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Tesis doctoral

Caracterización e inclusión de subproductos de plataneras en piensos sostenibles para acuicultura

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Characterization and inclusion of banana by-products in sustainable feeds for aquaculture

PhD thesis

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La vida es una unión simbiótica y cooperativa que permite triunfar a los que se asocian

Lynn Margulis

A mi madre, Saro Bolaños

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List of Abbreviations

AB	Bacteriolytic Activity
ABS	Absorbance
ARA	Arachidonic Acid
BF	Banana flower
BFE	Banana flower extract
BFR	Banana flower residue
BHI	Brain heart infusion
BHT	Butylated hydroxytoluene
BP	Banana Pseudo-stem
BPS	Banana Pseudo-stem
CAT	Catalase
CFU	Colony formers unit
cm	Centimetres
Ct	Threshold Cycle
Da	Dalton
DAD	Diode-Array Detection
DBF	Dry Banana Flower
DBPS	Dry Banana Pseudo-stem
DEPC	Diethylpyrocarbonate
DHA	Docosahexaenoic Acid
DIFF	Difference
dw	Dry Weight
EC	European Commission
EDTA	Ethylene Diamine Tetra Acetate
EPA	Eicosapentaenoic Acid
EPP	Extractable Polyphenols
EU	European Union
FAA	Fatty Acids
FAMEs	Fatty Acids Methyl Ester
FAO	Food and Agriculture Organization
FCR	Food Conversion Ratio
FEFAC	European Feeds Manufacturers´ Federation
FI	Feed Intake
FW	Final Weight
HBSS	Hans´ Balanced Solution
HK	Headkidney
hpi	Hours Post Infection
HPLC	High Performance Liquid Chromatography
HPP	Hydrolysable Polyphenols
HSI	Hepatosomatic Index
HUFAS	High Unsaturated Fatty Acids
IFNs	Interferons
L	Liter
L/h	Liter per hour
LC-PUFAS	Long Chain Polyunsaturated Fatty Acids
MDA	Malonaldehyde
Min	Minutes
mL	Mililiter
mL/min	Mililiter per minute
mM	Milimolar
MOS	Mannanoligosacharydes
MS	Mass Spectrophotometry
MUFA	Monounsaturated Fatty Acid
NEPA	Non Extractable Proantocyannidins
NEPP	Non Extractable Polyphenols
nm	Nanometers
NSP	Non Starch Polysaccharides
°C	Celsius degrees
PBS	Phosphate-Buffered Saline
PER	Protein efficiency ratio

PUFAs	Polyunsaturated Fatty Acids
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
SDGs	Sustainable Development Goals
SFA	Saturated Fatty Acid
SW	Saltwater
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TLR3	Toll-like Receptor 3
Tn	Tons
U/mL	Units per mililiter
UN	United Nations
UV	Ultraviolet
v/v	Volume/volumen
VSI	Viscerosomatic Index
w/v	Weight/Volumen
µL	Microliter
µm	Micrometers
µM	MicroMolar

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Chapter 1. Introduction

1.1. Feed raw materials

European regulations on animal feed are extensive and constantly being renewed, as they have a considerable weight, representing 13% of global feed production in 2020, with the main European producers being Spain, Germany and France (FEFAC, 2021). Currently, the directive that regulates animal feed and raw materials defines each of these concepts: *compound feeds* are those products intended for animal feed made up of two or more raw materials with or without the presence of additives, while *raw materials* are defined as products of plant or animal origin that meet the nutritional needs of animals and products derived from their industrial processing (EC Nº 767/2009). Raw materials are themselves listed in EC regulation 2017/1017, which is constantly being updated. The concept of *by-product* is more recently introduced into the legislation and is more up-to-date, being a substance or object resulting from a production process whose primary purpose is not the production of that substance or object, complying with the following: that it is to be used subsequently, that it can be used directly without having to undergo further processing, that the substance is produced as part of a production process and that it is not harmful to human health or the environment (7/2022, BOE 2022). Likewise, it can be considered as a *secondary by-product* the residue from the processing of the *primary by-product* in another industry different from the original one, as there is no clear legal definition established.

These raw materials are subject to the global market, which includes those destined for animal feed, human food and other uses. In this respect, the evolution of the different raw materials used in the production of animal feed over the last 30 years indicates an increase in the use of cereals, with soya, maize and wheat leading the way, following the 2001 crisis, and a reduction in the use of animal meal (**Figure 1.1**). Concerning food safety, the use of animal by-products in livestock feed has been restricted since 2001 due to bovine spongiform disease, and although the use of plant by-products has no evidence of being able to cause disease and threaten food safety, there may be risks of cross-contamination with pathological agents (Salami *et al*., 2019).

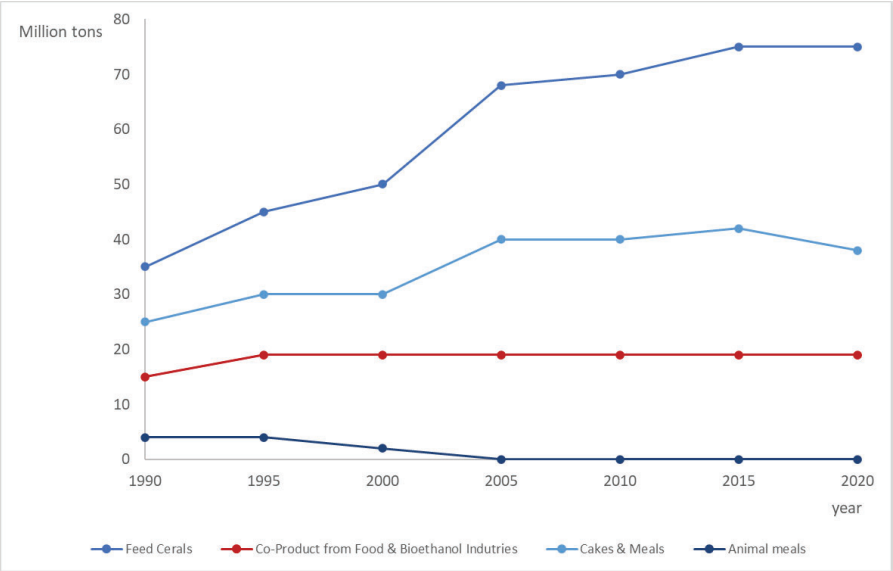

Figure 1.1. Development of raw materials consumption by the EU compound feed industry (adapted from FEFAC, 2021).

Interestingly, what remains stable over this period is the use of by-products from the food industry and biorefineries intended for animal feeds, although enormous economical and research efforts it is been behind, as seen in summary in **Table 1.1**. The pre-treatment of plant ingredients is of utmost importance for the bioaccessibility of nutrients and the elimination of contaminating factors (Drew *et al*., 2007). One of the utilities of by-products from crops has been used for animal feed directly (Federici *et al*., 2009; Guil-Guerrero *et al.,* 2016; Wang *et al.,* 2016; Sivilai & Preston, 2017), as fodder. That is the case of banana production discharges, for which this traditional use has been naturally occurring throughout the world and in the Canary Islands (Padam *et al*., 2014). However, since the early 2000s, research reports have been pointing out the potential of plant materials and fruit by-products as functional ingredients for the human and animal food industry (Schieber *et al*., 2001). This has led to the search for new uses for by-products from other industries, be it food, alcoholic beverages or bioethanol production (**Table 1.1**), especially in the last twenty years. For the subsequent use of these by-products, preservation and pre-treatment is essential to maintain their properties and prevent the proliferation of microorganisms (Al Khawli *et al*., 2019).

Table 1.1. Origin and feed uses of diverse residues from different industries.

Residue	Origin	Feed Uses	Reference
Lignocellulosic residues	Agro-industry	Rumiant and non rumiant	Vilas-Boas *et al*., 2002
Shrimp heads	Shrimp culture and fishing	Domestic animals	Coward-Kelly *et al*., 2006
Grape pomace	Wine industry	Animals	

Yeast lees	Wine industry	Fish	Nerantzis & Tataridis, 2006
Bioaqueous stream	Palm oil industry	Animals	Tan *et al*., 2007
Sesame coat	Sesame processing	Animals	Elleuch *et al*., 2007
Distillers grains plus solubles	Ethanol industry	Cattles	Klopfenstein *et al*., 2008
Olive leaves Olive cake	Oil production	Rumiant	Molina-Alcaide & Yáñez-Ruiz, 2008
Coffee pulp Coffee husks Coffee sliver skin spent coffee	Coffee industry	20% for cattles, 5% for chickens, 16% for pigs and 3% for birds	Murthy & Naidu, 2012
Cassava peel Cassava root Cassava leaves Sweet potato peel Sweet potato leaves	Food industry	Poultry Pigs Fish	Apata & Babalola, 2012
Chicken by-products	Poultry industry	Livestock, Pet, Aquafeeds	Lasekan *et al.,* 2012
Potato peels	Food industry	Farm animals	Mohdaly *et al*., 2013
Sugar beet pulp	Sugar industry	Cattle	
Sesame cake	Oil production	Animals	
wheat bran wheat middlings	Wheat flour	Pigs	Rosenfeldr *et al.,* 2013
Distillers grains	Bioethanol	Livestock	
Citrus meal Citrus fines	Citrics juices	Single cell protein, Dietary fiber	Mamma & Chirstakopoulos, 2014
Pseudostem Leaves Inflorescence Fruit stalk Rhizome Peels	Banana production	Sheeps, umiants, rabbits	Padam *et al.,* 2014
Seafood protein hydrolisated	Seafood industry	Pigs	Martínez-Álvarez *et al*., 2015
Poultry by-products	Poultry industry		
Grape seed	Wine industry	Poultry	Brenes *et al*., 2016
Grape pomace		Pigs, rabbits	
Plants by-products	Food industry	livestock	Guil-Guerrero *et al*., 2016

Mango peel	Fruit processing	Broiler chicken	Okino Delgado & Fleuri, 2016
Bones hids and skin faneras Ruminant digesta	Meat industry	Animals Cattles, poultry, lamb and catfish	Alao *et al*., 2017
tomato seeds grape extracts mango residues	Fruit processing	Poultry Dairy cows cattle	Kowalska *et al*., 2017
Aquaculture by-products: Frames Skin	Salmon production	Pets	Stevens *et al*., 2018
Camelina residues	Camelina oil extraction	Dairy cows, sheeps, goats	Halmemies-Beauchet-Filleau *et al.,* 2018
Plants by-products	Brewery, biofuel, sugar production, fruit and vegetable production, oil production, herbal and tree processing	Rumiant	Salami *et al*., 2019
Fish heads Seals Skin Shills Viscera	Fishing industry	Animals	Al Khawli *et al*., 2019

Most of the primary products need to be further processed into secondary products that can be later used in other industries (Alao *et al*., 2017; Al Khawli *et al*., 2019). Several authors describe the use in animal feed of by-products from primary products, such as blood and viscera that become hydrolysed protein concentrate after fish oil extraction (Stevens *et al*., 2018), olive cake ash from olive cake (Molina-Alcaide & Yáñez-Ruiz, 2008), grape seed extract after grape pomace extraction (Brenes *et al*., 2016), or fine solids from the bioaqueous stream of palm oil extraction (Tan *et al*., 2007).

1.2. Raw materials, primary and secondary by-products in aquafeeds

Aquaculture is a fundamental part of the blue transformation promoted by the FAO (FAO, 2022a), whose objective is the maximum contribution from sustainable aquaculture production towards food security and the SDGs related to the eradication of hunger and poverty in the 2030

Agenda. To achieve this, among other actions, it is necessary to continue with the lines of research open to make the aquaculture industry a more sustainable industry, in which aquafeed materials has a direct impact.

The contribution of aquaculture to the world of feed is very small, so much so that it is not even differentiated in the statistics of European feed producers (FEFAC, 2021). Despite equalling the contribution of extractive fisheries in 2020 (FAO, 2022b), the demand for fishmeal and fish oil has not increased in animal feed due to the increased use of common vegetable raw materials, such as soya, maize, fish oil and wheat that compete by the way in other markets (Rana *et al*., 2009). This is forcing the industry to innovate within new raw materials that do not compete with other industries, such as from livestock and bioethanol production and discharge materials from the human food processing like packed vegetables or fruits juice. When considering protein ingredients specifically, and alternatives to fishmeal and its counterpart soy concentrate in aquaculture feeds is protein hydrolysates, mainly from aquaculture by-products such as viscera, skin and remains of fish that are processed for the market (Martínez-Álvarez *et al*., 2015; Stevens *et al*., 2018; Hinchcliffe *et al*., 2019). Other alternative protein sources that have been tested in recent years are the generation of fungal biomass from biorefinery waste (Karimi *et al*., 2018), and polychaetes grown from macroalgae agar extraction residues (Stabili *et al*., 2019). As evidenced in **Table 1.2,** there are still few studies of secondary by-products for aquaculture feed, although it is increasingly on the rise.

The trend towards including land-based ingredients in aquaculture feeds may reduce pressure on marine resources, but may increase competition for land, causing social and environmental conflicts (Malcorps *et al.,* 2019), as well as being at the mercy of the consequences of climate change, which affects terrestrial crops to a greater extent (Rana *et al.,* 2009). Thus, the land-based trend should better insight and transfer knowledge regarded by-products, primary but also secondary origin.

Tilapia and sea bass: target species for novel feed by-products

Tilapia and sea bass represent two target species for working with these primary and secondary by-products. Both species are of high commercial importance, with tilapia being the second most produced freshwater species in the world (Bartley, 2022) along with carp, with a 2018 production of 4.525 million tonnes (FAO, 2020a). Sea bass, on the other hand, is the second most produced marine species in Europe, after salmon (FAO, 2020a), been Spain is the second largest producing country after Greece (APROMAR, 2021) with a production in 2020 of 21,709 tonnes.

On one side, Tilapia (**Figure 1.2**) is a well-known freshwater omnivorous species, although it can also be farmed in brackish water and able to survive even in salt water (El-Leithy *et al*., 2019). Due to its biology, it is a species that can exploit different plant quality resources for food. Thus, only in recent years, there are several studies of fish oil substitution by different vegetable oils such as palm oil (Peng *et al*., 2016; Larbi Ayisi *et al*., 2018; Nakharuthai *et al*., 2020), soybean oil (Nassef *et al*., 2019; Nakharuthai *et al*., 2020), linseed oil (Li *et al*., 2016; Peng *et al*., 2016; El Asely *et al.,* 2020; Nakharuthai *et al*., 2020), chia oil (Montanhes *et al.,* 2015), corn oil (El Asely *et al.,* 2020), sunflower oil (El Asely *et al.,* 2020), rapeseed (Peng *et al*., 2016) oil and microalgae oil (Sarker *et al*., 2016), although corn oil seems the best demonstrated substitutes, and above all, linseed oil, which even improves growth by weight when fully replacing fish oil (El Asely *et al*., 2020). This is due to the ability to synthesise essential fatty acids such as EPA and DHA from 18-carbon fatty acids by stimulation of elongases and desaturases enzymes (Teoh *et al.,* 2011).

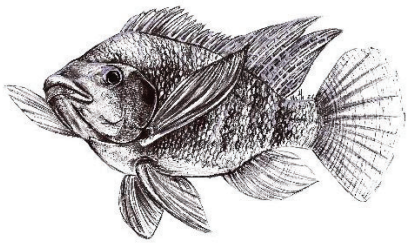

Figure 1.2. Nile tilapia (*Oreochromis niloticus*). *Source: ©Nathalia Hernández.*

In the case of protein sources, there have been many studies for more than twenty years exploring ways of substituting fishmeal in tilapia feed (El Sayed, 1998; El Sayed, 1999). Nowadays Tilapia is a species consider that does not rely on fishmeal for amino acids requirements. The main alternative to fish protein is soybean, due to its optimal essential amino acid profile (O'Keefe, 2003), with soybean concentrate able to substitute 100% fish meal in tilapia diets (Zhao *et al.,* 2010). But soy has a major disadvantage, which is that it is used by the livestock market and in human food, making it the second most expensive ingredient in aquaculture feeds, behind only fishmeal (Brown *et al*., 2008). Because of this, there is a need to replace soya in turn with other more affordable ingredients. In this regard, there are numerous studies reported in the last decade by using different raw materials and novel by-products to replace fishmeal and soy protein, such as soybean by-products (Vidal *et al.,* 2017), fermented bean meal (Valdez-González *et al*., 2017), insect meal (Sánchez-Muros *et al*., 2016; Agbohessou *et al*, 2020), cashew nut meal (Pradhan *et al*., 2020), microalgae blend (Sarker *et al.,* 2020), cassava roots

Table 1.2. Studies carried out during the last years in aquaculture with the use of primary by-products and secondary by-products.

Cultivated Sp	Primary By-product	Origin-Processing	Secondary By-product	Effects	Reference
Salmo salar	Meat Meal Blood Meal Feather Meal	Meat industry		Used for decades	Martínez-Álvarez *et al.*, 2015
Acipenser baerii	Olive pomace	Olive oil industry		↑DHA in muscle	Banavreh *et al.*, 2018
General Aquafeed	Salmon heads Salmon frames Salmon skins Salmon viscera	Salmoniculture Oil extraction Hydrolyzed protein	Silages	↑PUFAs ↑ Minerals	Stevens *et al.*, 2018
General Aquafeed	Palm oil waste Stillage Wheat bran Lignocellulose	Industrial waste Biorefinery	Fungal biomass	High proteins	Karimi *et al.*, 2018
Dicentrarxus labrax	Macroalgae	Bioremediation process	Polychaetes	No negative effects ↑ Palatability	Stabili *et al.*, 2019
General Aquafeed	Head Frames Viscera	Herring fishing Combinations pH-shift	Proteins concentrates	Residual LC-PUFAs and Minerals Gelation capacity remains	Hinchcliffe *et al.*, 2019
Sparus aurata	Agar waste Ethanolic extraction waste	Gracilaria production Agar extraction Ethanol extraction		Improve stress response oxidative stress resistance	Silva-Brito *et al.*, 2020
Oncorhynchus mykiss *Sparus aurata*	Spent yeast Spent grain	Beer industry		Similar growth	Nazzaro *et al.*, 2021
Sparus aurata	Spent yeast	Beer industry		Better growth	Estévez *et al.*, 2021
Liza aurata	Aloe vera residue	Aloe vera processing		Enhance Fatty Acid metabolism	Quirós-Pozo *et al.*, 2021
Oreochromis niloticus	Banana peel	Banana processing		Lower lipids digestibility	Yossa *et al.*, 2021
Oncorhynchus mykiss	Poultry By-Products			No negative effects	Palomba *et al.*, 2022

(Obasa *et al*., 2021), macroalgae (Eissa *et al.,* 2021) and Sacha inchi by-product (Khieokhajoukhet *et al*., 2022).

On the other hand, sea bass (**Figure 1.3**) is a mainly carnivorous saltwater species. Since the beginning of the 21st century, studies on fish meal and fish oil substitution in sea bass feed have been carried out, mainly using rapeseed oil, linseed oil, soybean oil and palm oil (Montero *et al*., 2005; Mourente *et al*., 2005; Mourente & Bell, 2006; Richard *et al*., 2006; Castro *et al.*, 2016), but olive oil and canola oil have also been used with good results (Mourente *et al.,* 2005; Wassef *et al*., 2016). These studies have established that 60% to 70% of fish oil can be replaced with vegetable oils, although this may affect the fatty acid profile of sea bass, as C18 fatty acid levels tends to increase while n-3 HUFA decreases (Montero *et al*., 2005; Mourente *et al*., 2005; Machado *et al*., 2019). This is due to the low enzymatic activity of elongase and desaturase enzymes (Mourente & Dick, 2002), in the same way that, diets without EPA and DHA stimulate the expression of the fads2 gene but do not increase enzymatic activity, which decreases the ability to convert PUFA to HUFA (Geay *et al.,* 2010).

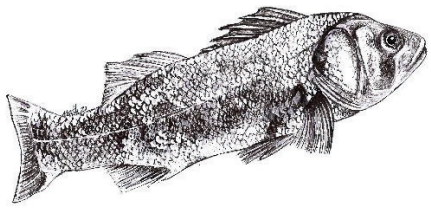

Figure 1.3. European seabass (*Dicentrarchus labrax). Source: ©Nathalia Hernández.*

For the replacement of fishmeal as a protein source in the seabass diet, soybean and soybean concentrate are used, but many other vegetable flours such as wheat gluten, extruded pea meal, wheat sprout, and meals from insect or yeast are also well assayed (Bonaldo *et al.,* 2008; Messina *et al*., 2013; Rimoldi *et al*., 2015; Torrecillas *et al*., 2018; Pérez-Pascual *et al.,* 2019; Reis *et al*., 2019). With soybean meal and wheat gluten, substitutions levels between 70% and 100% have been achieved (Messina *et al*., 2013; Rimoldi *et al.,* 2015; Pérez-Pascual *et al*., 2020), although in the case of total substitution with soybean meal, the addition of 0.4% MOS as an additive has been shown to improve the generalized negative effects produced by soy on the intestine and immune system of fish (Torrecillas *et al*., 2018). From all the above data, sea bass is a carnivorous marine fish species that tolerate very high inclusion levels of vegetable raw

materials in the diet, making it an ideal species to continue exploring new raw materials derived from novel sources of plant by-products.

1.3 Banana cultivation: Applying the Circular Economy

The banana tree (*Musa acuminata var Cavendish*) is an herbaceous tree-like plant, in which the pseudo-stem is made up of layers of new leaves that remain rolled up and 95% of its composition is water.

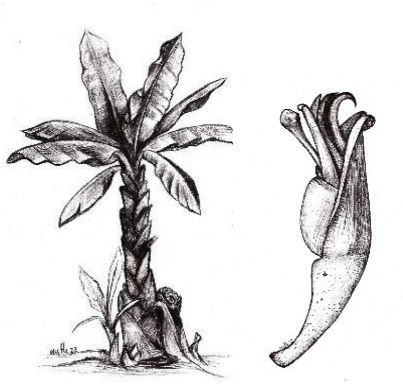

Figure 1.4. Banana by-products: pseudo-stem and flower. *Source: ©Nathalia Hernández.*

The mature pseudo-stem is very robust and can support the weight of bunches of more than 50kg (Robinson *et al*., 2012). The inflorescence is composed of a peduncle with female flowers, which produce the fruit, hermaphrodite flowers and male flowers, which never emerge from the bracts of the inflorescence (**Figure 1.4**). Farmers cut the stalk at the base of the hermaphrodite flowers and remove it, avoiding stalk growth and possible breakage of the pseudo-stem by the weight of the fruit cluster (Robinson *et al*., 2012; Lau *et al.,* 2020).

Banana is the most produced fruit in the world and reached 116 million tonnes in 2019 (FAO, 2019). In Europe it is the most consumed fruit, being the region with the highest banana imports in the world. European banana production is concentrated in the tropical and subtropical regions of the French West Indies, the Canary Islands and Madeira (FAO, 2021a). In the Canary Islands, banana production in 2021 was 411,732 Tn (ASPROCAN, 2022), making it the largest European producer.

In banana plantations, a large amount of waste is generated by the way the fruit is grown and harvested: part of the stalk and flowers are removed to allow the female flowers to develop and ripen into fruit, and, once the banana bunch is harvested, the pseudo-stem is cut down, leaving the roots for the daughter plant to develop (Lau *et al*., 2020). These residues represent 80% of

the total biomass (Padam *et al*., 2014), the management of these residues can generate not only an environmental problem, related to soil toxicity and high carbon footprint (Adsal *et al*., 2020), but can also be a food security problem (Campos *et al*., 2020). The management of these wastes is essential, and they have a large number of potential uses as by-products for industries such as biorefinery, textile industry, plastic replacement in packaging, automotive industry and reinforcement for construction materials (Ortega *et al*., 2016; Campos *et al*., 2020; Pryadarshana *et al.,* 2020; Rodríguez *et al*., 2020).

Traditionally, banana pseudo-stem remains have been directly used for livestock feed (Wang *et al*., 2016), and also, together with the flower and rhizome, are used by populations around the world in traditional medicine for the treatment of gastric diseases, diabetes, bronchitis, ulcers and menstrual cramps (Lau *et al.,* 2020). Other uses associated with banana crop residues include the use of discards in diets for lambs (Menezes *et al*., 2018), and the use of the flower and pseudo-stem as dietary supplements for diabetic patients (Bhaskar *et al*., 2011).

They also contain a wide variety of bioactives (Lau *et al*., 2020), although there are not many characterisation studies of pseudo-stem and banana flowers. The only study we found so far with the characterisation of these two by-products together is Bahskar *et al*. (2012), which uses the spice *Musa sp. Elakki bale* and concludes that they are a good source of dietary fibre with associated polyphenols. Other studies on banana flowers corroborate the amount of polyphenols present (Schmidt *et al*., 2015), as well as the important presence of minerals such as magnesium, iron and copper, in addition to well-balanced essential amino acids (Sheng *et al.,* 2010).

Based on the above, both pseudo-stem and banana flowers are two wastes that can be used as by-products, primary or secondary in the case, and assimilated into the dynamics of the Circular Economy within the framework of the SDGs (FAO, 2018), with the aim of obtaining new value-added products and reducing the ecological footprint of crops.

1.4 Polyphenols: Bioactives in plant residues

Polyphenols are dietary antioxidants present mainly in vegetables, which have one or more phenolic groups in their structure (Pérez-Jiménez *et al.,* 2013). These bioactives are present in agricultural residues, along with other compounds such as sterols and triterpenes (Singh *et al*., 2016; Lau *et al.,* 2020), and show biological activity of different types such as radical scavenging, modulation of microbiota, regulation of glucose homeostasis, related to obesity and type II

diabetes, lipid metabolism and as a natural preservative in food (Cao *et al*., 2019; Fraga *et al*., 2019; Lau *et al*., 2020; Ma & Chen, 2020; Hamed & Abdel-Tawwab, 2021).

The different polyphenols are classified according to their structures and thus to the classes and subclasses established in the Phenol-Explorer tool (Neveu *et al*., 2010), as it is shown in **Table 1.3**. The wide variety of structures of phenolic compounds means that new structures are still being identified (Nguyen *et al.,* 2019).

Table 1.3. Polyphenol classification according to their molecular structure.

Polyphenol Class	Flavonoids	Lignans	Phenolic acids	Stilbenes	Other polyphenols
Polyphenol Sub-Class	Anthocyanins Chalcones Dihydrochalcones Flavanols Flavanones Flavones Flavonols Isoflavonoids	Lignans	Hydroxybenzoic acids Hydroxycinnamic acids Hydroxyphenylacetic acids Hydroxyphenylpropanoic acids Hydroxyphenylpentanoic acids	Stilbenes	Alkylmethoxyphenols Alkylphenols Curcuminoids Furanocoumarins Hydroxybenzaldehydes Hydroxybenzoketones Hydroxycinnamaldehydes Hydroxycoumarins Hydroxyphenylpropenes Methoxyphenols Naphtoquinones Phenolic terpenes Tyrosols

Apart form that, and according to the extraction method, they can be classified into extractable polyphenols (EPP), which are those that can be obtained with aqueous and organic solvents, and non-extractable polyphenols (NEPP). The latter include hydrolysable polyphenols (HPP), low molecular weight phenolic compounds associated with polysaccharides and proteins, and non-extractable proanthocyanidins (NEPA), which are high molecular weight structures. NEPPs are metabolised in the gut by the gut microbiota and release secondary metabolites to perform different functions (Pérez-Jiménez & Saura-Calixto, 2015). Traditionally, the most studied and most interesting polyphenols have been EPPs, although NEPPs are gaining relevance in more recent studies (Pérez-Jiménez *et al*., 2013).

Phenolic compounds previously identified in bananas, skin, pseudostem, flowers and rhizomes are phenolic acids, flavonol, epicatechins, gallic acid and protocatechuic acid (Bhaskar *et al*., 2012; Kandasamy & Aradhya, 2014; Tsamo *et al*., 2015; Pico *et al*., 2019). Health-related effects caused by these polyphenols have been described: protocatechuic acid improves the response to some types of cancer, type I and II diabetes, regulates hyperglycaemia, and has a

neuroprotective effect against pollutants (Talagavadi *et al*., 2016; Erukainure *et al*., 2017; D'Archivio *et al*., 2018; Adedara *et al.,* 2019; Al Olayan *et al*., 2020; Yuliana *et al.,* 2020). Nonetheless only a few studies of these specific banana bioactive polyphenols have been reported in fish, no in the target species commented before.

1.5. Objectives

The main objective of this study is the valorisation of banana by-products as alternative and sustainable ingredients in aquaculture feeds.

Specific objectives:

1. Biochemical and functional characterisation of banana by-products Pseudo-stem and flower.

2. Effects on fish growth, histomorphology and oxidative status by the inclusion of banana pseudo-stem with a pre-treatment in the diet for juvenile tilapia.

3. Effects on growth, histomorphology and oxidative status by the inclusion of banana pseudo-stem without pre-treatment in a low fish meal and fish oil diet for sea bass.

4. Effects on growth, histomorphology, oxidative status and stress resistance by the inclusion of banana flower meal in the diet for juvenile tilapia.

5. Effect on growth, histomorphology, oxidative status and ex-vivo immune parameters by the inclusion of banana flower meal at the low fish meal and fish oil diet for juvenile sea bass.

6. Characterization of the organic extract of banana flower and the generated residue to assayed which of these banana flower containing bioactives fraction may higher affect on oxidative status, stress resistance and immune parameters in both species.

Chapter 2. Methodology

2.1. Feedstocks collection: by-products from local producers

Banana by-products were supplied by local Canarian producers of banana cultivars in the framework of the UE project LIFEBAQUA (code: LIFE15 ENV/ES/000157).

The banana pseudo-stem was processed at the University of Las Palmas de Gran Canaria, Fabricación Integrada y Avanzada Research Group facilities, to mechanically separate the external long fibre (patent: WO2014/174115) from the residue that remained in the machine, which represent up to 76% of the dry pulp. This high quantity of secondary by-product was considered pseudo-stem fibre to be studied as a novel dietary fish ingredient.

The banana flowers were freeze-dried (Lyobeta, Spain) previous to finally ground in a mill (Ultra Centrifugal Mill ZM200, Retsch, Germany) down to 125-250 microns.

Tilapia were obtained by natural reproduction in recirculated systems at the aquaculture facilities of the Ecoaqua Institute belong to the University of Las Palmas de Gran Canaria, while all seabass were supplied by a local producer (Aquanaria S.L.).

2.2. Experimental design

In the first phase, the characterisation of the by-products was carried out, performing proximate biochemical analysis, fatty acid composition and characterisation of the polyphenols present.
In the second phase of product valorisation, a methodology consisting of three experiments was designed, the first two feeding experiments with the inclusion of different levels of banana pseudo-stem and banana flower, where growth was evaluated, the biochemical and fatty acid composition of liver and muscle was determined, histopathological analysis of liver and intestine was carried out and, in the case of the flower, stress parameters were analysed. The third and final experiment was carried out with the organic extract from the flower and the residue of this extract, the proximal and fatty acid composition of liver, liver and gut morphology, and certain parameters related to stress and the immune system of the fish were determined. This methodology was applied to two different species, one freshwater (*Oreochromis niloticus*) and one saltwater (*Dicentrarchus labrax*) (**Figure 2.1**).

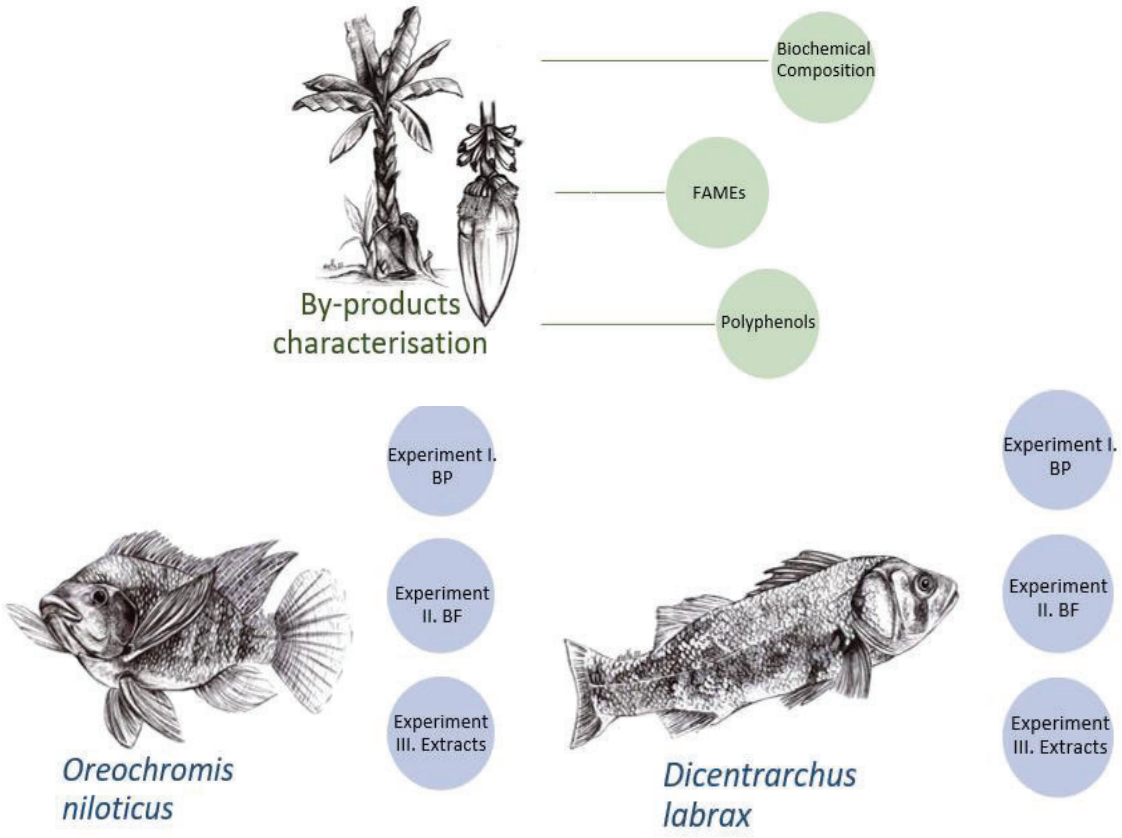

Figure 2.1. Experimental design

All the experiments followed the standard bioethics protocol by the Bioethics Committee of the University of Las Palmas de Gran Canaria (Real Decreto 53/2013).

2.3. Production of experimental diets

All diets were produced in the Pilot Product and Processing Plant facilities at Ecoaqua-ULPGC Science and Technology Park in Taliarte, Telde, Spain. All ingredients for the differetn diets were weighed, mixed in the mixer (MA 60, Ecych, Spain), pelletised (CL3, CPM, Spain) to the correspondent grain size accoding to the fish under the trials. Pellets were then air dried in a chamber at 38°C for 16h and storage at 11 °C until use.

