used to test differences between diets containing 14 and 28 % of red beet and 0.9 and 1.63 % betaine corresponding to the different experimental treatments. Differences among means with $P<0.05$ were accepted as representing statistically significant differences.

2.5. Ethical statement

The rainbow trout *Oncorhynchus mykiss* (Walbaum) study complied with both European Union Council Directive 2010/63/UE, which lays down minimum standards for the protection of animals, and Spanish national legislation (Spanish Royal Decree 53/2013) protecting animals used in experimentation and for other scientific purposes and approved by Animal Ethics Committee of Agro-Technological Institute of Castilla y León (Spain).

Fish in the tanks were checked on a daily basis. At the end of the trials, fish were weighed individually and their health status was assessed by observation, after sedation with MS222 dissolved in water (MS222®; 200 mg L^{-1}) to minimize animal suffering. Animals were euthanized by excess of MS222 (300 mg L^{-1}) and then dissected.

3. RESULTS

3.1. Cortisol

Before stress (basal levels) plasma cortisol levels were low without significant differences among the experimental diet groups (Figure 32A). Basal levels range from 3.32 to 6.69 ng mL^{-1}. Thirty minutes after the stress, plasma cortisol levels significantly increased ($P<0.05$) with values more than 30 times higher than basal values. This significant increase occurred in all groups but fish fed with diets containing red beet and

betaine (107.94-134.88 ng mL^{-1}) showed significantly higher values compared with the control group (73.99 ng mL^{-1}). After 6 and 12 hours from stress, plasma cortisol levels significantly decreased ($P<0.05$) in all groups returning to basal values, except in fish fed with diet RB281 (28 % red beet and 0.9 % betaine) where cortisol plasma levels remained higher (36.51 ng mL^{-1}) than basal values (6.00 ng mL^{-1}; $P<0.05$) only at 12 hours. Some uncontrolled stress probably occurred after 6 hours as the basal levels were recovered after 6 hours.

The effect of red beet and betaine on fin cortisol levels of rainbow trout after acute stress challenge and subsequent recovery are shown in Figure 32B. Results showed that different red beet and betaine concentrations had no effect on fin cortisol levels during the stress experimental trial. Fin cortisol levels were low before the stress (basal levels) showing values ranging from 1.48 to 2.44 ng mg^{-1}. As occurred in cortisol plasma levels, 30 minutes after the stress fin cortisol concentration significantly increased ($P<0.05$) in all experimental groups, although the increase was only 7 times higher than basal values. Also, fish groups fed with red beet and betaine recovered overtime without significant differences with the control, although final values were higher than the levels before stress.

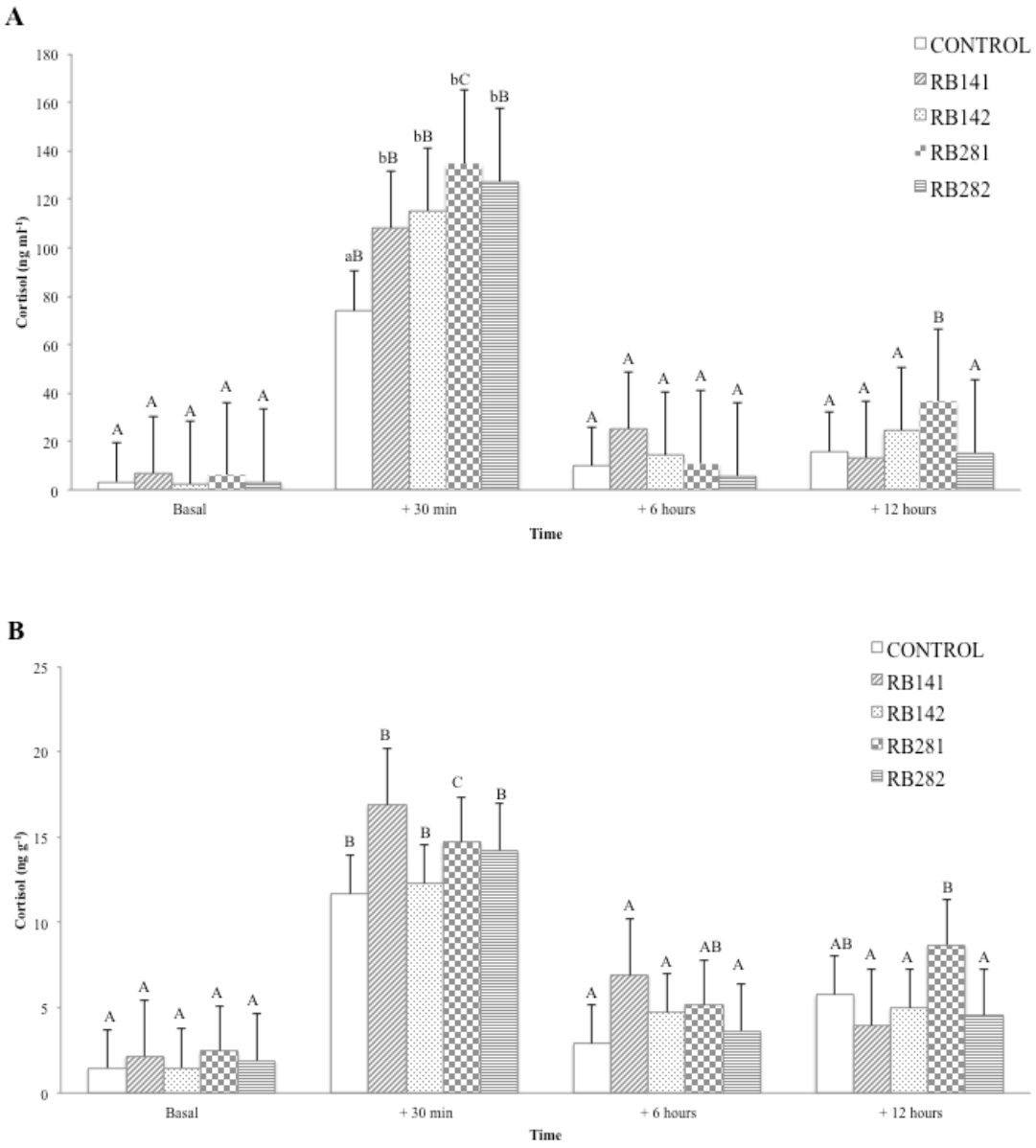

Figure 32. Effect of red beet and betaine on plasma cortisol (A) and fin cortisol (B) content of rainbow trout before the acute stress challenge (basal), 30 minutes after stress and 6 and 12 hours after stress.

Data were expressed as least-square means ± SEM, n=12. Different capital letters above the bars indicate significant differences ($p<0.05$) at different time points of the same group and different small letters above the bars indicate significant differences ($p<0.05$) between different experimental diets in the same time point. CONTROL (0% red beet, 0% betaine), RB141 (14% red beet, 0.9% betaine), RB142 (14% red beet, 1.63% betaine), RB281 (28% red beet, 0.9% betaine) and RB282 (28% red beet, 1.63% betaine) are the different experimental diets.

3.2. Plasma glucose and lactate

Results of plasma glucose levels are shown in Figure 33A. Basal levels range from 45.08 to 57.72 mg dL^{-1} and were not affected by the diet. After acute stress challenge (+ 30 minutes) there was a significant increase ($P<0.05$) of glucose reaching values 1.5 times higher than basal values. The recovery overtime (6 and 12 hours after the stress) did not follow the same pattern for every experimental group and fish fed diet RB141 did not recover basal levels after 12 hours.

Figure 33B shows that basal plasma lactate levels were significantly affected ($P<0.05$) by the diet. Control group showed the highest lactate level (39.43 mg dL^{-1}) and the lowest was observed in those fish fed with the diets with 0.9 % betaine (diets RB141 and RB281, 23.57 mg dL^{-1} and 21.42 mg dL^{-1}, respectively). Thirty minutes after the acute stress challenge, a significant increase ($P<0.05$) of lactate in all groups was observed, except for the control group. The diet had a significant effect, the least effected being fish fed with diet RB281, while lactate values were significantly lower in fish fed with the control diet than fish fed with diets at higher betaine concentration. After 6 and 12 hours of recovery fish of all groups recovered basal lactate levels.

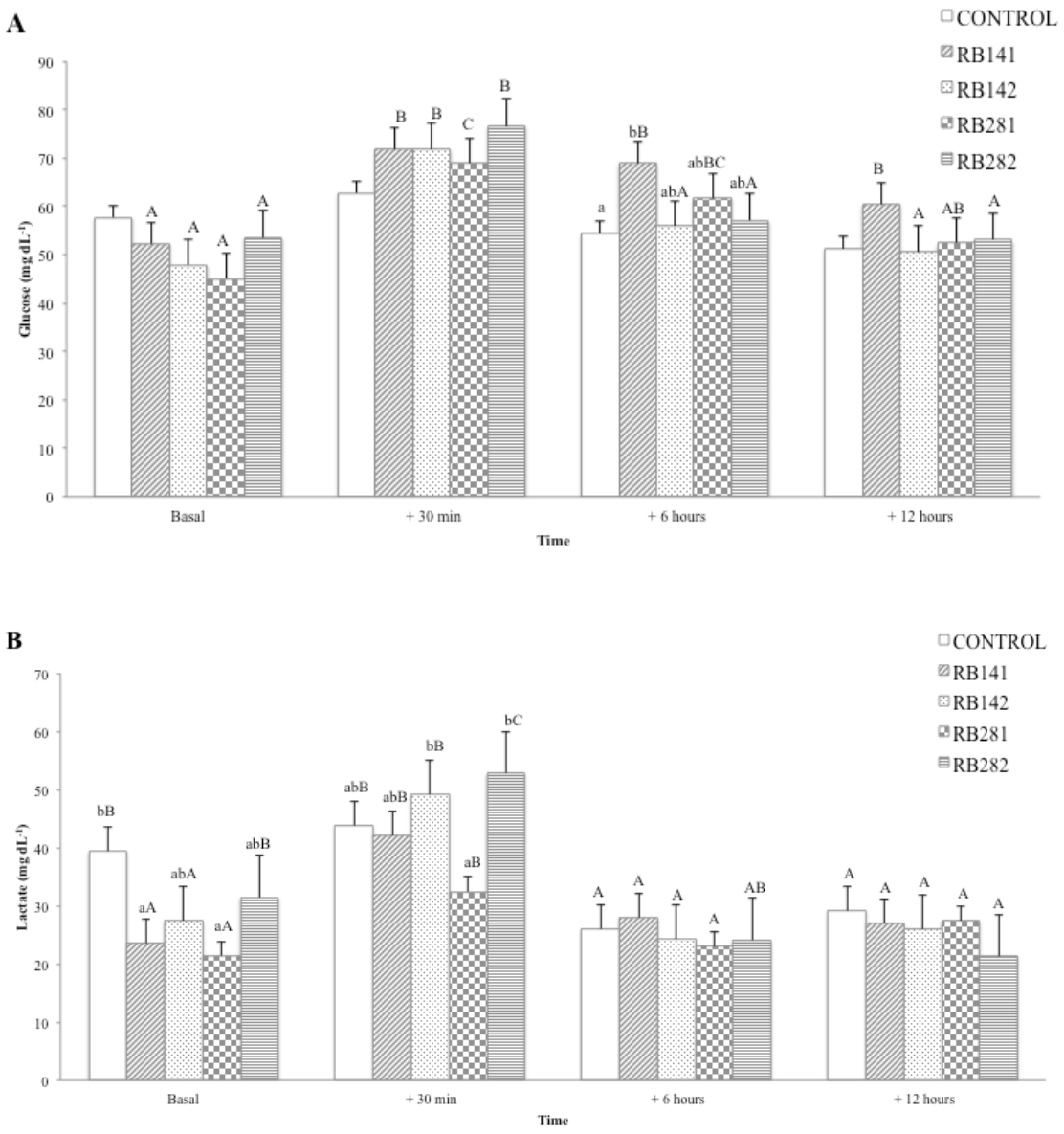

Figure 33. Effect of red beet and betaine on plasma glucose (A) and lactate (B) content of rainbow trout before the acute stress challenge (basal), 30 minutes after stress and 6 and 12 hours after stress.

Data were expressed as least-square means ± SEM, n=12. Different capital letters above the bars indicate significant differences ($p<0.05$) at different time points of the same group and different small letters above the bars indicate significant differences ($p<0.05$) between different experimental diets in the same time point. CONTROL (0% red beet, 0% betaine), RB141 (14% red beet, 0.9% betaine), RB142 (14% red beet, 1.63% betaine), RB281 (28% red beet, 0.9% betaine) and RB282 (28% red beet, 1.63% betaine) are the different experimental diets.

3.3. Lipid peroxidation

Before the stress challenge no significant differences on MDA values were observed regardless of the diet (P>0.05). Thirty minutes after the stress fish fed with diet RB141 (14 % RB and 0.9 % betaine) showed a significantly increase on MDA concentration. Although no significant differences were observed with the control group, lower MDA values were observed on fish fed at higher RB and betaine concentration (RB282, 28 % RB and 1.63 % betaine). Overtime the recovery period (6 and 12 hours after the stress), no significant differences were observed regardless of the diet (Figure 34).

Data showed that high RB concentrations (28 % RB) did not produce an increase on MDA values, meanwhile, fish fed with the control diet and lower RB concentrations (14 % RB) showed an impact on MDA values 30 minutes after the stress but recover basal levels after 12 hours (Figure 34).

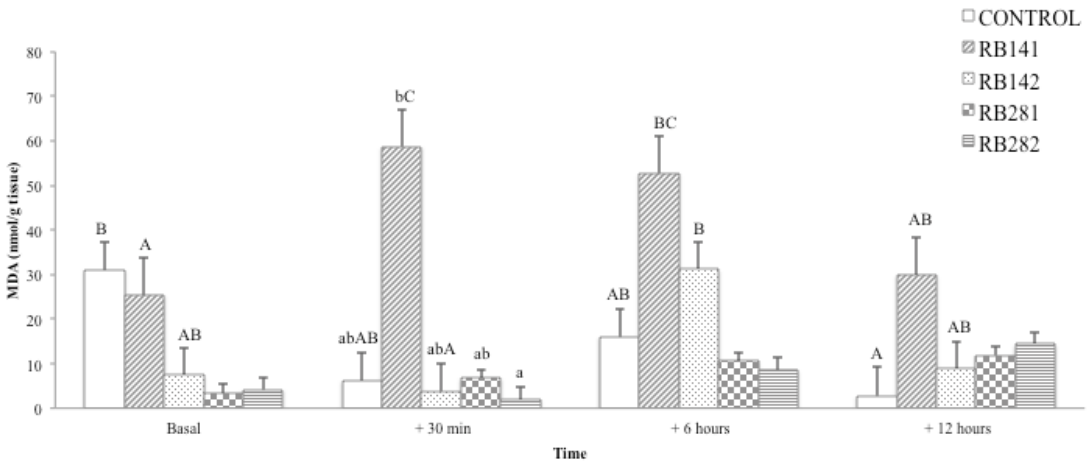

Figure 34. Effect of red beet and betaine on MDA (nmol per g of tissue) of rainbow trout before the acute stress challenge (basal), 30 minutes after stress and 6 and 12 hours after stress.

Data were expressed as least-square means ± SEM, n=12. Different capital letters above the bars indicate significant differences (p<0.05) at different time points of the same group and different small letters above the bars indicate significant differences (p<0.05) between different experimental diets in the same

time point. CONTROL (0% red beet, 0% betaine), RB141 (14% red beet, 0.9% betaine), RB142 (14% red beet, 1.63% betaine), RB281 (28% red beet, 0.9% betaine) and RB282 (28% red beet, 1.63% betaine) are the different experimental diets.

4. DISCUSSION

Results of the present study showed that the incorporation of red beet and betaine on rainbow trout diets had a significant effect on fish under acute stress challenge responses and recovery. It can be observed differences between the used indicators and matrixes although their trend along the stress challenge was similar.

Basal cortisol plasma values (about 4 ng mL^{-1}) were similar to values reported by other authors (Tintos et al. 2006, Bertotto et al. 2010) confirming the state of no stress of the fish before the stress challenge. The cortisol levels increased significantly 30 minutes after the stress in all groups and regardless of the diet. It is well reported an increase in plasma cortisol after various stress conditions in teleost species (Bertotto et al. 2010, Ming et al. 2012, Pérez-Jiménez et al. 2012). Cortisol, together with the catecholamines, are involved in adaptive mechanisms developed by fish to maintain oxygen supply to the tissues under hypoxia situations (Pichavant et al. 2002). However, in the present study, the higher values were recorded in fish fed diets containing red beet in comparison with the control diet suggesting that this ingredient did not enhance the tolerance of rainbow trout to a stress challenge although all groups recover with the same trend. On the other hand, fin cortisol levels showed a similar trend but it was not affected by the diet. Nevertheless, fin cortisol levels were about 10 times lower than plasma cortisol values. These differences could be explained by the different diffusion rates in the various matrices even if the kinetics of the hormone in the various matrices should be better-understood (Bertotto et al. 2010). Plasma and fin cortisol levels returned to control levels regardless of the diet 6 hours after the exposure and remained

low after 12 hours. Similar results were observed by Sadoul et al. (2015) in rainbow trout submitted to stress confinement and by Fast et al. (2008) in Atlantic salmon after a heat-stress with recovery in 5-6 hours.

Plasma glucose basal values were not affected by the inclusion of red beet and betaine. However, plasma lactate basal values were significantly affected by the inclusion of red beet and betaine. Significantly lower values were observed in fish fed with red beet and betaine compared to fish fed with the control diet. Plasma glucose and lactate levels increased after the stress since it has been verified that the increase of cortisol and catecholamines released by the perception of a stressor produce the mobilisation of energy stores to provide metabolic fuel, usually in the form of glucose and lactate, to overcome a stress challenge (Ings et al. 2012). In fish, it is generally accepted that catecholamines are mostly responsible for the increase of glycogenolysis while cortisol is believed to induce gluconeogenesis and its role on promoting glycogenolysis, if any, is less clear (Janssens and Waterman 1988, Mommsen et al. 1999In this study, 30 minutes after the acute stress challenge, as expected, fish glucose plasma levels significantly increased compared with the basal levels, regardless of the diet. However, lactate basal values significantly increased and surprisingly fish fed with diets containing red beet and betaine showed significantly higher values than fish fed with the control diet. This suggests that these ingredients did not enhance the tolerance of rainbow trout to a stress challenge even though all fish recover basal levels in the same way regardless of the diet. At 6 hours, the inclusion of red beet and betaine on diets had a significant effect on rainbow trout plasma glucose level recovery. Fish fed with diets containing red beet and betaine presented significant higher glucose levels than fish fed with the control diet. Only fish fed the highest betaine and red beet concentration (diets RB142, RB281 and RB282) returned to basal values by 12 hours,

while fish fed with diets with 14 % red beet and 0.9 % betaine (diets RB141) maintained plasma glucose levels higher than the basal ones even after 12 hours. Overall, as occurred with plasma cortisol values, higher values were recorded in fish fed diets containing red beet in comparison with those fed with the control diet.

The response dynamic of cortisol, glucose and lactate levels as stress markers was as expected and similar to the results reported by several authors (Aluru and Vijayan 2006, Fast et al. 2008, Ming et al. 2012, Gesto et al. 2013, 2015). However for all parameters it could be observed that red beet and betaine did not have an enhancing effect, although the recovery had a similar trend for every stress index.