2.4. Sampling

Pseudo-stem and flowers were supplied by local producers of banana cultivars (*Musa accuminata cavendish*), in two different times in 2017 and 2018 for both materials. All the

analysis were performed in triplicates and results are given in dry weight (dw) basis and are expressed as mean ± standard deviation.

Before starting the fish experiments, fish were anaesthetised with clove essential oil (1mL/100L) and individually weighed. To measure growth performance during the experiments, intermediate weight and length sampling was carried out, in tilapia every 15 days and in sea bass every 30 days, and the weight and total and furcal length of each individual were recorded. At the end of each experiment, fish were anaesthetised, weighed and measured. Fish for sampling were sacrificed with an overdose of clove oil, except for fish for blood sampling, which was sacrificed by a blow to the head. At the end of each experiment, samples from 3 fish per tank were taken for the different analysis: liver for biochemical composition, FAMEs, oxidation, histopathology and enzymatic; muscle for biochemical composition, oxidation and FAMEs; intestine for histopathology; headkidney for cell culture and immune parameters; and blood for serum and plasma for stress parameters.

2.5. Growth parameters

FW: Final weight (g)

FI: Feed intake (g) per fish for the experimental-day period

FCR: Feed conversion ratio = Feed intake (g) / Weight increase (g)

PER: Protein efficiency rate= weight gain / protein intake (g)

SGR: Specific growth rate = ((Ln Final weight – Ln Initial weight)/ nº days) x 100

K: Condition Factor= (Final Weight (g) / Total Length $(cm)^3$) x 100

PI: Protein intake (g)

VSI: Viscerosomatic index = (Weight of whole fish (g) − Weight of fish without viscera (g)) x 100

HIS: Hepatosomatic index = 100 × wet liver weight /body weight.

2.6. Biochemical Analysis

Moisture

Weigh about 0.5 g of sample on a crystal beaker (A), note the weight and place it in an oven at 100°C. After 24 hours, remove the crystal beaker from the oven, allow it to cool in a desiccator and weigh it (B). It is put back in the oven for one hour and the procedure is repeated until the final weight is constant (AOAC, 2000). The following formula is used to obtain the percentage of moisture:

100 x (B-A)/(A-weight of crystal beaker)

Lipids

This protocol was established by Folch *et al*. (1957). Weigh 0.5g of the sample in a glass tube, and add 5mL chloroform:methanol (2:1) with 0.01% BHT. Homogenise for 5 minutes on ice to avoid degradation, wash with 5mL chloroform:methanol (2:1) with 0.01% BHT, add a further 2mL of 0.88% KCl and centrifuge for 5 minutes at 2000 rpm. The upper phase is discarded and the lower phase is transferred to a tube, previously weighed, and passed through a filter with anhydrous sodium sulphate and soaked with chloroform. Finally, it is evaporated with N_2 until completely dry, weighed and the amount of wet lipids is obtained. The formula applied is:

% lipids= (gr lipids/gr sample) x100.

FAMEs

The method is based on the transesterification of fatty acids (Christie, 1982) and the identification and quantification by gas chromatography described by Izquierdo *et al.* (1989).

In detail, 1mL of toluene with BHT (50mg/L) is added to a maximum of 80mg of previously extracted lipids. Add 2 mL of methanol:sulphuric acid at 1% by volume. Shake, fill the tube with N_2 and seal and incubate for 16h at 50°C on a heating mantle in the dark. After this time, allow to cool, add 3.5mL ultrapure water and 4mL Hexane:diethyl ether 1:1 with 0.01% BHT, shake the tube and centrifuge at 2000 rpm for 5 minutes. Transfer the upper phase to a second tube, add 4 mL of Hexane:diethyl ether 1:1 without BHT to the original tube, shake and centrifuge at 2000rpm for 5 min. Pool the two upper phases obtained and add 3 mL of 2% $KHCO_3$, shake and centrifuge for 5 min at 2000rpm. Transfer the upper phase to a third pre-weighed tube and evaporate with N_2. Dissolve the FAMEs with 1-2ml of HPLC grade hexane, pass through the NH_2 sep-pack, evaporate to dryness and diluted with hexane to a concentration of 40mg/mL. It is passed through gas chromatography to obtain the fatty acid profile.

Proteins

Protein determination is carried out by the Kjeldahl method (AOAC, 2000). This method is used for the determination of crude protein and is based on the digestion of the sample with sulphuric acid and a catalyst to convert all the N_2 present in the sample into $(NH_4)_2SO_4$, the NH_3 is released

by adding NaOH in excess and distilled in 1% boric acid, finally, it is titrated with HCl to determine the NH_3 released.

First, 0.4 g of sample is weighed into a watch glass and placed together with a catalyst tablet and 10 mL H_2SO_4 in a digestion tube. The tube is placed in the digester for 1h at 400°C. Once the sample is digested, it is left to cool and the amount of proteins is evaluated with the Kjeldahl destilator, which carries out the distillation with distilled water and NaOH in excess. After this distillation, titrate with 0.1N HCl until the colour changes. The formula is applied:

% protein= ((mL HCl sample - mL HCl blank) x 0.1x 14.007 x 6.25 x 100)/Sample weight in mg

Ash

About 0.5g of sample is weighed into a crucible and placed in a muffle furnace (CARBOLITE Gero, Madrid, Spain) at 450°C overnight, removed, allowed to dry in the desiccator and weighed on a precision balance.

The formula ash (%)= Aw/Fw x 100 is applied.

Aw: weight of ash

Fw: dry weight of the sample

TBARs

The thiobarbituric acid reaction protocol is based on Burk *et al*. (1980).

Dilute total lipids with chloroform:methanol 2:1 with BHT to a concentration of 10mg/mL. Take 200uL of this dilution and add 50uL of 0.2% BHT in absolute ethanol, 0.5mL of 0.288 % TBA and 0.5mL of 10% TCA. A blank without a sample is also prepared. Incubate at 100°C for 20 minutes in the dark. After the time has elapsed, the reaction is stopped by placing the tubes on ice for 5 minutes. Centrifuge at 2000G for 5 min and measure in the spectrophotometer at 532nm. The formula applied is:

Nmol malonaldehyde (MDA)/g lipid= (abs λ532nm/0.156) x (1/g lipids)

2.7. Polyphenols analysis

Polyphenols determination

For the determination of polyphenols, three protocols were used, one for each type of polyphenol. These techniques were acquired during my stay at ICTAN-CSIC in Madrid, with Dr Jara Pérez Jiménez.

The extraction technique for extractable polyphenols (EPP) is based on the work described by Pérez-Jiménez et al. (2008). First, 0.5 g of sample was extracted by shaking at room temperature with 20 mL of methanol/water (50:50 v/v, pH 2) and then with 20 mL of acetone/water (70:30, v/v). The two supernatants were combined, which corresponded to the extractable phenolic compounds fraction. For the extraction of the hydrolysable polyphenols (HPP), the methods followed were established by Arranz *et al*. (2009) and Hartzfeld *et al*. (2002). The residue from the extractable phenolic compounds extraction was treated with 20 mL of methanol and 2 mL of sulfuric acid (12M) at 85 °C for 20 h. After washing with distilled water, an aliquot of the extract was adjusted to pH 5.5.
In addition, to determine the nonextractable proanthocyanidins (NEPA), the residue from the extractable phenolic compounds extraction was treated with butanol/HCl (97.5:2.5, v/v) with 0.1% $FeCl_3$ at 100 °C for 1h, following the protocol established by Pérez-Jiménez *et al*. (2009).

The total content of phenolic compounds in the fractions was determined by spectrophotometry performing the Folin-Ciocalteau assay (Singleton *et al*., 1998) to EPP and HPP. The results were expressed as g of gallic acid equivalents/100 g dw. NEPA content was determined by measuring the sum of absorbance at 450 and 555 nm (Zurita *et al*., 2012) and the results were expressed as mg NEPA/100g dw, using a standard curve from carob pod concentrate. The total percentage of non-extractable polyphenols was determined by the formula (HP+NEPA)/total polyphenols x100. All these measurements were carried out in a 96-well plate reader (Synergy MX, Bio Tek, Winooski, Vermont, USA).

Identification by Mass spechtrophotometry

For HPLC−MS analysis, the EPP and HPP fractions from DBF, as well as HPP fraction of DBPS, were concentrated (6:1) with a N_2 stream; EPP fraction of pseudo-stem was discarded after previous evaluation of spectrophotometry results. For separation, the HPLC apparatus (Agilent 1200, Agilent Technologies, Santa Clara, CA) with DAD (Agilent G1315B) and a QTOF mass analyzer (Agilent G6530A) with an atmospheric pressure electrospray ionization (ESI) was used. The column used was a 100A 50mm × 2 mm i.d., 5 µm, Luna C18 (Phenomenex, Torrance, CA).

Gradient elution was performed with a binary system consisting of 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The following gradient was applied at a flow rate of 0.4 mL/min: 0 min, 8% B; 10 min, 23% B; 15 min, 50% B; 20 min, 50% B; 23 min, 100% B; followed by a re-equilibration step. The injection volume was 20 µL, and the column temperature was 25 °C. Data were acquired using negative ion mode with a mass range of 100−1200 Da and using a source temperature of 325°C and a gas flow of 10 L/h. Peak identity was established by comparison with the retention times of commercial standards when available. Also, the molecular formula proposed by the MassHunter Workstation software version 4.0 for the different signals obtained in the MS experiments were compared with previously reported phenolic compounds in banana and other vegetal materials, and a maximum error of 10 ppm was accepted. For MS/MS experiments, the auto MS/MS acquisition mode was used; the main fragments were compared with the fragmentation patterns reported for phenolic compounds.

A relative quantitation was performed based on UV signals, using the calibration curve of a commercial standard for each class of phenolic compounds. Hydroxybenzoic acid was monitored at 280 nm and directly quantified, other hydroxybenzoic acids were monitored at 280 nm and quantified with protocatechuic acid, *p*-coumaric acid was monitored at 320 nm and directly quantified, other hydroxycinnamic acids were monitored at 320 nm and quantified with ferulic acid, flavonols were monitored at 365 nm and quantified with kaempferol.

2.8. Histopathology and Image analysis

Samples were embedded in 4% formaldehyde, fixed, placed in a cassette and placed in the processor for dehydration by embedding in alcohols in ascending proportion and fixation in paraffin (STP 120-2, Fisher Scientific, Spain). A modular embedding system consisting of a 63°C paraffin bath (MEDITE TES 99, Medizine Technik, Burgdorf, Germany) and the paraffin dispensing unit (MEDITE TES 99, Medizine Technik, Burgdorf, Germany) is used to mount the paraffin blocks, the sample is placed in the block and allowed to cool. The slices are made on the microtome (AUTOCUT JUNG 2055, LEICA, Lyon, France) with a thickness of 3µm, placed in a bath at 50°C (J.P. Selecta, Barcelona, Spain), then several slices are placed on the slides and left to dry. Before staining, the samples are placed in an oven for 30 minutes at 100°C (Memmert 300, Memmert GmbH, Schwabach, Germany).

For haematoxylin and eosin staining, the protocol of Martoja & Martoja-Pearson, (1970) was followed, starting with hydration of the samples with a battery of alcohols starting with xylol

and ending in distilled water. Alcian blue staining was performed following the Lev (1964) protocol.

Visual assessments were performed with the optical microscope (CX41, Olympus Optical, PA, USA) and photos for image analysis with the camera incorporated (Olympus DP50, Olympus Optical Co. LTD, Shinjuku-ku, Tokyo, Japan), measurements were taken with the ImageJ program (Schenider *et al*., 2012). In the liver, the maximum and minimum diameter measurements were taken, making an intersection in the nucleus of the hepatocytes, and the total area of the hepatocyte. In the intestine the length and width of the villi, the width of the lamina propria and the number and area of goblet cells.

2.9. Enzymatic analysis

Catalase activity

Extraction was performed from the fish liver with PBS buffer (1mM EDTA, pH 7.4) at a ratio of 1.5:10 (w/v) and dilutions were performed to obtain the absorbance range adjusted to the calibration line.

The Catalase Assay Kit (Cayman Chemicals, USA) was used to determine the catalase activity. In the 96-well plate, 100µL of diluted assay buffer, 30 µL of methanol and 20uL of standard, positive control or sample, where appropriate, are poured into the 96-well plate. To start the reaction, 20µL of diluted hydrogen peroxide is added to all wells. Incubate on the shaker for 20 min at room temperature and add 30µL of potassium hydroxide to stop the reaction. Add 30µL of catalase purpald chromophore. Incubate on the shaker for 10 min at room temperature. When finished, add 10µL of catalase potassium periodate, incubate for another 5 min and read the absorbance at 540nm. The following formulae are used to determine the enzyme activity:

$$\text{Formaldehyde (µM)} = (\text{Sample abs} - (\text{y-intercept})/\text{slope}) \times (0.17\text{mL}/0.02\text{mL})$$

$$\text{CAT activity} = \text{µM sample}/20\text{ min} \times \text{Sample dilution} = \text{nmol/min/mL}$$

2.10. Serum parameters

Bacteriolytic activity

(Sunyer and Tort, 1995) a culture of *Listonella anguillarum* is prepared in BHI. A colony of *L. anguillarum* is inoculated in 20mL of liquid BHI and incubated at 25°C with constant agitation

for 16 hours. A 1:10 dilution of the culture is made and measurements are made at 620nm until 0.5-0.8 absorbance is reached to start the assay. In the microplate, wells are seeded with 100uL of bacterial culture to measure absolute growth, wells corresponding to serum samples are seeded with 100µL of culture and 100µL of the sample, and wells corresponding to the negative control are seeded with 100µL of serum. Measurements are made at 0, 12 and 24 hours and the following formula is applied:

%AB= (Abs positive control-(Abs sample-Abs negative control))/Abs positive control x 100

Peroxidase activity

According to Quade and Roth (1997), in a 96-well microplate, 30µL of serum is added to each well (a duplicate of each sample) and distilled water is added for the blank. Add 120µL of Hans' Balancing Solution (HBSS), 50µL of TMB (20 mM) and 50µL of H2O2 (5mM). After 2 minutes, 50µL H_2SO_4 (2M) is added to stop the reaction and the absorbance is measured at 450nm.

Lysozyme

The method is based on the reduction of absorbance due to the lysis of *Micrococcus sp* by the lysozyme present in the serum. A dilution of lysozyme of 47700 units/mL is prepared with distilled water. From this dilution a calibration curve is prepared with the following values: 2385U/mL, 1192.5U/mL, 596.25U/mL, 298.125 U/mL, 149.062 U/mL, 74.531 U/mL, 37.27 U/mL, 18.63 U/mL, 9.315 U/mL, 4.6575 U/mL. In addition, a suspension of lyophilised *Micrococcus sp* is prepared in a phosphate/NaCl dilution (pH 6.3).

In the 96-well plate, 10 µL of standard or sample solution and 10 µL of distilled water in the blanks are placed. Then 200 µL of *Micrococcus sp* solution is added to each well. Readings are taken on the plate reader at 620 nm every 5 minutes until minute 60. The time at which the regression curve is the tightest is selected and the amount of lysozyme present in the samples is obtained as a function of absorbance.

(Abs t15- Abs t0/ 15 min) – 0.1846 / -0.0001

2.11. Plasma parameters

Cortisol and glucose determination were performed in an external certified laboratory (Animal Lab, Las Palmas de Gran Canaria, Spain).

2.12. Cellular assays

Lymphocyte extraction and fixation

The headkidney of three fish per tank was extracted and kept cold in supplemented L-15 medium (serum 2% and 1% antibiotic: penicillin 100U·mL^{-1} + streptomycin 100mg·mL^{-1} and gentamicin 5ug·mL^{-1}). Lymphocytes were extracted following the modified protocol of Secombes (1990). Tissue was manually processed with sterile micro pistons and filtered with a Nylon membrane (100µm) with supplemented L-15 medium. The cell suspension was centrifuged at 450g for 10 minutes at 4°C, the supernatant was discarded and the cells were resuspended in 2mL of L-15 supplemented medium. The 2mL of cell suspension was put in Lymphoprep 1:1 and centrifuged at 1100g for 30 minutes at 4°C without acceleration. The interface was removed and put in a sterile glass tube, the lymphocytes were washed with 1mL of L-15 medium and centrifuged (450g, 10 minutes, 4°C). The supernatant was removed, and the cells suspension was mixed with erythrocytes lysis solution (1:3 v/v) (0.862% w/v, NH_4Cl, 1mM EDTA, 10mM $KHCO_3$), after 10 minutes at room temperature, was centrifuged for 10 minutes (2500rpm, 4°C), the supernatant was removed and cells were resuspended in 2mL of PBS. 10 minutes of centrifugation (2500rpm, 4°C), remove supernatant, and resuspended in 1 mL of no supplemented L-15 medium. Cell counting was performed in the Neubauer chamber with Trypan Blue stain for which, 10uL of the cell suspension was stained with 40µL of Trypan Blue. The lymphocytes were plated in 24-well plates with 15 mm diameter lenses, at a concentration of 10^5 diluted in L-15 medium without serum and with the antibiotic. After 3h incubation at 22ºC, unfixed cells were washed with L-15 medium and left until the next day with L-15 medium with 2% serum and antibiotic.

Poly I:C stimulated Mx expression

Lymphocytes in 24-well plates were incubated with Poly I:C to simulate virus infection and with PBS as a negative control. Mx gene expression was measured at 0, 6, 24 and 48h incubation by qPCR (quantitative PCR). Two wells were used for each tank.

RNA extraction from lymphocytes was performed with the EZNA® Total RNA Kit I (Omega Bio-tek) and cDNA synthesis was performed with the cDNA iScript cDNA synthesis kit (Biorad®). SYBR as fluorochrome (Bio-Rad Laboratories S.A., California, EEUU) and a thermal cycler iQ^{TM}5 (Multicolor Real-Time PCR Detection System, Bio-Rad Laboratories S.A., California, EEUU) were used to amplified Mx gene from the cDNA.

For cDNA synthesis, 5µL of 5x buffer, 12 µL DEPC water and 2 µL of RNA were mixed and placed in the thermal cycler for 10 min at 70°C without reverse transcriptase. Then, 1 µL of enzyme and 5 µL of water were added and cycled for 10 min at 25°C, 50 min at 42°C and 15 min at 70°C.

For qPCR, 2 µL of the cDNA synthesised in the previous step was taken, 10 µL of fluorochrome, 0.5 µL of the corresponding primers and milli-Q water were added to a final volume of 25 µL. The protocol in RT-qPCR was:

1x 5 minutes at 95°C
45x 15 seconds 95°C
1x 30 seconds 60°C (hybridisation temperature)
1x 1 minute 70°C
81x 30 seconds from 55°C to 95°C (Melting curve)

At the end of the hybridisation phase of each cycle, the value for the threshold cycle (Ct) was estimated, where a statistically significant increase of the emitted fluorescence was detected. The result obtained was the expression of the Mx gene relative to the expression of β-actin (housekeeper gene) used as a reference (**Table 2.1**), following the Livak method (Livak & Schmittgen, 2001) which compares the Ct ($2^{-\Delta\Delta Ct}$).

Table 2.1. Sequences of the Mx and β-actin genes.

Gene	**Sequence**	
Mx	Forward	5´-GACAGGGAGCGGCATTGTTAC-3´
	Reverse	5´-TCGTCCAGCTCTTCCTCGTG-3´
β-actin	Forward	5´-TCTGTCTGGATCGGAGGCT-3´
	Reverse	5´-AAGCATTTGCGGTGGACG-3´

Phagocytic capacity of lymphocytes

First, a 20 mm diameter lens was placed in the well of the plates. Then, 500 µL aliquots of 10^7 cells/mL in supplemented medium L-15 were prepared and placed in the lenses and homogeneously distributed. They were incubated with 10 µL of 10^9 CFU/mL (MOI 1:1) *Vibrio anguillarum* for 1 hour at 22°C. After this time, wash with PBS and air dry the lenses. Next, proceed with rapid Diff-Quik staining of each lens:

1 minute in fixing solution (triarylmethane dye and methanol)
Wash in distilled water
1 minute in the solution I (Eosin and pH buffer)
Wash in distilled water
1 minute in the solution II (Methylene blue)
Wash in distilled water
Dry in air

After drying, the lenses were put on a slide with 20 µL of glue and sealed with varnish. To determine the phagocytic capacity, one hundred lymphocytes per lens were counted, indicating those with phagocytic vacuoles and those without them.

2.13. Statistical analysis

Data were processed with GraphPad Prism 8 software. First, it was determined whether the data followed a normal distribution using the Shapiro-Wilk test. For the comparison of means, in those data populations that followed a normal distribution, homogeneity of variance was tested with Levene's test; for populations that met the above precepts, ANOVA analysis was used. For populations that were normal but did not comply with the homogeneity of variance, the difference between means was determined with the Brown-Forsythe statistics. Finally, for data populations that do not follow a normal distribution, the analysis of means was performed using non-parametric analyses, such as Student's t-test. The results were expressed as mean ± standard deviation.

Chapter 3. Potential of banana flower and pseudo-stem as novel ingredients rich in phenolic compounds

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3.1 Introduction

World banana production in 2017-2019 reached 116 million tons, making it the most important fruit production worldwide (FAO, 2019). Due to the way bananas are harvested, the amount of waste generated in the plantations reaches 80% of the total biomass (Padam *et al*., 2014), constituting both an environmental problem related to soil toxicity and elevated carbon print (Adsal *et al*., 2020) and a food safety problem derived from food waste management (Campos *et al.,* 2020). Interest in banana residues has been renewed in recent times mostly due to their variety of bioactive compounds (Lau *et al*., 2020), apart from the emerging uses of banana plant fibre in several industries (Ortega *et al*., 2016; Rodríguez *et al*., 2020); all these usages and its processes generate waste as a secondary by-product after processing. The utilization of these secondary by-products fit with the current FAO SDGs actions on the Circular Economy (FAO, 2018).

Polyphenols are the most common bioactive compounds in plant residues (Lau *et al*., 2020; Singh *et al*., 2016). They are secondary metabolites with biological activities such as radical scavenging ability or microbiota modulation, which results in health benefits related to glucose homeostasis, obesity, type II diabetes, systemic inflammation or lipid metabolism, besides their

use as a natural preservative (Cao *et al*., 2019; Fraga *et al*., 2019; Lau *et al*., 2020). Interest in polyphenols have been traditionally focused on the so-called extractable polyphenols (EPP), i.e., low molecular weight compounds present free in vegetal cells, although increasing evidence is showing the relevance of non-extractable polyphenols (NEPP) or macromolecular antioxidants, which are high molecular weight polyphenols or small ones associated with macromolecules such as protein or dietary fibre (Pérez-Jiménez *et al*., 2013).

Among agricultural residues from banana production are the banana flowers, which arise upon the bracts axis of the inflorescence (Ram *et al*., 1962) and cut down when they reach a specific size so that they do not interfere with the ripening and growth of the fruit (Lau *et al*., 2020), and the whole pseudo-stem, which is left on the ground of the plantation. These by-products have been widely used in traditional medicine (Lau *et al*., 2020; Mathew & Negi, 2017). There are currently several studies on the inclusion of banana by-products in animal and human diets. For instance, banana leftovers were used to replace up to 75% of the cornmeal in lamb diets without causing adverse effects (Menezes *et al*., 2018). Likewise, several studies with pseudo-stem show that it can be considered as an alternative source to traditional livestock fodder (Wang *et al*., 2016). The banana flower has been studied for its antioxidant and anti-diabetic capacity (Lau *et al*., 2020); thus Bhaskar *et al*. (2012) showed that both, banana pseudo-stem and banana flower used as a dietary supplement in patients with diabetes may reduce associated complications.

Nevertheless, studies on the phytochemical characterisation of banana flower or banana pseudo-stem are still scarce. Thus, Bashkar *et al*. (2012) is the only found study with these two banana by-products from *Musa sp. elakki bale*, where HPLC analysis was performed. Also, Schmidt *et al*. (2015) have reported some characterisation of banana male flower and bracts from *Musa cavendish* in which EPP content by spectrophotometry was measured. Moreover, most of the studies performed with banana residues do not differentiate the banana flower from the inflorescence being the composition and effects indistinctly attributed to both parts of the plant (Lau *et al*., 2020).

The present study aims to perform a preliminary approach to evaluate the potential of the use of banana flower and pseudo-stem as novel food ingredients, for which a nutritional characterisation of both materials was performed, including HPLC-MS analysis of polyphenol fractions.

3.2 Material and methods

Chemicals

The Folin-Ciocalteu reagent was from Panreac (Castellar del Vallés, Barcelona, Spain). Gallic acid, kaempferol, myricetin, ferulic acid, 2-hydroxybenzoic acid, protocatechuic acid, 1-caffeoylquinic acid, *p*-coumaric acid, (+)-catechin and (-)-epicatechin were all obtained from Sigma-Aldrich (St. Louis, MO). All the reagents used for the preparation of phenolic compounds fractions and spectrophotometric determinations were of analytical grade, while for MS analysis, they were of MS grade. Condensed tannin concentrate from mediterranean carob pods (*Ceratonia siliqua L*) was supplied by Nestlé Ltd. (Vers-chez-les Blancs, Switzerland).

Samples

Pseudo-stem and flowers were supplied by local producers of banana cultivars (*Musa accuminata cavendish*) from 2017 and 2018 harvests. Pseudo-stem was processed at University of Las Palmas de Gran Canaria facilities to mechanically separate the external long fibre for other studies (patent: WO2014/174115), the residue that remained in the machine was the pseudo-stem fibre considered for the present study. Dried banana flower (DBF) and dried pseudo-stem (DBPS) were freeze-dried and ground in a mill (Ultra Centrifugal Mill ZM200, Retsch, Germany) to a particle size of 0.5mm. All the analysis were performed in triplicates for each harvest; results are given on dry weight (dw) basis and are expressed as the mean of the two harvest ± standard deviation.

Proximate composition and Fatty acids profile

Moisture, crude protein and ash content were determined according to AOAC (1995). Lipids were determined according to Folch et *al*. (1957). The total carbohydrate content was calculated by the difference method. FAMEs (Fatty Acid Methyl Esters) were obtained by transmethylation of total lipids following Christie (1982) protocol and separated and quantified by liquid chromatography (Izquierdo *et al*., 1989).

Extractable Polyphenols (EPP)

0.5 g of sample was extracted by shaking at room temperature with 20 mL of methanol/water (50:50 v/v, pH 2) and then with 20 mL of acetone/water (70:30, v/v) for 1 hour. The two supernatants were combined, which corresponded to the extractable phenolic compounds fraction (Pérez-Jiménez et *al*., 2008).

Hydrolyzable Polyphenols (HPP):

The residue from the extractable phenolic compounds extraction was treated with 20 mL of methanol and 2 mL of sulfuric acid (12M) at 85 °C for 20 h. After washing with distilled water, an aliquot of the extract was adjusted to pH 5.5 (Arranz *et al*., 2009; Hartzfeld *et al*., 2002).

Nonextractable Proanthocyanidins (NEPA)

The residue from the extractable phenolic compounds extraction was treated with butanol/HCl (97.5:2.5, v/v) with 0.1% FeCl3 at 100 °C for 1h (Pérez-Jiménez *et al*., 2009).

Determination of EPP, HPP and NEPA content

The total content of phenolic compounds was determined by spectrophotometry. The Folin-Ciocalteau assay (Singleton *et al*., 1999) was applied to EPP and HPP, the results being expressed as g of gallic acid equivalents/100 g dw. NEPA content was determined by measuring the sum of absorbance at 450 and 555 nm (Zurita *et al*., 2012) and the results were expressed as mg NEPA/100g dw, using a standard curve from carob pod concentrate. The total percentage of non-extractable polyphenols was determined by the formula (HP+NEPA)/total polyphenols x100. All these measurements were carried out in a 96-well plate reader (Synergy MX, Bio Tek, Winooski, Vermont, USA).

Phenolic profile by HPLC−ESI−QTOF

The EPP and HPP fractions from DBF, as well as HPP fraction of DBPS, were concentrated (6:1) with a N_2 stream; EPP fraction of pseudo-stem was discarded after previous evaluation of spectrophotometry results. For separation, the HPLC apparatus (Agilent 1200, Agilent Technologies, Santa Clara, CA) with DAD (Agilent G1315B) and a QTOF mass analyzer (Agilent G6530A) with an atmospheric pressure electrospray ionization (ESI) was used. The column used was a 100A 50mm × 2 mm i.d., 5 µm, Luna C18 (Phenomenex, Torrance, CA) at 25°C. Gradient elution was performed with a binary system consisting of 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The following gradient was applied at a flow rate of 0.4 mL/min: 0 min, 8% B; 10 min, 23% B; 15 min, 50% B; 20 min, 50% B; 23 min, 100% B; followed by a re-equilibration step. The injection volume was 20 µL. Data were acquired using negative ion mode with a mass range of 100−1200 Da and using a source temperature of 325 °C and a gas flow of 10 L/h. Peak identity was established by comparison with the retention times of commercial standards when available. Also, the molecular formula proposed by the MassHunter Workstation software version 4.0 for the different signals obtained in the MS experiments were compared with previously reported phenolic compounds in banana and other vegetal materials, and a maximum error of 10 ppm was accepted. For MS/MS experiments, the

auto MS/MS acquisition mode was used; the main fragments were compared with the fragmentation patterns reported for phenolic compounds.

A relative quantitation was performed based on UV signals, using the calibration curve of a commercial standard for each class of phenolic compounds. Hydroxybenzoic acid was monitored at 280 nm and directly quantified, other hydroxybenzoic acids were monitored at 280 nm and quantified with protocatechuic acid, *p*-coumaric acid was monitored at 320 nm and directly quantified, other hydroxycinnamic acids were monitored at 320 nm and quantified with ferulic acid, flavonols were monitored at 365 nm and quantified with kaempferol.

Statistical analysis

The distribution of the results obtained in each batch was analysed with Rcmdr: R Commander (R package version 2.6-2, Fox J, Bouchet-Valat M, 2020) with Shapiro-Wilk test to determine the normality and also the descriptive statistics where performed.

3.3 Results and Discussion

This study aims to explore the potential of DBF and DBPS as possible new functional ingredients, as a way to valorise these by-products in a circular economy context. For this, the proximate composition of these materials were obtained, completing previous data and, especially, a comprehensive analysis of polyphenols was performed.

Proximate composition of DBF and DBPS are provided in **Table 3.1.** There were some differences with previous studies, which reported lower values in proteins and ash content (Bhaskar *et al*., 2012; Wang *et al.*, 2016) in pseudo-stem, or proteins, lipids and ash in the flower (Sheng *et al*., 2010). This may be related to the differences between varieties and the samples that were taken; for instance, the part of the pseudo-stem that was analysed in the present study was the remain fibrous pulp after the extraction of most of the fibre, instead of the whole pseudo-stem.

Table 3.1 Proximate composition of dried banana flower and dried banana pseudo-stem.

	Banana flower	**Banana pseudo-stem**
(% dw)		
Lipids	8.66 ± 0.23	1.01 ± 0.32
Ash	18.07 ± 0.76	15.97 ± 2.67
Protein	13.59 ± 0.41	7.25 ± 2.48
Moisture	7.23 ± 1.89	8.97 ± 1.01
Carbohidrates	59.68 ± 1.26	76.09 ± 5.58

(mean ± sd. deviation of the two harvest)

The detailed composition of the fat fraction of each material was evaluated (**Table 3.2**). The two samples presented marked different fatty acid profile. The overall profile for DBF agreed with that described in another study (Lau *et al*., 2020), although other authors described a lower proportion of unsaturated fatty acids (Vilela *et al*., 2014). DBPS composition differs from the one described by Ramu *et al*. (2017), where palmitic acid content was lower while linoleic acid was higher than in this preliminary study. Regarding the potential benefits of fatty acids present in these residues, since DBPS exhibits a low amount of lipids in this product, the actual impact on health would be irrelevant. On the contrary, the DBF does present an appreciable quantity of lipids that can influence health. Thus, a portion of 20g of DBF can provide 197.3 mg of linoleic acid, which is 18% of the recommended dietary amount; DHA quantity present in 20g of DBF is the equivalent of half DHA present in 100g of salmon; also, the content of n-3 fatty acid is similar to the recommended portion which is 450 mg/day (Kris-Etherton *et al*., 2009; Ytrestøyl *et al*., 2015). Furthermore, eicosadienoic acid, a precursor of DHA (Lupette & Benning, 2020), is present with 157.2 mg/portion. The benefits of MUFA and PUFA are widely described in the literature, such as mediation in the innate immune system, reduction of cardiovascular disease risk and the redistribution of adipose tissue (Lau *et al.,* 2020; Tvrzicka *et al*., 2011).

Therefore, these results show that both DBF and DBPS may have an interesting nutritional composition, especially regarding proteins and lipids, which could make them suitable to be tested in different formulations.

Table 3.2. Fatty acid profile from dried banana flower and dried banana pseudo-stem.

FAA (%)	**Banana flower**	**Banana pseudo-stem**
14:00	0.16	1.55
14:1n-5	0.06	0.77
15:00	0.26	2.29
15:1n-5	0.03	0.11
16:00	12.90	39.89
16:1n-7	0.21	1.04
16:1n-5	0.03	0.08
16:2n-6	0.62	0.00
17:00	0.04	0.30
16:3n-4	0.03	0.21
16:3n-3	0.02	0.05
16:3n-1	0.03	0.10
16:4n-3	0.26	0.16
18:00	2.19	6.17
18:1n-9	2.04	9.21
18:1n-7	0.73	3.93
18.1n-5	0.04	0.18
18:2n-9	0.03	0.09
18:2n-6	11.47	15.46
18:2n-4	1.02	0.25

18:3n-6	0.46	0.90
18:3n-3	5.68	0.22
18:4n-3	0.37	8.39
20:00	1.15	1.92
20:1n-9	0.12	0.39
20:1n-7	1.55	0.48
20.1n-5	0.20	0.09
20:2n-9	0.09	0.07
20:2n-6	9.14	0.46
20:3n-6	1.24	1.02
20:4n-6	0.00	0.46
20:3n-3	0.41	0.07
20:4n-3	0.23	0.12
20:5n-3	0.17	0.16
22:1n-11	0.41	0.25
22:1n-9	19.11	1.10
22:4n-6	0.79	0.15
22:5n-6	0.31	0.40
22:5n-3	0.36	0.00
22:6n-3	26.04	0.94
∑SFA	16.70	52.12
∑MUFA	24.47	16.86
∑PUFA	58.77	29.68
n-3	33.54	10.11
n-6	24.03	18.85
n-9	21.39	10.86

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

From phenolic compound analysis (**Table 3.3**), DBF emerged as a potentially relevant source of these compounds. Regarding EPP fraction (not detected in DBPS), the number of phenolic compounds in the flower agreed with that reported by Padam *et al*. (2012) and, interestingly, was much higher than that described in the fruit (Pico *et al.,* 2019). In agreement with this higher phenolic compound content in DBF than in DBPS, HPP fraction was between 6-fold and 8-fold higher in the latter. Previous data showed that a relevant fraction of phenolic compounds in DBF was associated with hemicellulose A (Bhaskar *et al*., 2012), thus corresponding to the HPP fraction. But the most relevant fact of phenolic compounds analysis in these by-products was the determination, by the first time, of NEPA content. A very high content (about 10% dw) was determined in DBF, indeed constituting 90% of the total phenolic compound content in this material. This novel result is in concordance with previous descriptions of a very high NEPA content in banana fruit (Pérez-Jiménez & Saura-Calixto, 2015) and banana peel (Pérez-Jiménez & Saura-Calixto, 2018), being commonly underestimated. The difference observed between the spectrophotometry and the HPLC-MS determination was within the ranges observed in other characterisations of vegetal materials (Pérez-Ramírez *et al*., 2018), where the

spectrophotometry determinations were higher due to the unspecific of the technique and are not directly comparable techniques.

Table 3.3 Total content (mg/100 g dw) for extractable polyphenols (EP), hydrolysable polyphenols (HP) and non-extractable proanthocyanins (NEPA), and percentage of non-extractable polyphenols (NEPP) in dried banana flower and dried banana pseudo-stem.

	Banana flower	**Banana pseudo-stem**
Fraction		
EP	1075.02 ± 20.60	nd
HP	735.23 ± 103.91	150.75 ± 53.54
NEPA	11200.00 ± 3357.61	nd
%NEPP	91.73	100

(mean ± sd. deviation of the two harvest)

Detailed phenolic compounds profile was evaluated by HPLC-ES-QTOF MS technique analysis in EPP fraction of DBF (**Table 3.4**), and HPP fractions in DBF (**Table 3.5**) and DBPS (**Table 3.6**). NEPA, due to the butanol medium, exhibit problems for HPLC analysis and, besides, due to the depolymerization process information about original structures would be completely lost (Pérez-Jiménez & Torres, 2011).

The identity of individual phenolic compounds in DBF and DBPS was performed for the first time by MS.

In BBF EPP fraction (**Table 3.4**) and both HPP fractions (**Table 3.5, Table 3.6**), phenolic acids were the most relevant constituents, although some flavonol was also detected, which agrees with previous results in banana fruit and peel (Tsamo *et al*., 2015). Previous studies also identified epicatechin and gallic acid in the banana flower associated with hemicellulose and in green banana flour (Bhaskar *et al*., 2012; Pico *et al*., 2019) or protocatechuic acid in banana rhizome (Kandasamy & Aradhya, 2014). Nevertheless, for most detected phenolic compounds, identity could not be assigned, since they corresponded to structures not present in common databases nor literature on banana. In the EPP fraction from DBF, 45 non identified compounds were detected, and 28 compounds in HPP fraction. In the HPP fraction from DBPS 12 non identified were detected (data not shown). The wide diversity of structures in the phenolic compounds family makes that new structures are still being identified (Nguyen *et al*., 2019); this indicates the need of further structural analysis in DBF and DBPS.

Table 3.4. Phenolic compunds identified by HPLC-ESI QTOF MS analysis in the extractable polyphenols (EPP) fraction of dried banana flower of the two harvest.

ID	Compound	Formula	(M-H)-teo	(M-H)-cal	Diff	Concentration (mg/100g dw)
Hydroxybenzoic acids						
1	3,5-Dihydroxybenzoic acid	$C_7H_6O_4$	153.0187	153.0195	2.17	0.44 ± 0.02
	Gentisic acid	$C_7H_6O_4$	153.0187	153.0195	2.17	0.44 ± 0.02
2	Protocatechuic acid*	$C_7H_6O_4$	153.0187	153.0185	4.28	0.43 ± 0.04
3	Hydroxybenzoic acid*	$C_7H_6O_3$	137.0238	137.0237	4.86	6.61 ± 2.74
Hydroxybenzaldehydes						
4	4-Hydroxybenzaldehyde	$C_7H_6O_2$	121.0289	121.0291	2.98	0.24 ± 0.02
	Benzoic acid	$C_7H_6O_2$	121.0289	121.0291	2.98	0.24 ± 0.02
Hydroxycinnamic acids						
5	Caffeic acid	$C_9H_8O_4$	179.0344	179.0345	2.78	10 ± 3.38
6	*p*-Coumaric acid*	$C_9H_8O_3$	163.0398	163.0395	1.54	0.38 ± 0.05
7	Ferulic acid*	$C_{10}H_{10}O_4$	193.0507	193.0500	4.11	1.64 ± 0.57
Flavonols						
8	Quercetin 3,4'-O-diglucoside	$C_{27}H_{30}O_{17}$	625.1404	625.1440	-4.24	1.01 ± 0.83
	Quercetin 3-O-sophoroside	$C_{27}H_{30}O_{17}$	625.1404	625.1440	-4.24	1.01 ± 0.83

*identified by standard. cal, calculated; exp, experimental.

Table 3.5. Phenolic compounds indentified by HPLC-ESI-QTOF MS analysis in the hydrolizable polyphenols (HPP) fraction of dried banana flower of the two harvest

ID	Compound	Formula	(M-H)-teo	(M-H)-cal	Diff	Concentration (mg/100g dw)
Hydroxybenzoic acids						
2	Protocatechuic acid*	$C_7H_6O_4$	153.0187	153.0195	1.16	48.68 ± 0.62
9	Gallic acid 3-o-gallate	$C_{14}H_{10}O_9$	321.0246	321.0227	7.68	0.43 ± 0.01
Hydroxycinnamic acids						
7	Ferulic acid*	$C_{10}H_{10}O_4$	193.0500	193.0498	4.4	0.71 ± 0.17
10	Isoferulic acid	$C_{10}H_{10}O_4$	193.0500	193.0505	0.64	5.74 ± 0.16

*identified by standard. cal. calculated; exp. experimental. nd, non-detected.

Table 3.6. Phenolic compounds identified by HPLC-ESI-QTOF MS analysis in the hydrolizable polyphenols (HPP) fraction of dried banana pseudo-stem of the two harvest

ID	Compound	Formula	(M-H)-cal	(M-H)-exp	Diff	Concentration (mg/100g dw)
Hydroxybenzoic acids						
2	Protocatechuic acid*	$C_7H_6O_4$	153.0187	153.0191	4.24	49.02 ± 27.64
Hydroxycinnamic acids						
6	*p*-Coumaric acid*	$C_9H_8O_3$	163.0395	163.0395	3.34	0.48 ± 0.12
7	Ferulic acid*	$C_{10}H_{10}O_4$	193.0500	193.0508	-1.4	1.26 ± 0.29
11	*p*-Coumaric acid ethyl ester	$C_{11}H_{12}O_3$	191.0708	191.0720	-3.76	0.05 ± 0.01

*identified by standard; cal. calculated; exp. experimental. nd, non-detected.

Due to the total concentration and profile of individual phenolic compounds in DBF, this product, as an additional source of phenolic compounds in the context of the whole diet, might have health benefits. For instance, protocatechuic acid, for which health effects related to cancer, type I and II diabetes and neuroprotection have been reported (Adedara *et al*., 2019; Al Olayan *et al*., 2020), was detected in DBF at a concentration higher than that observed in most common foods, according to the Phenol-Explorer database (Neveu *et al*., 2010). Although the potential effects would be mostly derived from the combination of all phenolic compounds present in this product, as shown in a study in diabetic rats where phenolic extracts of banana pseudo-stem and flowers modulated the antioxidant defence enzymes, decreasing the complications associated with diabetes (Bhaskar *et al*., 2011). Also, regarding the high NEPA content detected in DBF, it should be remarked that other products rich in this phenolic compound fraction, such as grape pomace, have shown several health-related properties, for instance regarding fasting insulin (Martínez-Maqueda *et al*., 2018).

In conclusion, this study provides preliminary new data on proximate composition and, especially, phenolic compounds profile, in DBF and DBPS, two by-products from banana industry currently neglected. In the light of the results obtained, future comprehensive studies need to be performed to establish an accepted range of the main constituent of these by-products, taking into account the possible difference between batches of the same species due to environmental growing conditions (Lau *et al*., 2020; Vinson *et al*., 2018); and thus, the real potential of the DBF and DBPS as novel food ingredients as well as the technological aspects for their incorporation into foods and their potential health effects.

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Chapter 4. Assessing the performance of juvenile tilapia (*Oreochromis niloticus*) with the inclusion of new banana by-products in starter diets

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Abstract

The high worldwide amount of residues derived from the banana harvest is an opportunity to create synergies between the banana industry and aquaculture, from a circular economy perspective. The present work objective is to evaluate the inclusion of banana by-products, such as banana pseudo-stem and banana flower in tilapia feeds, and to explore the extracts from the banana flower on the development and health in tilapia juveniles**.** Three consecutive feeding trials were performed to evaluate the test by-products inclusions: Experiment I) with 0, 2.5, 5, 15 and 20% of banana pseudo-stem; Experiment II) with 0, 0.2, 0.5, 1 and 3% of banana flower; and Experiment III) with 3% of banana flower against its correspondent organic extract and residue of the extraction. Salinity stress challenges were conducted after the feeding trials in Experiments II and III. From the obtained results, up to 5% of banana pseudo-stem and 3% of banana flower inclusion were suitably regarded the fish growth and the liver health, also, as the essential fatty acids proportion in the muscle, despite the reduction in total lipid percentage. Regarding the stress challenges, the flower and more specific, the remaining residue from its organic extraction, appears to regulate the levels of plasma cortisol and glucose and reduced the oxidation parameters in fish liver and muscle, which may be due to the polyphenols present in both, the whole banana flower and in its organic extraction residue.

4.1. Introduction

In the next decade, new challenges are emerging as is shown in the UN Sustainable Development Goals, which address the degradation of freshwater ecosystems and declining water availability and the need for sustainable and secure food production, among others (U.N., 2021). It has been reported that aquaculture, through its sustainable development, contributes to most of the UN´s SDO through Blue Growth (Bartley, 2020), representing moreover one of the most resilient productive sectors due to the variety of species cultivated and the diverse farming methods (FAO, 2021b).

Among fish species, and according to FAO (2020a), tilapia spp. represents the second most-produced in aquaculture worldwide, due to its high adaptation capacity to different ambient, fast growth and low technology dependence under culture in most cases. The production of tilapia is mostly extended in tropical areas worldwide but becoming interesting as emergent species in many other geographical areas due to the increase in consumption in countries such as the USA, Canada and Central Europe (Prabu *et al.*, 2019; FAO, 2020a). The adaptability of the species to increasing salinity water enables the adaptation from the fresh aquaculture to brackish or even salt water (El-Leithy *et al*., 2019), which gives it versatility in dealing with the problem of lack of fresh water. Furthermore, opportunities for using this species in alternative and recirculated production systems, like aquaponics (Kloas *et al*., 2015), extend tilapias' sustainable production research worldwide. Apart from that, it is well known the capacity of feeding tilapia with low-cost feeds including a high variety of regular vegetable materials like different products from corn, wheat, rice and cassava (Ng & Wee, 1989; Chiayvareesajja *et al*., 1990; Liti *et al*., 2005), but also the demonstrated opportunity of using against the global trade ingredients, other locally available by-products such as aloe vera, *Azadirachta indica*, sacha inchi or sweet orange peel in tropical areas (Obaroh & Nzeh, 2014; Acar *et al*., 2015; Gabriel *et al*., 2015; Khieokhajonkhet *et al*., 2021).

Bananas are the most sought-after fruit worldwide (FAO, 2021a). The European Union is the region with more importation of banana fruit, with 90% of the home production developed in the Canary Islands (Spain) and the French West Indias (FAO, 2021a). The harvesting process generates a high amount of residues, almost 80% of the total plant mass (Padam *et al*., 2014). The male flowers fall off during the development of the fruits and when the banana is harvested, the rest of the plant has to be cut to let the rest of the plantain grow up again (Lau *et al*., 2020). There is a long tradition of using banana residues as feed for livestock (Marie-Magdelein *et al*., 2010; Wadhwa *et al*., 2015; Wang *et al*., 2016; Menezes *et al*., 2017), with also some studies in rats to evaluate the bioactives present in the pseudo-stem and the flower (Bahskar *et al*., 2012).

On the aquaculture side, a few studies have reported the utilization of banana by-products, most of them on green bananas discard during packaging (Giri *et al*., 2016; Palintorn *et al*., 2019; Felix e Silva *et al*., 2021; Karaket *et al*., 2021; Yossa *et al*., 2021), but to our knowledge, there is no one focus on the utilization of banana pseudo-stem and banana flower in fish, nor on tilapia feeds. The interest in these two by-products under aquaculture perspectives is not only due to the high availability throughout the year, but to the interesting nutritional and polyphenol profile described in a previous study (Ramírez-Bolaños *et al*., 2021), where it was established the potential use of both, the banana pseudo-stem and banana flower as ingredients.

The potential synergy between the two industries can be developed and may have several benefits, on the one hand by reducing the waste generated in banana cultivation, and on the other hand by increasing the list of alternative available raw materials, moreover, reducing the need for imported ingredients in some tilapia production regions, thus decreasing the impact of food and feed production on the environment. The circular economy is one of the paths toward sustainability that must be taken to solve the problems that lie ahead.

The main objective of this work is to evaluate the dietary inclusion of different banana by-products such as banana pseudo-stem and banana flower and to explore the extracts from the banana flower in the development and health of tilapia juveniles.

4.2. Materials and Methods

Raw materials

Banana pseudo-stem and banana flower were supplied by local Canarian producers of banana cultivars in the framework of the UE project LIFEBAQUA (code: LIFE15 ENV/ES/000157). The banana pseudo-stem was processed at the University of Las Palmas de Gran Canaria, Fabricación Integrada y Avanzada Research Group facilities, to mechanically separate the external long fibre (patent: WO2014/174115) normally used for bioplastic purposes, from the residue that remained in the machine, which represent up to 76% of the dry pulp. This high quantity of secondary by-product was considered pseudo-stem fibre (BP), to be studied as a dietary fish ingredient. The proximate composition of BP and banana flower (BF) was described in previous work (Ramírez-Bolaños *et al*., 2021) being 7.23% and 13.59% protein, 1% and 8.66% lipid, 15.97% and 18.07% ash and 76% and 60% carbohydrates, respectively. According to its carbohydrates content, as reported to be for banana pseudo-stem 26% cellulose, 19% hemicellulose, 8% lignin and 22% starch (Díaz *et al.*, 2021), a pre-treatment was proposed to make the carbohydrate and fibre fraction present in this waste more accessible and thus favour fermentation in the digestive tract of the juveniles. For this, acid hydrolysis was performed under diluted acid conditions

(H_2SO_4 2% w/w, 500g/L wet weight, 100°C) for 30 minutes (Souza *et al*., 2014). Then, the product obtained was neutralized, dried, and milled, generating the BP meal used in this study (0.93 ± 0.36 lipids (%), 1.91 ± 0.35 proteins (%), 50.32 ± 0.67 ash (%), 4.12 ± 0.06 moisture (%) and 44.51 ± 0.10 carbohydrates (%). The banana flower was freeze-dried (Lyobeta, Spain) to finally ground in a mill (Ultra Centrifugal Mill ZM200, Retsch, Germany) down to 125-250 microns. Moreover, based on the interesting banana flower polyphenol content and profile determined in the previous work (Ramírez-Bolaños *et al*., 2021), an organic extract from the banana flower was performed with methanol:water and acetone:water; then the organic solvents were evaporated in a rotary evaporator (Laborota 4000 Efficient, Heidolph, Germany) until only the aqueous fraction remained, and the residues from that extraction were dried in an oven at 37°C (Digitheat-TFT, J. P. Selecta, Spain), being both stored at -80°C until use.

Experimental conditions

Three different experiments were carried out at the aquaculture facilities of the Ecoaqua Institute belonging to the University of Las Palmas de Gran Canaria. Triplicate groups of fish obtained by natural reproduction on-site were randomly distributed in recirculated systems (80L/tank, 3 per treatment) for the 3 experiments, being fish manually fed to apparent satiation twice a day, six days a week during the trials. All the experiments followed the bioethics protocol followed by the Bioethics Committee of the University of Las Palmas de Gran Canaria (Real Decreto 53/2013).

A previously tested diet in our facilities based on a comprehensive review (**Table 4.1**), where used as control from which cornmeal was substituted with increasing levels of BP in Experiment I (0%, 2.5%, 5%, 15% and 20%), and increasing levels of BF (0.2%, 0.5%, 1% and 3%) in Experiment II. In Experiment III the 3% BF diet was assayed against a diet containing the organic extract corresponding to 3% BF and a diet with its solid remaining residue (**Table 4.2**).

Table 4.1. Formulation of basal diet for the tilapia trials.

Formulation (%)	
Fish meal	21.00
Corn meal	20.00
Corn gluten	7.00
Soy meal	26.00
Wheat meal	9.00
Linseed Oil	6.00
Vitamin mix[a]	1.00
Mineral mix[b]	2.00
Bi-Calcium Phosphate	0.50
Bread Meal	5.00
α-Cellulose	1.50

[a]Vitamin mix (g kg^{-1}) = thiamine 0.02, riboflavin 0.025, pyridoxine 0.02, calcium pantothenate 0.0585, nicotinic acid 0.1, biotin 0.0005, cyanocobalamin 0.0003, choline chloride 1.350, myo-inositol 1, ascorbic acid 2.5, α-tocopherol 0.125, menadione 0.01, cholecalciferol 0.0025, retinyl acetate 0.0125, ethoxyquin 0.1
[b]Mineral mix (g kg^{-1}) = $Ca(H_2PO)$ 1.605, $CaCO_3$ 4, $FeSO_4 \times 7H_2O$ 1.5, $MgSO_4 \times 7H_2O$ 1.605, K_2HPO_4 2.8, $Na_2PO_4 \times H_2O$ 1, $Al(SO_4)_3 \times 6H_2O$ 0.02, $ZnSO_4 \times 5H_2O$ 0.24, $CuSO_4 \times 5H_2O$ 0.12, $MnSO_4 \times H_2O$ 0.08, KI 0.02, $CaSO_4 \times 7H_2O$ 0.08

Table 4.2. Proximate composition of experimental diets including hydrolysed banana pseudo-stem (Experiment I), banana flower (Experiment II) and banana flower extract and residue (Experiment III).

	Diet				
	Control	**BP2.5**	**BP5**	**BPT15**	**BPT20**
Experiment I (%dw)					
Protein	37.32 ± 1.41	38.12 ± 0.40	38.31 ± 0.60	38.72 ± 0.14	38.69 ± 1.53
Lipids	11.09 ± 0.61	10.70 ± 0.18	10.60 ± 0.09	10.05 ± 0.27	10.33 ± 0.28
Ash	8.71 ± 0.04	9.76 ± 0.07	10.75 ± 0.05	15.67 ± 0.06	18.52 ± 0.90
CHO & fibre	48.04 ± 2.07	46.20 ± 0.58	45.14 ± 0.77	41.11 ± 0.61	38.42 ± 0.87
	Control	**BF 0.2**	**BF 0.5**	**BF 1**	**BF 3**
Experiment II (%dw)					
Protein	32.93	36.03 ± 1.08	35.96 ± 1.13	34.01 ± 1.10	36.43 ± 1.20
Lipids	13.64	13.70	12.94 ± 0.85	13.29 ± 0.09	12.72 ± 0.16
Ash	8.64 ± 0.04	8.66 ± 0.33	8.54 ± 0.11	8.77 ± 0.13	8.84 ± 0.08
CHO & Fibre	47.45 ± 0.02	44.13 ± 0.64	44.66 ± 0.94	46.17 ± 0.21	43.86 ±0.33
	Control	**BF3**	**BF3R**	**BF3E**	
Experiment III (% dw)					
Protein	41.46 ± 0.30	40.92 ± 0.09	35.80 ± 0.35	39.79 ± 1.02	
Lipids	13.77 ± 0.77	13.54 ± 0.41	15.17 ± 1.12	13.81 ± 0.27	
Ash	8.75 ± 0.16	9.87 ± 0.15	9.01 ± 0.13	8.65 ± 0.13	
CHO & fibre	40.39 ± 1.86	39.97 ± 1.40	43.91 ± 1.40	40.25 ± 0.16	

Experiment I: Hydrolysed Banana pseudo-stem (BP)

Triplicate groups of 12 fish per tank (3.9 ± 0.64 g) were manually fed with five diets with increasing levels of BP (0, 2.5, 5, 15 and 20%) for 47 days with 25.56 ± 0.61°C and 5.04 ± 0.62mg/L O_2 during the whole trial. At the end of the experiment, 9 samples (3 per replicate) for biochemical composition, histopathology and growth parameters were taken.

Experiment II: Banana flower (BF)

Triplicate groups of 12 fish per tank (7.85 ± 0.39 g) were manually fed for 47 days, with a control diet and four diets with BF flour inclusion (0.2%, 0.5%, 1% and 3%) regarded the polyphenols content in banana flower and the results obtained from Gabriel et al. (2015). Measured temperature and oxygen ranged between 22.65 ± 1.03°C and 5.32 ± 0.52 mg/L O_2, respectively. After the feeding trial, an osmotic challenge was performed (salinity increment to 21ppt), unfortunately, the fish from the BF1 diet were removed due to a failure in the system that could not be restored in time to perform the challenge. Blood samples (2 fish per tank, 6 fish per treatment) were collected at the beginning of the challenge (t0), and 3 hours (t3), 24h (t24) and 48h (t48) according to Kammerer *et al*. (2010). Individual samples were centrifuged, and each plasma was stored at -80°C for cortisol and glucose analysis. Also, survival was observed during the 48h hours and the time of fish death in the case was recorded.

Experiment III: Banana flower extracts (BF3E) and residue (BF3R)

Twenty fish per tank (0.94 ± 0.005 g) were fed in triplicate groups with four diets with 3% banana flower inclusion (BF3), against the organic extraction equivalent to that of 3% of banana flower (BF3E) and the residues remained from that extraction (BF3R). All diets were tested against the control diet during 45 days of feeding, at 25.73 ± 0.80°C and 5.61 ± 0.50 mg/L O_2 for temperature and oxygen respectively. After the feeding period, blood samples were taken from two fish per tank and cortisol and glucose were determined. To corroborate the results obtained in experiment II, an osmotic challenge was performed in two parts: first, the fish were placed in brackish water (18 ppt) and due to the lack of mortality, after five days in brackish water, the fish were placed in saltwater (35ppt) and mortality along 77 hours was again recorded.

Growth Performance

To evaluate the growth performance, fish were individually weighted every fifteen days until the end of each feeding experiment and the subsequent parameters were determined: FW, Final Weight (g); FI, Feed Intake (g/fish/day); FCR, Feed Conversion Ratio = Feed intake (g) / Weight increase (g); PER, Protein Efficiency Ratio= weight gain (g) / protein intake (g); SGR, Specific Growth Rate = ((Ln Final weight – Ln Initial weight)/ nº days)x100; K, Condition Factor= (Final Weight (g) / Total Length $(cm)^3$)x100; VSI, Viscerosomatic index (%) = (Weight of whole fish (g) - Weight of fish without viscera (g)/ Weight of whole fish (g)) x100; HSI, Hepatosomatic index (%) = (liver weight /body weight)x100.

Proximal composition

In each experiment, the lipid composition was analysed in muscle and liver. Moisture content was determined following AOAC (2000) protocol; lipids were determined according to Folch *et al.,* (1957) being fatty acids profiles obtained by transmethylation of total lipids according to Christie (1982) protocol and later separated and quantified by liquid chromatography (Izquierdo *et al*., 1989). In Experiment II and Experiment III, the protein and ash content in the muscle were determined by Kjeldahl and muffle furnace incineration, respectively (AOAC, 2000).

Histopathology

Liver for histopathological analysis was fixed with formaldehyde (4% formalin, 0.08M sodium phosphate, pH 7.0), dehydrated, included in paraffin, and stained with haematoxylin & eosin (Martoja & Martoja-Pearson, 1970). The vacuolization grade on the hepatocytes was evaluated following a scale from 1.0 (no evident presence of lipid vacuoles) to 2.5 (presence of lipid vacuoles, core displacement and cellular deformity). Micrographs from each stained slide were taken using an Olympus CX41 microscope (Olympus Optical, PA, USA) incorporated with an Olympus DP50 camera (Olympus Optical Co. LTD, Shinjuku-ku, Tokyo, Japan). The total area of 30 hepatocytes per specimen (180 hepatocytes per experimental diet) was measured, as well as maximum and minimum hepatocyte length with the program ImageJ (Schneider *et al*., 2012).

Oxidation (MDA & Liver catalase activity)

In the trial with banana flower (Experiment II) the malonaldehyde concentration (MDA) was determined in the lipids from fish muscle at t0 and t24 and in the liver at t24, as an indicator of oxidative status. The analysis was performed following the protocol from Burk *et al*. (1980).
The catalase activity analysis was performed with a catalase assay kit (Cayman Chemical, USA) on the livers at the beginning of the osmotic challenge (t0), three hours after (t3) and 24 hours after placing the fish in SW (21 ppm).

Plasma analysis (Cortisol and Glucose)

Blood samples were taken from six fish per treatment, at 0h, 3h and 24h from the caudal vein using a sterile syringe, during the challenge of Experiment II and after the feeding trial in Experiment III. Plasma cortisol and glucose were performed in an external certified laboratory.

Statistics

The results were expressed as mean ± standard deviation. All the data were analysed with GraphPad Prism 8.0.2 (GraphPad Software, San Diego, California USA, www.graphpad.com), normal distribution was determined with the Shapiro-Wilk test, one-way ANOVA analysis was performed on the normal data and non-parametric Kruskal-Wallis test was performed on the data without normal distribution. The significance was established with a p-value = 0.05.

4.3 Results

Tilapia´s performance was barely affected by the inclusion of banana residues. In experiment I, banana pseudo-stem decreased tilapia's performance (**Table 4.3**), but to a higher extent for BP15 and BP20 concerning BP2.5 and BP5, with about 50% and 30% less final fish weight for the higher and lower substitution levels, respectively. Feed acceptance, as shown by feed intake for the whole trial, and the condition factor (K) was not reduced among the increasing banana pseudo-stem inclusion. The FCR values maintain close to control by up to 5%, being significantly higher in the case of 15% and 20% inclusion (**Table 4.3**). The protein efficiency ratio was reduced with the banana pseudo-stem inclusion up to 5%. Regarding body indexes, all BP inclusion levels and banana flower extract and its residue, increased the VSI concerning control fish, while only the 20% of BP in the case of the liver (**Table 4.3**).

Table 4.3. Fish growth and performance parameters from Experiment I with hydrolysed banana pseudo-stem inclusion.

	DIET				
	Control	**BP2.5**	**BP5**	**BP15**	**BP20**
FW (g)	30.70 ± 6.60^{a}	20.59 ± 4.11^{b}	21.15 ± 5.99^{b}	14.99 ± 4.27^{c}	18.38 ± 4.04^{b}
[1]FI (g fish^{-1})	84.78 ± 0.54	92.77 ± 2.02	86.93± 0.40	92.47 ± 5.91	93.22 ± 4.84
[2]FCR	0.97 ± 0.00^{c}	1.13 ± 0.03^{abc}	1.07 ± 0.01^{bc}	1.28 ± 0.10^{a}	1.18 ± 0.09^{ba}
[3]PER	3.01 ± 0.11^{a}	2.52 ± 0.08^{ab}	2.66 ± 0.07^{bc}	2.22 ± 0.17^{c}	2.39 ± 0.23^{bc}
[4]SGR	6.62 ± 0.14^{a}	5.50 ± 0.03^{b}	5.42 ± 0.24^{b}	4.14 ±0.12^{c}	5.01 ± 0.31^{b}
[5]K	1.79 ± 0.14	1.75 ± 0.08	1.81 ± 0.27	1.76 ± 0.14	1.71 ± 0.13
[6]HSI (%)	0.79 ± 0.01^{b}	0.96 ± 0.13^{ab}	0.96 ± 0.09^{ab}	0.86 ± 0.14^{b}	1.27 ± 0.95^{a}
[7]VSI	10.71 ± 0.17^{b}	13.44 ± 0.21^{a}	12.38 ± 0.70^{a}	13.73 ± 0.51^{a}	12.69 ± 0.95^{a}

Different letter in same arrow indicates significant differences (p-value<0.05)

[1]FI= Feed intake (g) per fish for the experimental-day period

[2]FCR= Feed intake (g) / Weight increase (g)

[3]PER= weight gain / protein intake (g)

[4]SGR= ((Ln Final weight – Ln Initial weight)/ nº days) x 100

[5]K= (Final Weight (g) / Total Length (cm)^{3}) x 100

[6]HIS (%) = 100×wet liver weight /body weight.

[7]VSI (%) =((Weight of whole fish (g) − Weight of fish without viscera (g))/ Weight of whole fish (g))x 100

In experiment II, the banana flower inclusion did not produce any effect on the growth of tilapia juveniles but did affect some of the productive parameters (**Table 4.4**). The protein efficiency ratio decreased with the dietary inclusion of banana flower being significantly higher for BF1 and above.

Table 4.4. Fish growth and performance parameters from Experiment II with banana flower inclusion.

	DIET				
	C	**BF0.2**	**BF0.5**	**BF1**	**BF3**
FW(g)	20.65 ± 7.33	17.58 ± 5.70	18.12 ± 7.60	16.53 ± 2.33	17.28 ± 6.44
[1]FI (g fish-1)	79.14 ± 2.07	78.65 ± 2.75	77.36 ± 1.36	80.02 ± 4.14	82.14 ± 2.03
[2]FCR	1.33 ± 0.11	1.41 ± 0.09	1.35 ± 0.10	1.46 ± 0.13	1.55 ± 0.10
[3]PER	2.42 ± 0.21^{a}	2.04 ± 0.17^{ab}	2.14 ± 0.16^{ab}	1.90 ± 0.22^{b}	1.74 ± 0.18^{b}
[4]SGR	1.96 ± 0.27	1.74 ± 0.09	1.81 ± 0.14	1.69 ± 0.11	1.62 ± 0.21
[5]K	1.68 ± 0.01^{b}	1.67 ± 0.11^{b}	1.67 ± 0.14^{b}	1.81 ± 0.06^{a}	1.78 ± 0.03^{b}
[6]VSI	14.86 ± 3.65	13.38 ± 1.40	13.63 ± 0.13	15.08 ± 1.80	14.29 ± 1.05

Different letter in same arrow indicates significant differences (p-value<0.05)

[1]FI= Feed intake (g) per fish for the experimental-day period

[2]FCR= Feed intake (g) / Weight increase (g)

[3]PER= weight gain / protein intake (g)

[4]SGR= ((Ln Final weight – Ln Initial weight)/ nº days) x 100

[5]K= (Final Weight (g) / Total Length (cm)^{3}) x 100

[6]VSI (%) = ((Weight of whole fish (g) − Weight of fish without viscera (g))/ Weight of whole fish (g))x 100

In experiment III, the banana flower extract and residue did not affect fish growth, but the BF3R diet showed a reduction in condition factor (K), also, BF3 in the present trial and similarly BF3R decreased the protein efficiency ratio compared to the control diet (**Table 4.5**).

Table 4.5. Fish growth and performance parameters from Experiment III with the inclusion of banana flower extract and residue.

	DIET			
	C	**BF3**	**BF3R**	**BF3E**
FW (g)	7.09 ± 0.52	6.19 ± 0.28	6.26 ± 0.04	7.09 ± 0.78
[1]FI (g fish-1)	81.07 ± 4.47	89.64 ± 2.76	98.82 ± 26.41	86.98 ± 5.90
[2]FCR	0.94 ± 0.05	1.06 ± 0.02	1.16 ± 0.31	1.00 ± 0.08
[3]PER	1.03 ± 0.09^{a}	0.79 ± 0.04^{b}	0.74 ± 0.01^{b}	0.92 ± 0.12^{ab}
[4]SGR	4.48 ± 0.17	4.18 ± 0.10	4.21 ± 0.00	4.48 ± 0.23
[5]K	1.73 ± 0.04^{a}	1.73 ± 0.06^{a}	1.61 ± 0.02^{b}	1.75 ± 0.02^{a}
[6]HSI (%)	0.99 ± 0.39	1.43 ± 0.66	0.87 ± 0.20	1.07 ± 0.13
[7]VSI	10.73 ± 2.89^{b}	12.56 ± 1.51^{ab}	15.68 ± 2.26^{a}	13.46 ± 4.04^{a}

Different letter indicates significant differences (p-value<0.05)

[1]FI= Feed intake (g) per fish for the experimental-day period

[2]FCR= Feed intake (g) / Weight increase (g)

[3]PER= weight gain / protein intake (g)

[4]SGR= ((Ln Final weight – Ln Initial weight)/ nº days) x 100

[5]K= (Final Weight (g) / Total Length (cm)^{3}) x 100

[6]HIS (%) = 100×wet liver weight /body weight.

[7]VSI (%) = ((Weight of whole fish (g) − Weight of fish without viscera (g))/ Weight of whole fish (g))x 100

Proximate composition

The lipids composition of muscle and liver decreased with the inclusion of hydrolysed banana pseudo-stem (**Table 4.6 and 4.7**) in Experiment I and in those fish fed with the extract of banana flower (BF3E) (Experiment III), in which a decrease in muscle moisture was observed (**Table 4.10**). Otherwise, in Experiment II, none of the experimental diets did affect the composition of the muscle and liver (**Table 4.8 and 4.9**) and non also on the fish liver in Experiment III diets (**Table 4.11**).

Table 4.6. Muscle lipids, moisture and fatty acids composition from tilapia fed with hydrolysed banana pseudo-stem inclusion in Experiment I.