Regarding lipid peroxidation levels, expressed as MDA, results showed that the inclusion of red beet and betaine did not significantly affect before the stress challenge. After the stress (30 minutes) red beet and betaine significantly affect MDA values. The stress had a significant effect on the control group and on fish fed with 14 % RB, but all groups recovered basal values 12 hours after the stress challenge. Oxidative stress is common under stressful conditions (Pérez-Jiménez et al. 2012). This oxidative stress produced free radicals that may attack polyunsaturated fatty acid producing lipid peroxidation (Chagas and Val 2006, Ming et al. 2012) and several studies reported an increase of lipid peroxidation under hypoxia (Lushchak et al. 2005, Pérez-Jiménez et al. 2012). Interestingly and in accordance with the current results, Leveelahti et al. (2014) in a study on three fish species (the epaulette shark, threespine stickleback and rainbow trout) exposed to hypoxia, reported that in general, fish do not show an increase in redox-active antioxidant defence in response to oxidative stress associated with hypoxia. Rather, the changes in antioxidant defences during hypoxia are very much specie- and tissue-specific and are not linked to the level of hypoxia tolerance of the fish species. It is well known that the response of MDA is very tissue-specific (Lushchak

and Bagnyukova 2006) and depends on the type of stress.

5. CONCLUSIONS

In conclusion, results show that the inclusion of red beet and betaine on rainbow trout diets followed the normal pattern of any stress; an increase of cortisol, glucose and lactate levels after the acute stress challenge followed by a decrease on these values after a recovery period. However, the inclusion of red beet and betaine did not enhance the tolerance to the acute stress challenge because no differences were observed compared to the control group. Soon after the stress challenge, MDA values showed significantly lower values on fish fed with 28 % RB and 1.69 % betaine than fish fed with 14 % RB and 0.9 % betaine. Also, high RB concentrations (28 %) avoid the effect of stress on MDA after the stress challenge (30 minutes), while fish fed with the control diet and lower RB concentrations (14 %) suffered the effect of the stress. Although the level of ingredients is important, it could be also be added that this effect suggests a possible antioxidant effect of red beet and betaine but further studies should be done to confirm this effect of the ingredient.

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CHAPTER 10. RED BEET AND BETAINE HAVE A POSITIVE EFFECT ON HISTOLOGICAL PARAMETERS OF RAINBOW TROUT BEFORE AND AFTER AN ACUTE HYPOXIA CHALLENGE.

ABSTRACT

The present study investigated the effect of red beet and betaine on liver and gut histology parameters of rainbow trout before and after an acute hypoxia challenge. Rainbow trouts were fed using 5 diets for 45 days. A control diet was compared to 4 experimental diets in which two red beet levels (14 and 28%) and two betaine levels (0.9 and 1.63%) were incorporated according to a factorial design. After that period, fish were submitted to an acute hypoxia by decreasing oxygen concentration from 6.34 to 4 mg L^{-1} during 10 minutes. Results showed that before the stress, lower betaine concentrations showed higher hepatocyte areas and lengths and at higher red beet and betaine levels significantly decreased liver cell vacuolation. Thirty minutes after the stress, hepatocytes size (areas and lengths) was significantly lower on fish fed with the lowest red beet concentration, however, at higher red beet and betaine levels liver cell vacuolation and the incidence of appearance of lymphocytic foci significantly decreased. The interactive effect between diet and stress was studied and results showed that fish hepatocytes were more regular shaped after the stress. The posterior intestine was not affected neither by red beet and betaine concentration nor by stress, for this reason the study suggests that both ingredients had not a negative effect on rainbow trout gut histology.

***Keywords**: Red beet, betaine, rainbow trout, hypoxia, liver and gut histology and morphology*

1. INTRODUCTION

A sustainable aquaculture development seeks to maintain the best conditions for maximizing production and fish welfare (Pérez-Jiménez et al. 2012). Fish gastrointestinal tract is composed by organs that interact with the environment and can be affected by morphologic alterations when some environmental changes occur (Tran-Ngoc et al. 2017), such as a decrease on the water-dissolved oxygen. Stress is evident in the liver due to its important role in energy storage and metabolism (Harper and Wolf 2009). Wolf and Wolfe (2005) reported that these alterations could be macroscopically visible as changes in liver size. Under intensive rearing of Atlantic salmon, a decrease on the water-dissolved oxygen creates a stress on fish increasing the permeability of the intestinal walls (Olsen et al. 2002, Sundh et al. 2010, Mosberian-Tanha et al. 2017). Several studies have reported the effects of an acute hypoxia on growth, digestibility, oxygen consumption and stress parameters in European sea bass and juvenile turbot (Pichavant et al. 2001) or rainbow trout (Pinedo-Gil et al. 2018) but without mentioning its impact on liver and gut morphology and histology. There is a lack of data on the impact of water oxygen concentration on fish liver and gut histology and morphology.

Diet is an important stress factor in fish that can affect liver and gut characteristics (Caballero et al. 2004, Martínez-Llorens et al. 2012, Jalili et al. 2013, Baeza-Ariño et al. 2014, Mosberian-Tanha et al. 2017). For this reason, the use in fish feed of alternative components can contribute to animals to enhance their health and to overcome adverse situations such as oxygen depletion that is one of the main problems in aquaculture closed systems.

Dietary carbohydrates have been widely used in fish diets to improve the feed quality and to provide a source of energy (Vielma et al. 2003, Pinedo-Gil et al. 2017a). An excess level of carbohydrates may reduce the growth rate and produce a poor feed

utilization, produce metabolic disturbances (Hemre 2002) and changes in the gastrointestinal tract and influence the stress response (Vielma et al. 2003), although for rainbow trout, the effect of different CHO sources on histological parameters and their utilization is not deeply studied.

During the last years, most studies have focused their attention on the use of natural substances from fruits, vegetables and herbs as natural antioxidants and functional nutrients (Ganessan et al. 2011). Red beet (*Beta vulgaris* L.) is a source rich in natural betaine but also other important nutrients such as magnesium, sodium, vitamin C and betalains (Pinedo-Gil et al. 2017b). Also, red beet is rich in valuable active compounds such as carotenoids, polyphenols, flavonoids, betalains and betaines. All these compounds make red beet one of the most potent functional vegetable (Nistor et al. 2017). Betaines and betalains have been widely studied for their nutritional and health benefits as antioxidant, antimicrobial and antiviral activities (Pedreno and Escribano 2001, Attia et al. 2013). It has been demonstrated that betaines and betalains antioxidant activity is higher than that provided by ascorbic acid (Stintzing et al. 2005) and have a potential use in protecting against stress factors (Kujala et al. 2002, Ganessan et al. 2011, Rabeh 2015). For this reason, these components have been widely used as a dietary feed supplement in animal nutrition.

Few studies have been reported about the use of red beet as a source of betaine and betalains in fish. To the best of our knowledge the use of red beet on aquaculture has been limited mainly due to their high fibre content that reduce fish digestibility (Hemre et al. 2002, Krogdahl et al. 2004, Tan et al. 2006, Enes et al. 2006, Wu et al. 2007, Cui et al. 2010); maybe due to a low palatability which would lead to a depletion of fish intake and growth; or maybe due to the probability of the presence of some antinutritional component such as tannins, oxalates or phytates which can promote to a

reduction on rearing parameter (Francis et al. 2001, Pinedo-Gil et al. 2017b). However, red beet, as a natural source of betalains would had several advantages as promoting feed intake in some fish species (Tiril et al. 2008, Lim et al. 2015), due to its potential as feed attractant, but also is a natural osmoprotector. Betaine has been extensively used in salmon feeding to protect them from the stress related to salinity variations (Virtanen 1995). And also, betaine plays an important role in cell metabolism substituting the aminoacid choline (Guérin 2000).

There are several works in which the effect of the diet on hepatic and gut histology is studied (Martínez-Llorens et al. 2012, Baeza-Ariño et al. 2014, Liu et al. 2017). Also, some of them study the effect of hypoxia on liver and gut histology (Scott and Rogers 1980, Harper and Wolf 2009). However, to the best of our knowledge there is a lack of works focused on the study of both factors and their interaction (Tran-Ngoc et al. 2016, Mosberian-Tanha et al. 2017). For this reason, in the present work the main purpose was to evaluate the effect of different red beet and betaine concentration on liver and gut histological parameters before and after an acute hypoxia.

2. MATERIAL AND METHODS

2.1. Experimental design

A factorial design (2x2) was used to evaluate the effects of an acute hypoxia and diet composition (2 red beet concentrations and 2 betaine concentrations and a control diet without the experimental ingredients) on liver and gut histology and morphology of rainbow trout. Juveniles of rainbow trout were obtained from a local fish farm.

The current study was complied with European Union Council Directive 2010/63/UE, which lays down minimum standards for the protection of animals, was also in accordance with Spanish national legislation (Spanish Royal Decree 53/2013)

protecting animals used in experimentation and for other scientific purposes and approved by Animal Ethics Committee of Agro-Technological Institute of Castilla y León (Spain).

2.2. Diets

Five isoproteic and isolipidic diets were formulated using red beet and betaine as experimental ingredients. The composition and proximate analysis of the diets were described in previous papers published by authors (Pinedo-Gil et al. 2017b). Control diet was prepared with the same ingredients as experimental diets but without red beet and betaine on the formulation. It was not a commercial diet. Diets A (14% red beet and 0.9% betaine), B (14% red beet and 1.63% betaine), C (28% red beet and 0.9% betaine) and D (28% red beet and 1.63% betaine) were the experimental diets. Diets were prepared according the extrusion process described in Pinedo-Gil et al. 2017a. The experimental diets were assayed in two groups.

2.3. Rearing conditions and feeding

A total of 400 rainbow trout were used. Fish were randomly allocated in 10 cylindrical fiberglass tanks (500 L) and 40 fish (initial stocking density 19.9 ± 0.1 kg m^{-3}) per tank. The tanks were all connected in a recirculation system, which allowed online real and cumulative control of water flow, oxygen concentration, temperature, pH and conductivity. During the experiments water temperature remained constant at 15.06 ± 0.30 ºC (mean ± SD). The level of dissolved oxygen was 6.20 ± 0.61 mg L^{-1}. All tanks were equipped with aeration and an oxygen probe. Water pH was 7.96 ± 0.22 and ammonia and nitrites concentration in water were 0.93 ± 0.46 and 0.97 ± 0.74 mg L^{-1} respectively. Water flow was 10.35 ± 0.80 L h^{-1}. The photoperiod consisted on 12 hours light and 12 hour dark intervals. All tanks had identical light conditions.

The fish were fed by hand twice a day (8:00 and 15:00), 6 days per week to apparent satiation level during the whole experimental period. The pellets were distributed slowly to allow all fish to eat. Prior to the feeding trial, all fish were acclimated to the indoor rearing conditions for 1 week and fish were fed once a day (8:00) to apparent satiation using exclusively the control diet. The study lasted 45 days.

2.4. Acute hypoxia

At the end of the experimental period (after 45 days of feeding), fish were controlled stressed by decreasing oxygen concentration from 6.34 to 4 mg L^{-1} (acute stress, hypoxia). The concentration of oxygen was decreased by lowering water level to a volume of 50 L and removing the aeration. When the levels of dissolved oxygen in water reached 4 mg L^{-1}, it started to count 10 min in these conditions, reaching levels of < 2mg L^{-1}. After 10 min of hypoxia, tanks were fulfilled with water and aerated. Before applying the stress all fish were starved for 2 days.

2.5. Sampling procedure

Samples were taken before the stress (basal levels) and 30 minutes after the stress (starting when oxygen level in water reached 4 mg L^{-1}). For each sampling time 6 fish per tank (n=12) were sacrificed using 300 mg L^{-1} MS222 (100% w/w; PHARMAQ®) (in order fish died immediately). After, the liver and gut of fish were taken for histological analysis. They were preserved in phosphate buffered formalin (4% pH 7.4). Three sections were obtained from each liver and from the posterior intestine (two transversal and one longitudinal); these were embedded in paraffin. For histological analysis, 4 μm tissue slices were stained with hematoxylin-eosin and alcian-blue. Liver slices were evaluated for the infiltration of peripancreatic fat, hepatocytes area and length and presence of lymphocytes foci. Gut slices were evaluated for vacuolation, incidence of mucus cells and presence of cell infiltration. This evaluation

was carried out individually. Microphotographs were taken with Nikon Microphot-FXA microscope and Olympus DP50 camera. The liver photographs (100X) were used to measure the area and maximum (Lmax) and minimum (Lmin) lengths of randomly selected hepatocytes passing through nucleus. For each fish, 15 hepatocytes were measured. All measurements were made with the aid of Image Pro® Plus software (media Cybernetics, Inc.; MD, USA).

2.6. Statistical analysis

Statistical analysis was performed using SAS version 9 (SAS Institute Inc., Cary, North Carolina, USA). All data were tested for normality and homogeneity of variance. Least-squares means (LSM) ± the standard error of the mean (SEM) were calculated for the hepatocytes areas and lengths using one-way analysis of variance (ANOVA). T-Student test was used to find significant differences.

The infiltration of peripancreatic fat and the presence of lymphocytes foci on liver, gut vacuolation, incidence of mucus cells and cell infiltration were analysed using the CATMOD procedure, and the significance was determined using a chi-square test at $P < 0.05$.

3. RESULTS

3.1. Effect of red beet and betaine on liver and gut histology and morphology

Liver morphological studies showed that the inclusion of red beet and betaine significantly affects hepatocyte size before the stress challenge.

Lower betaine concentrations (diets A and C) showed higher hepatocyte areas and lengths (Table 22) than the control diet. The biggest hepatocytes appeared on fish fed with diet A (14% red beet and 0.9% betaine). Liver cell vacuolation was lower on

fish fed with diets at higher red beet and betaine concentrations (Figure 35). And liver tissue examination revealed a liver degeneration by the appearance of inflammation characterized by the presence of lymphocytic cells foci, without significant differences regarding the diet (Figure 36).

Table 22. Effect of red beet and betaine on hepatocytes areas, maximum (LMax) and minimum (LMin) lengths (μm) before the stress. (values are least-squares means ± SEM, n=4).

	DIETS[1]						
	CONTROL	**A**	**B**	**C**	**D**	**SEM**	**P-value**
Area	0.151^{a}	0.502^{c}	0.160^{a}	0.254^{b}	0.139^{a}	0.022	< 0.0001
Lmax	0.706^{a}	1.278^{c}	0.754^{a}	0.911^{b}	0.704^{a}	0.045	< 0.0001
Lmin	0.285^{a}	0.501^{c}	0.304^{b}	0.361^{b}	0.280^{a}	0.020	< 0.0001

[1] Control (0% red beet, 0% betaine); A (14% red beet, 0.9% betaine); B (14% red beet, 1.63% betaine); C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets. Data in the same row not sharing a common superscripts letter are significantly different ($P < 0.05$). SEM: standard error of the mean.

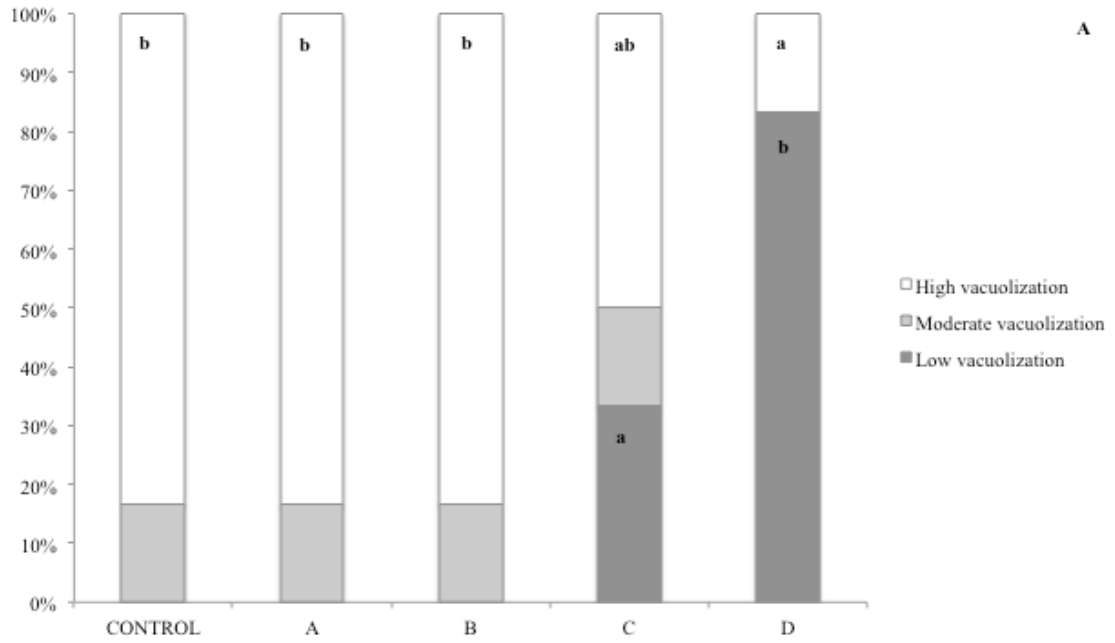

Figure 35. Hepatocytes vacuolization of fish fed different experimental diets.

Control (0% red beet, 0% betaine); A (14% red beet, 0.9% betaine); B (14% red beet, 1.63% betaine); C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets. Different letters indicate significant differences ($P < 0.05$) between different experimental diets

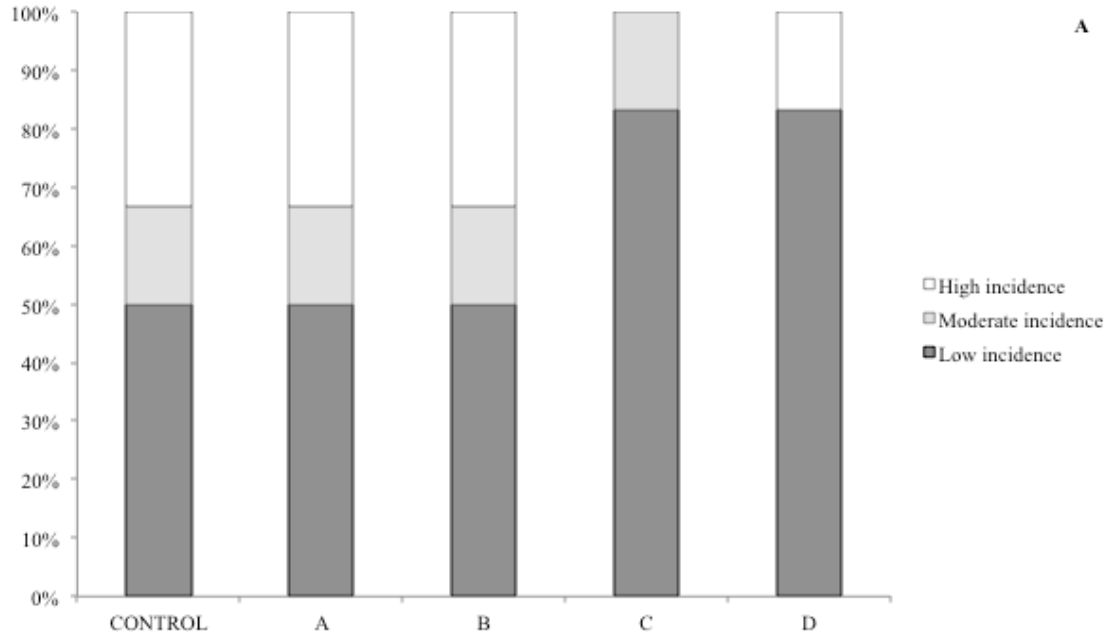

Figure 36. Incidence of lymphocytic foci appearance in hepatocytes of fish fed different experimental diets before the hypoxia challenge.