	Diet				
	Control	**BP2.5**	**BP5**	**BP15**	**BP20**
Lipid (% dw)	9.01 ± 1.20^{a}	4.92 ± 0.40^{b}	5.02 ± 0.41^{b}	5.04 ± 0.75^{b}	6.10 ± 0.13^{b}
Moisture (%dw)	80.13 ± 0.67	80.73 ± 0.39	80.93 ± 0.45	79.39 ± 1.44	79.16 ± 1.05
Fatty acid (%)					
14:00	1.56 ± 0.36	1.14 ± 0.34	1.12 ± 0.01	1.50	1.36 ± 0.22
14:1n-7	0.05 ± 0.01	0.05 ± 0.00	0.05 ± 0.00	0.03	0.03 ± 0
14:1n-5	0.07 ± 0.01	0.08 ± 0.02	0.07 ± 0.01	0.09	0.12 ± 0.01
15:00	0.19 ± 0.03	0.22 ± 0.03	0.24 ± 0.01	0.27	0.30 ± 0.01
15:1n-5	0.03 ± 0.01	0.04 ± 0.02	0.04 ± 0.01	0.02	0.02 ± 0.00
16:OISO	0.06 ± 0.01^{b}	0.07 ± 0.01^{ab}	0.09 ± 0.01^{ab}	0.10	0.13 ± 0.01^{a}
16:00	15.97 ± 3.13	14.83 ± 1.25	15.64 ± 2.17	19.57	19.11 ± 1.21

16:1n-7	2.25 ± 1.65	2.00 ± 0.73	1.61 ± 0.07	2.22	2.45 ± 0.23
16:1n-5	0.22 ± 0.01^{b}	0.30 ± 0.02^{ab}	0.37 ± 0.05^{ab}	0.47	0.54 ± 0.06^{a}
16:2n-4	0.06 ± 0.01	0.05 ± 0.02	0.05 ± 0.00	0.06	0.06 ± 0.00
17:00	0.02 ± 0.02	0.05 ± 0.01	0.06 ± 0.00	0.05	0.06 ± 0.01
16:3n-4	0.25 ± 0.01^{a}	0.18 ± 0.05^{b}	0.17 ± 0.00^{b}	0.22	0.30 ± 0.00^{a}
16:3n-3	0.08 ± 0.00^{c}	0.13 ± 0.01^{b}	0.15 ± 0.03^{ab}	0.15	0.18 ± 0.01^{a}
16:3n-1	0.01 ± 0.01^{b}	0.03 ± 0.01^{a}	0.04 ± 0.01^{a}	0.03	0.03 ± 0.00^{a}
16:4n-3	0.03 ± 0.01^{ab}	0.04 ± 0.01^{ab}	0.04 ± 0.01^{a}	0.03	0.02 ± 0.00^{b}
16:4n-1	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01	0.01 ± 0.00
18:00	5.02 ± 5.02^{b}	13.83 ± 1.37^{ab}	19.26 ± 4.30^{a}	16.86	16.69 ± 1.70^{a}
18:1n-9	18.96 ± 10.97^{b}	17.82 ± 3.91^{ab}	19.09 ± 0.44^{a}	23.14	23.43 ± 0.22^{a}
18:1n-7	0.90 ± 0.90	1.98 ± 0.19	2.41 ± 0.49	2.64	3.19 ± 0.12
18:1n-5	0.86 ± 0.76	0.10 ± 0.01	0.11 ± 0.02	0.10	0.13 ± 0.01
18:2n-9	0.12 ± 0.02	0.10 ± 0.03	0.08 ± 0.00	0.08	0.08 ± 0.01
18:2n-6	6.11 ± 6.11	11.70 ± 1.55	11.6 ± 0.61	10.67	11.45 ± 0.60
18:2n-4	0.08 ± 0.00^{b}	0.10 ± 0.02^{ab}	0.10 ± 0.00^{ab}	0.11	0.13 ± 0.01^{a}
18:3n-6	0.27 ± 0.00	0.30 ± 0.04	0.25 ± 0.07	0.16	0.20 ± 0.00
18:3n-4	0.04 ± 0.00^{b}	0.05 ± 0.00^{ab}	0.06 ± 0.01^{a}	0.07	0.06 ± 0.00^{ab}
18:3n-3	8.79 ± 0.07	7.78 ± 2.10	6.86 ± 1.40	6.70	7.45 ± 1.33
18:4n-3	0.17 ± 0.02	0.17 ± 0.03	0.15 ± 0.05	0.08	0.09 ± 0.00
18:4n-1	0.05 ± 0.00	0.05 ± 0.01	0.04 ± 0.02	0.04	0.03 ± 0.01
20:00	0.18 ± 0.01^{b}	0.25 ± 0.05^{ab}	0.38 ± 0.09^{a}	0.46	0.39 ± 0.05^{a}
20:1n-9	0.03 ± 0.03^{b}	0.05 ± 0.01^{ab}	0.05 ± 0.00^{ab}	0.12	0.08 ± 0.01^{a}
20:1n-7	0.63 ± 0.01^{b}	0.56 ± 0.00^{b}	0.66 ± 0.16^{b}	1.28	0.94 ± 0.03^{a}
20:1n-5	0.07 ± 0.01^{b}	0.08 ± 0.00^{b}	0.10 ± 0.02^{ab}	0.14	0.12 ± 0.01^{a}
20:2n-9	0.05 ± 0.01^{a}	0.04 ± 0.00^{ab}	0.04 ± 0.01^{b}	0.06	0.04 ± 0.00^{b}
20:2n-6	0.37 ± 0.01^{b}	0.71 ± 0.17^{a}	0.76 ± 0.11^{a}	0.98	0.89 ± 0.10^{a}
20:3n-9	0.01 ± 0.01^{b}	0.05 ± 0.02^{a}	0.05 ± 0.00^{a}	0.07	0.03 ± 0.00^{ab}
20:3n-6	0.22 ± 0.01^{a}	0.53 ± 0.15^{b}	0.44 ± 0.02^{b}	0.41	0.36 ± 0.05^{ab}
20:4n-6	0.92 ± 0.00^{b}	2.44 ± 0.64^{a}	2.16 ± 0.45^{a}	1.47	0.96 ± 0.11^{b}
20:3n-3	1.00 ± 0.12^{c}	1.84 ± 0.39^{ab}	1.62 ± 0.12^{ac}	2.35	2.51 ± 0.41^{a}
20:4n-3	0.18 ± 0.03	0.32 ± 0.07	0.25 ± 0.05	0.20	0.22 ± 0.05
20:5n-3	0.59 ± 0.08^{b}	1.26 ± 0.24^{a}	0.87 ± 0.26^{ab}	0.39	0.50 ± 0.00^{b}
22:1n-11	0.04 ± 0.00^{b}	0.04 ± 0.00^{b}	0.06 ± 0.02^{b}	0.37	0.11 ± 0.02^{a}
22:1n-9	0.09 ± 0.00^{c}	0.10 ± 0.03^{bc}	0.21 ± 0.03^{ab}	0.81	0.32 ± 0.08^{a}
22:4n-6	0.11 ± 0.00^{b}	0.30 ± 0.10^{a}	0.22 ± 0.01^{ab}	0.16	0.12 ± 0.03^{b}
22:5n-6	0.14 ± 0.00^{b}	0.38 ± 0.13^{a}	0.30 ± 0.07^{ab}	0.17	0.15 ± 0.03^{b}
22:5n-3	0.64 ± 0.11^{b}	1.70 ± 0.54^{a}	1.03 ± 0.30^{ab}	0.67	0.77 ± 0.20^{b}
22:6n-3	5.49 ± 0.25^{b}	16.15 ± 6.16^{a}	11.01 ± 4.69^{ab}	4.41	3.79 ± 0.90^{b}
[1]Saturades	22.94 ± 8.58^{b}	30.32 ± 0.23^{ab}	36.70 ± 6.57^{ab}	38.70	37.91 ± 3.19^{a}
[2]Monoenoics	24.10 ± 12.84	23.08 ± 4.51	24.63 ± 1.14	30.62	31.14 ± 0.48
[3]∑n-3	16.97 ± 0.55^{b}	29.38 ± 5.24^{a}	21.97 ± 6.61^{ab}	14.98	15.54 ± 2.88^{b}
[4]∑n-6	8.15 ± 6.08^{b}	16.38 ± 0.40^{a}	15.74 ± 1.11^{ab}	14.02	14.15 ± 0.69^{ab}
[5]∑n-9	19.26 ± 11.03	18.16 ± 3.90	19.53 ± 0.47	24.29	23.98 ± 0.14
[6]∑n-3 HUFA	7.90 ± 0.59^{b}	21.26 ± 7.39^{a}	14.77 ± 5.19^{ab}	8.02	7.79 ± 1.56^{b}
ARA/EPA	1.54 ± 0.20^{b}	1.94 ± 0.15^{b}	2.50 ± 0.27^{a}	3.78	1.93 ± 0.20^{b}
DHA/EPA	9.26 ± 0.77^{ab}	12.86 ± 2.58^{a}	12.70 ± 1.76^{ab}	11.38	7.65 ± 1.87^{a}
DHA/ARA	6.00 ± 0.27	6.62 ± 0.86	5.09 ± 1.18	3.01	3.95 ± 1.41
[7]Total PUFA	25.79 ± 5.61^{b}	46.43 ± 4.72^{b}	38.37 ± 7.75^{ab}	29.76	30.49 ± 3.58^{a}

n-3/n-6	1.55 ± 1.69	1.79 ± 0.36	1.40 ± 0.32	1.07	1.10 ± 0.15

Different letter indicates significant differences (p-value<0.05)

[1]14:00,15:00, 16:00, 17:00, 18:00, 20:00

[2]14:1n-7; 14:1n-5; 15:1n-5; 16:1n-7 16:1n-5; 18:1n-9; 18:1n-7; 18:1n-5; 20:1n-9; 20:1n-7; 20:1n-5; 22:1n-11; 22:1n-9.

[3]16:3n-3; 18:3n-3; 18:4n-3; 20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3.

[4]18:2n-6; 18:3n-6; 20:2n-6; 20:3n-6; 20:4n-6; 22:4n-6; 22:5n-6

[5]18:1n-9; 18:2n-9; 20:1n-9; 20:2n-9; 20:3n-9

[6]20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

[7]18:2n-9; 18:2n-6; 18:2n-4; 18:3n-6; 18:3n-4; 18:4n-3; 18:4n-1; 20:2n-9; 20:2n-6; 20:3n-9; 20:3n-6; 20:4n-6; 20:3n-3, 20:4n-3; 20:5n-3; 22:4n-6, 22:5n-6; 22:5n-3; 22:6n-3

Table 4.7. Liver lipids, moisture and fatty acids composition from tilapias fed with hydrolysed banana pseudo-stem inclusion in Experiment I.

	Diet				
	Control	**BP2.5**	**BP5**	**BP15**	**BP20**
Lipid (% dw)	34.42 ± 6.52[a]	22.64 ± 2.16[b]	17.80 ± 1.36[b]	20.47 ± 3.44[b]	18.71 ± 3.03[b]
Moisture (% dw)	72.54 ± 0.89	76.59 ± 1	75.91 ± 1.34	76.37 ± 0.49	72.65 ± 1.13
Fatty acid					
14:00	1.27 ± 0.21[a]	0.39 ± 0.06[b]	0.38 ± 0.09[b]	0.45 ± 0.05[b]	0.69
14:1n-7	0.08 ± 0.00[ab]	0.12 ± 0.01[a]	0.12 ± 0.04[a]	0.06 ± 0.01[b]	0.02
14:1n-5	0.06 ± 0.01[a]	0.02 ± 0.00[b]	0.04 ± 0.01[ab]	0.03 ± 0.02[ab]	0.09
15:00	0.18 ± 0.03	0.13 ± 0.02	0.16 ± 0.01	0.16 ± 0.03	0.19
15:1n-5	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.02
16:OISO	0.05 ± 0.01[a]	0.03 ± 0.00[b]	0.04 ± 0.00[ab]	0.03 ± 0.01[ab]	0.09
16:00	17.21 ± 2.43[a]	16.60 ± 1.44[b]	15.18 ± 0.41[b]	15.15 ± 0.49[b]	11.54
16:1n-7	2.78 ± 0.21[a]	0.66 ± 0.11[b]	0.85 ± 0.12[b]	0.91 ± 0.11[b]	1.65
16:1n-5	0.21 ± 0.04	0.14 ± 0.02	0.20 ± 0.04	0.17 ± 0.05	0.41
16:2n-6	0.02 ± 0.02	0.01 ± 0.01	0.02 ± 0.02	0.01 ± 0.00	0.00
16:2n-4	0.08 ± 0.02	0.04 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.06
17:00	0.05 ± 0.02	0.04 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05
16:3n-4	0.21 ± 0.04[a]	0.08 ± 0.02[b]	0.11 ± 0.01[b]	0.10 ± 0.02[b]	0.24
16:3n-3	0.09 ± 0.03	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.12
16:3n-1	0.19 ± 0.02[b]	0.56 ± 0.00[a]	0.56 ± 0.17[a]	0.45 ± 0.08[a]	0.03
16:4n-3	0.23 ± 0.07[b]	0.74 ± 0.04[a]	0.63 ± 0.21[a]	0.59 ± 0.13[a]	0.04
16:4n-1	0.03 ± 0.02	0.05 ± 0.01	0.07 ± 0.02	0.04 ± 0.01	0.01
18:00	11.10 ± 0.38[b]	14.98 ± 1.32[a]	13.74 ± 1.41[ab]	11.55 ± 0.48[b]	10.79
18:1n-9	28.06 ± 1.56[a]	15.08 ± 2.26[b]	17.14 ± 1.56[b]	14.22 ± 1.25[b]	17.67
18:1n-7	2.62 ± 0.33	2.90 ± 0.13	2.97 ± 0.17	2.93 ± 0.33	2.35
18:1n-5	0.10 ± 0.02[a]	0.06 ± 0.01[b]	0.07 ± 0.01[ab]	0.06 ± 0.01[b]	0.11
18:2n-9	0.12 ± 0.02[a]	0.07 ± 0.01[b]	0.09 ± 0.01[ab]	0.05 ± 0.00[b]	0.06
18:2n-6	12.58 ± 0.53[a]	9.80 ± 0.54[b]	10.84 ± 1.35[ab]	11.16 ± 1.21[ab]	11.64
18:2n-4	0.11 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.14
18:3n-6	0.22 ± 0.04	0.17 ± 0.08	0.14 ± 0.02	0.15 ± 0.01	0.26
18:3n-4	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.00	0.07
18:3n-3	7.83 ± 0.89[ab]	4.03 ± 0.51[c]	5.44 ± 1.08[bc]	8.77 ± 1.17[a]	11.46

18:3n-1	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.01 ± 0.00	0.02
18:4n-3	0.11 ± 0.04	0.08 ± 0.01	0.13 ± 0.02	0.14 ± 0.02	0.17
18:4n-1	0.03 ± 0.01	0.02 ± 0.00	0.04 ± 0.01	0.03 ± 0.00	0.05
20:00	0.30 ± 0.05^{b}	0.40 ± 0.06^{ab}	0.49 ± 0.07^{a}	0.36 ± 0.01^{ab}	0.24
20:1n-9	0.08 ± 0.01	0.07 ± 0.03	0.10 ± 0.02	0.07 ± 0.01	0.07
20:1n-7	0.91 ± 0.11	0.83 ± 0.10	0.97 ± 0.15	0.72 ± 0.10	0.76
20:1n-5	0.10 ± 0.01	0.11 ± 0.03	0.12 ± 0.03	0.10 ± 0.00	0.10
20:2n-9	0.07 ± 0.01	0.05 ± 0.01	0.08 ± 0.04	0.04 ± 0.00	0.05
20:2n-6	0.66 ± 0.12^{b}	1.07 ± 0.06^{a}	1.09 ± 0.07^{a}	0.99 ± 0.06^{a}	0.95
20:3n-9	0.03 ± 0.01^{c}	0.05 ± 0.01^{a}	0.04 ± 0.00^{bc}	0.04 ± 0.00^{ab}	0.05
20:3n-6	0.41 ± 0.06^{b}	0.81 ± 0.11^{a}	0.73 ± 0.03^{a}	0.73 ± 0.06^{a}	0.55
20:4n-6	1.20 ± 0.19^{b}	2.60 ± 0.23^{a}	2.37 ± 0.23^{a}	2.03 ± 0.24^{a}	2.03
20:3n-3	1.42 ± 0.35^{b}	1.98 ± 0.30^{ab}	2.21 ± 0.20^{a}	2.55 ± 0.22^{a}	3.57
20:4n-3	0.22 ± 0.05^{c}	0.30 ± 0.04^{bc}	0.38 ± 0.02^{ab}	0.42 ± 0.05^{a}	0.52
20:5n-3	0.67 ± 0.21^{b}	1.50 ± 0.22^{a}	1.74 ± 0.32^{a}	1.72 ± 0.17^{a}	1.13
22:1n-11	0.09 ± 0.03^{b}	0.13 ± 0.02^{b}	0.24 ± 0.05^{a}	0.12 ± 0.03^{b}	0.09
22:1n-9	0.23 ± 0.02	0.33 ± 0.06	0.40 ± 0.04	0.32 ± 0.09	0.23
22:4n-6	0.18 ± 0.04^{c}	0.44 ± 0.05^{a}	0.33 ± 0.04^{b}	0.36 ± 0.04^{ab}	0.29
22:5n-6	0.25 ± 0.07^{b}	0.73 ± 0.07^{a}	0.73 ± 0.10^{a}	0.58 ± 0.05^{a}	0.37
22:5n-3	1.10 ± 0.30^{b}	3.20 ± 0.54^{a}	3.38 ± 0.31^{a}	4.01 ± 0.57^{a}	2.87
22:6n-3	6.37 ± 2.18^{b}	18.31 ± 2.57^{a}	15.23 ± 1.61^{a}	17.27 ± 3.73^{a}	16.09
[1]Saturades	30.11 ± 3.06	32.54 ± 2.81	30.01 ± 1.60	27.72 ± 0.89	23.50
[2]Monoenoics	35.12 ± 1.59^{a}	20.15 ± 2.36^{b}	22.83 ± 1.93^{b}	19.41 ± 1.42^{b}	23.33
[3]∑n-3	18.05 ± 3.78^{b}	30.22 ± 4.12^{a}	29.22 ± 2.76^{a}	35.52 ± 2.85^{a}	35.97
[4]∑n-6	15.52 ± 0.87	15.64 ± 0.90	16.25 ± 1.28	16.00 ± 0.97	16.10
[5]∑n-9	28.59 ± 1.56^{a}	15.65 ± 2.30^{b}	17.84 ± 1.63^{b}	14.75 ± 1.17^{b}	18.13
[6]∑n-3 HUFA	9.78 ± 3.01^{b}	25.29 ± 3.63^{a}	22.94 ± 2.06^{a}	25.96 ± 4.03^{a}	24.18
ARA/EPA	1.80 ± 0.25^{a}	1.74 ± 0.12^{a}	1.36 ± 0.21^{ab}	1.18 ± 0.05^{b}	1.80
DHA/EPA	9.56 ± 2.18	12.21 ± 0.65	8.74 ± 1.99	10.06 ± 1.29	14.27
DHA/ARA	5.33 ± 1.15^{b}	7.03 ± 0.67^{ab}	6.42 ± 0.42^{ab}	8.52 ± 1.26^{a}	7.94
[7]Total PUFA	34.49 ± 4.56^{b}	46.95 ± 4.98^{a}	46.72 ± 3.57^{a}	52.52 ± 2.01^{a}	52.84
n-3/n-6	1.16 ± 0.19^{b}	1.93 ± 0.17^{a}	1.80 ± 0.17^{a}	2.22 ± 0.30^{a}	2.23

Different letter indicates significant differences (p-value<0.05)

[1]14:00,15:00, 16:00, 17:00, 18:00, 20:00

[2]14:1n-7; 14:1n-5; 15:1n-5; 16:1n-7 16:1n-5; 18:1n-9; 18:1n-7; 18:1n-5; 20:1n-9; 20:1n-7; 20:1n-5; 22:1n-11; 22:1n-9.

[3]16:3n-3; 18:3n-3; 18:4n-3; 20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3.

[4]18:2n-6; 18:3n-6; 20:2n-6; 20:3n-6; 20:4n-6; 22:4n-6; 22:5n-6

[5]18:1n-9; 18:2n-9; 20:1n-9; 20:2n-9; 20:3n-9

[6]20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

[7]18:2n-9; 18:2n-6; 18:2n-4; 18:3n-6; 18:3n-4; 18:4n-3; 18:4n-1; 20:2n-9; 20:2n-6; 20:3n-9; 20:3n-6; 20:4n-6; 20:3n-3, 20:4n-3; 20:5n-3; 22:4n-6, 22:5n-6; 22:5n-3; 22:6n-3

Table 4.8. Muscle proximate composition and fatty acids content from tilapias fed with banana flower inclusion in Experiment II.

	Diet				
	Control	**BF0.2**	**BF0.5**	**BF1**	**BF3**
Lipid (% dw)	6.34 ± 0.87	5.65 ± 0.37	6.06 ± 0.88	6.37 ± 0.82	5.41 ± 0.45
Ash (%dw)	7.22 ± 0.69	8.12 ± 0.94	7.29 ± 0.16	7.42 ± 0.57	8.64 ± 0.26
Protein (%dw)	91.71 ± 6.34	91.55 ± 0.77	90.21 ± 4.3	93.16 ± 2.28	93.34 ± 0.48
Moisture (% dw)	78.93 ± 0.63	78.91 ± 0.94	79.95 ± 0.16	79.63 ± 0.26	79.32 ± 0.67
Fatty acid (%)					
14:00	0.59 ± 0.38	0.67 ± 0.29	1.01 ± 0.47	0.52 ± 0.35	0.46 ± 0.36
14:1n-7	0.10 ± 0.09	0.54 ± 0.58	0.18 ± 0.09	0.20 ± 0.25	0.11 ± 0.14
14:1n-5	0.11 ± 0.12	0.41 ± 0.39	0.05 ± 0.03	0.06 ± 0.03	0.07 ± 0.06
15:00	0.23 ± 0.14	0.47 ± 0.40	0.20 ± 0.05	0.14 ± 0.08	0.18 ± 0.12
15:1n-5	0.13 ±0.16	0.36 ± 0.31	0.06 ± 0.03	0.05 ± 0.03	0.07 ± 0.06
16:OISO	0.14 ± 0.17	0.34 ± 0.24	0.06 ± 0.04	0.06 ± 0.04	0.07 ± 0.06
16:00	16.03 ± 7.12	13.14 ± 1.09	16.96 ± 5.51	12.45 ± 6.07	16.61 ± 4.81
16:1n-7	0.82 ± 0.49	1.20 ± 0.20	1.42 ± 0.80	1.10 ± 0.63	0.97 ± 0.37
16:1n-5	0.18 ± 0.10	0.56 ± 0.53	0.16 ± 0.05	0.16 ± 0.11	0.19 ± 0.14
16:2n-4	0.28 ± 0.20	0.78 ± 0.47	0.16 ± 0.04	0.15 ± 0.03	0.17 ± 0.04
17:00	0.13 ± 0.14	0.88 ± 1.01	0.05 ± 0.02	0.08 ± 0.04	0.07 ± 0.03
16:3n-4	0.17 ± 0.11	1.18 ± 1.34	0.17 ± 0.09	0.19 ± 0.06	0.14 ± 0.04
16:3n-3	0.14 ± 0.11	0.49 ± 0.35	0.08 ± 0.03	0.07 ± 0.03	0.08 ± 0.03
16:3n-1	0.89 ± 0.27	0.86 ± 0.22	0.39 ± 0.08	0.47 ± 0.25	0.68 ± 0.17
16:4n-3	1.21 ± 0.27	1.35 ± 0.17	0.65 ± 0.11	0.70 ± 0.35	1.08 ± 0.24
16:4n-1	0.12 ± 0.12	0.55 ± 0.34	0.08 ± 0.03	0.09 ± 0.04	0.12 ± 0.02
18:00	12.07 ± 4.96	8.48 ± 0.42	9.85 ± 1.79	10.82 ± 2.10	11.65 ± 2.06
18:1n-9	12.55 ± 3.79	11.27 ± 3.44	15.96 ± 4.16	15.42 ± 2.14	12.74 ± 2.56
18:1n-7	2.67 ± 0.90	2.61 ± 1.01	2.44 ± 0.50	2.82 ± 0.39	2.77 ± 0.26
18.1n-5	0.16 ± 0.12	0.52 ± 0.30	0.08 ± 0.01	0.10 ± 0.04	0.09 ± 0.02
18:2n-9	0.18 ± 0.18	0.81 ± 0.61	0.14 ± 0.03	0.13 ± 0.00	0.10 ± 0.03
18.2n-6	9.36 ± 1.66	8.74 ± 1.80	11.75 ± 1.97	12.38 ± 0.93	10.67 ± 1.04
18:2n-4	0.23 ± 0.16	0.75 ± 0.66	0.12 ± 0.04	0.14 ± 0.07	0.19 ± 0.04
18:3n-6	0.67 ± 0.06	1.88 ± 0.14	1.16 ± 0.58	0.82 ± 0.17	0.68 ± 0.08
18:3n-4	0.16 ± 0.18	0.53 ± 0.41	0.07 ± 0.03	0.09 ± 0.04	0.08 ± 0.03
18:3n-3	5.24 ± 0.29	4.98 ± 1.79	8.00 ± 1.78	8.98 ± 1.88	7.13 ± 1.70
18:4n-3	0.25 ± 0.19	0.86 ± 0.63	0.25 ± 0.05	0.30 ± 0.09	0.28 ± 0.06
18:4n-1	0.15 ± 0.15	0.55 ± 0.49	0.06 ± 0.01	0.08 ± 0.05	0.06 ± 0.04
20:00	0.41 ± 0.08	0.24 ± 0.34	0.33 ± 0.04	0.33 ± 0.13	0.27 ± 0.08
20:1n-9	0.22 ± 0.19	0.71 ± 0.54	0.10 ± 0.03	0.11 ± 0.03	0.10 ± 0.06
20:1n-7	0.99 ± 0.07	1.68 ± 0.68	0.91 ± 0.03	0.90 ± 0.25	0.77 ± 0.23
20.1n-5	0.21 ± 0.16	0.62 ± 0.54	0.09 ± 0.04	0.09 ± 0.04	0.07 ± 0.03
20:2n-9	0.16 ± 0.13	0.84 ± 0.38	0.17 ± 0.08	0.11 ± 0.03	0.09 ± 0.05
20:2n-6	1.13 ± 0.13	1.28 ± 0.55	0.96 ± 0.24	1.01 ± 0.28	0.98 ± 0.20
20:3n-9	1.11 ± 0.26	1.31 ± 0.35	0.95 ± 0.12	1.18 ± 0.14	0.99 ± 0.04
20:3n-6	0.25 ± 0.25	0.82 ± 0.30	0.10 ± 0.04	0.12 ± 0.08	0.10 ± 0.09
20:4n-6	2.85 ± 0.67	2.64 ± 0.75	2.53 ± 0.60	3.44 ± 0.49	3.03 ± 0.72
20:3n-3	1.94 ± 0.35	1.77 ± 0.07	1.85 ± 0.55	1.98 ± 0.47	1.99 ± 0.17
20:4n-3	0.38 ± 0.08	1.19 ± 0.90	0.36 ± 0.01	0.52 ± 0.12	0.38 ± 0.18

20:5n-3	0.79 ± 0.29	1.52 ± 0.09	0.88 ± 0.38	1.00 ± 0.05	0.92 ± 0.40
22:1n-11	0.36 ± 0.25	0.97 ± 0.68	0.24 ± 0.09	0.23 ± 0.09	0.21 ± 0.10
22:1n-9	0.73 ± 0.28	0.83 ± 0.49	0.40 ± 0.03	0.43 ± 0.19	0.45 ± 0.08
22:4n-6	1.17 ± 0.67	1.54 ± 0.91	0.79 ± 0.24	0.95 ± 0.14	0.75 ± 0.23
22:5n-6	1.47 ± 0.87	2.06 ± 0.87	1.01 ± 0.53	1.15 ± 0.14	1.01 ± 0.21
22:5n-3	3.29 ± 2.34	2.75 ± 0.94	2.43 ± 1.28	2.87 ± 0.29	3.02 ± 1.21
22:6n-3	17.58 ± 14.61	11.48 ± 7.91	14.33 ± 12.3	14.99 ± 0.97	17.38 ± 8.09
[1]Saturades	29.61 ± 12.06	24.21 ± 2.28	28.46 ± 7.61	24.4 ± 5.06	29.3 ± 7.43
[2]Monoenoics	19.96 ± 4.79	23.11 ± 1.29	22.49 ± 5.53	22.11 ± 2.48	19.05 ± 3.54
[3]∑n-3	30.82 ± 17.02	26.39 ± 8.57	28.84 ± 12.89	31.41 ± 1.97	32.25 ± 10.38
[4]∑n-6	16.90 ± 0.6	18.96 ± 0.23	18.3 ± 0.25	19.87 ± 1.89	17.21 ± 0.16
[5]∑n-9	14.95 ± 3.53	15.78 ± 1.07	17.73 ± 4.14	17.39 ± 2.31	14.48 ± 2.69
[6]∑n-3 HUFA	23.98 ± 17.53	18.7 ± 7.93	19.85 ± 14.51	21.36 ± 0.48	23.69 ± 9.65
ARA/EPA	3.70 ± 0.45	1.76 ± 0.59	3.01 ± 0.51	3.42 ± 0.34	3.60 ± 1.04
DHA/EPA	20.18 ± 9.83	7.71 ± 5.65	14.5 ± 6.17	14.98 ± 1.64	18.99 ± 3.5
DHA/ARA	5.68 ± 3.41	4.08 ± 1.84	5.17 ± 3.23	4.44 ± 0.91	5.51 ± 1.53
[7]Total PUFA	51.17 ± 16.75	53.51 ± 3.07	49.45 ± 13	53.92 ± 2.7	52.1 ± 10.22
n-3/n-6	1.81 ± 0.94	1.39 ± 0.47	1.57 ± 0.68	1.59 ± 0.21	1.88 ± 0.61

Different letter indicates significant differences (p-value<0.05)

[1]14:00,15:00, 16:00, 17:00, 18:00, 20:00

[2]14:1n-7; 14:1n-5; 15:1n-5; 16:1n-7 16:1n-5; 18:1n-9; 18:1n-7; 18:1n-5; 20:1n-9; 20:1n-7; 20:1n-5; 22:1n-11; 22:1n-9.

[3]16:3n-3; 18:3n-3; 18:4n-3; 20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3.

[4]18:2n-6; 18:3n-6; 20:2n-6; 20:3n-6; 20:4n-6; 22:4n-6; 22:5n-6

[5]18:1n-9; 18:2n-9; 20:1n-9; 20:2n-9; 20:3n-9

[6]20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

[7]18:2n-9; 18:2n-6; 18:2n-4; 18:3n-6; 18:3n-4; 18:4n-3; 18:4n-1; 20:2n-9; 20:2n-6; 20:3n-9; 20:3n-6; 20:4n-6; 20:3n-3, 20:4n-3; 20:5n-3; 22:4n-6, 22:5n-6; 22:5n-3; 22:6n-3

Table 4.9. Liver lipids, moisture and fatty acids content from tilapias fed with banana flower inclusion in Experiment II.