Control (0% red beet, 0% betaine); A (14% red beet, 0.9% betaine); B (14% red beet, 1.63% betaine); C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets. Different letters indicate significant differences ($P < 0.05$) between different experimental diets

When the posterior intestine was examined before the hypoxia challenge, the incidence of appearance of cell infiltration (Fig. 1 – supplementary data), mucus cells (Fig. 2 – supplementary data) and vacuolation (Fig. 3 – supplementary data) of the posterior intestine did not show significant differences between diets (data not shown).

3.2. Effect of the stress on liver and gut histology and morphology

Thirty minutes after the stress, hepatocytes size (areas and lengths) significantly ($P < 0.05$) decreased (Figure 37). Liver cell vacuolation (Fig. 4 – supplementary data) and the incidence of appearance of lymphocytic foci (Fig. 5 – supplementary data) did not change regardless the stress (data not shown).

Results showed that the stress did not have a significant effect on the incidence of appearance of cell infiltration (Fig. 6 – supplementary data), on the vacuolation (Fig. 7 – supplementary data) and mucus cells (Fig. 8 – supplementary data) of the posterior intestine (data not shown).

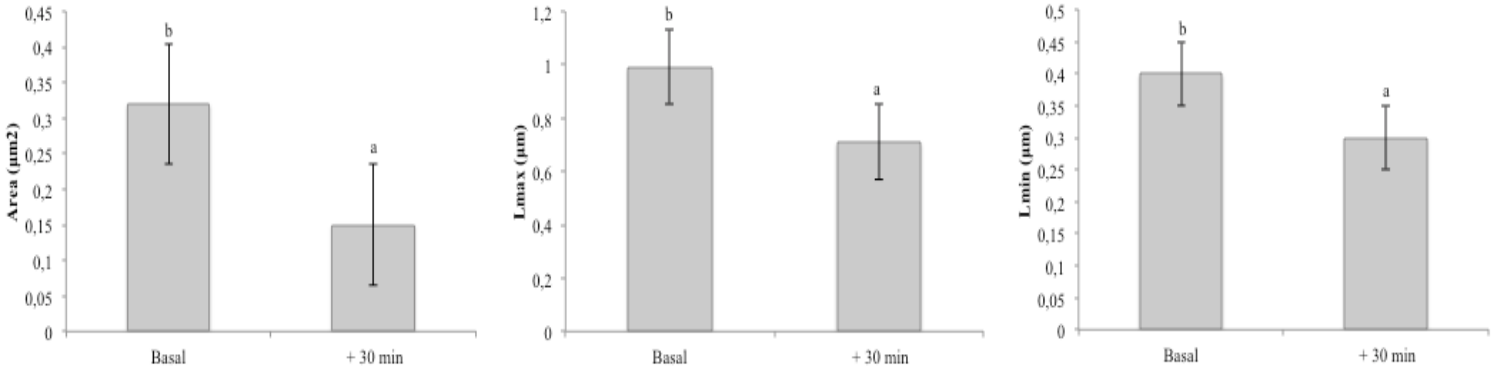

Figure 37. Effect of the stress on hepatocytes areas, maximum (L_{max}) and minimum (L_{min}) lengths.

Different letters indicate significant differences ($P < 0.05$).

3.3. Interaction between diets and stress on liver and gut histology and morphology

Fish fed with 14% of red beet concentration (diets A and B) showed significantly higher ($P < 0.05$) hepatocytes areas and diameters than fish fed with control diet and at higher red beet levels (Table 23). Liver cell vacuolation was lower on fish fed with diets at higher red beet and betaine concentrations (Figure 38) than control. And the incidence of appearance of lymphocytic foci significantly decreased ($P < 0.05$) with increasing levels of red beet and betaine on the diet (Figure 39).

Table 23. Effect of red beet and betaine on hepatocytes areas, maximum (LMax) and minimum (LMin) lengths (μm) after the stress (values are least-squares means ± SEM, n=4).

	DIETS[1]						
	CONTROL	**A**	**B**	**C**	**D**	**SEM**	**P-value**
Area	0.140^{ab}	0.156^{bc}	0.168^{c}	0.132^{a}	0.164^{bc}	0.009	0.0225
Lmax	0.696	0.706	0.727	0.688	0.765	0.032	0.4241
Lmin	0.296^{ab}	0.327^{bc}	0.345^{c}	0.280^{a}	0.319^{abc}	0.016	0.0453

[1] Different experimental diets as explained in Table 1.

Data in the same row not sharing a common superscripts letter are significantly different ($P < 0.05$). SEM: standard error of the mean.

In general, it was observed that fish hepatocytes were more regular shaped after the stress. No significant differences were observed on liver cell vacuolation, except on fish fed the highest red beet and betaine concentration (diet D) where liver cell vacuolation significantly decreased 30 minutes after the hypoxia challenge. And the inclusion of red beet and betaine significantly decreased the incidence of appearance of lymphocytic foci after the stress period.

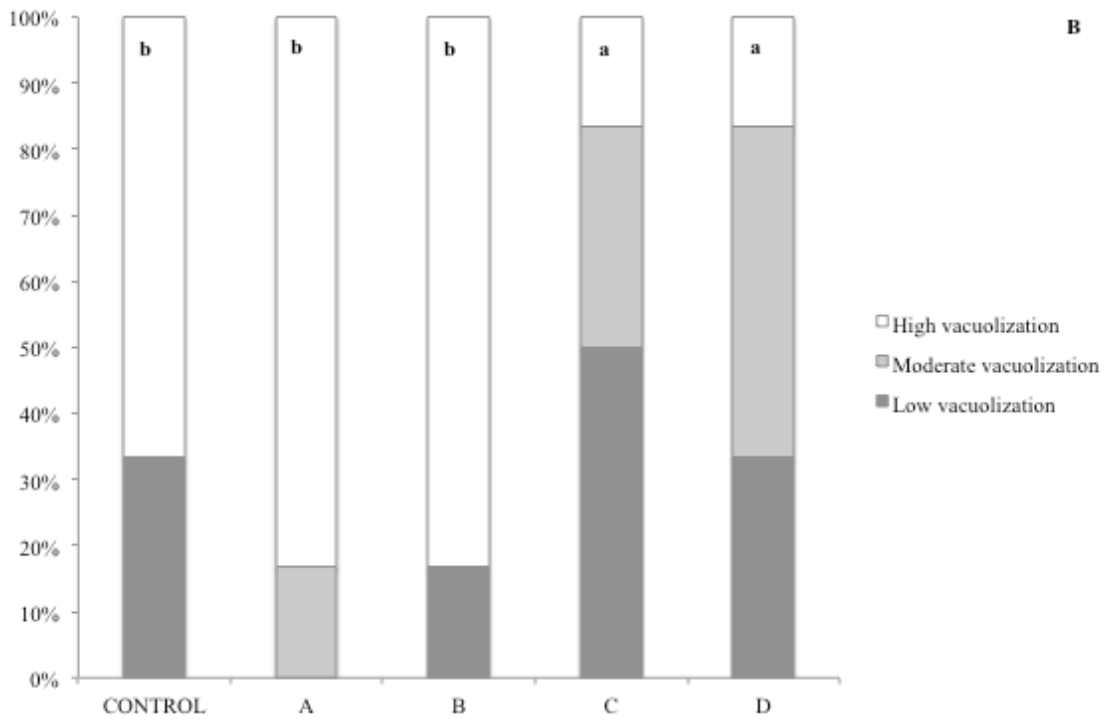

Figure 38. Hepatocytes vacuolization of fish fed different experimental diets 30 minutes after the hypoxia challenge.

Control (0% red beet, 0% betaine); A (14% red beet, 0.9% betaine); B (14% red beet, 1.63% betaine); C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets. Different letters indicate significant differences ($P < 0.05$) between different experimental diets.

The interaction between diets and stress did not show significant modifications on the incidence of appearance of cell infiltration (Fig. 9 – supplementary data), on the vacuolation (Fig. 10 – supplementary data) and mucus cells (Fig. 11 – supplementary data) of the posterior intestine (data not shown).

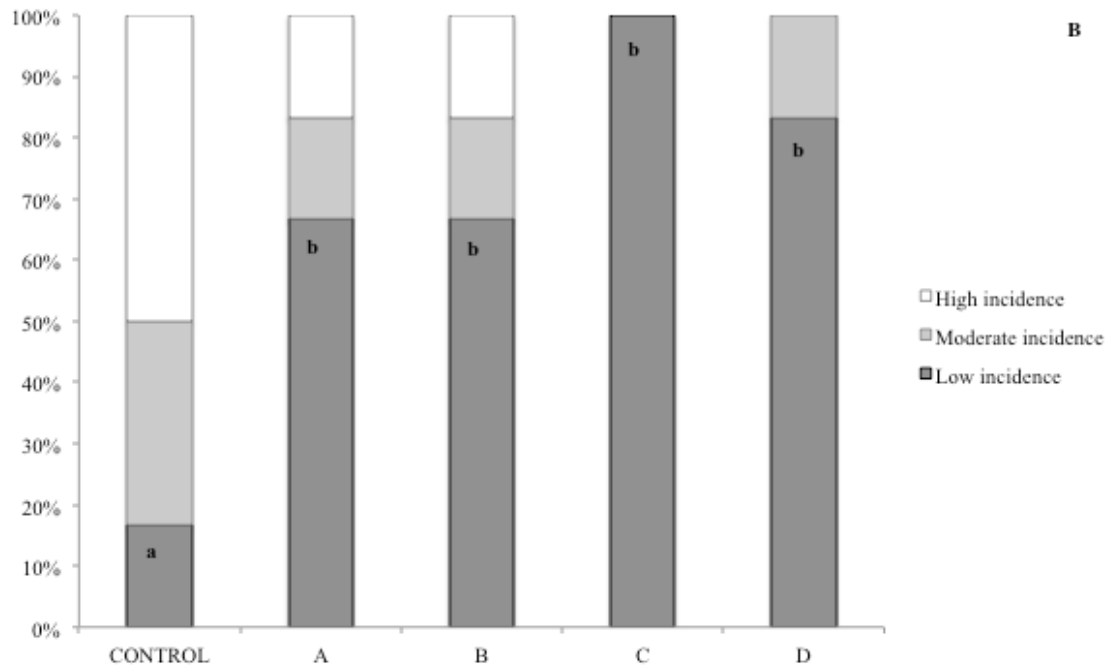

Figure 39. Incidence of lymphocytic foci appearance in hepatocytes of fish fed different experimental diets after the hypoxia challenge.

Control (0% red beet, 0% betaine); A (14% red beet, 0.9% betaine); B (14% red beet, 1.63% betaine); C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets. Different letters indicate significant differences ($P < 0.05$) between different experimental diets

4. DISCUSSION

Red beet and betaine concentrations had a significant effect on liver histology. Lower betaine concentrations showed higher hepatocyte areas and lengths than the control diet and higher betaine levels and liver cell vacuolation was lower on fish fed with diets at higher red beet and betaine concentrations. Bigger hepatocytes are more vacuolized than smaller hepatocytes as it was observed by other authors (Rusell et al. 2001, Pereira et al. 2002, Figueiredo-Silva et al. 2005). Similar results were observed by other authors feeding different fish species with different ingredients. Soybean meal in carp diets induce apoptosis of hepatocyte cells (Liu et al. 2017); and carob seed meal cause an excessive vacuolation and nuclear displacement in sea bream (Martínez-Llorens et al. 2012). The hepatocellular vacuolation observed was caused by the

accumulation of lipid droplets, which reflects the higher hepatosomatic and viscerosomatic index observed on Pinedo-Gil et al. (2017b). Nonetheless, certain liver cell vacuolation that makes fish hepatocytes being bigger sized than wild fish was found normal for species under rearing conditions regardless of the diet (Figueiredo-Silva et al. 2005). Liver tissue examination showed the appearance of inflammation characterized by the presence of lymphocytic cells foci without significant differences regarding the diet. This degeneration on liver appears also in the control group and may be due, in part, to an excessive hepatocellular vacuolation own to fish growth under rearing conditions. According to Mosconi-Bac (1990), the accumulation of lipids in fish liver is a hepatocyte reaction to a new metabolic state that can finish in liver necrosis if the diet is not corrected. The inclusion of higher red beet concentration than 14% reduces hepatocellular vacuolation, possibly due to a mobilisation of storage lipids, however, it produced a positive effect as preventing from an increasing appearance of lymphocytic foci, this positive effect of red beet and betaine may be due to the antioxidant and bioactive properties of both ingredients (Nistor et al. 2017).

Results of the present study showed that 30 minutes after the stress, hepatocytes size (areas and lengths) significantly decreased compared to basal values. Stress responses may be evident in the liver because its important role in energy storage and metabolism. Often, quantitative alterations in hepatic energy storage are visible macroscopically as changes in liver size and coloration, and histologically as variations in hepatocellular vacuolation and tinctorial staining characteristics (Harper and Wolf 2009, Wolf and Wolfe 2005). This decrease on hepatocytes size may be due to a more demand of energy produced by the lack of oxygen. The stress produced a modification on liver size, however, results showed that this change was less significant on those fish fed with the lowest red beet concentration. Wolf and Wolfe (2005) also reported that

stress produce liver histological modifications as variations in hepatic vacuolation and histopathologic changes. Decreased vacuolation can result from loss of cytoplasmic glycogen and/or lipid caused by insufficient energy intake relative to need and/or glucocorticoid-induced glycogenolysis (Harper and Wolf 2009, Wolf and Wolfe 2005). Results showed that fish fed with diets at higher red beet and betaine concentrations showed significantly lower hepatocellular vacuolation than control and lower red beet and betaine levels that is also correlated with a lower energy intake of those fish. This means that red beet concentrations higher than 14% may had a negative effect on fish on their tolerance to a lack of oxygen and crowding. On the other hand, the incidence of appearance of lymphocytic foci significantly decreased with the inclusion red beet and betaine on the diet. The presence of lymphocytic foci after a stress period, such as hypoxia, causing necrotic areas in liver is normal due to a lack of oxygen on several areas of the organ (Scott and Rogers 1980). However, the inclusion red beet and betaine on rainbow trout diets protect the liver to this fact throughout the own antioxidant and bioactive properties of both ingredients.

When the red beet and betaine concentration effect on posterior intestine was examined, the incidence of appearance of cell infiltration, mucus cells and vacuolation did not show significant differences. Mucus cells are involved in the secretion of mucus, which are very related with the immune system, acting as a lining, avoiding injuries and preventing drying (Monge-Ortiz et al. 2016). On the contrary, several studies reported an increase number of mucus cells when fish are fed with plant ingredients (Heidarieh et al. 2013, Baeza-Ariño et al. 2014), however, red beet and betaine concentration produced a positive effect as they did not affect the appearance of mucus cells.

Results showed that the stress did not have a significant effect on the incidence of appearance of cell infiltration, on the vacuolation and mucus cells of the posterior

intestine. It is well known that not only diet, but also, environmental conditions play an important role on gut histology and morphology (Lakani et al. 2013, Tran-Ngoc et al. 2017). In contrast to the results observed on the present study, Sundh et al. (2010) reported that when Atlantic salmon was submitted to hypoxia tended to shorten villi height, presented changes on the appearance of the intestinal segments and showed a widen subepithelial mucosa of the enterocyte in the distal intestine. The lack of significant differences on gut histology and morphology before and after the stress suggests that the presence of red beet and betaine did not worsen gut health. This effect is more evident after the stress as it was not observed a significant change with fish fed with control diet and the inclusion of plant ingredients promote the incidence of appearance of cell infiltration and mucus cells, that could be aggravated with the hypoxia challenge.

5. CONCLUSION

The current study showed that both diet composition and the lack of oxygen caused changes in liver morphology. It was observed that fish hepatocytes were more regular shaped after the stress. The inclusion of higher red beet concentrations than 14% on rainbow trout diets reduces the hepatocellular vacuolation. The posterior intestine was not affected neither by red beet and betaine concentration nor by stress, for this reason the study suggests that both ingredients had not a negative effect on rainbow trout gut histology.

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Supplementary data

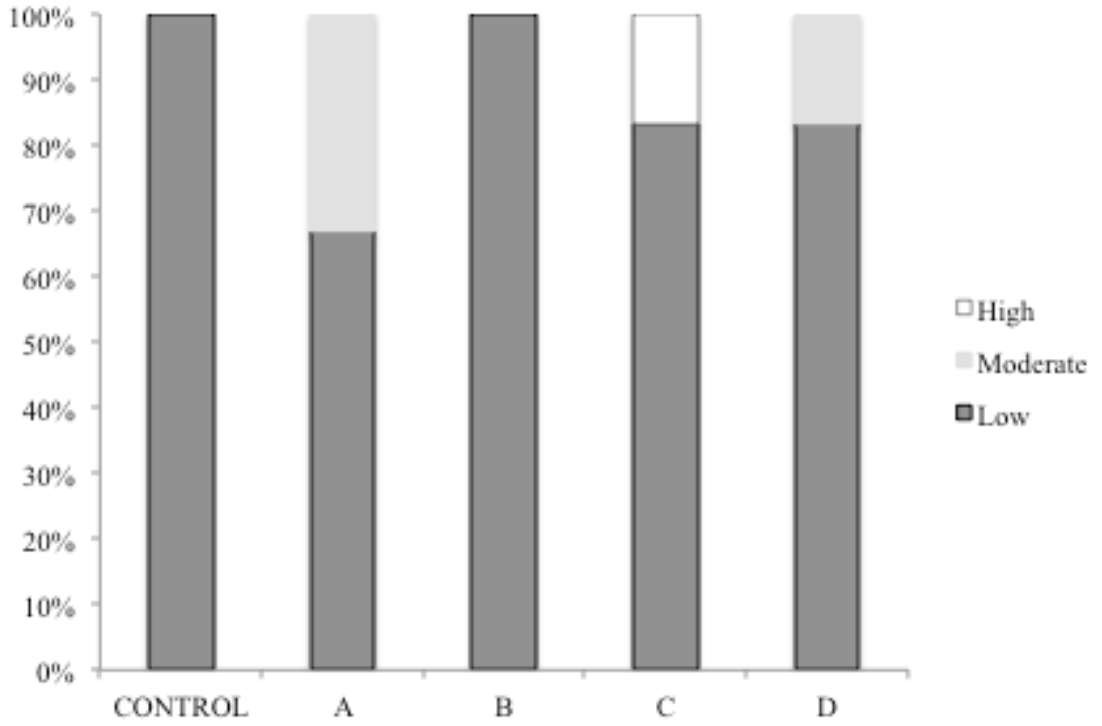

Fig. 1. Effect of red beet and betaine on the incidence of appearance of cell infiltration on the posterior gut. Control (0% red beet, 0% betaine); A (14% red beet, 0.9% betaine); B (14% red beet, 1.63% betaine); C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets. The lack of different letters indicate no significant differences ($P > 0.05$) between different experimental diets

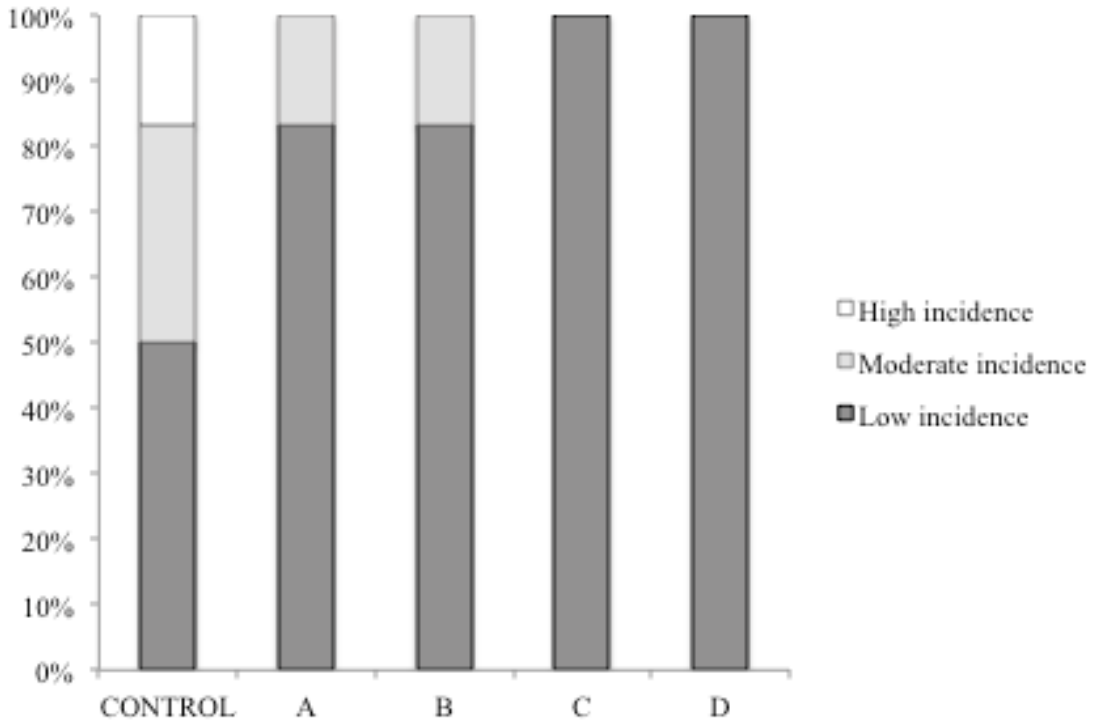

Fig. 2. Effect of red beet and betaine on the presence of mucus cells on the posterior gut. Control (0% red beet, 0% betaine); A (14% red beet, 0.9% betaine); B (14% red beet, 1.63% betaine); C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets. The lack of different letters indicate no significant differences ($P > 0.05$) between different experimental diets

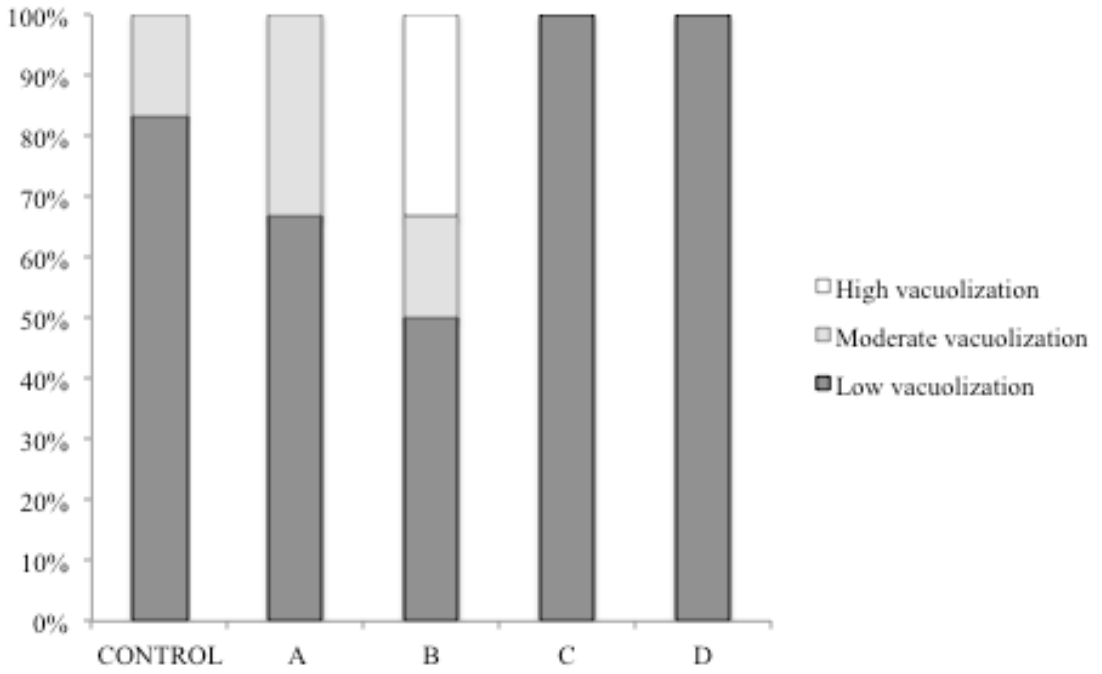

Fig. 3. Effect of red beet and betaine on posterior gut vacuolation. Control (0% red beet, 0% betaine); A (14% red beet, 0.9% betaine); B (14% red beet, 1.63% betaine); C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets. The lack of different letters indicate no significant differences ($P > 0.05$) between different experimental diets.

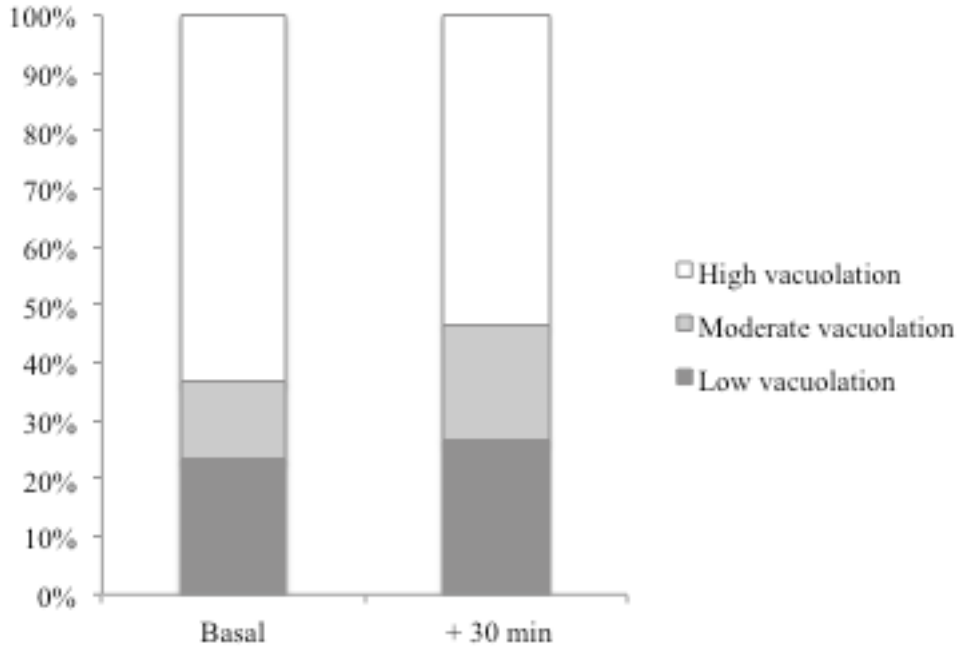

Fig. 4. Effect of stress on liver cell vacuolation. Absence of different letters indicate no significant differences ($P > 0.05$).

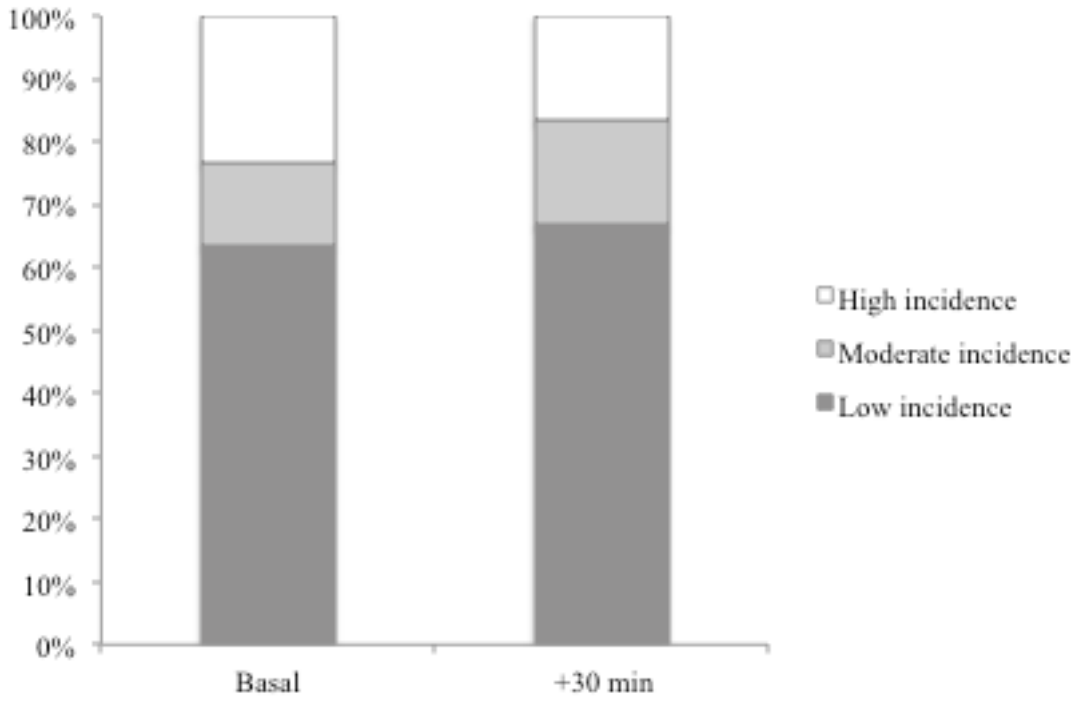

Fig. 5. Effect of the stress on the incidence of lymphocytic foci appearance in hepatocytes of fish. Absence of different letters indicate no significant differences (P > 0.05).

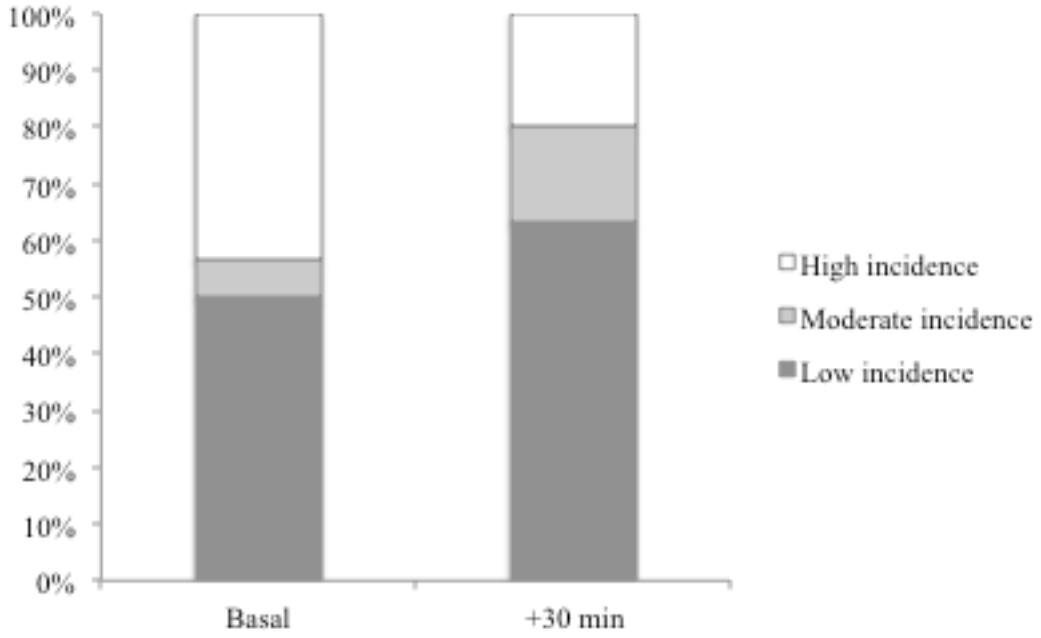

Fig. 6. Effect of stress on the incidence of appearance of cell infiltration on posterior gut. Absence of different letters indicate no significant differences (P > 0.05).

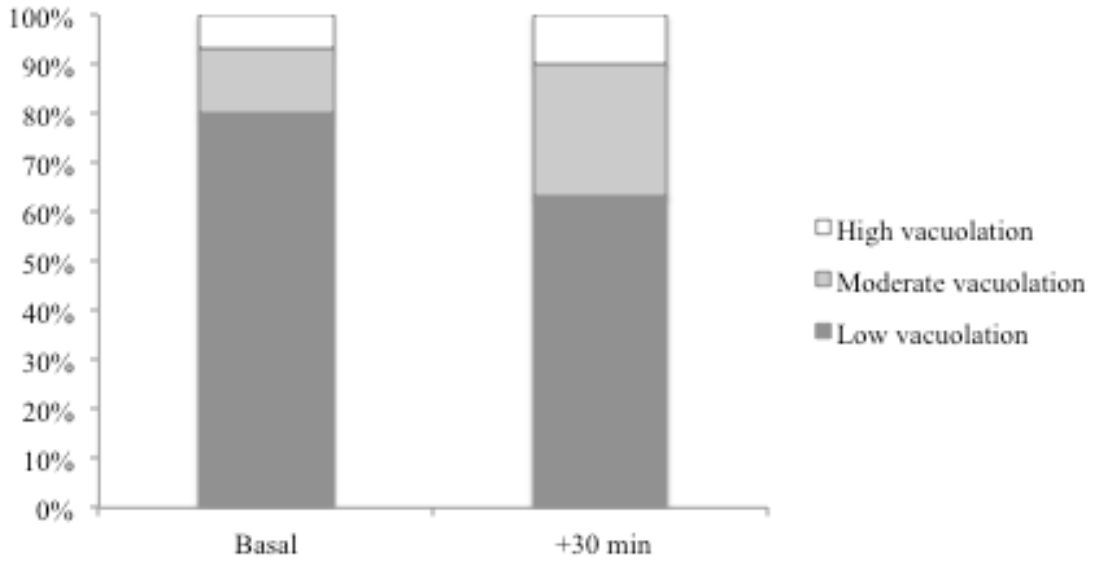

Fig. 7. Effect of stress on posterior gut vacuolation. Absence of different letters indicate no significant differences (P > 0.05).

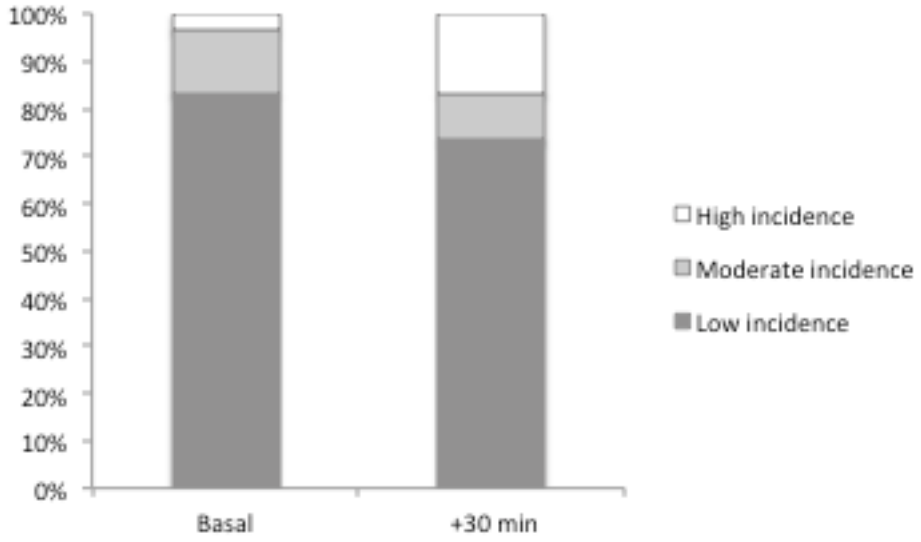

Fig. 8. Effect of stress on the presence of mucus cells on the posterior gut. Absence of different letters indicate no significant differences (P > 0.05).

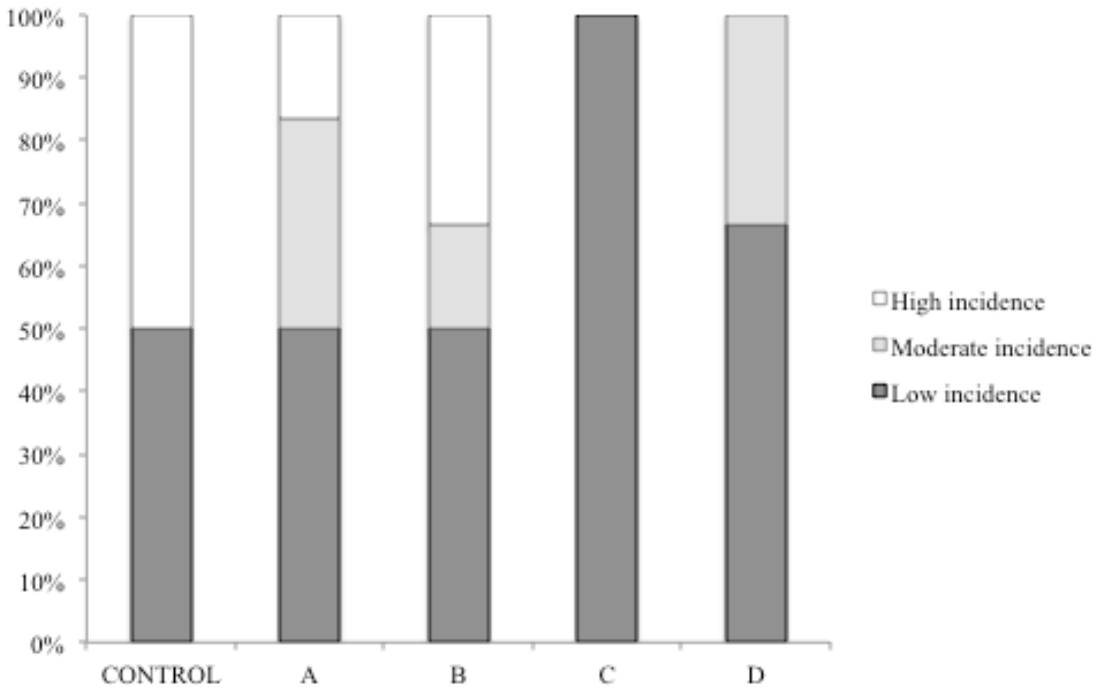

Fig. 9. Effect of red beet and betaine on the appearance of cell infiltration on posterior gut after the stress challenge. Control (0% red beet, 0% betaine); A (14% red beet, 0.9% betaine); B (14% red beet, 1.63% betaine); C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets. The lack of different letters indicate no significant differences ($P > 0.05$) between different experimental diets.