	Diet				
	Control	**BF0.2**	**BF0.5**	**BF1**	**BF3**
Lipid (% dw)	17.02 ± 2.53	21.22 ± 4.45	24.28 ± 2.12	20.76 ± 2.35	22.84 ± 1.42
Moisture (% dw)	70.71 ± 5.33	68.06 ± 3.47	72.25 ± 5.60	70.86 ± 1.19	71.77 ± 1.01
Fatty acid (%)					
14:00	0.74 ± 0.37	0.91 ± 0.19	1.27 ± 0.63	1.49 ± 0.09	1.09 ± 0.49
14:1n-7	0.14 ± 0.22	0.03 ± 0.01	0.05 ± 0.03	0.06 ± 0.01	0.04 ± 0.02
14:1n-5	0.24 ± 0.35	0.03 ± 0.01	0.03 ± 0.00	0.04 ± 0.02	0.04 ± 0.02
15:00	0.21 ± 0.18	0.08 ± 0.01	0.13 ± 0.07	0.13 ± 0.03	0.15 ± 0.08
15:1n-5	0.11 ± 0.17	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.05 ± 0.02
16:OISO	0.03 ± 0.03	0.06 ± 0.03	0.04 ± 0.00	0.05 ± 0.01	0.05 ± 0.01
16:00	9.38 ± 0.98	10.42 ± 2.69	12.99 ± 3.67	13.46 ± 0.53	10.91 ± 2.03
16:1n-7	1.81 ± 0.79	3.15 ± 0.56	3.37 ± 1.72	3.98 ± 0.19	2.76 ± 1.00
16:1n-5	0.28 ± 0.11	0.13 ± 0.03	0.13 ± 0.03	0.21 ± 0.06	0.19 ± 0.05
16:2n-4	0.09 ± 0.07	0.14 ± 0.00	0.13 ± 0.01	0.14 ± 0.02	0.16 ± 0.02

17:00	0.14 ± 0.17	0.05 ± 0.02	0.06 ± 0.01	0.05 ± 0.00	0.06 ± 0.01
16:3n-4	0.29 ± 0.19	0.24 ± 0.01	0.25 ± 0.04	0.25 ± 0.05	0.24 ± 0.05
16:3n-3	0.23 ± 0.24[a]	0.08 ± 0.00[ab]	0.07 ± 0.01[ab]	0.07 ± 0.01[b]	0.07 ± 0.02[ab]
16:3n-1	0.19 ± 0.26[a]	0.05 ± 0.04[ab]	0.04 ± 0.01[ab]	0.03 ± 0.01[b]	0.03 ± 0.01[ab]
16:4n-3	0.25 ± 0.28[a]	0.05 ± 0.02[ab]	0.05 ± 0.02[ab]	0.06 ± 0.01[b]	0.06 ± 0.02[ab]
16:4n-1	0.01 ± 0.01	0.02 ± 0.02	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.02
18:00	10.88 ± 3.55	9.35 ± 2.11	8.07 ± 1.09	9.21 ± 0.38	9.18 ± 0.63
18:1n-9	18.28 ± 2.58	25.48 ± 0.73	27.2 ± 5.52	26.01 ± 1.45	22.86 ± 1.30
18:1n-7	2.35 ± 0.30	2.68 ± 0.10	2.49 ± 0.53	2.69 ± 0.16	2.29 ± 0.32
18.1n-5	0.23 ± 0.21	0.11 ± 0.02	0.09 ± 0.02	0.11 ± 0.01	0.10 ± 0.01
18:2n-9	0.25 ± 0.18	0.18 ± 0.00	0.17 ± 0.08	0.24 ± 0.04	0.14 ± 0.03
18.2n-6	8.71 ± 0.36	9.77 ± 0.70	10.50 ± 2.49	8.75 ± 0.71	11.16 ± 1.49
18:2n-4	0.18 ± 0.21	0.08 ± 0.03	0.06 ± 0.01	0.04 ± 0.01	0.07 ± 0.02
18:3n-6	0.77 ± 0.07	0.7 ± 0.06	0.69 ± 0.21	0.70 ± 0.11	0.70 ± 0.15
18:3n-4	0.16 ± 0.18	0.08 ± 0.02	0.08 ± 0.02	0.05 ± 0.01	0.05 ± 0.02
18:3n-3	5.75 ± 0.58	7.21 ± 1.98	7.21 ± 2.19	5.9 ± 0.78	8.75 ± 2.00
18:4n-3	0.34 ± 0.21	0.25 ± 0.11	0.21 ± 0.10	0.19 ± 0.02	0.22 ± 0.08
18:4n-1	0.16 ± 0.24	0.05 ± 0.05	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.02
20:00	0.43 ± 0.26	0.28 ± 0.02	0.25 ± 0.07	0.23 ± 0.02	0.25 ± 0.10
20:1n-9	0.26 ± 0.31	0.17 ± 0.08	0.15 ± 0.03	0.12 ± 0.01	0.12 ± 0.03
20:1n-7	1.17 ± 0.38	1.42 ± 0.11	1.25 ± 0.06	1.08 ± 0.13	1.04 ± 0.33
20.1n-5	0.17 ± 0.17	0.11 ± 0.04	0.08 ± 0.01	0.07 ± 0.01	0.09 ± 0.03
20:2n-9	0.25 ± 0.31	0.16 ± 0.05	0.12 ± 0.04	0.13 ± 0.03	0.08 ± 0.03
20:2n-6	1.10 ± 0.12	0.93 ± 0.06	0.87 ± 0.39	0.71 ± 0.09	0.93 ± 0.21
20:3n-9	1.08 ± 0.09	1.14 ± 0.03	0.84 ± 0.13	1.01 ± 0.04	0.92 ± 0.11
20:3n-6	0.17 ± 0.21	0.07 ± 0.03	0.10 ± 0.03	0.07 ± 0.05	0.08 ± 0.02
20:4n-6	4.38 ± 1.25	3.14 ± 0.42	2.64 ± 0.63	3.29 ± 0.15	3.24 ± 0.24
20:3n-3	2.31 ± 0.71	2.15 ± 0.27	2.05 ± 1.01	1.76 ± 0.29	2.60 ± 0.33
20:4n-3	0.59 ± 0.07	0.59 ± 0.04	0.43 ± 0.14	0.51 ± 0.13	0.51 ± 0.11
20:5n-3	1.14 ± 0.17[a]	0.76 ± 0.05[b]	0.56 ± 0.06[b]	0.52 ± 0.06[b]	0.78 ± 0.16[ab]
22:1n-11	0.72 ± 1.04	0.19 ± 0.04	0.17 ± 0.05	0.10 ± 0.01	0.20 ± 0.17
22:1n-9	0.47 ± 0.68	0.12 ± 0.02	0.10 ± 0.03	0.10 ± 0.02	0.13 ± 0.05
22:4n-6	1.09 ± 0.43	0.70 ± 0.12	0.50 ± 0.14	0.72 ± 0.12	0.60 ± 0.17
22:5n-6	1.28 ± 0.39	0.73 ± 0.02	0.56 ± 0.19	0.72 ± 0.10	0.68 ± 0.29
22:5n-3	2.33 ± 0.11	2.04 ± 0.58	1.42 ± 0.30	1.92 ± 0.42	1.78 ± 0.38
22:6n-3	18.89 ± 4.67	13.92 ± 2.12	12.42 ± 4.08	12.94 ± 0.47	14.5 ± 3.47
[1]Saturades	21.80 ± 1.76	21.15 ± 4.93	22.82 ± 5.15	24.63 ± 0.27	21.70 ± 3.09
[2]Monoenoics	26.70 ± 6.99	33.78 ± 1.06	35.25 ± 7.50	34.71 ± 1.91	30.03 ± 2.08
[3]∑n-3	31.82 ± 5.36	27.03 ± 5.19	24.43 ± 6.57	23.87 ± 1.45	29.28 ± 1.86
[4]∑n-6	17.51 ± 0.74	16.03 ± 0.57	15.85 ± 3.39	14.97 ± 0.90	17.40 ± 0.83
[5]∑n-9	20.58 ± 3.54	27.24 ± 0.55	28.59 ± 5.57	27.60 ± 1.51	24.25 ± 1.51
[6]∑n-3 HUFA	25.25 ± 5.39	19.44 ± 3.07	16.9 ± 5.30	17.66 ± 0.68	20.18 ± 3.92
ARA/EPA	3.84 ± 1.37	4.15 ± 0.83	4.70 ± 1.20	6.29 ± 0.44	4.15 ± 0.64
DHA/EPA	16.53 ± 4.70	18.38 ± 1.56	22.12 ± 6.88	24.71 ± 3.33	18.54 ± 7.16
DHA/ARA	4.31 ± 0.35	4.43 ± 1.28	4.70 ± 0.57	3.93 ± 0.28	4.47 ± 0.99
[7]Total PUFA	51.98 ± 4.91	45.19 ± 6.01	42.03 ± 9.93	40.75 ± 1.93	48.4 ± 1.19
n-3/n-6	1.82 ± 0.23	1.69 ± 0.26	1.54 ± 0.10	1.59 ± 0.10	1.68 ± 0.19

Different letter indicates significant differences (p-value<0.05)

[1]14:00,15:00, 16:00, 17:00, 18:00, 20:00

[2]14:1n-7; 14:1n-5; 15:1n-5; 16:1n-7 16:1n-5; 18:1n-9; 18:1n-7; 18:1n-5; 20:1n-9; 20:1n-7; 20:1n-5; 22:1n-11; 22:1n-9.

[3]16:3n-3; 18:3n-3; 18:4n-3; 20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3.

[4]18:2n-6; 18:3n-6; 20:2n-6; 20:3n-6; 20:4n-6; 22:4n-6; 22:5n-6

[5]18:1n-9; 18:2n-9; 20:1n-9; 20:2n-9; 20:3n-9

[6]20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

[7]18:2n-9; 18:2n-6; 18:2n-4; 18:3n-6; 18:3n-4; 18:4n-3; 18:4n-1; 20:2n-9; 20:2n-6; 20:3n-9; 20:3n-6; 20:4n-6; 20:3n-3, 20:4n-3; 20:5n-3; 22:4n-6, 22:5n-6; 22:5n-3; 22:6n-3

Table 4.10. Muscle proximate composition and fatty acids content from tilapias fed with banana flower extract and the residue inclusion in Experiment III.

	DIET			
	Control	**BF3**	**BF3R**	**BF3E**
Lipid (% dw)	8.00 ± 0.71	8.13 ± 1.14	9.79 ± 1.48	6.67 ± 3.24
Ash (%dw)	5.11 ± 5.62	6.02 ± 5.81	5.17 ± 2.07	7.40 ± 8.59
Protein (%dw)	92.51 ± 0.73	96.34 ± 0.55	89.19 ± 0.09	85.82 ± 2.33
Moisture (% dw)	79.77 ± 1.50^{a}	79.11 ± 1.53^{ab}	79.58 ± 0.63^{ab}	76.90 ± 2.75^{b}
Fatty acid (%)				
14:00	0.15 ± 0.05	0.20 ± 0.03	0.29 ± 0.12	0.09 ± 0.02
14:1n-5	0.05 ± 0.04	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01
14:1n-7	0.06 ± 0.05	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.01
15:00	0.08 ± 0.00	0.06 ± 0.01	0.08 ± 0.02	0.03 ± 0.01
15:1n-5	0.04 ± 0.04	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01
16:OISO	0.05 ± 0.03	0.01 ± 0.01	0.02 ± 0.00	0.01 ± 0.01
16:00	10.23 ± 4.54	12.39 ± 2.5	13.73 ± 1.74	7.31 ± 1.31
16:1n-7	1.01 ± 0.47	1.33 ± 0.01	1.31 ± 0.24	0.85 ± 0.07
16:1n-5	0.09 ± 0.00	0.07 ± 0.01	0.11 ± 0.02	0.08 ± 0.03
16:2n-4	0.05 ± 0.03	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
17:00	0.07 ± 0.03	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.01
16:3n-4	0.15 ± 0.01	0.13 ± 0.00	0.13 ± 0.01	0.13 ± 0.01
16:3n-3	0.08 ± 0.02	0.04 ± 0.01	0.03 ± 0.00	0.04 ± 0.01
16:3n-1	0.60 ± 0.10	0.50 ± 0.07	0.48 ± 0.05	0.49 ± 0.06
16:4n-3	0.96 ± 0.10	0.88 ± 0.19	0.88 ± 0.09	0.87 ± 0.04
16:4n-1	0.07 ± 0.01	0.06 ± 0.01	0.04 ± 0.03	0.06 ± 0.00
18:00	11.93 ± 0.64	9.28 ± 1.74	10.97 ± 1.35	10.41 ± 0.63
18:1n-9	24.46 ± 0.97^{a}	20.34 ± 1.93^{b}	22.87 ± 1.53^{ab}	25.00 ± 0.77^{a}
18:1n-7	3.47 ± 0.21	3.51 ± 0.79	3.32 ± 0.41	2.96 ± 0.03
18:1n-5	0.12 ± 0.03	0.07 ± 0.00	0.10 ± 0.01	0.07 ± 0.01
18:2n-9	0.23 ± 0.05	0.17 ± 0.02	0.22 ± 0.02	0.16 ± 0.03
18:2n-6	12.04 ± 0.18^{a}	11.64 ± 0.80^{a}	11.92 ± 0.51^{a}	13.96 ± 0.26^{ab}
18:2n-4	0.12 ± 0.04	0.08 ± 0.01	0.07 ± 0.01	0.11 ± 0.01
18:3n-6	0.68 ± 0.18	0.58 ± 0.01	0.55 ± 0.05	0.62 ± 0.02
18:3n-4	0.09 ± 0.04	0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
18:3n-3	8.00 ± 0.28^{a}	9.50 ± 1.02^{a}	8.86 ± 0.51^{a}	14.85 ± 1.10^{b}
18.3n-1	0.05 ± 0.04	0.01 ± 0.00	0.01 ± 0.01	0.02 ± 0.00
18:4n-3	0.28 ± 0.06	0.38 ± 0.07	0.23 ± 0.01	0.37 ± 0.04
18:4n-1	0.09 ± 0.06	0.02 ± 0.00	0.04 ± 0.02	0.04 ± 0.02
20:00	0.48 ± 0.11	0.30 ± 0.01	0.37 ± 0.05	0.39 ± 0.05
20:1n-9	0.18 ± 0.01	0.13 ± 0.05	0.12 ± 0.01	0.14 ± 0.02
20:1n-7	1.78 ± 0.09^{a}	1.33 ± 0.16^{b}	1.44 ± 0.14^{b}	1.37 ± 0.13^{b}

20.1n-5	0.11 ± 0.03	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.00
20:2n-9	0.18 ± 0.05	0.12 ± 0.03	0.12 ± 0.02	0.09 ± 0.01
20:2n-6	0.99 ± 0.11	0.7 ± 0.01	0.90 ± 0.11	0.83 ± 0.04
20:3n-9	0.01 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.03 ± 0.02
20:3n-6	1.07 ± 0.13	1.00 ± 0.04	1.12 ± 0.03	0.96 ± 0.02
20:4n-6	2.41 ± 0.43^{ab}	2.78 ± 0.45^{a}	2.36 ± 0.11^{ab}	1.82 ± 0.10^{b}
20:3n-3	1.54 ± 0.17^{a}	1.30 ± 0.02^{a}	1.53 ± 0.02^{b}	1.98 ± 0.09^{b}
20:4n-3	0.51 ± 0.10	0.54 ± 0.06	0.46 ± 0.04	0.66 ± 0.05
20:5n-3	1.04 ± 0.21	1.25 ± 0.03	0.84 ± 0.08	0.88 ± 0.08
22:1n-11	0.47 ± 0.03	0.40 ± 0.26	0.24 ± 0.02	0.24 ± 0.07
22:1n-9	0.46 ± 0.09	0.45 ± 0.04	0.40 ± 0.09	0.38 ± 0.03
22:4n-6	0.67 ± 0.13	0.76 ± 0.03	0.67 ± 0.06	0.45 ± 0.05
22:5n-6	0.82 ± 0.16^{ab}	1.03 ± 0.04^{a}	0.89 ± 0.10^{a}	0.57 ± 0.05^{b}
22:5n-3	1.99 ± 0.31^{a}	2.70 ± 0.14^{b}	2.14 ± 0.35^{a}	2.15 ± 0.07^{a}
22:6n-3	10.01 ± 2.06^{ab}	13.73 ± 1.28^{a}	9.89 ± 0.97^{ab}	8.30 ± 0.51^{b}
[1]Saturades	22.97 ± 3.78	22.28 ± 4.28	25.49 ± 0.47	18.27 ± 1.75
[2]Monoenoics	32.30 ± 0.91^{a}	27.74 ± 1.64^{b}	30.03 ± 1.73^{ab}	31.21 ± 0.89^{ab}
[3]∑n-3	24.39 ± 3.31^{a}	30.31 ± 2.36^{b}	24.87 ± 1.77^{a}	30.09 ± 1.52^{b}
[4]∑n-6	18.69 ± 0.95	18.50 ± 0.33	18.41 ± 0.41	19.22 ± 0.37
[5]∑n-9	25.52 ± 0.79^{a}	21.23 ± 2.04^{b}	23.74 ± 1.46^{ab}	25.8 ± 0.82^{a}
[6]∑n-3 HUFA	15.09 ± 2.85^{ab}	19.51 ± 1.47^{a}	14.87 ± 1.41^{b}	13.96 ± 0.48^{b}
ARA/EPA	2.32 ± 0.05^{a}	2.22 ± 0.31^{a}	2.82 ± 0.16^{b}	2.08 ± 0.12^{a}
DHA/EPA	9.60 ± 0.09	11.01 ± 1.26	11.77 ± 0.51	9.56 ± 1.34
DHA/ARA	4.14 ± 0.12	5.08 ± 1.30	4.18 ± 0.23	4.58 ± 0.46
[7]Total PUFA	44.58 ± 4.69	49.85 ± 2.65	44.34 ± 1.28	50.39 ± 1.24
n-3/n-6	1.30 ± 0.11^{b}	1.64 ± 0.10^{a}	1.35 ± 0.12^{ab}	1.57 ± 0.10^{ab}

Different letter indicates significant differences (p-value<0.05)

[1]14:00,15:00, 16:00, 17:00, 18:00, 20:00

[2]14:1n-7; 14:1n-5; 15:1n-5; 16:1n-7 16:1n-5; 18:1n-9; 18:1n-7; 18:1n-5; 20:1n-9; 20:1n-7; 20:1n-5; 22:1n-11; 22:1n-9.

[3]16:3n-3; 18:3n-3; 18:4n-3; 20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3.

[4]18:2n-6; 18:3n-6; 20:2n-6; 20:3n-6; 20:4n-6; 22:4n-6; 22:5n-6

[5]18:1n-9; 18:2n-9; 20:1n-9; 20:2n-9; 20:3n-9

[6]20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

[7]18:2n-9; 18:2n-6; 18:2n-4; 18:3n-6; 18:3n-4; 18:4n-3; 18:4n-1; 20:2n-9; 20:2n-6; 20:3n-9; 20:3n-6; 20:4n-6; 20:3n-3, 20:4n-3; 20:5n-3; 22:4n-6, 22:5n-6; 22:5n-3; 22:6n-3

Table 4.11. Liver lipids and moisture from tilapias fed with banana flower extract and residue inclusion in Experiment III.

	Diet			
	Control	**BF3**	**BF3R**	**BF3E**
Lipids (%dw)	60.04 ± 18.19	40.99 ± 9.00	54.50 ± 7.81	49.81 ± 11.43
Moisture (%dw)	69.17 ± 0.96	72.57 ± 19.68	66.64 ± 9.03	65.99 ± 5.74

Fatty acids composition

BP inclusion affects the fatty acid profile of both liver and muscle. Up to 5% of inclusion of hydrolysed banana pseudo-stem the levels of EPA, ARA, DHA, n-3, n-6, n-3 HUFA and total PUFA were increased, which contribute to maintaining the same amount of these fatty acids as the control diet in the whole muscle, in compensation to the decreasing amount of the total lipids. Stearic acid, oleic acid and 20:3n-3 increase with BP inclusion in diets (Table 6). In the liver, the inclusion of BP decreases some saturated acids such as myristic and palmitic (14:00 and 16:00), monoenoic and n-9, with a reduction of oleic acid (18:2n-6) but increases total PUFA and n-3/n-6 ratio. In addition, BP2.5 and BP5 decrease the amount of linolenic acid (18:3n-3 HUFA) but increase stearic acid (18:00) (**Table 4.7**).

Regarded dietary banana flower (Experiment II), there was no effect observed in the case of the muscle being the closer results observed among fish from BF3 and the control diets (**Table 4.8**). Similarly, no effect on the livers by BF inclusion apart from a slight reduction in the EPA content among other HUFAs which affect the FAA ratios although not significantly (**Table 4.9**).

In experiment III, the fatty acid profile of the muscle was affected by the experimental diets: all diets with flower and derivatives decreased the fatty acid 20:1n-7 compared to the control diet. In addition, the BF3 diet decreased oleic acid, monoenoic acids and n-9 and increased 22:5n-3, DHA, total n-3 and the n-3/n-6 ratio. On the other hand, the BF3E diet decreased ARA and DHA levels and increased linoleic acid, linolenic acid (18:2n-6 and 18:3n-3), 20:3n-3 and total n-3. Fatty acids n-3 HUFA were reduced by the BF3R diet compared to the BF3 diet (**Table 4.10**).

Histopathology

In Experiment I, quantitative morphometric analysis of the liver revealed a significant ($P<0.05$) increase in hepatocellular area and maximum and minimum cell length in hepatocytes from fish fed with BP diets (**Table 4.12**). The fish-fed control diet had the smallest hepatocellular area, as well as maximum and minimum length, showing an eosinophilic appearance with the presence of lipid droplets, and nuclear displacement to the cell periphery with a normal cell distribution showing prominent basophilic nuclei aligned around sinusoidal spaces (**Figure 4.1**).

Table 4.12. Histopathological analysis of the liver from the Experiments I, II and III.

	Diet				
Experiment I	**Control**	**HBT2.5**	**HBT5**	**HBT15**	**HBT20**
Hepatocelular Area (µm)	38.21 ± 14.56^{a}	107.46 ± 34.57^{b}	83.37 ± 23.00^{c}	95.74 ± 30.22^{b}	35.84 ± 11.13^{a}
Max Length(µm)	7.61 ± 1.69^{a}	13.00 ± 2.53^{b}	11.74 ± 1.97^{c}	12.62 ± 2.33b^{c}	7.68 ± 1.51^{a}
Min Length(µm)	5.58 ± 1.28^{a}	9.82 ± 1.81^{b}	8.49 ± 1.55^{c}	9.08 ± 1.84^{c}	5.58 ± 0.99^{a}
Hepatocytes vacuolization grade	1.94^{a}	1.22^{b}	1.22^{b}	1.06^{b}	1.83^{a}
Experiment II	**Control**	**BF0.2**	**BF0.5**	**BF1**	**BF3**
Hepatocelular Area (µm)	147.19 ± 46.56^{b}	160.11 ± 59.22^{ab}	140.92 ± 37.64^{b}	164.30 ± 42.36^{a}	162.52 ± 37.53^{a}
Max Length (µm)	15.15 ± 2.73^{bc}	15.90 ± 3.15^{ba}	14.78 ± 2.03^{c}	16.67 ± 2.79^{a}	16.62 ± 2.40^{a}
Min Length (µm)	11.19 ± 2.17^{b}	11.74 ± 2.55^{ab}	11.36 ± 2.07^{ab}	11.95 ± 2.01^{a}	12.01 ± 2.09^{a}
Hepatocytes vacuolization grade	1.83 ± 0.82	1.88 ± 0.75	2.00 ± 0.35	2.25 ± 0.5	1.42 ± 0.58
Experiment III	**Control**	**BF3**	**BF3R**	**BF3E**	
Hepatocelular Area (µm)	127.03 ± 34.76^{b}	123.80 ± 36.40^{b}	125.40 ± 32.62^{a}	110.63 ± 37.68^{b}	
Max Length (µm)	13.81 ± 2.17^{b}	13.45 ± 2.26^{ab}	14.06 ± 2.11^{a}	13.02 ± 2.40^{b}	
Min Length (µm)	9.95 ± 2.50^{a}	10.11 ± 2.33^{a}	8.28 ± 2.65^{a}	9.36 ± 2.36^{b}	
Hepatocytes vacuolization grade	1.92 ± 0.49	1.63 ± 0.95	3.00 ± 0.00	2.70 ± 0.27	

Different letter indicates significant differences in each experiment (p-value<0.05)

In fish fed with alternative diets from diets 2.5 onwards, cell size increased showing progressively more marked basophilia, cell consolidation and a greater presence of peri hepatocyte detachment. Hepatocytes with a more regular morphology were characterized by a considerable reduction in cytoplasmic vacuolation without nuclear displacement to the periphery, offering a more glucogenic content appearance. The presence of sinusoidal spaces decreased but the normal nuclear organization was maintained. However, when feeding the BP20 diet, phenomena of hepatocyte dissociation accompanied by foci of necrosis occur and debris presence, although without drastically modifying the nuclear morphology. The peri hepatocyte detachment was maintained but it was less evident due to the loss of structure that increases eosinophilia, reminding us of the control diet. The count was conditioned by the presence of cells without preserved limits, therefore limited to those hepatocytes with intact cytoplasmatic limits. This less preserved structure also affected the vacuolization level counting.

With the banana flower diets in Experiment II, the size of the hepatocytes increases in general, but it showed variability inside animals that prevents to determine a significant tendency (**Table**

4.12). The changes in the liver morphology were less noticeable including those referred to vacuolization grade (**Figure 4.1**).

Finally, no changes were registered in the size, morphology, or cellular distribution during Experiment III with the Banana flower extract and residue diets although an increase was found with the banana flower residue in the size of hepatocytes (**Table 4.12**).

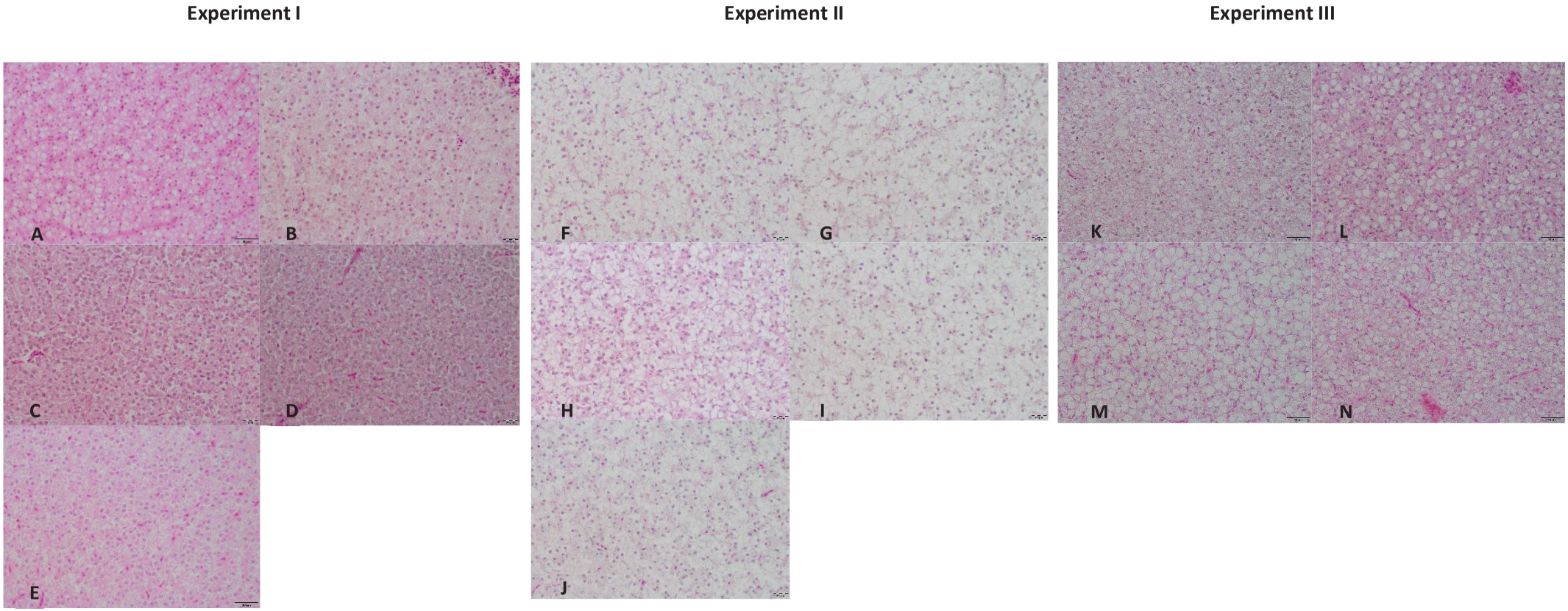

Figure 4.1. Liver´s micrograph (40x) from the three experiments. Experiment I: control diet (A), BP2.5 (B), BP5 (C), BP15 (d), BP20 (D). Experiment II: control diet (F), BF0.2 (G), BF0.5 (H), BF1 (I), BF3 (J). Experiment III: control diet (K), BF3 (L), BF3R (M), BF3E (N).

Osmotic Challenge

The fish survival during the osmotic challenge in experiment II was not significantly affected by the flower inclusion diets, during the osmotic challenge compared with the control diet (**Figure 4.2**), the BF1 diet was removed from the challenge due to a technical problem with the system that could not be solved in time to perform the challenge. In the first 24 hours of the challenge, what was observed is that the control and BF0.2 diets have the highest survival, although it is decreasing. In contrast, the BF3 and BF0.5 diets showed a stabilisation of mortality that was maintained over time. At the end of the challenge (48h), even though there were no significant differences, the diet with the highest survival was BF3 with 50% of the fish alive, followed by BF0.5 with 42% and the control diet and BF0.5 presented the lowest total survival with 25% and 17% each.

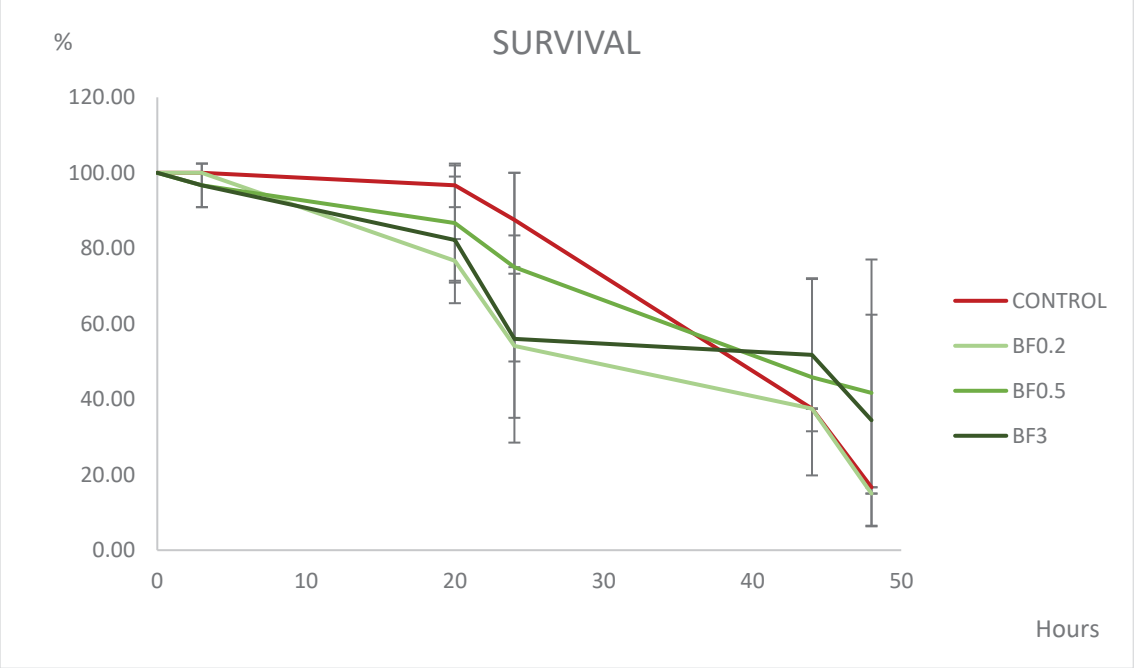

Figure 4.2. Survival measured during salinity challenge (21ppt) in the Experiment II. Different letter indicates significant differences (p-value<0.05).

In experiment III, in the first part of the challenge with 50% saltwater, no mortality was recorded after five days, that was the reason to increase salinity to observe the impact of the diets on the mortality during the osmotic challenge. The results obtained in the second part of the challenge with salt water 100% showed no significant influence but BF3 and BF3R gave 10% and 12% lower mortality relative to the control diet (**Figure 4.3**).

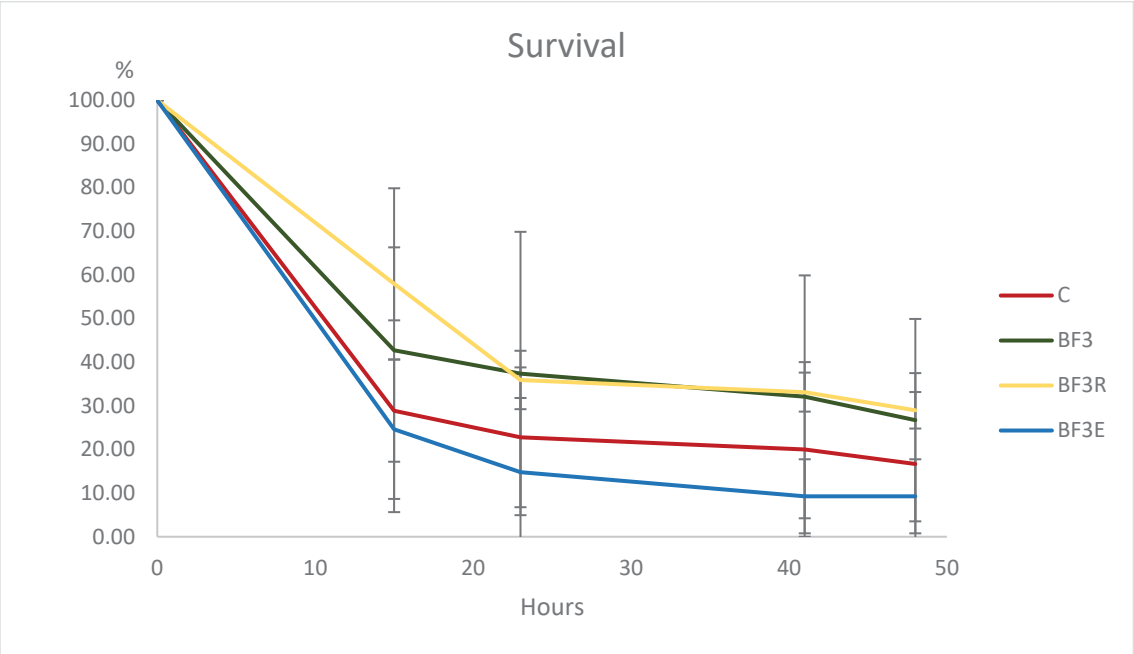

Figure 4.3. Survival measured during the salinity challenge (38 ppt) in the Experiment III.

Plasma parameters

No cortisol peak was observed at 3h of exposure in brackish water (21ppt), but there was an increase at 24h. The basal cortisol (t0) in experiment II presented a higher level in diets BF3 and BF1 but at 3h, only control, BF0.2 and BF0.5 were increased, BF1 and BF3 decreased the cortisol levels compared to 0h. BF0.5 maintained the cortisol level more stable during the challenge. Plasma glucose was increased as the time in brackish water increased. At 0h, the BF0.2 diet has the highest plasma glucose level, at 3h there were no differences between the diets and at 24h the BF1 diet had the highest level (**Table 4.13**). In Experiment III, a similar effect was observed between the control diet and BF3 diet, although there were no significant differences in any of the diets in the basal cortisol level. As in the previous experiment, there were no differences in glucose between the diets (**Table 4.14**).

Table 4.13. Plasma cortisol and glucose content during the osmotic challenge in the Experiment II (21 ppt salinity).

	DIET			
	Control	**BF0.2**	**BF0.5**	**BF3**
0h				
Cortisol (ug/dL)	10.85 ± 5.99[b]	9.35 ± 5.11[b]	16.02 ± 5.42[ab]	22.98 ± 6.61[a]
Glucose (mg/dL)	36.67 ± 4.46[b]	52.33 ± 3.33[a]	36.67 ± 5.72[b]	41.17 ± 8.30[b]
3h				
Cortisol (ug/dL)	13.20 ± 9.87	18.98 ± 11.16	16.62 ± 17.94	11.39 ± 4.57
Glucose (mg/dL)	103.50 ± 20.54	114.40 ± 11.55	107.17 ± 43.47	104.80 ± 22.92
24h				
Cortisol (ug/dL)	30.18 ± 4.28	26.07 ± 7.28	29.22 ± 9.95	26.02 ± 10.30
Glucose (mg/dL)	198.67 ± 53.45[a]	270.50 ± 54.45[a]	195.00 ± 7.07[b]	195.50 ± 55.94[ab]

Different letter indicates significant differences in each experiment (p-value<0.05)

Table 4.14. Plasma cortisol and glucose content at the end of the Experiment III.

	DIET			
	Control	**BF3**	**BF3R**	**BF3E**
Cortisol (ug/dL)	15.4 ± 5.85	23.81 ± 14.96	32.13 ± 8.26	22.53 ± 10.44
Glucose (mg/dL)	51.00 ± 6.99	41.29 ± 6.07	50.75 ± 6.99	45.50 ± 5.48

Muscle and liver oxidation in Experiment II (MDA)

At the end of the feeding trial (t0), no influence of the diets was observed on the muscle oxidation, but at 24h of being in brackish water (21ppt), oxidation was higher in tilapia fed the BF0.2 and BF0.5 diet meanwhile, as the BF inclusion was increased, oxidation was improved compared to the control diet. At the same time, the liver at 24h showed a higher concentration of MDA in the BF3 diet, while it decreased in the BF1 and BF0.5 diets (**Table 4.15**).

Table 4.15. Muscle and liver MDA concentration in lipids, as indicator of oxidation status at the end of the feeding trial (0h) and in muscle & liver after 24h in brackish water (21ppt) in Experiment II.