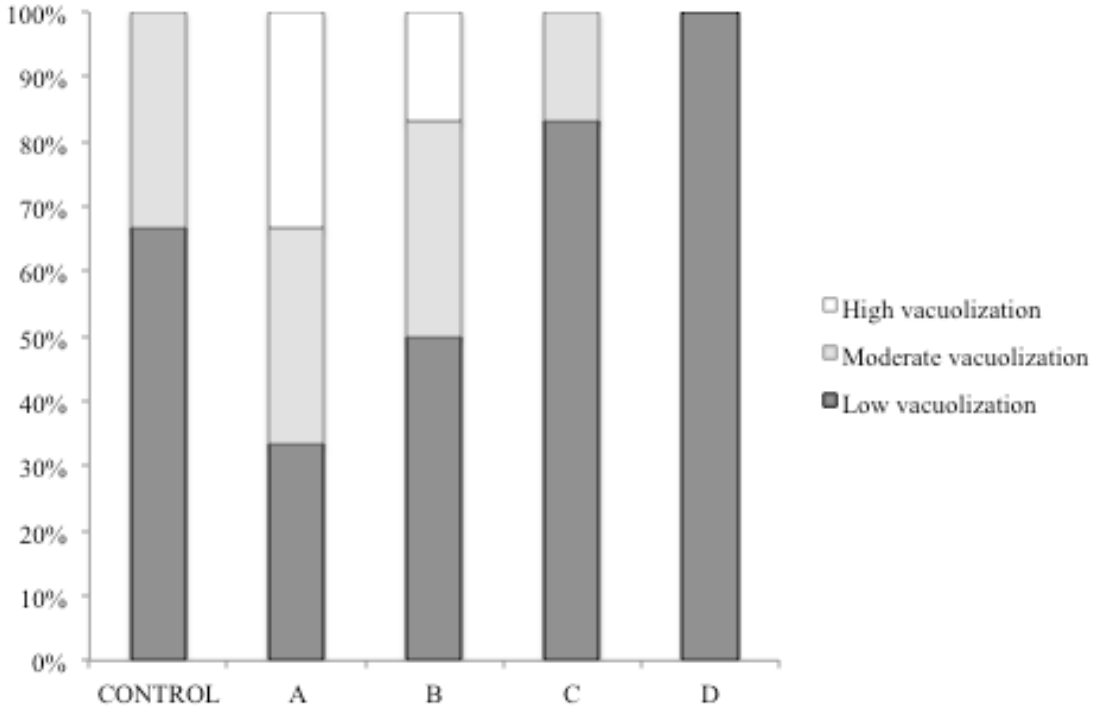

Fig. 10. Effect of red beet and betaine on posterior gut vacuolation after the stress challenge. Control (0% red beet, 0% betaine); A (14% red beet, 0.9% betaine); B (14% red beet, 1.63% betaine); C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets. The lack of different letters indicate no significant differences (P > 0.05) between different experimental diets.

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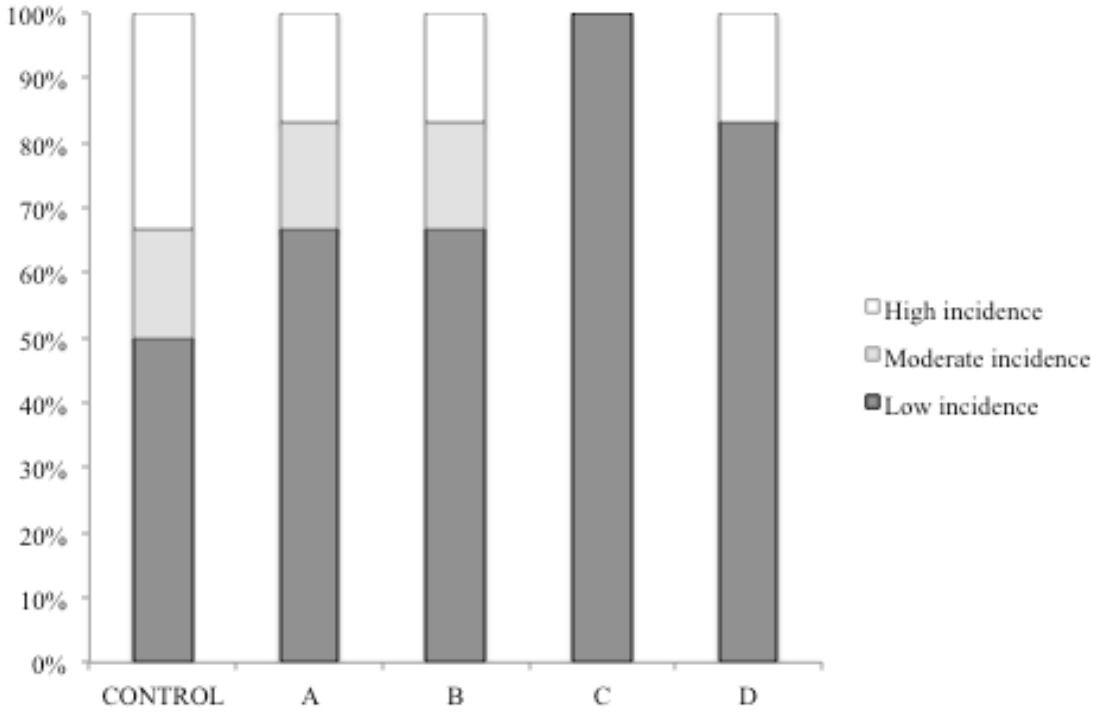
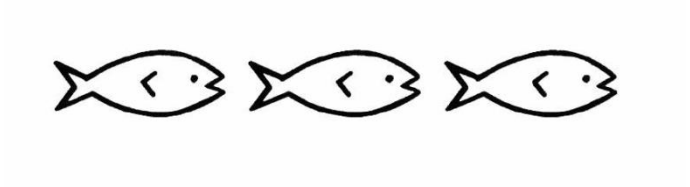


Fig. 11. Effect of red beet and betaine on the appearance of mucus cells on the posterior gut after the stress challenge. Control (0% red beet, 0% betaine); A (14% red beet, 0.9% betaine); B (14% red beet, 1.63% betaine); C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets. The lack of different letters indicate no significant differences (P > 0.05) between different experimental diets.

CHAPTER 11. REDUCTION OF LIPID OXIDATION AND ENHANCEMENT OF BIOACTIVITY THROUGH THE INCLUSION OF RED BEET AND BETAINE ON RAINBOW TROUT DIETS.



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ABSTRACT

The present study compares a control diet to 4 experimental diets in which 2 red beet (14 and 28%) and 2 betaine levels (0.9 and 1.63%) were incorporated on rainbow trout diets according to a factorial design. The effects of the inclusion of different red beet and betaine concentration on fatty acid profile, lipid peroxidation and antioxidant activity on rainbow trout fillets were investigated. Although no significant differences were observed with the control group, results indicated that red beet and betaine improved fish fillet fatty acid profile, producing an increase on PUFA, mainly DHA. Higher red beet and betaine concentrations increased flavonoid and phenolic content on the diets; however, no effects were observed on the antioxidant properties of rainbow trout fillets.

***Keywords**: Red beet, betaine, rainbow trout, fillet lipid oxidation, antioxidant activity*.

1. INTRODUCTION

Fish lipids contain high levels of polyunsaturated fatty acids (PUFA), which are susceptible to oxidation, resulting in a loss of fish quality (Chaiyapechara et al. 2003a, Baron et al. 2009, Pereira de Abreu et al. 2012, Gao and Koshio 2015). For this reason, fresh fish is a highly perishable product (Medina et al. 2009). Fish spoilage results from three basic mechanisms: enzymatic autolysis, oxidation and microbial growth (Aubourg 2008). However, these processes can occur alone or in combination and occurrence largely depends on fish species (size, lipid content, etc.), environmental conditions (feeding availability, temperature, etc.), post-mortem handling, storage and processing conditions (Medina et al. 2009, Fuentes et al. 2010). Rainbow trout (Oncorhynchus mykiss) is a fatty fish specie with high commercial value and very appreciated by European consumers (Rezaei and Hosseini 2008, Özogul et al. 2013). Trout as fatty fish variety, is very prone to deterioration (Pereira de Abreu et al. 2012, Özogul et al. 2013), precisely to its high oil/fat content (Fraser and Sumar 1998).

Different strategies have been proposed to prevent lipid and protein oxidation; some of these strategies are focused in processing process such as packaging and/or the use of antioxidants incorporated on the food products. However, recently, special attention has been paid in using antioxidant ingredients on fish diet. These ingredients have been reported as a strategy to maintain fish quality (Baron et al. 2009, García-Romero et al. 2014, Secci and Parisi 2016). Diet supplementation with antioxidants enables these substances to be incorporated into the phospholipid membrane, where they can effectively inhibit oxidation reactions (Lauridsen et al. 1997). Previous studies have reported the use of different antioxidants such as α-tocopherol, astaxanthin or canthaxanthin enhancing the quality of different fish species, by protecting fish muscle against oxidative degradation. Jensen et al. (1998) showed that the introduction of

astaxanthin on rainbow trout diets protects against lipid oxidation during the early stages of oxidative deterioration. Choubert et al. (2011) reported the same effect of astaxanthin on rainbow trout diets during long-term frozen storage. Other authors have confirmed the role of α-tocopherol or ascorbic acid as antioxidants when included as ingredient in different fish species: turbot (*Scophthahus maximus*) (Stéphan et al. 1995), rainbow trout (*Oncorhynchus mykiss*) (Chaiyapechara et al. 2003b), hybrid tilapia (*Oreochromis niloticus* x *O. aureus*) (Huang et al. 2003), red sea bream (*Pagrus major*) (Gao and Koshio 2015). New natural antioxidant have been utilised as feed additives such as thymol (Giannenas et al. 2012) or rosemary extracts (Álvarez et al. 2012, Hernández et al. 2014). However, the use of alternative natural ingredients, with bioactive compounds that can enhance fish quality and oxidative stability, have not been investigated. Previous work confirmed that the inclusion of barley on rainbow trout diets had an enhancing effect on quality parameters probably associated to the presence of antioxidant compounds (Pinedo-Gil et al. 2017A).

Red beet (*Beta vulgaris* L.) has gain relevance in recent years, especially by its health-promoting and bioactive properties (Clifford et al. 2015, Nistor et al. 2017). Red beet is rich in valuable active compounds such as carotenoids, polyphenols, flavonoids, betalains (which represents the principal pigment in red beet) and betaines. All these compounds make red beet an interesting source of antioxidant ingredients (Nistor et al. 2017). Betaines and betalains have been widely studied for their nutritional and health benefits; since present a high radical scavenging and antioxidant activity (Paciulli et al. 2016). More specifically, betaine has been reported widely to have antioxidant, antimicrobial and antiviral activities (Pedreno and Escribano 2001, Attia et al. 2013). Due to the beneficial effects found on red beet and betaine, in the present study was considered of interest to investigate the effect of these two ingredients on the quality of

fresh rainbow trout fillets, lipid stability and antioxidant activity when included as ingredient in the diet.

2. MATERIAL AND METHODS

2.1. Experimental design

Rainbow trout were provided by a local trout farm (Cien Fuentes Fishfarm, 19420, Cienfuentes, Gadalajara, Spain). The average fish weight for each fish was 69 ± 2.21 g (least-square mean ± SEM). Fish were fed with 5 isoproteic (40% crude protein) and isolipidic (18% crude fat) diets, which contained different red beet and betaine concentrations (0-28% red beet and 0-1.69% betaine). Diets formulation and composition are published on Pinedo-Gil et al. 2017B. There were five feeding treatment groups each in three replicates (n=9). Three fish per replicate were randomly sampled after 105 days (when fish reached commercial weight) for the determination of lipid oxidation and bioactivity parameters. For each fish, the skin was removed and skinless fillets were frozen until analysis.

2.2. Fatty acid profile (FAME)

Fatty acid profile (FA) was determined in red beet, diets and fish fillets. Blight and Dyer (B&D) method (Blight and Dyer 1959) was used for lipid extraction. Lipid-containing chloroform phase was separated and after evaporated. The remaining phase was dissolved in 1 mL of hexane and a methylated procedure was carried out by adding 100 µL of 0.5 M methanolic KOH and leaving the reaction for 10 min at room temperature (RT). The upper layer was transferred to a 2 mL vial. Analysis of FA methyl esters (FAME) was carried out on a gas chromatograph Agilent 7890A (Agilent Technologies, PA, California, USA) equipped with a flame ionization detector. For the analysis the method was run on helium, oven ramp temperature was set from 50 ºC to

200 ºC during the first 7 min at a rate of 3 ºC min^{-1} and held for 26 min. Injector and detector temperature were 250 ºC and 280 ºC, respectively. A sample volume of 1 µL was injected in split mode (ratio 25:1), and FAMEs were identified by comparison of retention times with those of 37 FAMEs standard mix (Supelco, Sigma-Aldrich, CO).

2.3. Alpha-tocopherol content

Alpha-tocopherol content in fish fillets was determined according to the AOCS official method (1992), using Agilent 1200 series HPLC equipped with a diode array detector. Two gramms of the B&D extract (Blight and Dyer 1959) was evaporated and resuspended in 2 mL of hexane with 20 µL of tocopherol acetate as internal standard. An aliquot (10 µL) was injected and a column (250 mm x 4.6mm 5 µm) (Teknokroma Anlítica S.A., Barcelona, Spain) was used. Elution was performed with an isocratic mixture of hexane:2-propanol (99.6:0.4; v:v) at a flow rate of 1.3 mL min^{-1}. Detection was set at 295 nm and 284 nm for tocopherol acetate. Results were expressed in µg tocopherol per gram of fillet.

2.4. Oxidative parameters

2.4.1. Peroxide value (PV)

Fish fillet PV was measured using the B&D extract according to the International IDF Standards method (1991). Results were expressed in meq of active oxygen per Kg of lipids.

2.4.2. Conjugated hydroperoxides (dienes and trienes)

Fish fillets conjugated hydroperoxides (B&D extract) were measured as described by Undeland et al. (1998). Results were calculated as mmol of hydroperoxides per Kg of lipids.

2.5. Antioxidant markers

2.5.1. Extract preparation

To measure the antioxidant activity, 1 g of blended sample was dissolved in 10 mL of 90% methanol. The extraction was accelerated using ceramic homogenizer on the test tubes by stirring for 30 s. Following samples were centrifuged at 1.635 x g for 10 min at 4 ºC and the supernatants were collected, filtered and stored at -80 ºC. All the extracts were used for antioxidant markers.

2.5.2. Phenolic characterization using HPLC

Phenolic characterization was determined on red beet and diets. Five grams of sample were mixed with 45 mL of 80% ethanol (v/v) and after it was sonicated in a water bath for 1 h. After centrifugation (5000 x g, 20 min., 10 ºC), the supernatant was removed and the extraction was repeated twice. Supernatants were mixed and then evaporated at 40 ºC under nitrogen until complete dryness; finally were reconstituted in 2 mL of 40% acetonitrile and then were filtered through 0.45 μm membrane for HPLC analysis (Bonoli et al. 2004, Zhao et al. 2006).

The phenolic compounds were separated and quantified using the method described by Schieber et al. (2001) with modifications, briefly as follows. Water Alliance 2795 Chromatography Separations Module (Waters Corp., Milford, USA) coupled to a Waters 2996 PDA detector fixed at 280 nm of wavelength was employed. Column equipped was a Zorbax sb-c18 Agilent (4.6 x 150 nm; 5 microns). The mobile phases consisted in 0.5% acetic acid (buffer A) and 20% (0.5% acetic acid):80% acetonitrile (buffer B). Initial gradient started with 5% of buffer B for 1 min, and then was increased up to a 55% for 50 minutes; the column was rinsed for 5 min by pumping 95% of buffer B and finally it was re-equilibrated for another 10 min. Calibration curves

were constructed using the following standards: gallic acid, chlorogenic acid, ferulic acid, p-cumaric acids, synaptic acid, 3-coumaric acid, 4-coumaric acid, syringic acid, maleic acid, transcinamic acid, vanilic acid, caffeic acid and 4-hydroxibenzoic acid at concentration of 5, 10, 20, 40 and 80 μg mL^{-1}.

2.5.3. Total Flavonoid determination (TFC)

TFC was determined using the method described by Lin and Tang (2007) for red beet and diets. Aliquots of 0.1 g of sample were dissolved in 1 mL of 10% aluminium chloride hexahydrate ($AlCl_3$), 0.1 mL of 1 M potassium acetate (CH_3COOK) and 2.8 mL of deionized water. After incubation at room temperature (RT) for 40 minutes the reaction was measured at 415 nm (Shimadzu PharmaSpec UV-1700. Milton Keynes, UK). The data were expressed quercetin equivalent (QE) per gram of sample based on the moisture content of lyophilized powder and "fresh sample".

2.5.4. Total phenols (TP)

TP were measured using the Folin-Ciocalteu method (Slinkard and Singleton 1977) on red beet, diets and fish fillets. Results were expressdd as mg of gallic acid per gram of dried weight (dw) sample.

2.5.5. Determination of the oxygen radical absorbance capacity (ORAC)

Oxygen radical absorbance capacity (ORAC) of fish fillets was measured following the procedure reported by Ou et al. (2001). Results were expressed as μmol of Trolox Equivalent (TE) per gram of sample (dw).

2.5.6. Trolox Equivalent Antioxidant Capacity (TEAC) and DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity

The measurement of total antioxidant capacity was determined following the procedure proposed by Serpen et al. (2007). One mg of fillet was mixed into 100 mg of

cellulose powder prior to measurement. TEAC results were expressed as mmol of Trolox Equivalent per gram of sample (dw) and DPPH as percentage of inhibition of the DPPH radical compared to a control with no red beet on diet.

2.5.7. Relative Antioxidant Capacity Index (RACI)

Relative antioxidant capacity index (RACI), a hypothetical concept, is created from the perspective of statistics by integrating the antioxidant capacity values generated from different in vitro methods, in this case TP, ORAC, DPPH and TEAC were evaluated (Sun and Tanumihardjo 2007).

2.6. Statistical analysis

Statistical analysis was performed using SAS version 9 (SAS Institute Inc., Cary, North Caroline, USA) by a GLM procedure for the variance analysis (ANOVA) followed by a t-Student test and considering significant differences between values when P-value < 0.05.

3. RESULTS AND DISCUSSION

Proximate composition and betaine content were analysed in red beet, experimental diets and fillets are reported elswhere (Pinedo-Gil et al. 2017B). The whole wheat portion substituted the highest red beet concentration. It was observed that all diets were isoproteic (40% protein) and isolipidic (18% lipids), red beet contained 0.65% betaine and natural betaine was added to reach diets with betaine concentrations range from 0.9 to 1.63%. In fillets, the inclusion of red beet and betaine significantly decreased fat content and increased betaine content.

3.1. Fatty acid profile

Table 24 shows the fatty acid profile of red beet and experimental diets. Red beet contained very small proportion of fat, so the proportion of fatty acids incorporated

by ingredient was very small. The most abundant fatty acids are palmitic acid (C16:0), linoleic (C18:2 n-6) and oleic acid (C18:1 n-9) with concentrations of 0.18, 0.37 and 0.27 g per 100 g^{-1} respectively. These results are in accordance with those reported by Neelwarne and Halagur (2012). And in agreement, USDA database, which showed that the most abundant fatty acids in red beet are palmitic acid, oleic and linoleic acids, the same as the results obtained in the present study.

The replacement of wheat with red beet and betaine resulted in a decrease in stearic acid (C18:0) and docosahexanoic acid (DHA, C22:6 n-3) in the experimental diets with high replacement level, compared with the control and the 14% replacement diets; however, the concentration of linoleic acid (C18:2 n-6) increased on the four replacement diets with the inclusion of red beet and betaine (Table 24).

Table 24. Red beet and experimental diets fatty acid profiles.

	Red beet	DIETS[1]				
		CONTROL	A	B	C	D
SFA						
C14:0	0.02	0.23	0.22	0.21	0.26	0.27
C16:0	0.18	2.40	2.40	2.35	2.41	2.44
C18:0	0.04	0.50	0.47	0.51	0.45	0.42
MUFA						
C16:1	0.02	0.26	0.25	0.21	0.25	0.26
C18:1 (n-9)	0.27	3.73	3.66	3.73	3.60	3.51
PUFA						
C18:2n6	0.37	5.29	5.66	6.00	6.02	5.62
C18:3n3	0.03	0.41	0.49	0.40	0.47	0.47
C20:5n3	0.02	0.21	0.21	0.18	0.18	n.d.
C22:6n3	0.02	0.34	0.35	0.30	0.31	0.31

[1] Experimental diets: CONTROL (0% red beet, 0% betaine); A (314% red beet, 0.9% betaine); B (14% red beet, 1.69% betaine); C (28% red beet, 0.9% betaine); D (28% red beet, 1.69% betaine).

SFA (saturated fatty acid); MUFA (monounsaturated fatty acid); PUFA (polyunsaturated fatty acid); DHA (docosahexaenoic acid, 22:6 n-3); EPA (Eicosapentaenoic acid, 20:5 n-3).

n.d. means not detected value.

On the other hand, although there was no fatty acid replacement in the diet formulation, some modifications were observed in the fillet fatty acid profile associated with the inclusion of red beet and betaine on the diet (Table 25). The inclusion of these ingredients showed a dose-dependent effect on myristic acid (C14:0) and polyunsaturated fatty acids (PUFA), specifically docosahexanoic acid (DHA; C22:6 n-3). Myristic acid (C14:0) reached the lowest values in those fish fed at higher red beet concentrations (diets C and D) and PUFA and DHA reached the highest values in those fish fed at higher red beet concentrations (diets C and D), although no significant differences were observed with the control diet. None of the other fatty acids were affected by diet. Results were in agreement with Welker et al. (2016) when fed rainbow trout with different varieties and concentrations of green tea. Also Ji et al. (2007) reported for Japanese flounder that fish fed with increasing levels of a mixture of herbs showed lower SFA and MUFA and higher PUFA in carcass. It seems that high content in phenolic compounds can contribute in decreasing SFAs and MUFAs, while increasing PUFAs content.

Table 25. Effect of red beet and betaine on the fatty acid profile of rainbow trout fillets. Data are shown as least-squares means ± standard error of the mean (SEM) of triplicate groups (n=9)

	DIETS					SEM	Sign.
	CONTROL	**A**	**B**	**C**	**D**		
Σ SFA	**21.50**	**21.77**	**21.33**	**20.72**	**21.10**	**0.34**	**N.S.**
C14:0	1.75^{b}	1.69^{ab}	1.72^{ab}	1.64^{a}	1.64^{a}	2.14	**
C16:0	14.58	14.76	14.37	13.96	14.23	0.26	N.S.
C18:0	4.04	4.26	4.20	4.05	4.17	0.09	N.S.
C20:0	0.81	0.72	0.70	0.72	0.72	0.03	N.S.
C22:0	0.31^{a}	0.33^{ab}	0.36^{b}	0.34^{ab}	0.33^{ab}	0.02	*
Σ MUFA	**34.44^{a}**	**34.30^{a}**	**35.72^{b}**	**34.43^{a}**	**33.66^{a}**	**0.43**	**
C16:1	2.66^{b}	2.64^{b}	2.59^{ab}	2.40^{a}	2.39^{a}	0.08	*
C18:1 n-9 trans	27.41	27.30	28.68	27.54	26.83	0.35	N.S.
C18:1 n-9 cis	1.91	1.90	1.96	1.97	1.97	0.07	N.S.
C20:1 n-9	1.14^{a}	1.21^{ab}	1.26^{b}	1.23^{b}	1.20^{ab}	0.03	*
C22:1	0.40	0.38	0.41	0.41	0.41	0.01	N.S.
C24:1	0.91	0.86	0.82	0.88	0.85	0.04	N.S.
Σ n-9	**30.46^{a}**	**30.41^{a}**	**31.90^{b}**	**30.73^{a}**	**29.99^{a}**	**0.38**	**
Σ PUFA	**44.07^{ab}**	**43.92^{ab}**	**42.95^{a}**	**44.85^{b}**	**45.25^{b}**	**0.59**	*
C18:2 n-6 cis	26.47	27.02	26.59	27.06	27.20	0.52	N.S.
C18:3 n-3	2.77	2.82	2.92	2.89	2.79	0.05	N.S.
C20:2	0.94^{a}	1.14^{b}	1.13^{b}	1.14^{b}	1.17^{b}	0.06	*
C20:3 n-6	0.75	0.85	0.80	0.78	0.88	0.05	N.S.
C20:3 n-3	0.18	0.15	0.15	0.16	0.17	0.02	N.S.
C20:4 n-6 (ARA)	1.11^{c}	0.89^{ab}	0.81^{a}	0.89^{ab}	1.05^{bc}	0.07	**
C20:5 (EPA)	2.44	2.23	2.23	2.37	2.39	0.09	N.S.
C22:6 DHA)	9.40^{bc}	8.84^{ab}	8.32^{a}	9.55^{bc}	9.60^{c}	0.32	*
Σ n-6	**28.34**	**28.75**	**28.20**	**28.73**	**29.13**	**0.55**	**N.S.**
Σ n-3	**2.94**	**2.97**	**3.07**	**3.06**	**2.96**	**0.05**	**N.S.**
n-6/n-3	**9.64**	**9.71**	**9.21**	**9.40**	**9.87**	**0.25**	**N.S.**
EPA/DHA	**0.26**	**0.25**	**0.27**	**0.24**	**0.25**	**0.02**	**N.S.**
ARA/EPA	**0.33**	**0.33**	**0.31**	**0.31**	**0.30**	**0.02**	**N.S.**

[1] Fish fed with experimental diets: Fish fed with CONTROL diet (0% red beet, 0% betaine); fish fed with A diet (14% red beet, 0.9% betaine); fish fed with B diet (14% red beet, 1.69% betaine); fish fed with C diet (28% red beet, 0.9% betaine); fish fed with D diet (28% red beet, 1.69% betaine).

SFA (saturated fatty acids); MUFA (monounsaturated fatty acids); PUFA (polyunsaturated fatty acids); ARA (arachidonic acid, 20:4 n-6); DHA (docosahexaenoic acid, 22:6 n-3); EPA (Eicosapentaenoic acid, 20:5 n-3).

Different superscript letters indicate significant differences ($P < 0.05$) between the experimental diets.

3.2. α-tocopherol content

Fish fillets α-tocopherol content was not significantly affected by different experimental diets (data not shown).

3.3. Oxidative parameters: peroxide value (PV) and conjugated hydroperoxides (dienes and trienes)

Peroxide value (PV) was evaluated on fish fillets since is one of the most common method for analysing primary lipid oxidation (Özogul et al. 2013). Similar to data observed on α-tocopherol content, PV and conjugated dienes and trienes hydroperoxides for fish fillets were not significantly affected by the concentration of red beet and betaine. Thus, these ingredients did not have any effect on fillet lipid oxidation (results not shown).

3.4. Antioxidant activity

3.4.1. Total flavonoid content (TFC) and total phenolic content (TP)

Ninfali et al. (2013) reported values of TFC on red beet root between 0.88 and 1.44 mg g^{-1}, which were in agreement with values of this study (1.82 μg QE g^{-1}) (Figure 41). When the total flavonoid content was determined in the different experimental diets it was observed that the substitution of wheat for red beet and betaine increased the TFC (Figure 40). Red beet improved the concentration of TFC on rainbow trout diets what could increase the bioactive properties of fish fillets.

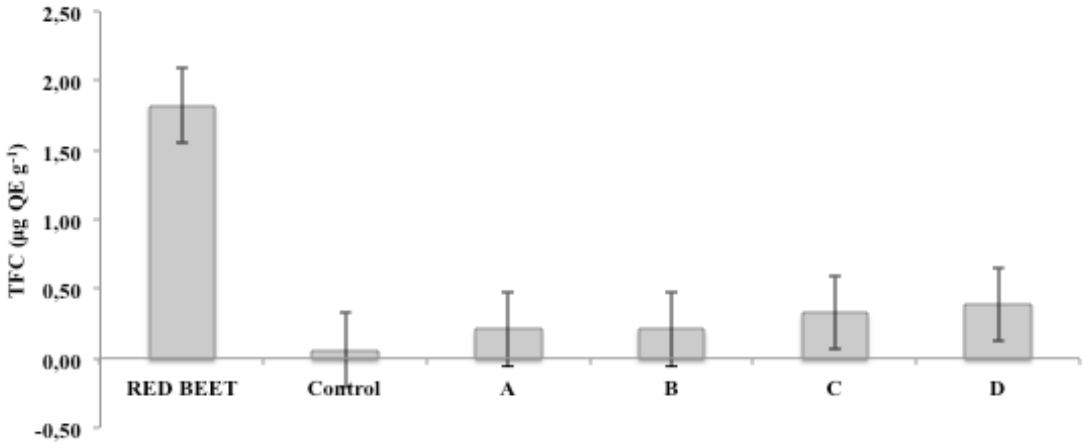


Figure 40. Red beet and experimental diets total flavonoid content (TFC).

CONTROL (0% red beet, 0% betaine); A (314% red beet, 0.9% betaine); B (14% red beet, 1.69% betaine); C (28% red beet, 0.9% betaine); D (28% red beet, 1.69% betaine) are the different experimental diets.

TP of red beet was 5.61 mg of GAE g^{-1} (Figure 41). Kujala et al. (2000) reported that the TP of red beet was 4.2 mg g^{-1} and Bavec et al. (2010) 4.94 mg g^{-1}, so results are in the same order of magnitude to previous findings. It is necessary consider that the value obtained in the present study was over dry samples and the values given by other authors were in fresh. Similar to TFC, TP also increased with increasing red beet concentrations on rainbow trout diets (Figure 41). The substitution of wheat for red beet on the experimental diets increase TFC and TP, which can provide bioactive properties to fish fed with those diets. However, TP of rainbow trout fillets was not affected by the concentration of red beet and betaine, contrary to what was expected (Figure 42).

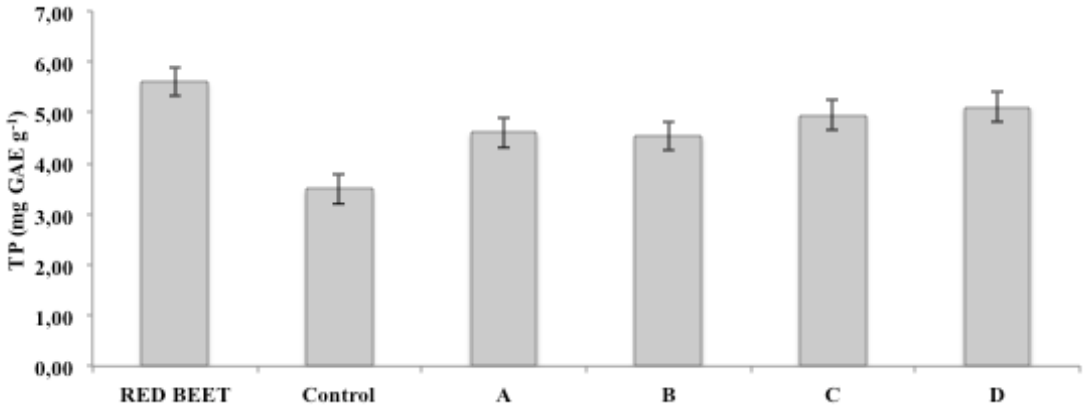


Figure 41. Red beet and experimental diets total phenolic content (TP).

CONTROL (0% red beet, 0% betaine); A (314% red beet, 0.9% betaine); B (14% red beet, 1.69% betaine); C (28% red beet, 0.9% betaine); D (28% red beet, 1.69% betaine) are the different experimental diets.

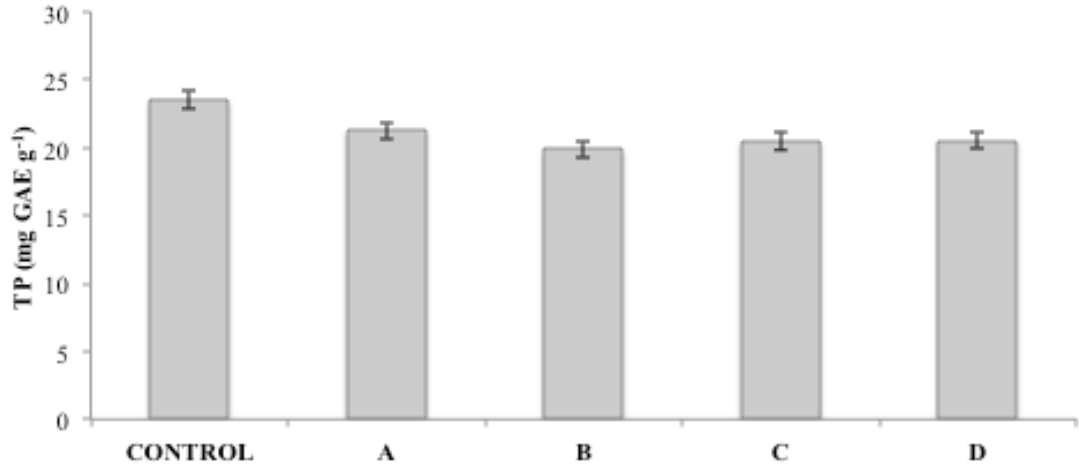


Figure 42. Fillets total phenolic content (TP) of fish fed with different experimental diets (n=9).

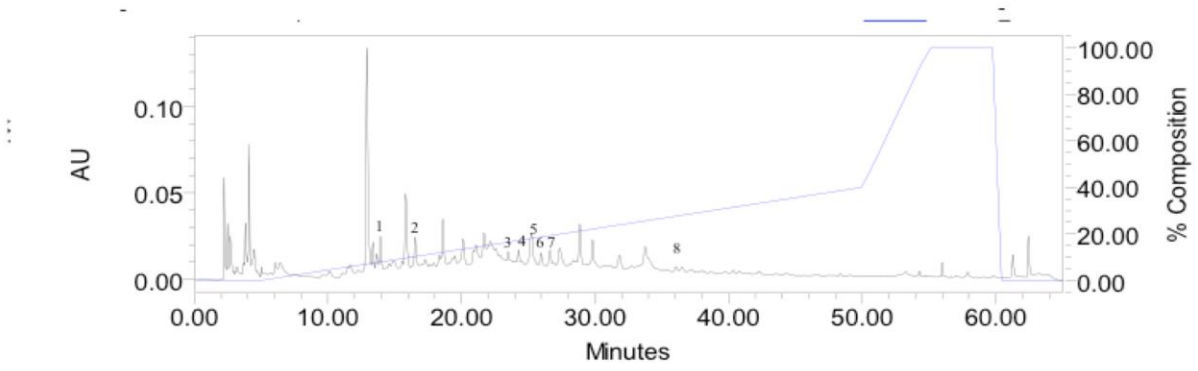
CONTROL (0% red beet, 0% betaine); A (314% red beet, 0.9% betaine); B (14% red beet, 1.69% betaine); C (28% red beet, 0.9% betaine); D (28% red beet, 1.69% betaine) are the different experimental diets. Absence of different small letters (a, b) correspond to no significant differences ($P > 0.05$) between different samples.

3.4.2. Individual phenolic compounds

Individual phenolic compounds were determined in red beet and experimental

diets. HPLC red beet profile appears in Figure 43. Eight phenolic compounds were identified and quantified in red beet. The results showed that maleic acid (198.57 μg mL^{-1}) was the main compound followed by syringic acid (26.47 μg mL^{-1}), chlorogenic acid (25.58 μg mL^{-1}), vanillic acid (17.18 μg mL^{-1}), gallic acid (16.45 μg mL^{-1}), 4-hydroxibenzoic acid (10.42 μg mL^{-1}), ferulic acid (3.30 μg mL^{-1}) and caffeic acid (2.59 μg mL^{-1}). All these phenolic compounds were also identified in red beet by Georgiev et al. (2010) and Ravichandran et al. (2012).

When the different phenolic compounds were quantified in the experimental diets results showed some differences compared with the control diet: for instance, 4-hydroxibenzoic acid was not detected on the control diet and increasing concentrations of this compound was observed in diets at higher red beet and betaine levels. Ferulic acid was only observed on those diets at higher red beet concentration (diets C and D). Vanillic acid content was higher at higher betaine concentration diets (B and D). And although transcinamic acid was not detected on red beet, it was on the experimental diets; probably these compounds are present due to the presence of other ingredients of the diet.



	Red beet	DIETS[1]				
		CONTROL	A	B	C	D
1 **Gallic acid**	16.45	307.86	288.04	278.39	107.74	320.64
2 **Maleic acid**	198.57	19.42	22.84	33.52	17.84	40.92
3 **4-hydroxybenzoic acid**	10.42	n.d.	0.06	0.32	9.37	10.29
4 **Chlorogenic acid**	25.58	9.35	31.91	0.06	4.13	18.66
5 **Vanillic acid**	17.18	5.36	n.d.	10.11	n.d.	15.10
6 **Caffeic acid**	2.59	2.45	2.54	2.45	2.49	2.68
7 **Syringic acid**	26.47	7.76	10.31	3.42	2.39	0.68
8 **Ferulic acid**	3.30	n.d.	n.d.	n.d.	0.67	0.78
9 **Transcinamic acid**	n.d.	4.52	17.17	1.76	5.32	11.38

Figure 43. HPLC chromatogram of phenolic compounds in red beet extracts.

1. Gallic acid; 2. Maleic acid; 3. 4-hydroxybenzoic acid; 4. Chlorogenic acid; 5. Vanillic acid; 6. Caffeic acid; 7. Syringic acid; 8. Ferulic acid; n.d. means not detected value.

3.4.3. Antioxidant activity of fish fillets

The antioxidant capacity of rainbow trout fillets was analysed by DPPH, ORAC, TEAC and RACI.

The antioxidant capacity measured by the different parameters was not significantly modified by the concentration of red beet and betaine on diet (Table 26). These results were different to what was expected, since fish fed with diets containing

higher betaine concentrations (B and D) presented significantly higher values of betaine on flesh than those with lower concentration (A and C) or control, and betaine is a compound with high antioxidant activity (Pedreno and Escribano 2001, Attia et al. 2013, Paciulli et al. 2016). Also, it was found that the inclusion of red beet and betaine increased TFC and TP of the experimental diets, which could be involved on the antioxidant activity of the rainbow trout fillets. However, probably these compounds although acted as antioxidant they are no incorporated in the fish that is why is not possible to observe any effect on the fish extracts.

Table 26. Effect of red beet and betaine on the antioxidant activity of rainbow trout fillets. Data are shown as least-squares means ± standard error of the mean (SEM) of triplicate groups (n=9)

	DIETS[1]					SEM	Sign.
	CONTROL	A	B	C	D		
DPPH	34.89	36.46	34.61	35.74	38.83	1.60	N.S.
TP	23.50	21.24	19.89	20.45	20.55	2.03	N.S.
ORAC	997.09	844.48	857.81	957.36	827.72	74.01	N.S.
TEAC	1266.68	1132.53	1032.19	1110.81	1054.79	99.27	N.S.
RACI	0.20	-0.01	-0.17	0.01	-0.01	0.22	N.S.

[1] Fish fed with experimental diets: Fish fed with CONTROL diet (0% red beet, 0% betaine); fies fed with A diet (314% red beet, 0.9% betaine); fish fed with B diet (14% red beet, 1.69% betaine); fish fed with C diet (28% red beet, 0.9% betaine); fish fed with D diet (28% red beet, 1.69% betaine).

DPPH (1,1-diphenyl-2-picrylhydrazyl); TP (Total phenols), ORAC (Oxygen radical absorbance capacity), TEAC (Trolox Equivalent Antioxidant Capacity); RACI (Relative antioxidant capacity index).