	Control	**BF0.2**	**BF0.5**	**BF3**
Muscle				
0h	82.44 ± 9.39	49.77 ± 32.75	119.73 ± 88.35	77.15 ± 59.02
24h	95.73 ± 35.42[b]	148.41 ± 25.64[a]	166.23 ± 42.42[a]	135.84 ± 59.84[ab]
Liver				
24h	73.25 ± 35.87[ab]	69.09 ± 62.99[ab]	34.66 ± 12.85[b]	123.44 ± 43.19[a]

Different letters indicate significant differences (p-value<0.05).

Liver catalase

Catalase activity was not affected by the diets during the osmotic challenge in experiment II until 24h, where BF0.2 was significantly lower than BF3. Values were in the range between 1712 and 4493 nmol/min/mL (**Figure 4.4**).

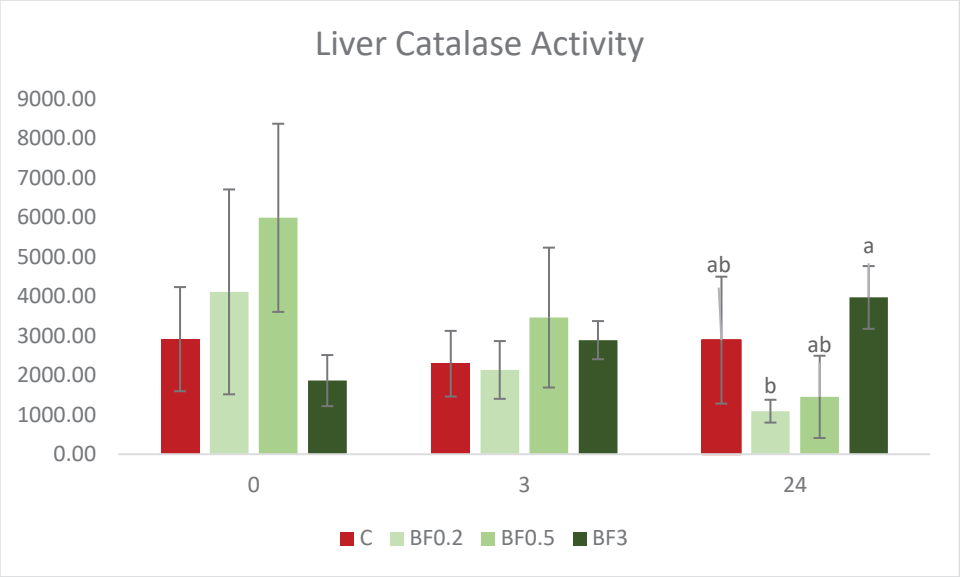

Figure 4.4. Liver catalase activity among the salinity challenge in the Experiment II. Different letter indicates significant differences (p-value<0.05).

4.4 Discussion

The use of banana by-products to replace cornmeal in feed for tilapia has some previous studies, but none have been carried out with secondary by-products from the processing of banana stems or with the male and hermaphrodite flowers of the banana inflorescence. The studies presented in this paper are the first attempt, to our knowledge, to explore this pathway of by-product recirculation. The inclusion of dietary plant ingredients has been shown to enhance the growth performance of tilapia in most cases, such as aloe vera, garlic or orange-peel oils (Metwally, 2009; Acar *et al.*,2015; Gabriel *et al.*, 2015).

In the case of banana discharges, reported results in tilapia showed different behaviours depending not only on the type of by-product but on the species under study. Thus, Karaket *et al*. (2021) found that despite the good acceptance of the feed by the fish only up to 5% of ripe bananas did not affect the growth of hybrid tilapia, with a similar size to the fish from Experiments I and II, while Yossa *et al*. (2021) obtained good results up to 30% in the case of banana peel tested in GIFT tilapia slightly larger in size (22g initial weight). In the same way, results in the present work in juvenile Nile tilapia depend on the materials under study, wherein

the maximum dietary level with no damage in growth parameters seems to be no more than 5% and 3% for hydrolysed banana pseudo-stem and banana flower, respectively. A factor to consider in Experiment I is the high amount of ash present in the BP meal, which could be explained due to the salt generation as a result of the neutralization process. Diet ash increases as the inclusion of BP increases, being from 15% higher than the control diet. The presence of this large amount of ash in the meal may be conditioning the response of the animals, thus with up to a maximum of 5% BP inclusion supported in the present study. According to Karaket *et al.* (2021) also in the tilapia trial, small levels of BP like 5% BP (about 10% whole dietary ash from which less than 2.5% comes from BP) in the present case may contribute to the whole dietary mineral balance, while over 5% BP levels (over 7.5 and 10% BP mineral contribution), the mineral content in diets increased and could prejudice the juveniles´ performance due to an excess of accessible minerals with high bioavailability that affected the digestibility of the nutrients in the diet (Lall & Kaushik, 2021). Moreover, the diluted sulphuric hydrolysis and final NaOH neutralization process, used towards a better carbohydrate bioavailability for the banana pseudo-stem, may also change and affect the minerals profile and minerals availability, but also, increasing digestive the pH content by increase the final salt mineral complex (Hu *et al*., 2014), affecting the reported normal pH in the tilapia digestive tract (Payne *et al*., 1978).

Also, the 3% extract of banana flower and the residue did not negatively affect the growth performance in Experiment III, even when the size of the juveniles was reduced, to see if the smaller fish respond to the diets in the same way. Thus, results for this novel dietary by-product are in line with previous ones, with no high inclusion levels supported by the fish towards the fish growth. A similar observation was made in the tambaqui (*Colossoma macropomum*), for with over 16% banana meal inclusion reduced growth (Felix e Silva *et al.,* 2020).

The liver does not appear to be damaged by the inclusion of any of the by-products, no lesions or signs of toxicity were observed in the treatments, and at least up to 15% banana pseudo-stem inclusion, which is consistent with results described in Nile tilapia fed mature banana (Palintorn *et al*., 2019). The only diet that showed some structural damage and necrosis foci was the 20% inclusion of banana pseudo-stem. The poor results obtained in this diet were conditioned by this fact, as the methodology is limited by the integrity of the cell structure at the time of measurement. Furthermore, these results were consistent with those observed with high soy feed (Khieokhajonkhet *et al.,* 2021) in hybrid tilapia hepatocytes, where as the level of soy inclusion increased, the vacuolisation also increased. The decrease in the degree of vacuolisation as by-product inclusion increases seems to be related to the lower lipid content in the liver which may be a response to a poor lipid digestibility coefficient as has been described for banana peel (Yossa *et al*., 2021). The fibre provided by BP may decrease lipid adsorption which is reflected in

the decrease of total lipids in both liver and muscle, which is in agreement with that found by Yossa *et al*. (2021), with a reduction of the lipid digestibility coefficient when feeding banana peel to GIFT tilapia. The undescribed amount of NSP which we assume to be high in BP, due to the chemical treatment that could facilitate the accessibility to the carbohydrates, influences juveniles' performance and lipid digestibility (Maas *et al*., 2020) related to possible modification of gut morphology and mucus viscosity, which is evident with inclusions higher than 5%. Lower inclusions, which show an increase in essential fatty acids, despite the decrease in total lipids in both muscle and fillet, do not show such a pronounced effect on growth, which according to Mass *et al*., (2020) may result from the fermentation of a fraction of the NSP present in the hydrolysed banana pseudo-stem, producing short fatty acids that could be rapidly absorbed and used to meet energy demands. A consequence of the decrease in total lipids is that the synthesis of LC n-3 PUFA from 18:2n-6 and 18:3n-3, especially EPA, ARA and DHA, increases (Olsen *et al*., 1990; Teoh *et al*., 2011; Chen *et al*., 2018), thus maintaining the proportions of these fatty acids despite the decrease in total lipids. Another sign of lipid mobilisation with both BP and flower extract and its residue is that VSI decreases with the inclusion of these ingredients. Even so, the inclusion of pseudo-stem can reduce the energy of diets affecting growth (Maina *et al*., 2003), which coincides with what was observed from diet BP15, where there is a decrease in growth. Furthermore, the BP20 diet fails to synthesise enough DHA to compensate for the drop in total lipids. This may be a sign of loss of functionality, which coincides with the necrosis observed in the histopathological analysis for fish fed on this diet.

The non-effect of the flower on the fatty acid profile may be due to insufficient inclusion levels to reflect the DHA supply from the flower meal (Ramírez-Bolaños *et al*., 2021), so although not the main objective in the present case, studies with higher inclusion would be interesting to see if there is an influence of the flower on the fillet quality of the tilapia. Since no effects on liver fatty acids were observed for BF, fatty acid profiling was not considered necessary in experiment III. Interestingly, in muscle, the different results obtained depend on the flower extract which may indicate the nature of the bioactive causing these effects. The decrease in all diets with banana flower and derivates of 20:1n-7 fatty acid, and monoenoic acids in the BF3 diet may indicate the use of these fatty acids in the animal's energy demand (Varga *et al*., 2020). In the diet with the flower extract, the decrease in ARA and DHA and the high levels of LNA and ALA indicate that there is no stimulation of desaturases and elongases for the production of essential fatty acids (Teoh *et al*., 2011). In light of these results, it can be inferred that the bioactive that enhances the increase of DHA in fish fed with the flower diets is located in the extract residue (BF3R).

A salinity challenge is a good option to determine whether the test materials used in this experiment may improve the response and adaptation of tilapia under a challenge and to determine where the benefit is in the flower. Immersion in water with a salinity of 25 ppt for at least 24h had an immunostimulatory effect on Mozambique tilapia (Jian *et al*., 2008); after 3h of exposure, cortisol increases in response to induced stress and may cause the observed changes in cell structure, allowing fish adaptation to that stress (Krammerer *et al*., 2010). In this study, the differences observed in t0 for higher flower inclusion disappeared during the challenge. At t3 the cortisol levels among the treatments were equal, but the reaction of the fish fed with BF1 and BF3 diets was to reduce the cortisol compared to t0. At t24h, all the treatments increased the cortisol levels above basal, except for BF1 and BF3 which maintained cortisol at the same basal level. This trend in cortisol behaviour at t0 was observed also in Experiment III, where all diets with banana flower, extract and residue slightly increased basal cortisol, being the residue the diet with the higher cortisol. This can be due to the presence of a component in the flower that could be in higher proportion in the residue. Based on the previous study performed with the banana flower characterization, the high quantity of polyphenols present in the banana flower could influence the stress response, as it was established in other studies where the high amount of polyphenols was determined as the cause of the decreasing cortisol with pomegranate peel (Hamed & Abdel-Tawwab, 2021) and also with specific studies with ferulic acid inclusion where the basal cortisol was decreased from 20mg/kg and after the heat stress, the lower values of cortisol corresponded to 80mg/kg of ferulic acid inclusion (Dawood *et al*., 2020).

Glucose increased with time in brackish water. In Experiment II (21 ppt), the banana flower inclusion increased plasma glucose related to the control diet, contrary to what was observed in previous studies (Khan *et al.,* 2022; Uma *et al*., 2022) with other plants by-products, but coincides with aloe effect as it increased serum glucose levels (Gabriel *et al.,* 2015). Otherwise, at 18ppt in Experiment III, BF3 and extract confirm the tendency of decreasing plasm glucose level. This may be due to the presence of a large number of polyphenols in banana flower that could act as glucose regulators as pomegranate peel (Hamed & Abdel-Tawwab, 2021), and, specifically, protocatechuic acid, which is the main polyphenol identified in the banana flower and its extract (Ramírez-Bolaños *et al.,* 2021), which has been observed to have a regulating effect on hyperglycaemia in mice (Talagavadi *et al.,* 2016; D'Archivio *et al*., 2018). Furthermore, other bioactive that could be present in the banana flower may act as a glucose regulator (Hamed & Abdel-Tawwab, 2021). Also, a high glucose plasma level may indicate an increasing demand for energy in the cells to respond to the stress as was reported by Hassaan *et al.,* (2019).

According to these results, survival during the challenges was not affected by the experimental diets, even the higher total survival observed at the end of the challenge in Experiment II with BF3 and BF0.5 (25% and 17% lower mortality concerning control diet) which may suggest that the benefits provided by the banana flower manifest itself over time. Other plant extracts like bougainvillaea and pineapple fibre increased survival in challenges with pathogens (Van Doan *et al*., 2021; Uma *et al*., 2022), so further and different studies must be carried out to clarify the possible benefits of banana flower under fish salinity resistance or even with other types of stress conditions. In experiment III, the results obtained agreed with the previous experiment, despite the smaller size of the fish (7g), and it also could indicate that the possible advantage of the banana flower may be located in the residue after the organic extraction. This brings up the possibility of increasing the amount of flower and residue to improve the potential benefits detected in these challenges.

Malonaldehyde concentration is an indicator of lipid oxidation status in the liver and in the muscle that increases with salinity (Sutti & Thaimuangphol, 2018; Mohamed *et al*., 2021). Polyphenols present in pomegranate and dietary ferulic acid decrease oxidation (Dawood *et al.,* 2020; Hamed & Abdel-Tawwab, 2021), which coincides with the results obtained in the current study, especially in the muscle, where the higher BF inclusions presented the best oxidation values with the control diet. CAT activity in the liver is an indicator of the hepatocytes reducing the reactive oxygen molecules in response to stress (Hegazi *et al*., 2010). At 24 hours, CAT activity was higher in fish fed the BF3 diet, which is under the results obtained in tilapia with pomegranate, probably due to the polyphenols present in the peel (Hamed & Abdel-Tawwab, 2021). Concerning the compounds present in the banana flower, the role of polyphenols and other bioactive in the regulation of cortisol and glucose levels and the influence on the oxidative status of fish needs to be studied more closely.

In conclusion, BP and BF, which account for a high amount of the discharges from banana production, can be introduced up to 5% and 3%, respectively, in juvenile tilapia feeds. Over 5% BP seems to be negatively affected mainly in two ways, by one hand due to the mineral content contribution from this by-product, which may affect mineral profile, digestive pH and nutrient digestibility. Moreover, the role of NSPs in gut morphology requires further investigation, as they influence the absorption of macronutrients such as lipids. Despite this, juvenile tilapia seems to be able to counteract the decrease in total lipids with the synthesis of essential fatty acids. BP processing should be approached not only concerning the bioaccessibility of carbohydrates but also to the amount and availability of minerals present in the raw material, by determining the mineral profile and determining the effect by including in fish diets. The

inclusion of the 3% flower also provides a benefit in terms of survival over time under osmotic stress. In this regard, it is of interest to determine the bioactive present in the banana flower extract that appears to stimulate the activity of fatty acid elongases and desaturases and also provides advantages during an osmotic challenge, as does the flower. For the BF opportunities of higher inclusions levels should be assayed.

4.5 Acknowledgement

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Chapter 5. Towards the sustainability of aquaculture: a complete study of novel banana by-products in high vegetable diets for juvenile seabass (*Dicentrarchus labrax)*

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In progress

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5.1 Introduction

Seabass (*Dicentrarchus labrax*) is one of the most important marine species in aquaculture production in southern Europe and the Mediterranean, with a production of 228,506 tonnes in 2020 (Apromar, 2021), with Spain as the second largest producer in Europe behind Greece. This specie represents, moreover, one among which more promising studies have been reported regarded high levels of fish meal and fish oil dietary substitution by vegetable ingredients, without effects on fish performance results (Montero *et al*., 2005; Mourente *et al*., 2005; Castro *et al*., 2016; Wassef *et al.,* 2016). In this sense, and in most of the cases as a result from high levels of dietary vegetable ingredients, one of the conditioning factors for the production of this species is stress resistance. Numerous studies are focused on obtaining functional ingredients that help fish under stressful situations (Machado *et al*., 2019; Islam *et al.,* 2020; Serradell *et al.,* 2020). These ingredients tend to benefit fish in reducing cortisol production and improving the innate immune system. Another problem faced by seabass aquaculture producers is the low resistance to diseases such as *Vibrio sp,* with these bacterial diseases being responsible for 74% of the diseases detected in sea bass (Muniesa *et al.,* 2021). As a result, there are numerous lines of research open, including tests of functional ingredients (Torrecillas *et al*., 2007), as well as different techniques which allow not sacrificing all fish with demonstrated in-vivo infections, but ex-vivo simulations to evaluate these effects, such as the use of POLY I: C for the stimulation of

the Mx gene (Acosta *et al*., 2004; Bravo *et al.,* 2013; Román *et al.*, 2013), or the phagocytic capacity of lymphocytes (Torrecillas *et al*., 2017), being these last cells fundamental in the immune system with the processes of phagocytosis and antigen presentation in lymphoid organs such as the anterior kidney (Carbone & Fagio, 2016).

Regarded the search for new functional materials for aquafeeds and the seabass specifically, the banana flower seems a good candidate due to its polyphenol composition (Ramírez-Bolaños *et al*., 2021), and results obtained in previous studies with tilapia (Ramírez-Bolaños *et al*., 2022 *in progress*). Natural polyphenols exhibit antioxidant, anti-inflammatory and hyperglycaemia-regulating properties (D'Archivio *et al*., 2018; Adedara *et al*., 2019; Al Olayan *et al*., 2020; Lau *et al*., 2020; Gao *et al*., 2021) in studies with rats and mice. Although there are several studies in (Meguro *et al.,* 2013; Magrone *et al.,* 2016; Arciuli *et al*., 2017; Long *et al*., 2017; Van Doan *et al*., 2020; Qian *et al.,* 2021) the effect of including polyphenol-rich materials in the diet for sea bass is a pathway for further exploration (Ahmadifar *et al*., 2021).

On the other hand, the search for new raw materials to be incorporated into aquaculture feeds is still one of the driving forces behind aquaculture research, presenting a great possibility for further progress in the sustainability of the industry (Hough, 2022). Progress are much needed in the replacement of feed flours with other no conventional flours that can be more sustainable, and that do not conflict with other uses for human consumption or other industries such as pharmaceuticals or the generation of biofuels (Kadam & McMillan, 2003). The use of agricultural by-products, or even secondary by-products from different agroindustries like in present case the use of waste generated by banana production, such as pseudo-stem and the discharged flowers. These wastes can not only be directly processed for integrating into the aquaculture feed industry, but, as in the case of the banana pseudo-stem, they can also be used in the textile or plastics industry (Ortega *et al*., 2016; Rodríguez *et al*., 2020); the secondary waste generated in that case could be later incorporated into the aquaculture production value chain.

In short, sea bass is a target species for the aquaculture production that it is well known to accept alternative material and by-products in their diet, not only from animal origin but also vegetable (Petereit *et al.,* 2022), making it a great candidate for the objective of this work, which is to evaluate the inclusion of these two novel banana by-products in sea bass feeds and their effect on fish development and welfare.

5.2 Material and Methods

For present experiment, the banana by-products were supplied by local producers in the framework of the European LIFE program through the BAQUA project (LIFE15 ENV/ES/000157).

In the facilities of Fabricación Integrada y Avanzada Research Group from the University of Las Palmas de Gran Canaria, the banana pseudo-stem was processed to mechanically separate the external fibre (patent: WO2014/174115). The secondary residue generated in this procedure was the pseudo-stem pulp considered in the present work which represent the 76% of dry pseudo-stem (LifeBaqua, 2019). The Banana flower (BF) and banana pseudo-stem (BPS) were freeze-dried and ground in a mill (Ultra Centrifugal Mill ZM200, Retsch, Germany). The banana flower extract (BFE) was performed with methanol:water and acetone:water, according to previous work (Ramírez-Bolaños *et al.,* 2021) based on the polyphenol profile, and the solvents were evaporated in a rotary evaporator. The residue left after the extraction (BFR) was dried in an oven (37°C) and grounded in a mill.

Experimental diets include banana by-products as corn meal substitution in a control diet (**Table 5.1**). For the Experiment I, banana pseudo-stem (BPS) was included in increasing amounts 2%, 4% and 8% (BPS2, BPS4 and BPS8) diets, respectively, thus up to 100% corn meal substitution. In Experiment II, banana flower (BF) inclusion levels were 1%, 3% and 6% (BF1, BF3 and BF6 diets). Finally, an Experiment III was proposed to better test properties for the banana flower regarded fish stress response and the immune system. Thus, in Experiment III the BF6 diet was assayed against their correspondent organic extract and the resultant residue, BFE and BFR diets respectively. The proximate composition and the fatty acid profile for all diets is shown in **Table 5.2**.

Table 5.1. Formulation of the basal diet for three experiment.

	Diet
Ingredients (%)	
Fish Meal	20.00
Blood meal	5.00
Rapessed Meal	8.00
Corn Meal	8.00
Soja Meal	20.00
Wheat Meal	4.00
Wheat Gluten	14.00
Fish Oil	5.00
Rapeseed Oil	6.00
Linseed Oil	4.00
[1]Vit mix	2.00
[2]Min mix	2.00
Bi-Calcium Phosphate	1.50
A-Cellulose	0.50

[1]Vitamin premix (g/kg): Tiamin 0.04,Riboflavin 0.05, Piridoxin 0.04, Calcium Pantotenate 0.1169, Nicotinic acid 0.001, Folic acid 0.01, Cyanocobalamin 0.0005, Choline 2.70, Myo-Inositol 2.00, Vitamin C 5.00, Vitamin E 0.25, Menadione 0.02, Cholecalciferol 0.005, retinol acetate 0.025, Etoxiquin 0.10

[2] Mineral premix (g/kg): $(H_2PO)_2Ca$ 1.605, $CaCO_3$ 4.00, $FeSO_4 \times 7H_2O$ 1.50, $MgSO_4 \times 7H_2O$ 1.605, K_2HPO_4 2.80, $Na_2PO_4 \times H_2O$ 1.00, $Al(SO_4)_3 \times 6H_20$ 0.02, $ZnSO_4 \times 5H_2O$ 0.24, $CuSO_4 \times 5H_2O$ 0.12, $MnSO_4 \times H_20$ 0.08, KI 0.02, $CoSO_4 \times 7H_2O$ 0.08

Table 5.2. Proximate composition and fatty acid profile of the experimental diets from the three experiments with banana by-products inclusion.

	Diet								
	C	**BT 2**	**BT4**	**BT 8**	**BF 1**	**BF 3**	**BF 6**	**BFR**	**BFE**
Lipids	21.44 ± 0.98	21.82 ± 0.07	22.18 ± 0.32	20.66 ± 0.32	20.55 ± 1.55	21.30 ± 0.24	21.52 ± 0.32	19.57 ± 0.61	16.98 ± 0.45
Ash	9.67 ± 0.16	8.68 ± 1.21	10.36 ± 0.15	10.87 ± 0.02	9.64 ± 0.14	9.98 ± 0.06	10.64 ± 0.75	10.02 ± 0.27	9.66 ± 0.13
Protein	45.76 ± 0.23	45.74 ± 0.81	46.26 ± 0.18	46.98 ± 0.25	44.99 ± 0.64	45.82 ± 0.53	44.42 ± 0.42	46.06 ± 0.36	47.95 ± 0.26
Moisture	6.08 ± 0.76	6.61 ± 0.20	6.53 ± 0.21	6.47 ± 0.23	13.83 ± 0.36	13.99 ± 0.17	10.22 ± 0.28	11.18 ± 0.89	8.8 ± 0.55
CHO + Fibre	21.72 ± 0.08	22.19 ± 0.32	19.81 ± 0.91	20.10 ± 0.33	21.38 ± 0.25	19.70 ± 0.62	21.03 ± 0.45	21.63 ± 2.23	23.17 ± 0.24
Fatty acids (%)									
14:00	0.87	0.42	0.97	0.50	1.00	0.62	0.90	0.58	0.77
14:1n-7	0.01	0.00	0.02	0.02	0.02	0.01	0.02	0.01	0.01
14:1n-5	0.03	0.03	0.04	0.03	0.04	0.02	0.03	0.02	0.03
15:00	0.14	0.11	0.16	0.10	0.15	0.11	0.15	0.13	0.15
15:1n-5	0.02	0.04	0.01	0.03	0.01	0.01	0.01	0.01	0.01
16:OISO	0.02	0.03	0.03	0.03	0.03	0.02	0.02	0.01	0.03
16:00	9.26	6.61	9.44	7.18	9.55	7.81	9.08	10.32	10.81
16:1n-7	1.42	1.11	1.47	1.10	1.32	1.14	1.32	1.39	1.50
16:1n-5	0.04	0.06	0.05	0.06	0.03	0.04	0.04	0.04	0.05
16:2n-4	0.06	0.06	0.07	0.07	0.06	0.05	0.06	0.05	0.05
17:00	0.04	0.05	0.04	0.04	0.05	0.03	0.04	0.03	0.03
16:3n-4	0.15	0.14	0.15	0.14	0.14	0.13	0.13	0.14	0.16
16:3n-3	0.05	0.06	0.06	0.07	0.05	0.05	0.05	0.05	0.06
16:3n-1	0.02	0.05	0.03	0.03	0.03	0.02	0.03	0.01	0.01
16:4n-3	0.04	0.06	0.06	0.05	0.07	0.03	0.06	0.03	0.03
18:00	3.69	3.56	3.58	3.61	3.24	3.66	3.44	4.25	4.28
18:1n-9	39.74	38.46	39.16	38.67	36.48	38.79	38.22	42.32	41.13
18:1n-7	2.73	2.73	2.72	2.69	7.59	2.66	2.57	3.02	2.82
18.1n-5	0.07	0.07	0.07	0.07	0.00	0.07	0.06	0.07	0.07
18:2n-9	0.03	0.04	0.03	0.04	0.02	0.02	0.02	0.02	0.02
18.2n-6	17.43	17.33	17.28	17.23	16.88	17.54	17.58	16.86	17.41
18:2n-4	0.06	0.08	0.07	0.08	0.03	0.06	0.06	0.06	0.06
18:3n-6	0.11	0.11	0.11	0.11	0.09	0.12	0.12	0.09	0.11
18:3n-4	0.08	0.06	0.10	0.10	0.07	0.08	0.09	0.05	0.99
18:3n-3	14.06	14.84	14.13	15.00	13.90	15.04	16.18	12.25	11.94
18.3n-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01
18:4n-3	0.23	0.24	0.23	0.26	0.26	0.24	0.23	0.15	0.18
18:4n-1	0.03	0.07	0.05	0.04	0.06	0.04	0.04	0.01	0.02
20:00	0.48	0.58	0.47	0.52	0.40	0.51	0.43	0.51	0.49
20:1n-9	0.25	0.28	0.24	0.29	0.21	0.27	0.21	0.23	0.23
20:1n-7	2.01	2.40	2.02	2.27	1.62	2.19	1.74	2.00	2.02
20.1n-5	0.10	0.13	0.13	0.14	0.09	0.12	0.08	0.10	0.10
20:2n-9	0.05	0.05	0.04	0.06	0.03	0.04	0.04	0.03	0.03
20:2n-6	0.38	0.45	0.37	0.42	0.30	0.43	0.37	0.41	0.34
20:3n-9	0.02	0.06	0.03	0.05	0.02	0.02	0.02	0.01	0.01
20:3n-6	0.11	0.15	0.13	0.13	0.09	0.12	0.10	0.09	0.08
20:4n-6	0.25	0.32	0.28	0.33	0.32	0.30	0.27	0.19	0.24
20:3n-3	0.14	0.2	0.15	0.17	0.15	0.16	0.13	0.11	0.12
20:4n-3	0.21	0.25	0.22	0.25	0.21	0.23	0.19	0.13	0.15
20:5n-3	0.96	1.34	1.00	1.27	1.08	1.23	1.03	0.64	0.74
22:1n-11	1.13	1.60	1.16	1.52	0.88	1.25	0.93	1.02	1.05
22:1n-9	0.37	0.52	0.38	0.53	0.31	0.50	0.46	0.44	0.35
22:4n-6	0.10	0.17	0.10	0.13	0.09	0.09	0.08	0.05	0.06

22:5n-6	0.13	0.25	0.12	0.24	0.16	0.14	0.11	0.07	0.10
22:5n-3	0.41	0.66	0.41	0.61	0.41	0.52	0.39	0.23	0.26
22:6n-3	2.44	4.18	2.63	3.72	2.51	3.48	2.87	1.76	1.87
[1]Saturades	14.50	11.36	14.69	11.98	14.42	12.76	14.06	15.83	16.56
[2]Monoenoics	47.92	47.43	47.47	47.42	48.60	47.07	45.69	50.67	49.37
[3]n-6	18.51	18.78	18.39	18.59	17.93	18.74	18.63	17.76	18.34
[4]n-3	18.54	21.83	18.89	21.4	18.64	20.98	21.13	15.35	15.35
[5]n-9	40.46	39.41	39.88	39.64	37.07	39.64	38.97	43.05	41.77
[6]n-3 HUFA	4.16	6.63	4.41	6.02	4.36	5.62	4.61	2.87	3.14
ARA/EPA	0.26	0.24	0.28	0.26	0.30	0.24	0.26	0.30	0.32
DHA/EPA	2.54	3.12	2.63	2.93	2.32	2.83	2.79	2.75	2.53
DHA/ARA	9.76	13.06	9.39	11.27	7.84	11.6	10.63	9.26	7.79
[7]Total PUFA	37.23	40.85	37.48	40.24	36.68	39.9	39.92	33.22	33.75
n-3/n-6	1.00	1.16	1.03	1.15	1.04	1.12	1.13	0.86	0.84

[1]14:00,15:00, 16:00, 17:00, 18:00, 20:00

[2]14:1n-7; 14:1n-5; 15:1n-5; 16:1n-7 16:1n-5; 18:1n-9; 18:1n-7; 18:1n-5; 20:1n-9; 20:1n-7; 20:1n-5; 22:1n-11; 22:1n-9

[3]18:2n-6; 18:3n-6; 20:2n-6; 20:3n-6; 20:4n-6; 22:4n-6; 22:5n-6

[4]16:3n-3; 18:3n-3; 18:4n-3; 20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

[5]18:1n-9; 18:2n-9; 20:1n-9; 20:2n-9; 20:3n-9

[6]20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

[7]18:2n-9; 18:2n-6; 18:2n-4; 18:3n-6; 18:3n-4; 18:4n-3; 18:4n-1; 20:2n-9; 20:2n-6; 20:3n-9; 20:3n-6; 20:4n-6; 20:3n-3, 20:4n-3; 20:5n-3; 22:4n-6, 22:5n-6; 22:5n-3; 22:6n-3

Experimental conditions

The three different experiments were all developed at the Ecoaqua Institute facilities (University of Las Palmas de Gran Canaria) and the fish were supplied by a local producer (Aquanaria S.L.). For the Experiment I with BPS, twenty-four fish (18.94 ± 1.42g) per tank were distributed in twelve 500L fibreglass tanks with open flow natural saltwater (18.56 ± 0.51°C) an continuous aeration for oxygen supply. Fish were manually fed to apparent satiation 3 times per day with 4 experimental diets (Control, BPS2, BPS4 and BPS8) for 60 days. At the end of the trial growth performance, the proximate composition of muscle and liver, histopathology analysis, liver and muscle oxidation analysis were performed. In the case of Experiment II (BF) thirty fish (19.43 ± 0.11g) per tank were distributed in twelve tanks, same conditions as in previous trial and a similar temperature (18.48 ± 0.44°C). Fish were fed 3 times a day for 30 days with 4 diets (Control, BF1, BF3 and BF6). Growth performance and samples for proximate composition, histopathology, oxidation, serum for immunological parameters and lymphocytes for Mx gene expression were taken. For the Experiment III, eighteen fish (51.14 ± 5.42g) per tank were randomly distributed in triplicate groups in same conditions twelve tanks with saltwater OpenFlow (18.63 ± 0.35°C, 500L, O2). Fish were fed twice a day for 30 days with experimental diets (Control, BF6, BFE and BFR). After the feeding time, a confinement challenge was performed for seven days based on Serradell *et al*. (2020). During the challenge, fish were divided and put in submerged cages (40cm x 20cm), three cages per treatment. Samples were

taken to analyse the proximate composition, histopathology, oxidation, plasm analysis during the challenge and phagocytic capacity of the lymphocytes.
All the experiments followed the standard bioethics protocol by the Bioethics Committee of the University of Las Palmas de Gran Canaria (Real Decreto 53/2013).

Growth & proximate composition

The growth performance in the experiments with the banana pseudo-stem and the banana flower was evaluated after each feeding trial. Fish were individually weighed, and the subsequent parameters were determined: fish growth in weight; growth in length; weight gain; feed intake; specific growth rate (SGR); feed conversion ratio (FCR); Hepatosomatic index (%) Visceral index (%) and protein intake (PI).

Three samples from each tank were taken. The liver and the muscle of each treatment were processed to determine the lipid composition following the Folch *et al*. (1957) protocol and fatty acids profiles were established by transmethylation of total lipids (Christie, 1982) and separated and quantified by liquid chromatography (Izquierdo *et al*., 1989). Moisture, ash content and protein were determined according to AOAC (2000) methods.

MDA (Oxidation)

The malonaldehyde concentration was determined in the lipids from muscle and liver at the end of the three feeding trials, as an indicator of oxidative status. The analysis was performed following the protocol from Burk *et al*. (1980) of determination of thiobarbituric reactive species.

Histopathology

At the end of the three feeding trials, three livers and intestines per tank were sampled and fixed in 4% formaldehyde and processed. The livers were stained with haematoxylin and eosin (Martoja & Martoja-Pearson, 1970) and the intestines were stained with Alcian-blue (Lev & Spicer, 1964). Primary visual analysis was performed to evaluate the status and the vacuolization grade on the hepatocytes, following a scale from 1 (no evident presence of lipid vacuoles) to 3 (presence of lipid vacuoles, nucleus displacement and cellular deformity). After that, image analysis was performed with the program ImageJ (Schneider *et al*., 2012) in micrograph from each slide taken with a camera (Olympus DP50, Olympus Optical Co. LTD, Shinjuku-ku, Tokyo,

Japan) incorporated in a Olimpus CX41 microscope (Olympus Optical, PA, USA). In the liver, measures of hepatocellular area, maximum and minimum hepatocyte length (with the intersection in the cell nucleus) were taken and in the intestine the length and width of the villi, the width of the lamina propria and the number and area of goblet cells.

Plasm analysis: Cortisol and Glucose

Plasm was collected in the third experiment with the banana flower 6%, and banana flower extract and residue. The samples were taken from two fish per tank in three different moments: just before the confinement challenge (0 hours), 2 hours after the confinement (2 hours) and after seven days in the cages (7 days), based on Serradell *et al*. (2020). Cortisol and glucose levels were measured in an external laboratory, according to the following procedures.