Absence of superscripts letters indicates no significant differences (P>0.05) between the different experimental diets.

4. CONCLUSION

Results indicated that the inclusion of red beet and betaine on rainbow trout diets decreased total fatty acids concentration on fish muscle, but increase their PUFAs content, mainly DHA. ON the other hand, although increasing concentration of red beet

and betaine on diet increased flavonoid and phenolic content, no effect was observed on the antioxidant and oxidative properties of rainbow trout fillets.

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CHAPTER 12. GENERAL DISCUSSION

The current doctoral Thesis has investigated the effect of barley and red beet on different productive and quality parameters of rainbow trout as carbohydrate sources (Figure 45). The new sources were used as alternative ingredients to wheat in order to add a carbohydrate fraction that enhance the final product, with bioactive compounds which can improve fish performance, palliate stress responses due to potential inmunomodulatory compounds present in barley and red beet and produce a product with differentiated quality.

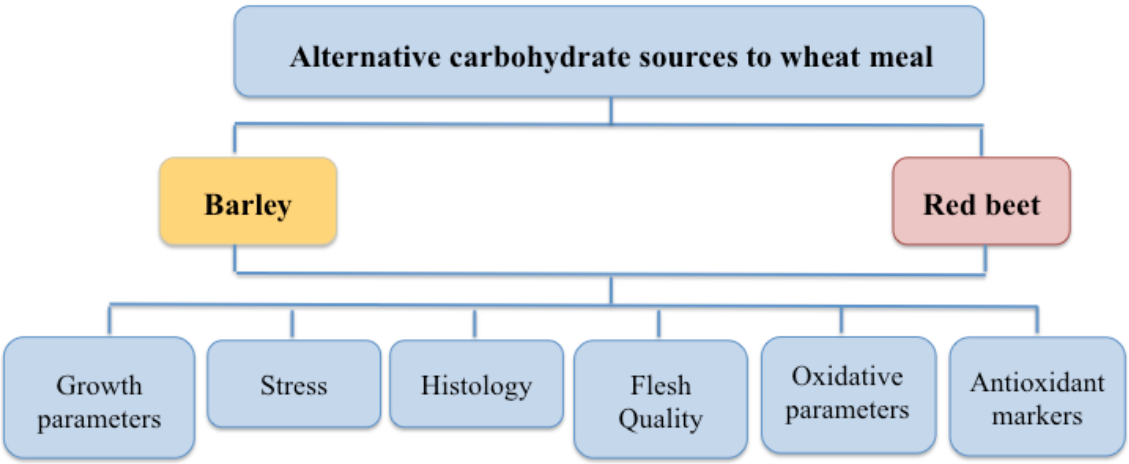

Figure 44. Summary of the different aspects studied in the current doctoral thesis.

1. Growth parameters

Global growth curves and a summery of the growth parameters results obtained on the barley and red beet experiments are shown in Figure 46 and Table 27, respectively.

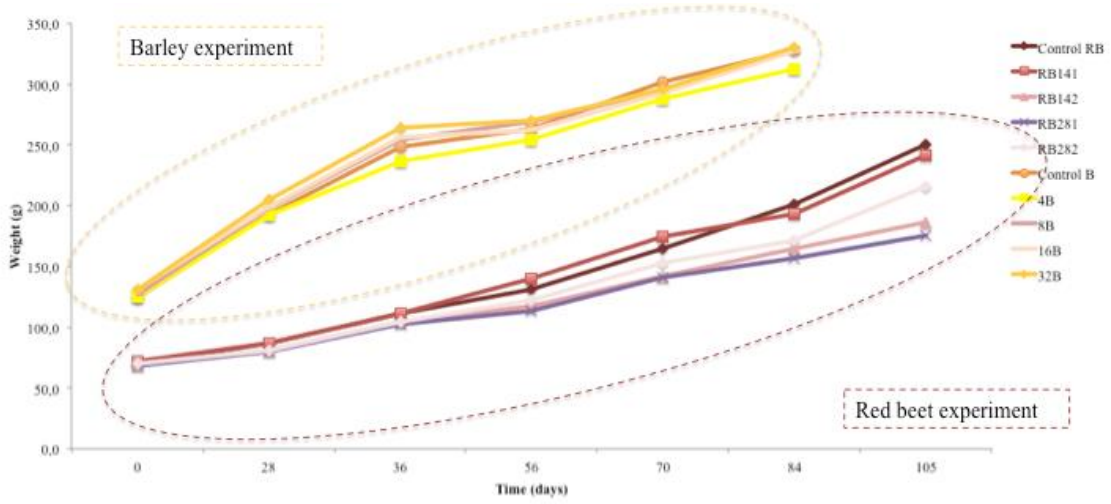

Figure 45. Growth curve in barley and red beet experiments.

Table 27. Growth and biometric parameters of rainbow trout fed at different barley and red beet and betaine concentrations.

	Diet	Wi	Wf	SGR	FI	FCR	CF	VSI	HSI
Barley experiment	Control	125	328	1.26	1.23	1.17	0.93	10.75	1.63
	40B	125	312	1.19	1.15	1.13	0.93	9.94	1.60
	80B	127	330	1.24	1.16	1.11	0.93	10.36	1.68
	160B	131	328	1.19	1.14	1.11	0.96	10.71	1.72
	319B	131	330	1.20	1.16	1.14	0.94	10.80	1.58
Red beet experiment	Control	69.82	**250.72^{d}**	**1.22^{d}**	1.05	**1.18^{a}**	**0.89^{b}**	**8.64^{a}**	**1.16^{a}**
	RB141	73,54	**241.47^{cd}**	**1.13^{cd}**	1.06	**1.19^{a}**	**0.86^{ab}**	**9.04^{a}**	**1.46^{b}**
	RB142	68.51	186.66^{ab}	0.95^{ab}	1.00	1.32^{b}	0.82^{a}	9.75^{ab}	**1.26^{a}**
	RB281	68.17	**175.27^{a}**	**0.90^{a}**	1.01	**1.50^{c}**	0.80^{a}	**10.56^{b}**	**1.45^{b}**
	RB282	69.78	215.52^{bc}	1.07^{bc}	1.02	1.28^{ab}	0.86^{ab}	9.44^{ab}	1.29^{ab}

Green and **red** values indicate the best and the worst values respectively for the growth, nutritive and biometric parameters.

Results showed that substituting wheat meal for barley did not alter growth performance of rainbow trout suggesting that both cereals (wheat and barley) could be used as carbohydrate source on rainbow trout diets. Similar results were reported by Sealey et al. (2008) who studied the effect of three barley genotypes on growth performance of rainbow trout and did not observe significant differences on final weight regardless barley concentration used. Comparing both carbohydrate sources, barley showed better growth results on rainbow trout growth than red beet. However, it is

important to mention that when fish start with smaller initial weight they used to grow more, so fish fed with red beet should have had a better growth. But it has not occurred in this way, this lower growth could be due to different factors, the fish batch, a bigger initial biomass in the tank, etc and not by the diet (as it could be observed in control diet since fish fed with the control diet did not have a better growth although they started with smaller initial weight), although for lower red beet and betaine concentrations, it seems to appear a positive interaction, showing no differences with the control. However, the inclusion of high red beet and betaine concentrations had a negative effect on growth and nutritive parameters. Similar results were reported in other fish species such as Atlantic salmon (Duston 1993), sea bass and sea bream (García-Alcáczar et al. 1994) or piauçu (Normandes et al. 2006) when they were fed with betaine.

Apparent digestibilities obtained in both experiments indicated adequate quality efficiency for the different experimental diets. ADC for protein and fat were higher than 80%. However, differences were observed between the use of barley or red beet and betaine compared to control diet. The use of barley on rainbow trout diets showed that protein digestibility coefficient was slightly lower in fish fed diets containing higher barley concentration, although due to the high percentage coefficients obtained in every experimental diet higher than 90%, it cannot be considered a negative effect of barley on protein digestibility. On the other hand, the use of dietary red beet on rainbow trout diets showed a significant decrease of fat digestibility coefficient. This effect may be associated to the higher VSI and HSI found in fish fed with higher red beet concentrations. The inclusion of red beet and betaine increased visceral adipose tissue mass and decreased growth, as it has been observed on Table 27. This effect has been reported by several authors with different carbohydrate sources and fish species (Tan et al. 2006, Wu et al. 2007, Cui et al. 2010). These authors agreed that carbohydrates not

used as an energy source, can be accumulated in the liver and transformed into lipids and glycogen which could lead to a higher HSI. More studies should be carried out to clarify if those negative effects on HSI and VSI on fish fed with red beet are attributable to the increase on the synthesis of lipids from carbohydrates, as this effect has not been observed on fish fed with other carbohydrates such as barley or wheat.

2. Stress

The incorporation of barley and red beet and betaine on rainbow trout diets had a significant effect on fish under acute stress challenge (acute hypoxia and crowding) responses and recovery.

In both experiments, basal cortisol plasma values were similar to values reported by other authors (Ellis et al. 2004, Tintos et al. 2006, Bertotto et al. 2010). Thirty minutes after the stress challenge it was observed a significant increase on cortisol levels in all fish groups as it was observed in other studies (Raaij et al. 1996, Bertotto et al. 2010, Ming et al. 2012, Pérez-Jiménez et al. 2012).

Results showed that carbohydrate source had a significant effect on plasma cortisol values after the acute stress challenge (30 minutes). Fish fed with 8% barley showed significantly lower values than fish fed with the control diet and other experimental diets. However, the inclusion of red beet and betaine had significantly higher plasma cortisol values than the control group. These results suggest that red beet did not enhance the tolerance of rainbow trout against a stress challenge, although all fish groups recover with the same trend. The inclusion of 8% barley concentration enhances the tolerance of fish to the same stress challenge. Unexpected, those fish also showed a better lipid oxidation stability and bioactivity, although no mechanisms have been proposed to explain this effect. Several studies (Jeney et al. 1997, Cain et al. 2003,

Dawood et al. 2015, Zeng et al. 2016 and Miest et al. 2016) have reported the effect of β-glucan on protecting fish against various stress factors. However, more studies should be done to verify this positive effect of barley.

Plasma glucose basal values were not affected by the inclusion of barley, red beet and betaine. On the other hand, plasma lactate basal values did not show any effect by the inclusion of barley, however, they were significantly affected by the inclusion of red beet and betaine. Significantly lower values were observed in fish fed with red beet and betaine compared to fish fed with the control diet.

In both experiments, as it was expected, plasma glucose and lactate levels increased after the stress (30 minutes) since it has been widely reported that plasma glucose and lactate increased during a stress challenge (Chagas and Val 2006, Pérez-Jiménez et al. 2012, Ings et al. 2012). Increased cortisol levels mediate the production of glucose (by increase glycogenolysis) (Janssens and Waterman 1988, Mommsen et al. 1999, Fast et al. 2008, Ming et al. 2012) and increase lactate due to muscle anaerobiosis. Thirty minutes after the stress the inclusion of barley, red beet and betaine had different effects on the glucose and lactate concentrations: fish fed at higher barley concentrations (32%) showed significantly the lowest glucose level and fish fed with 8% barley showed significantly the lowest lactate value; and fish fed with diets containing red beet and betaine showed significantly higher values than fish fed with the control diet. These suggest that barley may have a positive effect on the stress tolerance while red beet and betaine did not enhance the tolerance of rainbow trout to a stress challenge even though all fish recover basal levels in the same way regardless of the diet. The beneficial effect of barley on preventing the stress response could be due to its content on β-glucans; since previous studies have reported the same effect for other fish species: Welker et al. (2007) observed lower levels of cortisol and lactate in channel

catfish fed with an additive containing β-glucan after low-water stress; Cain et al. (2003) observed lower cortisol levels in Nile tilapia fed a 0.2% β-glucan diet after handling stress; Jeney et al. (1997) also observed reductions in cortisol in rainbow trout fed a 0.1% β-glucan diet after a transportation stress.

Results showed that the use of different carbohydrate sources had different effects on lipid peroxidation (MDA) levels before and after the stress. Before the stress the inclusion of red beet and betaine did not significantly affect MDA values, however, the inclusion of barley showed that fish fed with the highest barley concentration (31.9%) showed significantly the highest MDA values but without significant differences with the control. After the stress both ingredients suggest a protective effect from oxidative stress. The highest barley and red beet and betaine concentrations showed the lowest MDA values. These results are interesting since it has been widely reported that stress conditions contribute to lipid peroxidation (Lushchak et al. 2005, Chagas and Val 2006, Pérez-Jiménez et al. 2012, Ming et al. 2012).

3. Histology

Histological analysis of fish fed diets containing different barley, red beet and betaine concentrations exhibited morphological changes before and after an acute stress.

Before the stress, fish fed with the highest barley and betaine concentrations showed smaller hepatocytes areas and lengths than the control group. In general, as it has been previously observed by other authors, bigger hepatocytes are more vacuolized than smaller hepatocytes (Rusell et al. 2001, Pereira et al. 2002, Figueiredo-Silva et al. 2005). This result is in accordance with results observed in fish fed with red beet and betaine, where, results showed that hepatocytes of fish fed with the control diet and with diets lower in betaine levels were bigger and showed higher vacuolation, as it can be

observed in Figure 46, where in photos (1), (2) and (3) are observed a higher vacuolation than in photos (4) and (5). However, fish fed at higher barley concentration showed small hepatocytes with a big vacuolation. The higher lipid vacuolation is associated with the high VSI and HSI observed in the productive parameters. This effect was also reported by other authors feeding different fish species with other ingredients. Caballero et al. (2002) observed that rainbow trout fed at higher vegetable oils inclusions had varyng-size lipid vacuoles in livers and foci of non-stained swelling hepatocytes; and Figueiredo-Silva et al. (2005) reported for juvenile sea bass the accumulation of lipid in the liver when fed with soybean oil.

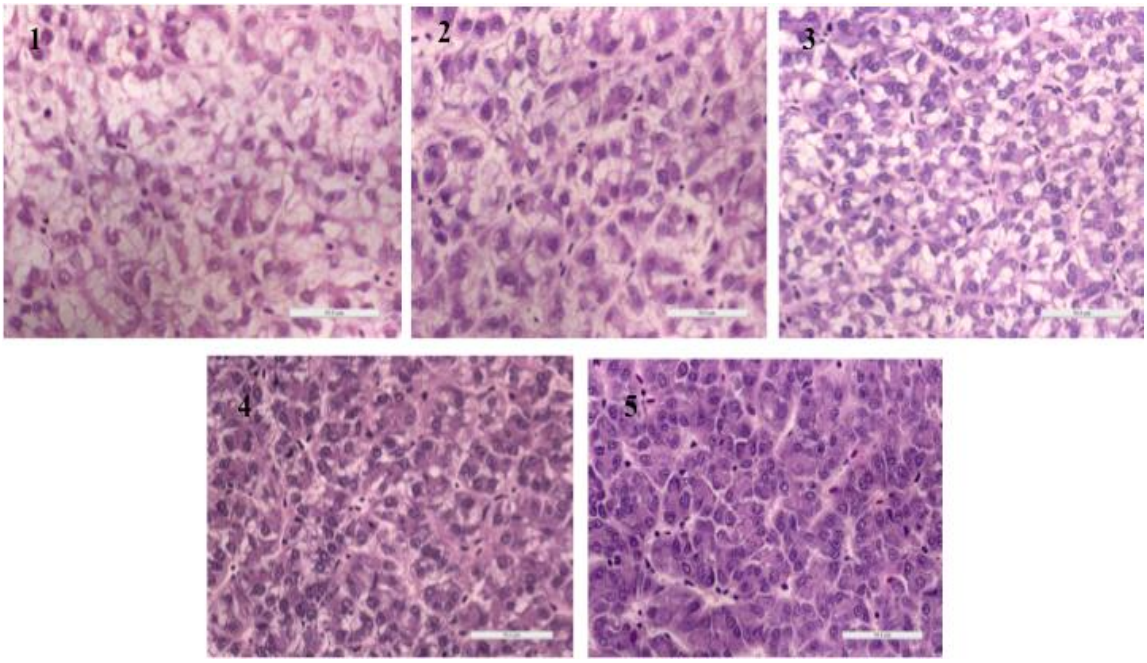

Figure 46. Hepatocites of fish fed with diets containing red beet and betaine before the stress trial. **(1)** shows hepatocytes of fish fed with control diet; **(2)** shows hepatocytes of fish fed with A diet (14% red beet, 0.9% betaine); **(3)** shows hepatocytes of fish fed with C diet (28% red beet, 0.9% betaine); **(4)** shows hepatocytes of fish fed with B diet (14% red beet, 1.63% betaine); **(5)** shows hepatocytes of fish fed with D diet (28% red beet, 1.63% betaine).

Liver tissue examination revealed the presence of lymphocytic cells foci in fish of all groups, regardless the carbohydrate source. This degeneration on liver appeared

also in the control group and may be due, in part, to an excessive hepatocellular vacuolation during fish growth under rearing conditions. Fish fed with red beet and betaine did not show significant differences between groups and intermediate barley levels (16B) showed the biggest liver degeneration (Figure 47). Figuere 47 (4) showed liver degeneration of fish fed with 16B diets, where all liver was invaded by an infiltration of cells, however in the rest of the pictures it can be observed insolated lymphocitic foci. Liver degeneration was more evident in those livers less vacuolized than when vacuolation was high, as it was also observed by Figuereido-Silva et al. (2005). Thus the presence of red beet and betaine and barley have a positive effect on liver vacuolation and degeneration maybe due to their antioxidant and bioactive properties linked to these ingredients (Nistor et al. 2017).

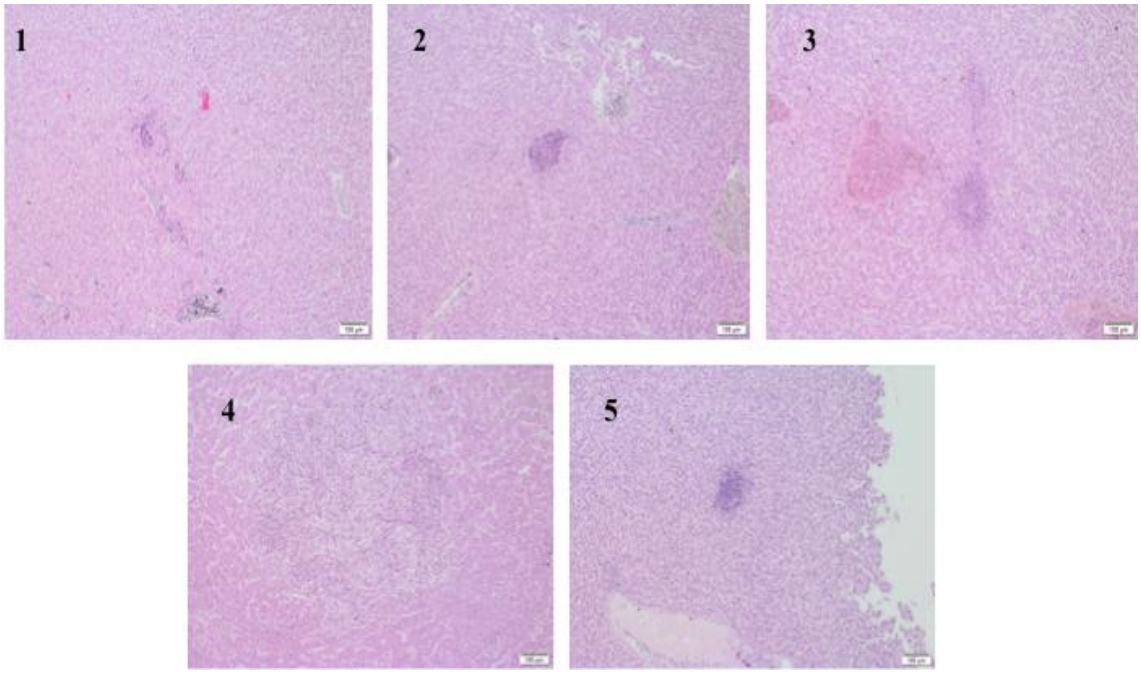

Figure 47. Liver degeneration of fish fed with diets containing barley before the stress challenge. **(1)** shows fish fed with control diet; **(2)** shows fish fed with 4B diet (4% barley); **(3)** shows fish fed with 8B diet (8% barley); **(4)** shows fish fed with 16B diet (16% barley); **(5)** shows fish fed with 32B diet (32% barley).