Serum analysis

For the bactericidal activity, the protocol by Sunyer & Tort (1995) based in the effect on the bacterial growth curve caused by the serum was used. In a microplate 100µl of bacterial culture (*Listonella anguillarum*) and 100µL of serum were mixed in each well and the absorbance was measured at 0, 12 and 24h. Wells with 100µL of bacterial culture only and with 100µL of serum only were used as control of absorbance. The bactericidal activity was determined by the formula

%AB= (Abs positive control – (Abs sample- Abs serum)) x 100/Abs positive control

The lysozyme activity method was based on the reduction of absorbance due to the lysis of *Micrococcus sp* by the lysozyme present in the serum. A calibration curve was prepared from a dilution of lysozyme (47700 units/mL) with distilled water. The suspension of lyophilised *Micrococcus sp* was prepared in a phosphate/NaCl dilution (pH 6.3). Standard or sample solution (10 µL) was placed in the 96-well plate and 200 µL of *Micrococcus sp* solution was added to each well. Readings are taken on the plate reader at 620 nm every 5 minutes until minute 60. The time selected according to the regression curve was 15 minutes, and the lysozyme activity was determined by the following function: (Abs t15- Abs t0/ 15 min) – 0.1846 / -0.0001

Peroxidase activity was determined following the protocol established by Quade and Roth, (1997). In a microplate 30µl of serum per sample was mixed with 120µl of HBSS solution and

50µL of TMB, 50uL of H_2O_2, after 2 minutes, 50µL of H_2SO_4 were added to stop the reaction and the absorbance at 450nm were measured

Lymphocytes' extraction

The extraction of the lymphocytes was performed by the method described by Secombes (1990), with some modifications. Cells were extracted from headkidney of 3 fish per tank. The tissue was maintained in cold L-15 supplemented medium (2% heat-inactivated fetal bovine serum, 1 U mL^{-1} penicillin, 1 mg mL^{-1} streptomycin and 5 µg mL^{-1} gentamicin) and then filtered through a membrane of 100 µm, with the tube in ice to prevent the heat. It was centrifuged at 450 xg during 10 minutes at 4°C, the supernatant was removed, and the cells were resuspended in 2mL of L-15 supplemented medium. The 2mL of cell suspension were layering onto 2mL lymphocyte separation medium (Lymphoprep™ Lonza™) and centrifuged at 1100 xg during 30 minutes at 4°C. The buffy coat layer was transferred to a sterile glass tube and washed with 1mL of L-15 medium and centrifuged for 10 minutes at 450xg and 4°C. The supernatant was then discarded and the pellet resuspended to be mixed with an erythrocytes lysis solution (1:3 (v/v); 0,826% (p/v) NH_4Cl, 1mM EDTA, 10mM $KHCO_3$), and after 10 minutes of incubation at room temperature, centrifuged (2500 rpm, 10 minutes, 4°C) to remove erythrocyte cell debris, and washed in 2mL of 1x PBS by centrifugation (2500rpm, 10 minutes, 4°C). After removing again the supernatant, lymphocytes were finally resuspended in 1 mL of L-15 medium no supplemented. Viable cell counts were performed by Trypan Blue staining using a Neubauer chamber.

In-vitro assay with Poly I:C

Lymphocytes cultures in 24-well plates were inoculated/incubated with Poly I:C to simulate virus infection, and with PBS as a negative control. *Mx* gene expression was measured at 0, 6, 24 and 48h incubation by quantitative PCR (qPCR). Two wells were used for each tank.

RNA extraction from lymphocytes was performed with the EZNA® Total RNA Kit I (Omega Bio-Tek) followed by cDNA synthesis with the iScript cDNA synthesis kit (Biorad®) using the thermal cycler. From this cDNA, *Mx* gene was amplified and quantified by qPCR using SYBR (Bio-Rad Laboratories S.A., California, EEUU) as fluorochrome and the thermal cycler iQ™5 (Multicolor Real-Time PCR Detection System, Bio-Rad Laboratories S.A., California, EEUU). The sequences of the primers used for qPCR analysis are shown in **Table 5.3**. At the end of the hybridisation phase of each cycle, the value for the threshold cycle (Ct) was estimated, where a statistically significant increase of the emitted fluorescence was detected. The result obtained was the expression of the *Mx* gene relative to the expression of *β-actin* (housekeeper gene) used as a reference, following the Livak method (Livak & Schmittgen, 2001) which compares the Ct ($2^{-\Delta\Delta Ct}$).

Table 5.3. Sequences of the Mx and β-actin genes

Gene	Sequence	
Mx	Forward	5´-GACAGGGAGCGGCATTGTTAC-3´
	Reverse	5´-TCGTCCAGCTCTTCCTCGTG-3´
β-actin	Forward	5´-TCTGTCTGGATCGGAGGCT-3´
	Reverse	5´-AAGCATTTGCGGTGGACG-3´

Phagocityc capacity

To determine the phagocytic capacity of lymphocytes from the seabass from the third experiment, a lens (20 mm diameter) was placed in each well of the plates and then, aliquots of 10^7 cells/mL in supplemented medium L-15 were placed in the lenses and homogeneously distributed. Next, the plate was incubated with *Vibrio anguillarum* (10uL, 10^9 CFU/mL, MOI 1:1) for 1 hour at 22°C. After this time, wash with PBS and air dry the lenses. Next, proceed with rapid Diff-Quik staining of each lens. After drying, the lenses were put on a slide. To determine the phagocytic capacity, one hundred lymphocytes per lens were counted, indicating those with phagocytic vacuoles and those without them.

Statistics

The results were expressed as mean ± standard deviation. All the data were analysed with GraphPad Prism 8.0.2 (GraphPad Software, San Diego, California USA, www.graphpad.com), normal distribution was determined with Shapiro-Wilk test, one way ANOVA analysis was performed to the normal data and non-parametric Kruskal-Wallis test were performed on the data without normal distribution. The significance was established with a p-value = 0.05.

5.3 Results

Growth

The influence on the seabass juvenile performance of the two raw by-products was tested in the first two experiments. Both BT and BF increased growth significantly. After two months of feeding, all diets with BT inclusion increased growth compared to the control diet with 4% BT inclusion having the highest final weight at the end of the feeding experiment (**Table 5.4**). Meanwhile, in the banana flower inclusion experiment, the 6% inclusion significantly increased weight after one month of feeding (**Table 5.6**). The rest of the production parameters were not affected by the inclusion of both by-products. Even in the last experiment, where growth was not the main objective, a small improvement in final weight was observed with 6% flower inclusion (**Table 5.7**).

Table 5.4. Growth parameters at the end of the feeding trial in the Experiment I with banana pseudo-stem inclusion.

	Diet			
	C	**BT2**	**BT4**	**BT8**
FW	19.82 ± 1.64[b]	20.49 ± 0.43[ab]	23.38 ± 1.56[a]	21.49 ± 1.50[ab]
[1]HSI	1.64 ± 0.14	1.79 ± 0.11	1.51 ± 0.12	1.67 ± 0.23
[2]VSI	12.14 ± 1.17	12.90 ± 1.06	11.85 ± 0.46	12.17 ± 1.72
[3]K	1.02 ± 0.04	1.07 ± 0.08	1.01 ± 0.08	1.02 ± 0.03
[4]FI	111.02 ± 8.71	110.09 ± 7.39	98.82 ± 13.05	102.16 ± 2.06
[5]FCR	3.59 ± 0.96	3.28 ± 0.24	2.39 ± 0.49	2.81 ± 0.45
[6]SGR	0.58 ± 0.14	0.61 ± 0.02	0.81 ± 0.11	0.69 ± 0.11
[7]PI	244.86 ± 3.53	229.96 ± 13.84	237.26 ± 15.45	240.92 ± 14.14

Different letters in same row indicates significant differences (p-value<0.05).

[1]HIS (%) = 100×wet liver weight /body weight.

[2]VSI (%) =((Weight of whole fish (g) − Weight of fish without viscera (g))/ Weight of whole fish (g))x 100

[3]K= (Final Weight (g) / Total Length (cm)^3) x 100

[4]FI= Feed intake (g) per fish for the experimental-day period

[5]FCR= Feed intake (g) / Weight increase (g)

[6]SGR= ((Ln Final weight – Ln Initial weight)/ nº days) x 100

[7]PI= (Protein (%)/ total feed) / 100

Table 5.5. Growth parameters at the end of the feeding trial in Experiment II with banana flower inclusion**.**

	Diet			
	C	**BF1**	**BF3**	**BF6**
FW	23.25 ± 0.7[b]	23.71 ± 1.19[ab]	23.66 ± 1.63[ab]	25.58 ± 0.89[a]
[1]HSI	1.68 ± 0.13	1.61 ± 0.04	1.44 ± 0.18	1.45 ± 0.23
[2]VSI	15.08 ± 2.12	15.67 ± 1.61	14.54 ± 0.77	15.79 ± 0.91
[3]K	1.32 ± 0.11	1.34 ± 0.04	1.26 ± 0.02	1.30 ± 0.06
[4]FI	40.95 ± 2.24	39.65 ± 3.74	38.46 ± 3.55	37.37 ± 1.43
[5]FCR	2.58 ± 0.54	2.28 ± 0.56	2.41 ± 0.95	1.56 ± 0.21
[6]SGR	0.49 ± 0.09	0.56 ± 0.16	0.53 ± 0.18	0.77 ± 0.11
[7]PI	121.26 ± 4.24	106.67 ± 9.52	103.48 ± 6.61	115.49 ± 8.76

Different letters in same row indicates significant differences (p-value<0.05).

[1]HIS (%) = 100×wet liver weight /body weight.

[2]VSI (%) =((Weight of whole fish (g) − Weight of fish without viscera (g))/ Weight of whole fish (g))x 100

[3]K= (Final Weight (g) / Total Length (cm)^3) x 100

[4]FI= Feed intake (g) per fish for the experimental-day period

[5]FCR= Feed intake (g) / Weight increase (g)

[6]SGR= ((Ln Final weight – Ln Initial weight)/ nº days) x 100

[7]PI= (Protein (%)/ total feed) / 100

Table 5.6. Growth parameters at the end of the feeding trial in Experiment III with banana flower and extract and residue.

	DIET			
	C	**BF6**	**BFR**	**BFE**
WEIGHT	48.79 ± 8.98	50.99 ± 11.37	48.96 ± 8.05	48.67 ± 7.58
TOTAL LENGTH	16.60 ± 0.93	17.13 ± 1.21	17.01 ± 1.25	16.69 ± 0.86
FURCAL LENGTH	15.46 ± 1.25	16.06 ± 1.15	15.77 ± 0.96	15.54 ± 0.97

Different letter indicates significant differences (p-value<0.05)

Proximate composition and FAMEs

The lipid content of the sea bass muscle was not affected by the experimental diets in the first two trials (**Table 5.7 and Table 5.9**), nor was the fatty acid profile. In the case of the livers, although the percentage of total lipids was not affected in any of the three experiments (**Table 5.8, Table 5.10 and Table 5.11**), but the fatty acid partner it was. Thus, the percentage of 18:3n-3 varies, in particular, increases up to double with the inclusion of BT (**Table 5.8**) in Experiment I, it also increases with the 6% inclusion of BF in Experiment II and Experiment III (**Table 5.10 and Table 5.11**), while the BF6E diet decreases it. Regarding other affected fatty acids, in Experiment II, BF6 significantly decreased the content of 14:00 and 16:1n-7, and in Experiment III, BF6R and BF6E increased the percentage of DHA almost twice as much as the control diet, but not statistically significantly.

Table 5.7. Lipids, moisture and fatty acids composition in fish muscle at the end of Experiment I with banana pseudo-stem inclusion**.**

	Diet			
	Control	**BT2**	**BT4**	**BT8**
Lipids (%dw)	21.68 ± 4.47	26.55 ± 2.67	28.80 ± 0.68	21.60 ± 8.19
Moisture (%dw)	74.55 ± 2.67	74.12 ± 0.58	72.55 ± 1.28	75.21 ± 1.65
Fatty Acids (%)				
14:00	1.65 ± 0.38	1.45 ± 0.66	1.81 ± 0.03	1.81 ± 1.12
14:1n-7	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.00	0.06 ± 0.06
14:1n-5	0.06 ± 0.01	0.05 ± 0.02	0.06 ± 0.00	0.08 ± 0.07
15:00	0.24 ± 0.02	0.20 ± 0.06	0.26 ± 0.02	0.26 ± 0.14
15:1n-5	0.02 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.05 ± 0.07
16:OISO	0.05 ± 0.01	0.03 ± 0.01	0.04 ± 0.00	0.07 ± 0.07
16:00	17.42 ± 1.51	15.71 ± 3.74	16.89 ± 0.74	18.92 ± 7.35
16:1n-7	2.94 ± 0.51	2.81 ± 0.83	3.01 ± 0.04	2.57 ± 0.36
16:1n-5	0.10 ± 0.01	0.11 ± 0.03	0.10 ± 0.00	0.13 ± 0.08
16:2n-4	0.10 ± 0.04	0.10 ± 0.01	0.12 ± 0.02	0.15 ± 0.07
17:00	0.06 ± 0.03	0.06 ± 0.01	0.09 ± 0.01	0.09 ± 0.03
16:3n-4	0.21 ± 0.02	0.23 ± 0.04	0.24 ± 0.01	0.23 ± 0.05
16:3n-3	0.10 ± 0.01	0.08 ± 0.02	0.09 ± 0.01	0.13 ± 0.05
16:3n-1	0.11 ± 0.08	0.09 ± 0.03	0.08 ± 0.02	0.11 ± 0.06
16:4n-3	0.13 ± 0.04	0.08 ± 0.04	0.09 ± 0.00	0.13 ± 0.02
16:4n-1	0.02 ± 0.01	0.14 ± 0.20	0.01 ± 0.02	0.05 ± 0.05
18:00	6.06 ± 1.02	6.90 ± 2.59	5.45 ± 0.31	6.63 ± 1.85

18:1n-9	39.30 ± 0.80	38.50 ± 2.95	39.45 ± 1.36	37.02 ± 4.09
18:1n-7	3.30 ± 0.20	2.63 ± 0.96	3.08 ± 0.03	3.23 ± 0.03
18.1n-5	0.14 ± 0.01	0.13 ± 0.02	0.14 ± 0.00	0.15 ± 0.04
18:2n-9	0.34 ± 0.02	0.35 ± 0.04	0.34 ± 0.04	0.32 ± 0.09
18.2n-6	11.73 ± 0.94	12.51 ± 0.98	12.69 ± 0.45	9.76 ± 4.33
18:2n-4	0.09 ± 0.00	0.10 ± 0.00	0.09 ± 0.00	0.12 ± 0.04
18:3n-6	0.23 ± 0.00	0.25 ± 0.00	0.25 ± 0.01	0.27 ± 0.01
18:3n-4	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.02	0.08 ± 0.02
18:3n-3	3.38 ± 1.25	4.97 ± 0.94	4.62 ± 1.07	3.55 ± 2.54
18.3n-1	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.05
18:4n-3	0.10 ± 0.07	0.17 ± 0.06	0.15 ± 0.11	0.20 ± 0.04
18:4n-1	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.05
20:00	0.42 ± 0.07	0.39 ± 0.05	0.36 ± 0.05	0.52 ± 0.19
20:1n-9	0.49 ± 0.07	0.59 ± 0.22	0.37 ± 0.02	0.43 ± 0.08
20:1n-7	4.23 ± 0.43	3.85 ± 0.78	3.75 ± 0.28	3.90 ± 0.69
20.1n-5	0.18 ± 0.02	0.17 ± 0.03	0.15 ± 0.01	0.18 ± 0.03
20:2n-9	0.09 ± 0.00	0.09 ± 0.01	0.08 ± 0.00	0.18 ± 0.14
20:2n-6	0.81 ± 0.06	0.88 ± 0.17	0.81 ± 0.03	0.81 ± 0.23
20:3n-9	0.10 ± 0.00	0.12 ± 0.03	0.10 ± 0.02	0.16 ± 0.03
20:3n-6	0.05 ± 0.02	0.04 ± 0.02	0.03 ± 0.00	0.12 ± 0.11
20:4n-6	0.23 ± 0.07	0.30 ± 0.06	0.26 ± 0.09	0.32 ± 0.06
20:3n-3	0.15 ± 0.02	0.18 ± 0.04	0.16 ± 0.03	0.24 ± 0.02
20:4n-3	0.10 ± 0.06	0.16 ± 0.07	0.12 ± 0.08	0.19 ± 0.06
20:5n-3	0.34 ± 0.26	0.64 ± 0.39	0.52 ± 0.45	0.63 ± 0.38
22:1n-11	2.77 ± 0.39	2.36 ± 0.51	2.08 ± 0.04	2.69 ± 0.51
22:1n-9	0.73 ± 0.17	0.66 ± 0.20	0.59 ± 0.05	0.82 ± 0.19
22:4n-6	0.08 ± 0.01	0.09 ± 0.04	0.08 ± 0.00	0.16 ± 0.07
22:5n-6	0.06 ± 0.03	0.09 ± 0.04	0.11 ± 0.00	0.16 ± 0.09
22:5n-3	0.08 ± 0.05	0.17 ± 0.12	0.14 ± 0.08	0.26 ± 0.10
22:6n-3	1.11 ± 0.12	1.38 ± 0.67	1.03 ± 0.42	1.97 ± 0.78
[1]Saturades	25.89 ± 2.06	24.76 ± 6.99	24.89 ± 1.15	28.30 ± 10.72
[2]Monoenoics	54.27 ± 1.42	51.90 ± 4.16	52.79 ± 1.72	51.32 ± 4.50
[3]n-6	13.17 ± 0.94	14.17 ± 1.26	14.23 ± 0.52	10.79 ± 4.10
[4]n-3	5.50 ± 1.44	7.83 ± 2.11	6.74 ± 2.24	7.31 ± 2.70
[5]n-9	41.06 ± 0.95	40.31 ± 2.92	40.93 ± 1.33	38.94 ± 3.91
[6]n-3 HUFA	1.79 ± 0.30	2.53 ± 1.28	1.97 ± 1.06	3.29 ± 1.13
ARA/EPA	0.90 ± 0.50	0.54 ± 0.17	0.68 ± 0.41	0.59 ± 0.22
DHA/EPA	5.60 ± 5.30	2.27 ± 0.56	2.62 ± 1.46	3.59 ± 2.04
DHA/ARA	5.36 ± 2.36	4.44 ± 1.34	3.93 ± 0.23	6.00 ± 1.68
[7]Total PUFA	19.07 ± 2.28	22.61 ± 3.21	21.68 ± 2.83	19.57 ± 6.63
n-3/n-6	0.41 ± 0.09	0.55 ± 0.11	0.47 ± 0.14	0.70 ± 0.14

[1]14:00,15:00, 16:00, 17:00, 18:00, 20:00

[2]14:1n-7; 14:1n-5; 15:1n-5; 16:1n-7 16:1n-5; 18:1n-9; 18:1n-7; 18:1n-5; 20:1n-9; 20:1n-7; 20:1n-5; 22:1n-11; 22:1n-9

[3]18:2n-6; 18:3n-6; 20:2n-6; 20:3n-6; 20:4n-6; 22:4n-6; 22:5n-6

[4]16:3n-3; 18:3n-3; 18:4n-3; 20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

[5]18:1n-9; 18:2n-9; 20:1n-9; 20:2n-9; 20:3n-9

[6]20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

[7]18:2n-9; 18:2n-6; 18:2n-4; 18:3n-6; 18:3n-4; 18:4n-3; 18:4n-1; 20:2n-9; 20:2n-6; 20:3n-9; 20:3n-6; 20:4n-6; 20:3n-3, 20:4n-3; 20:5n-3; 22:4n-6, 22:5n-6; 22:5n-3; 22:6n-3

Table 5.8. Lipids, moisture and fatty acids composition in fish liver at the end of Experiment I with banana pseudo-stem inclusion.

	Diet			
	Control	**BP2**	**BP4**	**BP8**
Lipids (%dw)	48.42 ± 2.59	55.42 ± 13.16	54.93 ± 4.36	51.63 ± 10.95
Moisture (%dw)	58.82 ± 8.11	55.24 ± 6.12	57.87 ± 2.26	57.24 ± 1.50
Fatty Acids (%)				
14:00	0.41 ± 0.30	0.67 ± 0.19	0.53 ± 0.15	0.49 ± 0.28
14:1n-7	0.02 ± 0.02	0.02 ± 0.00	0.09 ± 0.15	0.01 ± 0.00
14:1n-5	0.03 ± 0.00	0.02 ± 0.01	0.08 ± 0.11	0.02 ± 0.01
15:00	0.14 ± 0.05	0.12 ± 0.02	0.18 ± 0.13	0.12 ± 0.05
15:1n-5	0.02 ± 0.02	0.01 ± 0.00	0.07 ± 0.10	0.01 ± 0.00
16:OISO	0.04 ± 0.00	0.02 ± 0.01	0.09 ± 0.11	0.02 ± 0.01
16:00	13.06 ± 4.77	11.45 ± 0.95	8.65 ± 4.59	10.35 ± 1.42
16:1n-7	2.10 ± 1.11	2.15 ± 0.00	1.77 ± 0.83	1.94 ± 0.18
16:1n-5	0.11 ± 0.04	0.08 ± 0.00	0.12 ± 0.06	0.09 ± 0.02
16:2n-4	0.05 ± 0.02	0.05 ± 0.01	0.09 ± 0.06	0.04 ± 0.01
17:00	0.06 ± 0.01	0.06 ± 0.01	0.10 ± 0.07	0.07 ± 0.01
16:3n-4	0.24 ± 0.10	0.22 ± 0.02	0.22 ± 0.05	0.23 ± 0.01
16:3n-3	0.11 ± 0.02	0.06 ± 0.01	0.16 ± 0.15	0.06 ± 0.01
16:3n-1	0.02 ± 0.02	0.01 ± 0.00	0.09 ± 0.16	0.01 ± 0.00
16:4n-3	0.03 ± 0.02	0.02 ± 0.01	0.08 ± 0.09	0.02 ± 0.01
16:4n-1	0.01 ± 0.01	0.00 ± 0.00	0.06 ± 0.09	0.00 ± 0.00
18:00	5.63 ± 1.50	3.68 ± 0.26	3.89 ± 0.64	3.80 ± 0.36
18:1n-9	43.86 ± 0.87	43.74 ± 2.29	40.83 ± 4.91	41.98 ± 4.08
18:1n-7	3.50 ± 0.06	2.79 ± 0.07	3.04 ± 0.24	2.91 ± 0.17
18.1n-5	0.15 ± 0.05	0.10 ± 0.01	0.18 ± 0.11	0.11 ± 0.01
18:2n-9	0.80 ± 0.23	0.92 ± 0.25	1.08 ± 0.35	0.93 ± 0.17
18.2n-6	11.97 ± 0.97	13.16 ± 0.92	12.69 ± 0.73	14.17 ± 0.94
18:2n-4	0.09 ± 0.03	0.07 ± 0.00	0.15 ± 0.13	0.08 ± 0.01
18:3n-6	0.56 ± 0.30	0.56 ± 0.12	0.67 ± 0.13	0.65 ± 0.07
18:3n-4	0.10 ± 0.04	0.08 ± 0.01	0.15 ± 0.12	0.08 ± 0.00
18:3n-3	4.63 ± 1.41^{b}	8.43 ± 0.34^{ab}	7.80 ± 2.29^{ab}	8.61 ± 1.24^{a}
18.3n-1	0.02 ± 0.02	0.00 ± 0.00	0.08 ± 0.13	0.00 ± 0.00
18:4n-3	0.35 ± 0.19	0.61 ± 0.07	0.67 ± 0.17	0.62 ± 0.05
18:4n-1	0.05 ± 0.04	0.03 ± 0.00	0.10 ± 0.12	0.03 ± 0.00
20:00	0.26 ± 0.15	0.19 ± 0.01	0.32 ± 0.16	0.16 ± 0.01
20:1n-9	0.38 ± 0.02	0.28 ± 0.02	0.52 ± 0.22	0.29 ± 0.02
20:1n-7	2.97 ± 0.41	2.49 ± 0.42	3.50 ± 1.16	2.21 ± 0.11
20.1n-5	0.13 ± 0.04	0.09 ± 0.02	0.20 ± 0.15	0.08 ± 0.01
20:2n-9	0.10 ± 0.03	0.07 ± 0.00	0.19 ± 0.17	0.07 ± 0.01
20:2n-6	0.99 ± 0.17	0.70 ± 0.13	0.87 ± 0.33	0.73 ± 0.04
20:3n-9	0.00 ± 0.01	0.01 ± 0.00	0.07 ± 0.11	0.01 ± 0.00
20:3n-6	0.13 ± 0.01	0.12 ± 0.01	0.22 ± 0.13	0.14 ± 0.02
20:4n-6	0.36 ± 0.08	0.36 ± 0.04	0.55 ± 0.19	0.56 ± 0.20
20:3n-3	0.20 ± 0.04	0.17 ± 0.04	0.32 ± 0.27	0.20 ± 0.03
20:4n-3	0.21 ± 0.05	0.20 ± 0.07	0.34 ± 0.24	0.24 ± 0.05
20:5n-3	1.03 ± 0.11	1.23 ± 0.40	1.32 ± 0.48	1.39 ± 0.35
22:1n-11	0.83 ± 0.02	0.69 ± 0.26	1.43 ± 1.13	0.55 ± 0.13
22:1n-9	0.39 ± 0.13	0.34 ± 0.04	0.56 ± 0.20	0.29 ± 0.02
22:4n-6	0.06 ± 0.02	0.06 ± 0.01	0.23 ± 0.29	0.06 ± 0.01
22:5n-6	0.12 ± 0.04	0.11 ± 0.02	0.33 ± 0.36	0.14 ± 0.04
22:5n-3	0.38 ± 0.08	0.45 ± 0.14	0.68 ± 0.44	0.59 ± 0.21
22:6n-3	3.30 ± 0.03	3.29 ± 0.84	4.63 ± 2.13	4.85 ± 1.97
[1]Saturades	19.46 ± 3.43	16.19 ± 0.96	13.76 ± 4.81	15.02 ± 2.03
[2]Monoenoics	54.50 ± 0.29	52.82 ± 1.57	52.39 ± 3.75	50.48 ± 4.12
[3]n-6	14.19 ± 1.60	15.07 ± 1.01	15.56 ± 1.75	16.44 ± 1.21
[4]n-3	10.23 ± 1.36	14.46 ± 1.79	16.00 ± 5.85	16.56 ± 3.09
[5]n-9	45.54 ± 1.22	45.37 ± 2.48	43.25 ± 4.47	43.57 ± 4.26

[6]**n-3 HUFA**	5.11 ± 0.24	5.34 ± 1.49	7.29 ± 3.51	7.26 ± 2.6
ARA/EPA	0.36 ± 0.11	0.3 ± 0.07	0.44 ± 0.11	0.39 ± 0.05
DHA/EPA	3.22 ± 0.39	2.71 ± 0.2	3.47 ± 0.56	3.38 ± 0.66
DHA/ARA	9.31 ± 1.85	9.14 ± 1.41	8.15 ± 1.8	8.52 ± 0.69
[7]**Total PUFA**	25.45 ± 3.27	30.64 ± 2.52	33.15 ± 7.98	34.13 ± 4.14
n-3/n-6	0.72 ± 0.02	0.96 ± 0.05	1.01 ± 0.26	1.00 ± 0.11

[1]14:00,15:00, 16:00, 17:00, 18:00, 20:00
[2]14:1n-7; 14:1n-5; 15:1n-5; 16:1n-7 16:1n-5; 18:1n-9; 18:1n-7; 18:1n-5; 20:1n-9; 20:1n-7; 20:1n-5; 22:1n-11; 22:1n-9
[3]18:2n-6; 18:3n-6; 20:2n-6; 20:3n-6; 20:4n-6; 22:4n-6; 22:5n-6
[4]16:3n-3; 18:3n-3; 18:4n-3; 20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3
[5]18:1n-9; 18:2n-9; 20:1n-9; 20:2n-9; 20:3n-9
[6]20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3
[7]18:2n-9; 18:2n-6; 18:2n-4; 18:3n-6; 18:3n-4; 18:4n-3; 18:4n-1; 20:2n-9; 20:2n-6; 20:3n-9; 20:3n-6; 20:4n-6; 20:3n-3, 20:4n-3; 20:5n-3; 22:4n-6, 22:5n-6; 22:5n-3; 22:6n-3

Table 5.9. Lipids, moisture and fatty acids composition in fish muscle at the end of Experiment II with banana flower inclusion.

	Diet			
	Control	**BF1**	**BF3**	**BF6**
Lipids (%dw)	24.90 ± 3.53	29.92 ± 5.98	20.29 ± 6.28	22.69 ± 5.87
Moisture (%dw)	75.15 ± 1.35	73.6 ± 2.28	74.85 ± 1.80	75.09 ± 1.85
Fatty Acids (%)				
14:00	1.66 ± 0.74	1.50 ± 0.58	1.14 ± 0.33	1.03 ± 0.13
14:1n-7	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.00
14:1n-5	0.05 ± 0.02	0.05 ± 0.02	0.04 ± 0.01	0.04 ± 0.00
15:00	0.21 ± 0.06	0.19 ± 0.05	0.17 ± 0.03	0.17 ± 0.01
15:1n-5	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
16:OISO	0.05 ± 0.01	0.03 ± 0.01	0.06 ± 0.03	0.05 ± 0.02
16:00	13.95 ± 2.39	14.29 ± 2.38	12.85 ± 1.07	12.96 ± 0.44
16:1n-7	3.21 ± 0.76	3.25 ± 0.51	2.86 ± 0.44	2.77 ± 0.29
16:1n-5	0.09 ± 0.02	0.07 ± 0.00	0.07 ± 0.01	0.08 ± 0.00
16:2n-4	0.17 ± 0.04	0.16 ± 0.03	0.15 ± 0.02	0.15 ± 0.02
17:00	0.14 ± 0.03	0.13 ± 0.03	0.13 ± 0.01	0.13 ± 0.02
16:3n-4	0.20 ± 0.02	0.20 ± 0.01	0.18 ± 0.02	0.19 ± 0.01
16:3n-3	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
16:3n-1	0.04 ± 0.04	0.04 ± 0.01	0.07 ± 0.03	0.06 ± 0.02
16:4n-3	0.12 ± 0.03	0.1. ± 0.02	0.13 ± 0.02	0.11 ± 0.01
16:4n-1	0.03 ± 0.02	0.00 ± 0.00	0.01 ± 0.01	0.02 ± 0.01
18:00	3.95 ± 0.13	3.99 ± 0.14	3.94 ± 0.17	4.05 ± 0.16
18:1n-9	31.93 ± 0.24	32.51 ± 1.52	31.00 ± 1.38	32.03 ± 0.52
18:1n-7	2.40 ± 0.21	2.55 ± 0.19	2.61 ± 0.32	2.70 ± 0.12
18.1n-5	0.13 ± 0.00	0.13 ± 0.01	0.11 ± 0.01	0.12 ± 0.00
18:2n-9	0.39 ± 0.04	0.39 ± 0.03	0.37 ± 0.02	0.37 ± 0.01
18.2n-6	11.45 ± 0.65	11.37 ± 0.95	11.66 ± 0.16	12.61 ± 0.31
18:2n-4	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
18:3n-6	0.27 ± 0.01	0.26 ± 0.02	0.25 ± 0.03	0.29 ± 0.02
18:3n-4	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.02	0.11 ± 0.02
18:3n-3	5.10 ± 0.88	4.83 ± 1.01	5.31 ± 0.32	6.41 ± 0.42
18.3n-1	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
18:4n-3	0.68 ± 0.02	0.68 ± 0.02	0.69 ± 0.03	0.68 ± 0.04

18:4n-1	0.07 ± 0.01	0.07 ± 0.00	0.06 ± 0.01	0.06 ± 0.01
20:00	0.25 ± 0.02	0.27 ± 0.04	0.26 ± 0.02	0.26 ± 0.01
20:1n-9	0.22 ± 0.20	0.35 ± 0.05	0.39 ± 0.04	0.36 ± 0.02
20:1n-7	3.39 ± 0.30	3.32 ± 0.30	3.27 ± 0.31	3.14 ± 0.09
20.1n-5	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.02	0.13 ± 0.01
20:2n-9	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.01	0.11 ± 0.03
20:2n-6	0.77 ± 0.09	0.77 ± 0.06	0.81 ± 0.06	0.77 ± 0.03
20:3n-9	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01
20:3n-6	0.14 ± 0.01	0.14 ± 0.00	0.16 ± 0.00	0.15 ± 0.01
20:4n-6	0.57 ± 0.06	0.54 ± 0.06	0.66 ± 0.09	0.57 ± 0.05
20:3n-3	0.23 ± 0.02	0.23 ± 0.02	0.24 ± 0.03	0.23 ± 0.01
20:4n-3	0.47 ± 0.02	0.47 ± 0.03	0.48 ± 0.03	0.44 ± 0.01
20:5n-3	4.14 ± 0.27	4.11 ± 0.29	4.40 ± 0.35	3.85 ± 0.08
22:1n-11	2.04 ± 0.17	2.15 ± 0.29	2.15 ± 0.32	1.95 ± 0.14
22:1n-9	0.50 ± 0.06	0.51 ± 0.04	0.58 ± 0.08	0.55 ± 0.03
22:4n-6	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
22:5n-6	0.25 ± 0.04	0.24 ± 0.01	0.29 ± 0.04	0.25 ± 0.03
22:5n-3	1.06 ± 0.15	1.03 ± 0.05	1.19 ± 0.14	1.03 ± 0.08
22:6n-3	8.96 ± 1.58	8.38 ± 0.57	10.58 ± 1.69	8.71 ± 1.04
[1]Saturades	20.20 ± 3.13	20.4 ± 3.12	18.55 ± 1.23	18.65 ± 0.49
[2]Monoenoics	44.14 ± 0.59	45.07 ± 1.77	43.24 ± 1.32	43.89 ± 1.02
[3]n-6	13.55 ± 0.86	13.42 ± 0.97	13.94 ± 0.28	14.73 ± 0.31
[4]n-3	20.84 ± 2.81	19.91 ± 0.63	23.08 ± 2.28	21.54 ± 1.47
[5]n-9	33.16 ± 0.28	33.87 ± 1.63	32.44 ± 1.30	33.43 ± 0.52
[6]n-3 HUFA	14.86 ± 2.03	14.22 ± 0.90	16.88 ± 2.15	14.26 ± 1.19
ARA/EPA	0.14 ± 0.01	0.13 ± 0.01	0.15 ± 0.01	0.15 ± 0.01
DHA/EPA	2.15 ± 0.24	2.04 ± 0.03	2.40 ± 0.24	2.26 ± 0.22
DHA/ARA	15.65 ± 1.12	15.48 ± 0.80	15.99 ± 0.49	15.34 ± 0.60
[7]Total PUFA	35.02 ± 3.64	33.95 ± 1.42	37.59 ± 2.49	36.86 ± 1.38
n-3/n-6	1.53 ± 0.11	1.49 ± 0.10	1.65 ± 0.13	1.46 ± 0.11

[1]14:00,15:00, 16:00, 17:00, 18:00, 20:00

[2]14:1n-7; 14:1n-5; 15:1n-5; 16:1n-7 16:1n-5; 18:1n-9; 18:1n-7; 18:1n-5; 20:1n-9; 20:1n-7; 20:1n-5; 22:1n-11; 22:1n-9

[3]18:2n-6; 18:3n-6; 20:2n-6; 20:3n-6; 20:4n-6; 22:4n-6; 22:5n-6

[4]16:3n-3; 18:3n-3; 18:4n-3; 20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

[5]18:1n-9; 18:2n-9; 20:1n-9; 20:2n-9; 20:3n-9

[6]20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

[7]18:2n-9; 18:2n-6; 18:2n-4; 18:3n-6; 18:3n-4; 18:4n-3; 18:4n-1; 20:2n-9; 20:2n-6; 20:3n-9; 20:3n-6; 20:4n-6; 20:3n-3, 20:4n-3; 20:5n-3; 22:4n-6, 22:5n-6; 22:5n-3; 22:6n-3

Table 5.10. Lipids, moisture and fatty acids composition in fish liver at the end of Experiment II with banana flower inclusion.