When the posterior gut was studied different effects were observed regarding the

carbohydrate source. When the red beet and betaine concentration effect on posterior intestine was examined, the incidence of cell infiltration, mucus cells and vacuolation did not show significant effect and not differences with the control. However, when fish were fed with 8% barley concentration showed significantly the highest gut degeneration and mucus cells (Figure 48). Different authors have reported gut morphological changes including the appearance of inflammatory cells infiltration in the lamina propria when carnivorous fish are fed with plant ingredients (Krogdahl et al. 2000, Refstie et al. 2000, Borquez et al. 2011). An interesting indicator to detect inflammation in gut is the presence of mucus cells (Urán 2008 and Tran-Ngoc et al. 2016) and several studies reported an increase number of mucus cells when fish are fed with plant ingredients (Heidarieh et al. 2013, Baeza-Ariño et al. 2014). But those negative effects were only visible on fish fed with 8% barley, higher barley concentrations than 8% and the inclusion of red beet and betaine produce a positive effect, as they did not stimulate the appearance of mucus cells.

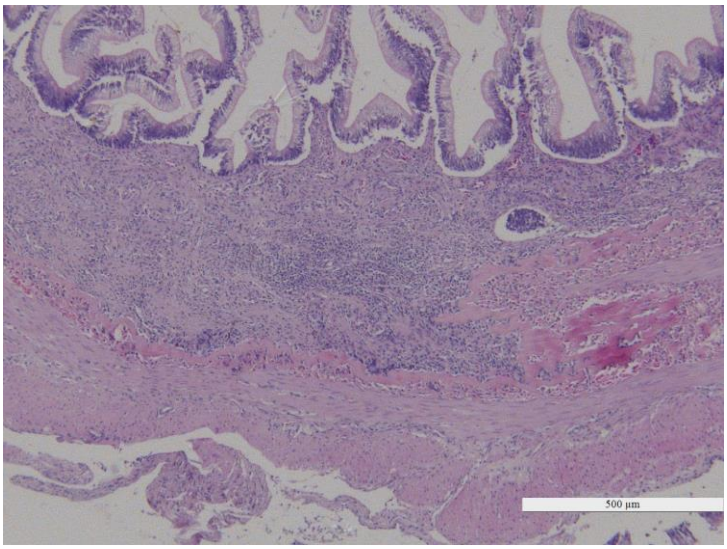

Figure 48. Gut cell infilitration of fish fed with diets containing 8% barley before the stress challenge.

Histologic and morphological parameters were studied 30 minutes after the

stress, parallel to the biochemical analysis, in order to observe the stress effect. Stress responses may be evident in the liver because its important role in energy storage and metabolism. Often, quantitative alterations in hepatic energy storage are visible macroscopically as changes in liver size and coloration, and histologically as variations in hepatocellular vacuolation and tinctorial staining characteristics (Harper and Wolf 2009, Wolf and Wolfe 2005). Thirty minutes is a very short time to produce any histologic or morphological change, but we checked if something happen at that level immediately after the stress. Thirty minutes after the stress, barley, red beet and betaine produced a significant effect on liver histology and morphology. Hepatocytes size (areas and lengths) significantly decreased compared to basal values. This decrease on hepatocytes size may be due to a more demand of energy produced by the lack of oxygen. Liver cell vacuolation was also lower and more evident on fish fed with diets at higher barley, red beet and betaine concentrations with exceptions. Fish fed with barley concentrations higher than 8% and red beet concentrations higher than 14% may have a negative effect on fish on their tolerance to an acute stress. Wolf and Wolfe (2005) reported that stress produce liver histological modifications as variations in hepatic vacuolation and histopathologic changes. Decreased vacuolation can result from loss of cytoplasmic glycogen and/or lipid caused by insufficient energy intake relative to need and/or glucocorticoid-induced glycogenolysis (Wolf and Wolfe 2005, Harper and Wolf 2009). On the other hand, the incidence of appearance of lymphocytic foci of fish fed with diets with barley concentrations of 4% was significantly lower than fish fed at higher barley concentrations, however, this significantly decreased with the inclusion red beet and betaine on the diet. The presence of lymphocytic foci after a stress period, causing necrotic areas in liver is normal due to a lack of oxygen on several areas of the organ (Scott and Rogers 1980). The decrease in oxygen availability to tissues can lead

to necrotic or apoptotic lesions in organs (Geng 2003, van der Meer et al. 2005, Harper and Wolf 2009). In cannel catfish, experimentally induced hipoxia was responsable for histopathologically necrosis in a variety of organs such as liver (Scott and Rogers 1980). It could be argued that these lesions are caused as a specific reaction to acute localized oxygen deprivation rather than to stress per se, it is posible that stress has contributed to that response in some way. However, the inclusion of red beet and betaine on rainbow trout diets protect the liver to this fact throughout the own antioxidant and bioactive properties of both ingredients.

And when the posterior intestine was examined after the stress the incidence of appearance of cell infiltration, the vacuolation and mucus cells did not show significant differences regardless of the diet. It is well known that not only diet, but also, environmental conditions play an important role on gut histology and morphology (Lakani et al. 2013, Tran-Ngoc et al. 2017). In contrast to the results observed on the present study, Sundh et al. (2010) reported that when Atlantic salmon was submitted to hypoxia tended to shorten villi height, presented changes on the appearance of the intestinal segments and showed a widen subepithelial mucosa of the enterocyte in the distal intestine. The lack of significant differences on gut histology and morphology suggested that the presence of barley or red beet and betaine did not produce a negative effect on gut health. However, it should be studied the effect of these ingredients after more than 30 minutes after the stress to get constant information on the different histological parameters.

4. Flesh quality

The proximal composition of the whole fish was not significantly affected by the CHO source, which is in accordance with previous studies in sea bass (Enes et al.

2006), white sturgeon and hybrid tilapia (Lin et al. 1997) and for rainbow trout (Tekinay and Davies 2001). However, other authors have reported a significant effect of the CHO source on the whole body proximal composition (Tan et al. 2006; Wu et al. 2007).

Also rainbow trout fillets proximate composition values were similar to those reported by other authors (Yildiz 2004; Popelka et al. 2014) and not affected by the carbohydrate source.

In the red beet experiment, as it was expected, the inclusion of red beet and betaine in fish diets increased betaine concentration in fish flesh compared to the control group.

Alpha-tocopherol has an important antioxidant activity and is well absorbed by rainbow trout when it is included on their diets (Timm-Heinrich et al. 2013, Valente et al. 2015). This compound was determined in fillets of both experiments in order to observe its effect on oxidative and antioxidant process. Differences on α-tocopherol were observed regarding the carbohydrate source. While fish fed with red beet fish α-tocopherol content was not significantly affected by different experimental diets, fish fed with diets containing 8% barley showed significantly higher α-tocopherol content than control and fish from the rest of experimental diets. Probably due to a higher α-tocopherol in red beet than in barley. However, α-tocopherol in the raw materials was not determined.

The concentration of barley had not a significant effect on fillets total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, however, red beet and betaine concentrations significantly affect fillet fatty acid profile. Several authors (Ozden 2005, Volpe et al. 2015) have reported a similar fatty acid composition

in rainbow trout fillets. Although FA composition of individual fish of the same specie can be different due to several factors, FA profile reflects the FA composition of the diet (Turchini et al. 2009, Volpe et al. 2015). For this reason, fish fed with barley and fish fed with red beet and betaine showed slightly different fatty acid profile. It seems that high content in phenolic compounds can contribute to the decreased of SFA and MUFA and increased of PUFA as it was observed in the present studies (Welker et al. 2016). In broiler chickens, purified phenolic compounds included on the diet decreased SFA and MUFA and increased PUFA (Kamboh and Zhu 2013). This is similar to the results obtained in rainbow trout fillets in the present thesis.

Lower water activity values were observed on fish fed with diets containing barley or red beet and betaine compared to control group. The water activity plays an important role in spoilage of fish (Ježek and Buchtová 2014). The reduction of aw would help to reduce lipid oxidation.

Lipid oxidation of fish meat was measured through TBARS. Trout fed with diets higher in barley concentration had a lower level of TBARS in fillets than those obtained from trouts fed with lower barley concentrations diets. However, lipid oxidation, TBARS values did not show significant differences between fillets from fish fed with different red beet and betaine concentrations. Although no significant differences were observed, the inclusion of both ingredients seems to reduce TBARS values (dose-dependent effect). This decrease on the TBARS index was correlated with the lower water activity, which probably reduced microbial and enzymatic activity and probably with a positive effect of different compounds of barley, red beet or betaine, which would act as endogenous antioxidants.

Peroxide values (PV) and conjugated dienes and trienes hydroperoxides for fish

fillets were not significantly affected by the concentration of red beet and betaine but fish fed at higher barley concentration showed significantly lower trienes values than control. Barley has a positive effect on the control of lipid oxidation, since oxidative markers were lower in fillets came from fish fed with barley. This result is correlated with the lower TBAR values and probably with the higher antioxidant activity observed on fillets fed at higher barley concentrations.

On the other hand, antioxidant activity was determined on fillets of fish fed with the different experimental diets. Total phenolic content (TPC) was not significantly affected by the concentration of red beet and betaine on diet and fish fed with control diets showed significantly higher TPC values than fish fed with barley. Results showed that other antioxidant markers (DPPH, ORAC, TEAC and RACI) were not significantly affected by the inclusion of red beet and betaine on the diet and ORAC was the only marker where differences were observed by barley concentration. ORAC showed the opposite trend from TPC, fillets from fish at higher barley concentration had significantly higher antioxidant activity than control. These results were different from expected results. The lack of correlation between ORAC and TPC on fish fed with barley could respond to the presence of non-phenolics compounds and probably there are some compounds that could interact in the determinations and not allow to show the betaine, phenolic and flavonoid antioxidant activity.

5. Sensory analysis

Experimental diets did not have a significant effect on acceptability of fish flesh, but, unexpected, during QIM analysis significant differences were observed regardless the carbohydrate source. Barley concentration significantly affected gill colour. Gills became pale on fish fed with 4% barley levels but at higher barley concentrations

enhanced the redness, so fish fed with 8% barley or higher enhanced fish freshness by making gills appear redder. Panellists detected that fish fed with diets with 28% red beet and 1.63% betaine presented a more rancid odour than fish fed with the other diets. These results were correlated with a loss of freshness in these fish.

On the other hand, QDM was evaluated on fish flesh. Fish fed with barley showed a significant effect on meat colour. Fish colour was redder in those fish fed with diets at higher barley concentrations. Texture was also affected by diets; fish fed with 8% barley showed a higher hardness than those fish fed with diets higher in barley concentrations. However, fish fed with red beet and betaine did not show a significant effect. When fish muscle texture was determined instrumentally some differences were observed compared to the panellists observations. Fillet gumminess significantly decreased on fish fed at higher barley concentration. However, fish fed at higher betaine concentration showed significantly higher elasticity than fish fed with control diet and lower betaine levels. Hardness and cohesiveness were not significantly affected by the diet.

6. Economic analysis

Another reason to consider the inclusión of those novel carbohydrate sources in rainbow trout diets is to consider their economical profitability. The price of the different experimental diets were very similar when barley was used, however, the use of red beet and betaine on rainbow trout diets, increased considerably the price of the diets, specially the diets with higher betaine concentration.

The inclusión of barley did not affect the different economic indexes (ECR and EPI) (Table 29).

Table 28. Results of economic parameters at the end of the trial using barley as experimental ingredient (n = 4).

	DIETS					SEM	P-value
	CONTROL	4B	8B	16B	32B		
Diet price (€ kg^{-1})	0.72	0.72	0.72	0.73	0.73		
ECR (€ kg^{-1})[1]	0.85	0.81	0.81	0.81	0.84	0.017	0.3391
EPI (€ fish^{-1})[2]	0.37	0.34	0.37	0.36	0.36	0.038	0.8709
EPI$_{st}$ (€ fish $^{-1}$)[3]	0.43	0.40	0.44	0.42	0.42	0.044	0.8709

The absence of superscript letters means that there are not significant differences (P-values > 0.05).

SEM: Standard Error of the mean

[1] CONTROL (0% barley), 4B (4% barley), 8B (8% barley), 16B (16% barley) and 32B (32% barley) are the different experimental diets.

[2] ECR (Economic Conversion Ratio) = FCR (kg diet kg^{-1} fish) x price of diet (€ kg^{-1} diet)

[3] EPI (Economic Profit Index) = [weight increase (kg fish^{-1}) x selling price (€ kg^{-1})] – [ECR (€ kg^{-1}) x weight increase (kg)]

[4] EPI$_{st}$ (Standarized Economic Profit Index) = 100 x (weight increase (kg fish^{-1}) / number of days] x [selling price (€ kg^{-1}) – ECR (€ kg^{-1})]

On the other hand, when wheat was replaced by red beet, and betaine was added, economic parameters, ECR, EPI and EPIst were affected (Table 30). The Economic Coversion Ratio (ECR) was significantly lower in fish fed with the control diet tan fish fed with the rest of the experimental diets. The Economic Profit Index (EPI) and the Standarized Economic Profit Index (EPI$_{st}$) were significantly higher in fish fed with the control and A diets (EPI, 0.32 and 0.29 € fish^{-1} respectively; EPIst, 0.31 and 0.28 € fish^{-1} 100 days respectively) than in fish fed with diets higher in red beet and betaine concentrations. The lack of significan differences between the control diet and diet A it is related also with the absence of difference on growth parameters.

Table 29. Results of economic parameters at the end of the trial using red beet and betaine as experimental ingredients (n = 4).

	DIETS[1]					SEM	P-value
	CONTROL	A	B	C	D		
Diet price (€ kg^{-1})	0.72	0.77	0.83	0.77	0.83		
ECR (€ kg^{-1})[2]	0.84^{a}	0.92^{b}	1.10^{cd}	1.16^{d}	1.07^{c}	0.024	< 0.0001
EPI (€ fish^{-1})[3]	0.32^{c}	0.29^{c}	0.18^{a}	0.16^{a}	0.23^{b}	0.025	< 0.0001
EPI$_{st}$ (€ fish $^{-1}$)[4]	0.31^{c}	0.28^{c}	0.17^{a}	0.15^{a}	0.22^{b}	0.024	< 0.0001

Means with different superscript letters are significantly different (P-values < 0.05).

SEM: Standard Error of the mean

[1] CONTROL (0% red beet, 0% betaine), A (14% red beet, 0.9% betaine), B (14% red beet, 1.63% betaine), C (28% red beet, 0.9% betaine) and D (28% red beet, 1.63% betaine) are the different experimental diets.

[2] ECR (Economic Conversion Ratio) = FCR (kg diet kg^{-1} fish) x price of diet (€ kg^{-1} diet)

[3] EPI (Economic Profit Index) = [weight increase (kg fish^{-1}) x selling price (€ kg^{-1})] – [ECR (€ kg^{-1}) x weight increase (kg)]

[4] EPI$_{st}$ (Standarized Economic Profit Index) = 100 x (weight increase (kg fish^{-1}) / number of days] x [selling price (€ kg^{-1}) – ECR (€ kg^{-1})]

Replacement of wheat by barley appears to be economically feasible. The cost of formulating present diets for rainbow trout did not affect too much. However, the replacement of wheat for red beet and betaine worsen considerably economic parameters. It is also difficult to make a real comparison between costs as there is not the replacement of the same ingredients and there is a lapse of time between studies and these issues affect costs. The inclusion of alternative ingredients of fish meal reduced the cost of the diets (Moutinho et al. 2017, Martínez-Llorens et al. 2012, Sánchez-Lozano et al. 2007). This is due to the high price of the fish meal in the market, however, wheat is one of the cheapest ingredients. The aim of the economic analysis in this study was not to lower the price of feed, but that the price does not increase with the introduction of other carbohydrate sources, that in addition to their binder effect in extruded feed can provide other functional characteristics to the diet, as would be the case of barley and red beet. In the case of barley study no significant differences were

observed on the economical parameters. However, in the red beet study the economic parameters evaluated were better on fish fed with the control diet and with the lowest red beet and betaine concentration than at higher concentration of these ingredients. The ECR seems to be highly dependent on diet price, and so it can be considered EPI_{st} a more suitable index than ECR to evaluate economic profitability, as it considers production, feed costs, and selling price, results suggest that there is a greater economic return using 14% red beet and 1% beataine on rainbow trout diets.

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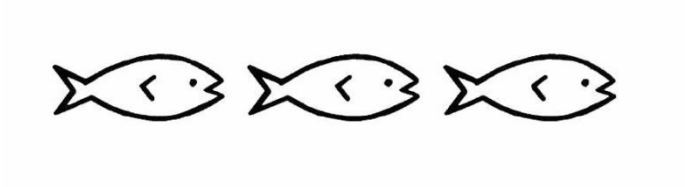
CHAPTER 13. CONCLUSIONS



The conclusions obtained from the different studies carried out on the current Doctoral Thesis have been:

1. Barley inclusion on rainbow trout diets did not produce any detrimental effect on growth parameters.
2. The inclusion of barley at concentrations of 8% and 32% controlled stress markers better than control diet, showing lower cortisol, glucose and lactate values in plasma and lower MDA levels in muscle.
3. Liver histological studies showed that intermediet inclusions of barley significantly decrease liver vacuolation.
4. The inclusion of 32% barley produced an improvement on fish fillets quality enhancing the textural properties and fresh colour reducing lipid oxidation of the fish fillets. And results were corroborated by sensory panel. This may associated with certain compounds present on barley, which could act as antioxidants.
5. Concentrations of 8% barley enhanced antioxidant properties, improving α-tocopherol content and antioxidant activity in fish fillets. However, concentrations higher than 8% produced a negative effect on fish fillets.
6. Red beet and betaine can be introduced on rainbow trout diets as carbohydrate source as long as the ingredients do not exceed 14% and 0.9%, respectively, higher concentrations showed evidence of adverse effects on growth parameters.
7. Red beet and betaine inclusion on rainbow trout diets did not enhance the tolerance to the acute stress challenge because no differences were observed compared to the control group.
8. Liver histological studies showed that the inclusion of red beet and betaine significantly decrease liver vacuolation and did not affect the histological parameters of the intestine.

9. The increment of red beet and betaine enhanced the quality of the product (texture and colour), but this inclusion did not reduce the lipid oxidation of fish compared to control or increase the antioxidant activity of final product.
10. The inclusion of red beet and betaine did not produce any effect under the point of view of the sensory panel

The results showed that the inclusion of barley at optimum concentration could be an alternative to conventional sources and enhance the quality of the final product. However, red beet and betaine seem not to be an alternative since could produce important negative effects on growth parameters although fish quality was not affected.