	Diet			
	Control	**BF1**	**BF3**	**BF6**
Lipids (%dw)	57.29 ± 10.00	53.23 ± 6.56	50.79 ± 13.26	50.49 ± 6.73
Moisture (%dw)	57.61 ± 1.36	55.84 ± 1.97	59.24 ± 4.66	58.70 ± 0.76
Fatty Acids (%)				
14:00	1.43 ± 0.13[a]	0.94 ± 0.37[ab]	1.22 ± 0.11[ab]	0.78 ± 0.22[b]
14:1n-7	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.00
14:1n-5	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.00
15:00	0.18 ± 0.03	0.14 ± 0.03	0.18 ± 0.02	0.12 ± 0.02

15:1n-5	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
16:OISO	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.01
16:00	14.77 ± 1.9	13.97 ± 1.14	13.51 ± 1.05	12.00 ± 2.00
16:1n-7	3.32 ± 0.08[a]	3.02 ± 0.54[ab]	2.90 ± 0.40[ab]	2.24 ± 0.26[b]
16:1n-5	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.02	0.10 ± 0.01
16:2n-4	0.09 ± 0.04	0.06 ± 0.03	0.07 ± 0.01	0.05 ± 0.01
17:00	0.09 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.01
16:3n-4	0.26 ± 0.01	0.25 ± 0.02	0.28 ± 0.03	0.23 ± 0.02
16:3n-3	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.00	0.05 ± 0.00
16:3n-1	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00
16:4n-3	0.04 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
18:00	3.70 ± 0.63	3.87 ± 0.46	3.58 ± 0.48	3.94 ± 0.29
18:1n-9	40.16 ± 4.60	42.56 ± 2.54	41.04 ± 2.76	41.57 ± 0.91
18:1n-7	2.43 ± 0.06	2.71 ± 0.23	2.25 ± 0.04	2.67 ± 0.14
18:1n-5	0.13 ± 0.02	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
18:2n-9	0.92 ± 0.37	1.13 ± 0.36	1.17 ± 0.29	1.36 ± 0.15
18.2n-6	11.75 ± 1.44	11.96 ± 0.67	12.58 ± 1.01	13.02 ± 0.91
18:2n-4	0.09 ± 0.02	0.09 ± 0.02	0.09 ± 0.00	0.09 ± 0.01
18:3n-6	0.53 ± 0.09	0.65 ± 0.16	0.75 ± 0.24	0.86 ± 0.04
18:3n-4	0.09 ± 0.02	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.02
18:3n-3	5.88 ± 0.94[b]	6.35 ± 0.66[a]	7.28 ± 0.29[ab]	8.41 ± 0.78[a]
18:4n-3	0.63 ± 0.11	0.65 ± 0.09	0.76 ± 0.16	0.84 ± 0.03
18:4n-1	0.04 ± 0.02	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.02
20:00	0.15 ± 0.02	0.14 ± 0.02	0.15 ± 0.01	0.15 ± 0.02
20:1n-9	0.32 ± 0.04	0.34 ± 0.04	0.29 ± 0.02	0.29 ± 0.03
20:1n-7	2.38 ± 0.26	2.15 ± 0.12	2.07 ± 0.1	2.07 ± 0.29
20.1n-5	0.09 ± 0.02	0.08 ± 0.00	0.08 ± 0.00	0.07 ± 0.01
20:2n-9	0.08 ± 0.00	0.08 ± 0.01	0.08 ± 0.02	0.08 ± 0.01
20:2n-6	0.65 ± 0.14	0.61 ± 0.02	0.65 ± 0.05	0.74 ± 0.11
20:3n-9	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.05
20:3n-6	0.13 ± 0.03	0.12 ± 0.01	0.13 ± 0.03	0.15 ± 0.02
20:4n-6	0.39 ± 0.12	0.40 ± 0.04	0.52 ± 0.18	0.52 ± 0.07
20:3n-3	0.19 ± 0.07	0.16 ± 0.03	0.16 ± 0.01	0.15 ± 0.02
20:4n-3	0.30 ± 0.17	0.22 ± 0.07	0.22 ± 0.02	0.19 ± 0.02
20:5n-3	1.94 ± 1.27	1.37 ± 0.42	1.42 ± 0.28	1.19 ± 0.11
22:1n-11	0.88 ± 0.33	0.65 ± 0.05	0.58 ± 0.04	0.51 ± 0.09
22:1n-9	0.29 ± 0.05	0.26 ± 0.03	0.28 ± 0.01	0.31 ± 0.05
22:4n-6	0.05 ± 0.02	0.05 ± 0.01	0.05 ± 0.00	0.07 ± 0.02
22:5n-6	0.12 ± 0.06	0.11 ± 0.03	0.13 ± 0.03	0.13 ± 0.01
22:5n-3	0.61 ± 0.39	0.51 ± 0.23	0.52 ± 0.1	0.47 ± 0.05
22:6n-3	4.56 ± 3.17	3.74 ± 1.24	4.37 ± 1.13	4.14 ± 0.35
[1]Saturades	20.36 ± 2.39	19.16 ± 1.40	18.73 ± 0.82	17.07 ± 2.36
[2]Monoenoics	50.21 ± 3.89	52.07 ± 2.30	49.80 ± 3.18	50.00 ± 1.02
[3]n-6	13.61 ± 1.61	13.91 ± 0.80	14.81 ± 1.51	15.50 ± 1.12
[4]n-3	14.22 ± 5.18	13.10 ± 2.01	14.83 ± 1.96	15.46 ± 1.33
[5]n-9	41.78 ± 4.89	44.39 ± 2.89	42.87 ± 2.46	43.65 ± 1.05
[6]n-3 HUFA	7.60 ± 5.06	6.01 ± 1.99	6.70 ± 1.53	6.14 ± 0.54
ARA/EPA	0.25 ± 0.12	0.31 ± 0.09	0.36 ± 0.06	0.43 ± 0.03
DHA/EPA	2.36 ± 0.28	2.71 ± 0.14	3.06 ± 0.21	3.46 ± 0.10
DHA/ARA	10.82 ± 4.65	9.35 ± 3.49	8.58 ± 0.90	8.08 ± 0.86
[7]Total PUFA	28.96 ± 6.10	28.36 ± 2.33	31.03 ± 3.73	32.58 ± 2.43
n-3/n-6	1.03 ± 0.31	0.94 ± 0.13	1.00 ± 0.05	1.00 ± 0.02

Different upper letters indicate significant differences (p-value<0.05)

[1]14:00,15:00, 16:00, 17:00, 18:00, 20:00

[2]14:1n-7; 14:1n-5; 15:1n-5; 16:1n-7 16:1n-5; 18:1n-9; 18:1n-7; 18:1n-5; 20:1n-9; 20:1n-7; 20:1n-5; 22:1n-11; 22:1n-9

[3]18:2n-6; 18:3n-6; 20:2n-6; 20:3n-6; 20:4n-6; 22:4n-6; 22:5n-6

[4]16:3n-3; 18:3n-3; 18:4n-3; 20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

[5]18:1n-9; 18:2n-9; 20:1n-9; 20:2n-9; 20:3n-9

[6]20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

[7]18:2n-9; 18:2n-6; 18:2n-4; 18:3n-6; 18:3n-4; 18:4n-3; 18:4n-1; 20:2n-9; 20:2n-6; 20:3n-9; 20:3n-6; 20:4n-6; 20:3n-3, 20:4n-3; 20:5n-3; 22:4n-6, 22:5n-6; 22:5n-3; 22:6n-3

Table 5.11. Lipids, moisture and fatty acids composition in fish liver at the end of Experiment III with banana flower, extract and residue inclusion.

	Diet			
	Control	**BF6**	**BFR**	**BFE**
Lipids (%dw)	51.40 ± 17.60	56.57 ± 10.74	52.42 ± 1.53	50.43 ± 8.56
Moisture (%dw)	43.79 ± 12.50	53.58 ± 6.34	56.78 ± 3.06	50.87 ± 3.17
Fatty Acids (%)				
14:00	1.16 ± 0.43	0.70 ± 0.02	0.95 ± 0.71	1.09 ± 0.22
14:1n-7	0.03 ± 0.02	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
14:1n-5	0.03 ± 0.01	0.02 ± 0.00	0.03 ± 0.02	0.04 ± 0.01
15:00	0.16 ± 0.03	0.11 ± 0.00	0.14 ± 0.09	0.16 ± 0.01
15:1n-5	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
16:OISO	0.04 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.04 ± 0.00
16:00	16.80 ± 1.59	14.01 ± 2.23	13.45 ± 3.52	17.61 ± 1.15
16:1n-7	3.99 ± 0.73	2.97 ± 0.17	3.00 ± 1.01	3.71 ± 0.15
16:1n-5	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
16:2n-4	0.06 ± 0.02	0.05 ± 0.01	0.07 ± 0.04	0.07 ± 0.01
17:00	0.06 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.00
16:3n-4	0.26 ± 0.01	0.24 ± 0.01	0.25 ± 0.01	0.25 ± 0.02
16:3n-3	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
16:3n-1	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00
16:4n-3	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.00
18:00	3.62 ± 0.25	3.73 ± 0.49	3.49 ± 0.73	3.90 ± 0.80
18:1n-9	40.76 ± 2.38	43.13 ± 2.53	37.5 ± 4.92	37.56 ± 4.36
18:1n-7	3.03 ± 0.14	3.09 ± 0.05	3.00 ± 0.30	2.82 ± 0.18
18.1n-5	0.15 ± 0.01	0.13 ± 0.02	0.16 ± 0.02	0.16 ± 0.02
18:2n-9	0.87 ± 0.18	1.09 ± 0.17	0.80 ± 0.35	0.60 ± 0.18
18.2n-6	10.24 ± 0.58	10.96 ± 1.41	11.57 ± 0.96	9.43 ± 0.66
18:2n-4	0.09 ± 0.01	0.10 ± 0.01	0.11 ± 0.02	0.12 ± 0.02
18:3n-6	0.47 ± 0.10	0.60 ± 0.10	0.50 ± 0.20	0.29 ± 0.05
18:3n-4	0.09 ± 0.01	0.08 ± 0.02	0.11 ± 0.03	0.09 ± 0.01
18:3n-3	4.76 ± 0.45^{ab}	5.90 ± 0.95^{a}	4.74 ± 1.38^{ab}	3.36 ± 0.40^{b}
18.3n-1	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
18:4n-3	0.46 ± 0.06	0.58 ± 0.09	0.55 ± 0.10	0.33 ± 0.01
18:4n-1	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.02	0.04 ± 0.01
20:00	0.13 ± 0.01	0.15 ± 0.01	0.14 ± 0.04	0.13 ± 0.03
20:1n-9	0.36 ± 0.03	0.37 ± 0.05	0.39 ± 0.01	0.34 ± 0.03
20:1n-7	2.24 ± 0.10	2.35 ± 0.24	2.44 ± 0.23	2.22 ± 0.07
20.1n-5	0.08 ± 0.01	0.08 ± 0.01	0.10 ± 0.02	0.09 ± 0.00
20:2n-9	0.08 ± 0.00	0.09 ± 0.01	0.09 ± 0.02	0.08 ± 0.01
20:2n-6	0.66 ± 0.04	0.76 ± 0.10	0.92 ± 0.08	0.80 ± 0.12
20:3n-9	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00
20:3n-6	0.11 ± 0.01	0.14 ± 0.02	0.17 ± 0.02	0.12 ± 0.03
20:4n-6	0.35 ± 0.02	0.42 ± 0.09	0.53 ± 0.01	0.45 ± 0.09

20:3n-3	0.19 ± 0.02	0.17 ± 0.06	0.26 ± 0.05	0.28 ± 0.09
20:4n-3	0.28 ± 0.07	0.26 ± 0.13	0.44 ± 0.11	0.48 ± 0.23
20:5n-3	1.70 ± 0.39	1.65 ± 0.82	2.95 ± 0.95	2.88 ± 1.33
22:1n-11	0.78 ± 0.09	0.77 ± 0.25	0.91 ± 0.02	0.80 ± 0.08
22:1n-9	0.28 ± 0.01	0.27 ± 0.04	0.30 ± 0.05	0.26 ± 0.03
22:4n-6	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.00	0.07 ± 0.01
22:5n-6	0.10 ± 0.02	0.11 ± 0.02	0.16 ± 0.02	0.14 ± 0.06
22:5n-3	0.66 ± 0.21	0.54 ± 0.26	1.00 ± 0.27	1.04 ± 0.52
22:6n-3	4.53 ± 1.09	3.95 ± 1.60	8.25 ± 2.70	7.83 ± 4.25
[1]Saturades	21.94 ± 2.04	18.25 ± 3.64	18.78 ± 2.72	22.95 ± 2.10
[2]Monoenoics	51.86 ± 1.64	47.96 ± 4.47	53.31 ± 2.25	48.13 ± 4.35
[3]n-6	11.98 ± 0.62	13.92 ± 1.23	13.04 ± 1.72	11.30 ± 0.84
[4]n-3	12.68 ± 1.44	18.31 ± 2.63	13.14 ± 3.43	16.30 ± 6.08
[5]n-9	42.37 ± 2.54	39.10 ± 5.29	44.96 ± 2.61	38.85 ± 4.55
[6]n-3 HUFA	7.37 ± 1.71	12.9 ± 4.07	6.56 ± 2.84	12.50 ± 6.42
ARA/EPA	0.21 ± 0.07	0.19 ± 0.05	0.28 ± 0.10	0.17 ± 0.05
DHA/EPA	2.69 ± 0.37	2.79 ± 0.01	2.47 ± 0.47	2.64 ± 0.26
DHA/ARA	13.10 ± 3.38	15.48 ± 5.17	9.23 ± 2.45	16.75 ± 6.18
[7]Total PUFA	25.73 ± 1.27	33.31 ± 1.06	27.48 ± 4.95	28.44 ± 6.45
n-3/n-6	1.06 ± 0.16	1.33 ± 0.32	1.00 ± 0.15	1.43 ± 0.50

Different upper letters indicate significant differences (p-value<0.05)

[1]14:00,15:00, 16:00, 17:00, 18:00, 20:00

[2]14:1n-7; 14:1n-5; 15:1n-5; 16:1n-7 16:1n-5; 18:1n-9; 18:1n-7; 18:1n-5; 20:1n-9; 20:1n-7; 20:1n-5; 22:1n-11; 22:1n-9

[3]18:2n-6; 18:3n-6; 20:2n-6; 20:3n-6; 20:4n-6; 22:4n-6; 22:5n-6

[4]16:3n-3; 18:3n-3; 18:4n-3; 20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

[5]18:1n-9; 18:2n-9; 20:1n-9; 20:2n-9; 20:3n-9

[6]20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

[7]18:2n-9; 18:2n-6; 18:2n-4; 18:3n-6; 18:3n-4; 18:4n-3; 18:4n-1; 20:2n-9; 20:2n-6; 20:3n-9; 20:3n-6; 20:4n-6; 20:3n-3, 20:4n-3; 20:5n-3; 22:4n-6, 22:5n-6; 22:5n-3; 22:6n-3

Muscle and liver oxidation

MDA concentration in the muscle did not vary with the inclusion of the raw materials tested in experiments I (BPS) and II (BF). In contrast, in the fish liver from Experiment I, a decrease in MDA concentration was observed with the BP2 and BP8 diets. The inclusion of flower did not seem to significantly affect the MDA concentration in the liver in experiments II and III, although in the last there was a tendency in BF6 and BF6E to reduce the concentration (**Table 5.12.**).

Table 5.12. MDA concentration in lipids from fish muscle and liver at the end of the experiments.

	DIET			
Muscle	**Control**	**BP2**	**BP4**	**BP8**
MDA (nmol/g lipids)	8.93 ± 2.05	10.37 ± 1.22	11.07 ± 4.33	6.24 ± 2.11
	Control	**BF1**	**BF3**	**BF6**
MDA (nmol/g lipids)	16.46 ± 4.08	15.11 ± 7.33	29.46 ± 0.48	19.58 ± 5.29
Liver	**Control**	**BT 2**	**BT4**	**BT8**
MDA (nmol/g lipids)	46.73 ± 1.45[a]	17.28 ± 6.16[c]	41.03 ± 2.61[ab]	27.90 ± 0.63[bc]
	Control	**BF1**	**BF3**	**BF6**
MDA (nmol/g lipids)	16.84 ± 6.97	12.98 ± 3.35	12.20 ± 4.34	20.96 ± 1.56
	Control	**BF6**	**BFR**	**BFE**
MDA (nmol/g lipids)	36.78 ± 10.59	25.11 ± 5.94	32.22 ± 4.89	23.33 ± 3.77

Different upper letters indicate significant differences (p-value<0.05)

Histopathological analysis

Histopathological analysis of the livers from the three experiments was shown in **Table 5.13**. In Experiment I, the size of the hepatocytes was only affected by 8% BP, which a significant size reduction. The degree of vacuolisation of the hepatocytes was not affected by the inclusion of the increased levels of banana pseudo-stem, although those determined for BP4 diet resulted with better values, as observed in **Figure 5.1** where it was corroborated that the displacement of the nucleus was lower in this diet.

Table 5.13. Histopathological analysis of the livers at the end of the three experiments with banana by-products.

	Diet			
	Control	**BT 2**	**BT 4**	**BT 8**
Hepatocelular Area	153.56 ± 53.08^{a}	168.75 ± 84.17^{a}	165.89 ± 65.27^{a}	140.07 ± 55.59^{b}
Max Length	14.46 ± 2.94^{ab}	15.37 ± 4.14^{a}	15.26 ± 3.65^{ab}	14.2 ± 3.02^{b}
Min Length	8.49 ± 2.95^{c}	9.44 ± 4.24^{b}	11.57 ± 2.86^{a}	9.37 ± 2.97^{b}
Hepatocytes vacuolization grade	2.5	2.28	1.83	2.38
	Control	**BF1**	**BF3**	**BF6**
Hepatocelular Area	179.51 ± 92.91^{a}	180.20 ± 84.51^{a}	161.37 ± 84.21^{b}	118.85 ± 45.90^{c}
Max Length	16.55 ± 4.33^{a}	16.64 ± 4.70^{a}	15.74 ± 3.95^{a}	13.66 ± 3.19^{b}
Min Length	11.87 ± 2.84^{a}	11.97 ± 3.27^{ab}	11.19 ± 3.25^{b}	9.01 ± 3.02^{c}
Hepatocytes vacuolization grade	1.72 ± 0.26	2.00 ± 0.25	2.11 ± 0.74	1.67 ± 0.25
	Control	**BF6**	**BFR**	**BFE**
Hepatocelular Area	143.14 ± 53.95^{b}	126.11 ± 44.70^{b}	164.36 ± 57.22^{a}	172.94 ± 86.79^{a}
Max Length	14.69 ± 3.34^{b}	13.94 ± 2.98^{b}	16.10 ± 3.20^{a}	16.03 ± 3.96^{a}
Min Length	9.07 ± 2.63^{c}	8.74 ± 2.68^{c}	11.68 ± 2.99^{a}	10.55 ± 3.53^{b}
Hepatocytes vacuolization grade	2.92 ± 0.20	2.58 ± 0.58	1.83 ± 0.82	2.92 ± 0.58

Different letters indicate significant differences in each experiment (p-value<0.05)

In Experiment II, from 3% BF inclusion, a decrease in hepatocyte size was also observed, being BF6 the diet with the smallest hepatocytes, although as in the previous experiment, the degree of vacuolisation was not affected. In this experiment, necrotic areas were observed in all diets, including the control diet.

In Experiment III, both the extract and the residue (BF6E and BF6R) had the opposite effect on hepatocyte size to that described above. Both diets increased hepatocyte size equally, with BF6 being the diet with the smallest size. The degree of hepatocyte vacuolisation was also unaffected by the experimental diets in this third test.

According to **Figure 5.1** it is difficult to recognise the significant differences obtained in the image analysis by the measurements of the cells.

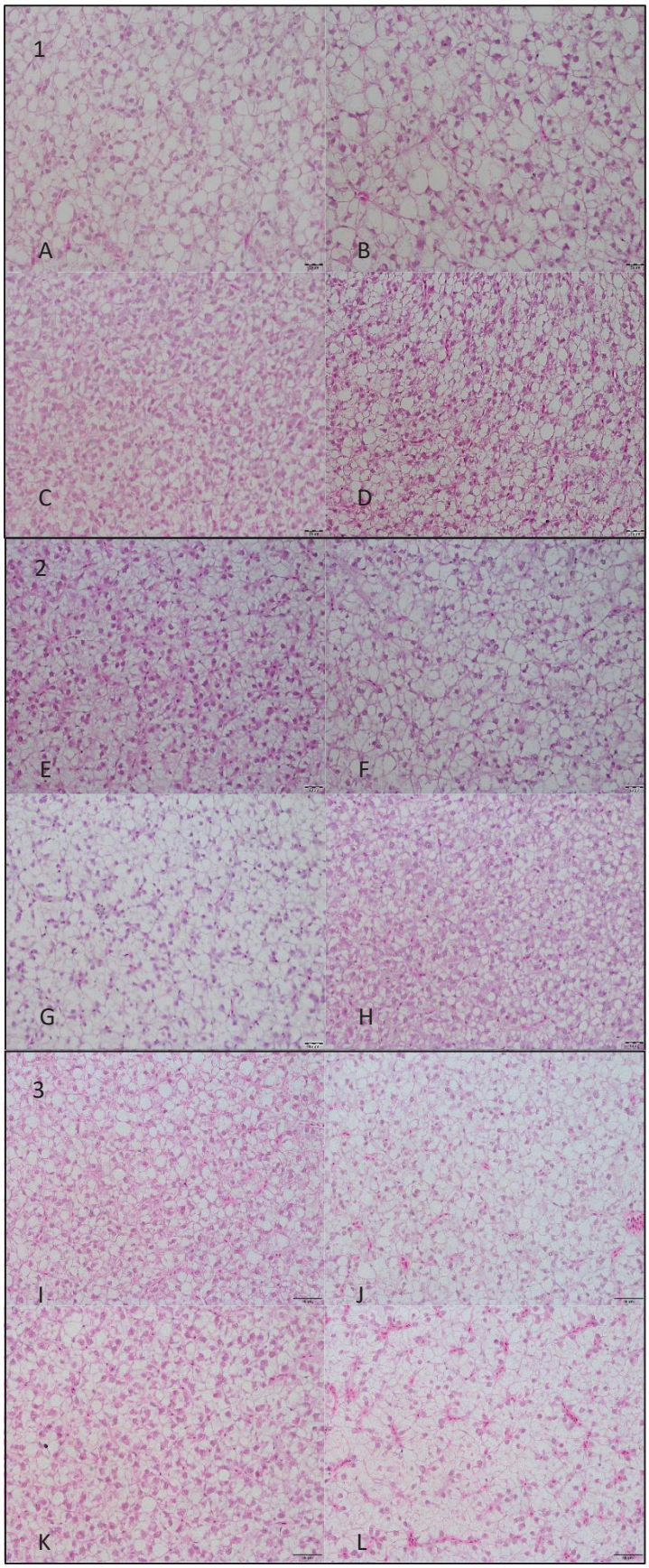

Figure 5.1. Micrograph from seabass livers fed with the experimental diets in the first Experiment (1): control diet (A), BT2 (B), BT4 (C) and BT8 (D). Liver from the Experiment II (2), control diet (E), BF1 (F), BF3 (G) and BF6 (H). Liver from the third Experiment (3) seabass: control diet (I), BF6 (J), BFR (K) and BFE (L).

Table 5.14. Histopathological analysis of the anterior intestine from seabass fed with experimental diets.

	Experiment 1		Experiment 2		Experiment 3	
	Control	BT4	Control	BF6	Control	BFR
Length (µm)	735 ± 235.38[b]	844.61 ± 289.31[a]	665.87 ± 299.15	610.95 ± 153.7	981.83 ± 302.12	1070.15 ± 374.92
Width (µm)	146.95 ± 50.9	155.55 ± 41.74	135.57 ± 46.99[a]	118.14 ± 32.35[b]	160.71 ± 54.18	163.58 ± 69.86
Lam propria (µm)	40.7 ± 14.74	40.72 ± 15.2	35.16 ± 12.99	32.94 ± 9.84	43.83 ± 21.18	45.17 ± 21.04
Goblets cells (nº)	38.75 ± 50.41	44.22 ± 42.07	59.59 ± 24.63	50.77 ± 36.38	94.29 ± 69.2	81.9 ± 81.03
Total goblets Area (px)	4157.08 ± 4852.2	6617.22 ± 6406.27	8085.66 ± 2575.97	8064.73 ± 5699.46	14328.71 ± 10866.94	13184.5 ± 13798.49
Average Area goblets (px)	106.81 ± 40.51[b]	154.21 ± 34.57[a]	141.2 ± 27.59	155.56 ± 27.92	148.12 ± 18.78	151.73 ± 20.08

Different letters indicate significant differences (p-value <0.05).

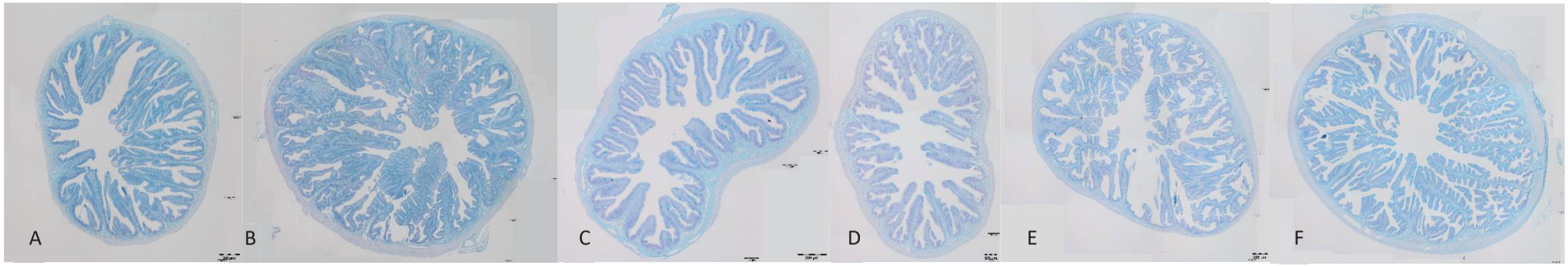

Figure 5.2. Micrograph from anterior intestine from seabass fed with the experimental diets: control Experiment I (A), BT4 (B), control Experiment II (C), BF6 (D), control Experiment III (E) and BFR (F).

The histopathological analysis of the anterior intestine of sea bass (**Table 5.14**) focused on the comparison of the control diets and the diet of each study that showed the highest growth (experiments I and II) and on the diet with the inclusion of fibrous residue (Experiment III).

In the first Experiment, the BP4 diet increased villus length and mean goblet cell area (**Figure 5.3**). In Experiment II, BF6 produced changes in villus morphology, although it only significantly affected the width of the villi, it reduced the size of the villi. In Experiment III, the BF6R diet did not affect any of the parameters measured.

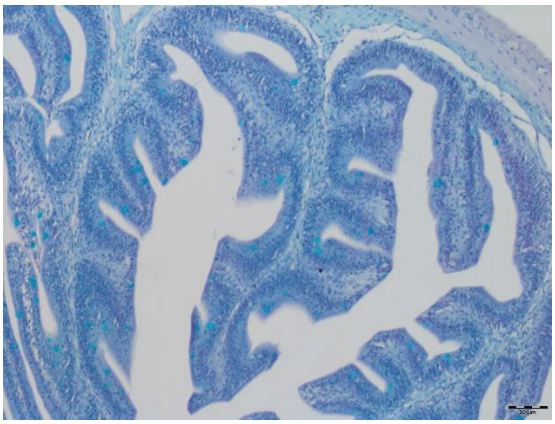

Figure 5.3. Micrograph of goblet cells detail (20x) from anterior intestine in seabass fed with diet BT4.

The differences observed in the image analysis above were not reflected in drastic changes in the morphology of the intestine, as can be seen in **figure 5.2**.

Serum parameters (Experiment II)

In the analysis of serum from Experiment II, although no significant differences in any of the parameters evaluated were detected, while the inclusion of 6% BF seemed to increase bactericidal activity (**Figure 5.4**).

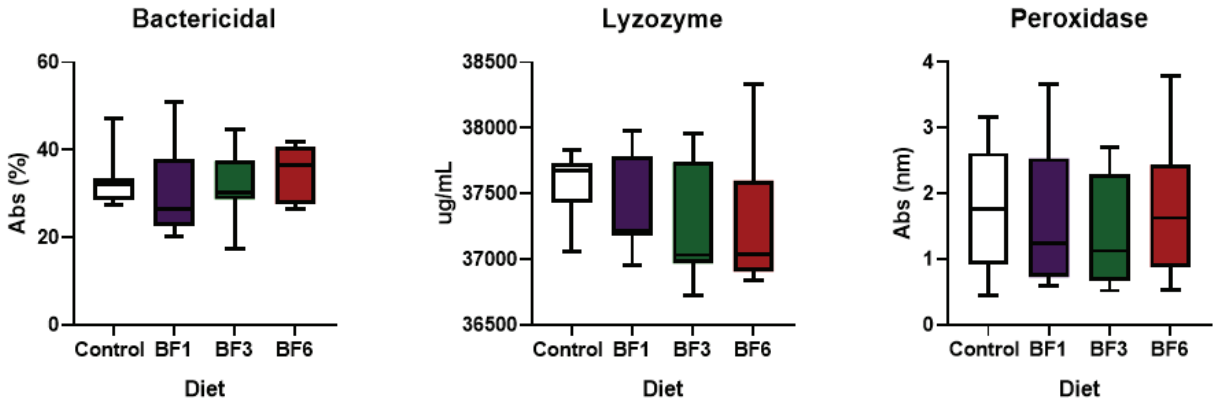

Figure 5.4. Serum parameters from experiment with banana flower inclusion**.**

HK Lymphocytes Mx Expression (Experiment II)

The lymphocytes from headkidney of the fish fed on the diet with 3% of banana flower expressed higher levels of Mx gene than those from the other diets studied in Experiment II, with a peak of expression at 24 hours post-inoculation in both the stimulated (Poly I:C) and non-stimulated (PBS) groups, with statistically significant differences, except for cells of the 6%BF diet group inoculated with Poly I:C (**Figure 5.5**). That is, an increase in the cellular response of cultures corresponding to 3% and 6% BF supplemented diets which can be observed after inoculation with Poly I:C, compared to the control diet (**Figure 5.5**). However, it seems not to have been induced by the immunostimulant, but as a result of the downregulation of Mx gene expression in the control diet group (**Figure 5.5**). Moreover, no differences were observed between the diet with lower concentration of banana flowers (1%) and the control diet, only some expression in cells inoculated with poly I:C after 24 hours of incubation, whereas at other times and in the control group (PBS) Mx was downregulated (**Figure 5.5**). On the other hand, as mentioned before, the addition of 3% and 6% of banana flowers to the diet of the animals led to a progressive increase in Mx expression in lymphocytes inoculated with poly I:C since inoculation time up to its maximum expression at 24 hpi, decreasing afterwards. The same pattern was observed with 3% BF diet even in the non-stimulated group (PBS) (**Figure 5.5**).

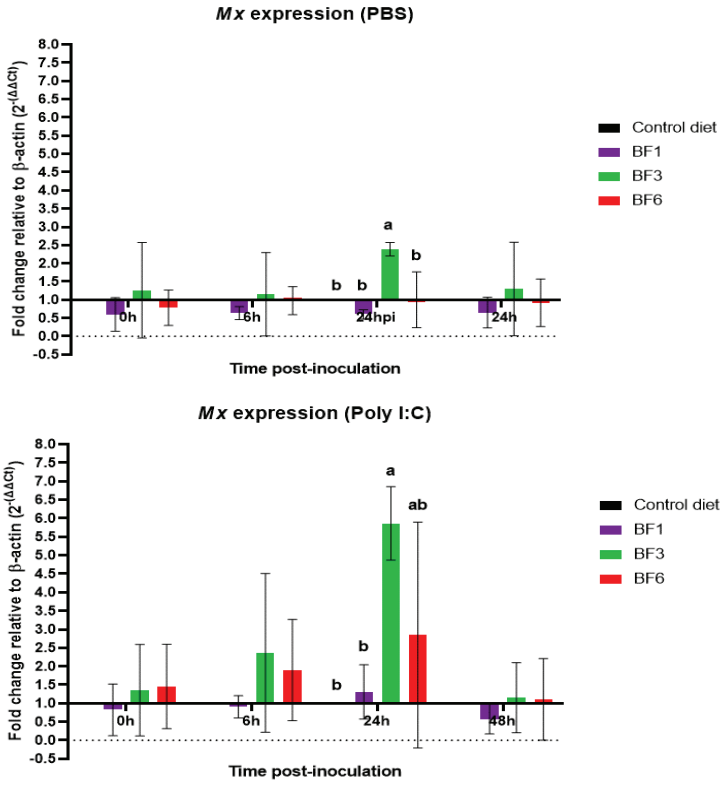

Figure 5.5. Mx gene expression of lymphocytes from experiment with banana flower inclusion without immunostimulant (PBS) and stimulated with Poly-C (Poly-C). Different letters indicate significant differences (p-value<0.05

HK Lymphocytes phagocytic capacity (Experiment III)

At the end of the feeding period, the phagocytic capacity of anterior kidney lymphocytes was assessed in Experiment III. This test showed that the BF6 diet slightly increased the phagocytic capacity of lymphocytes against *Listonella sp*. while both BF6R and BF6E did not differ from the control diet (**Figure 5.6**).

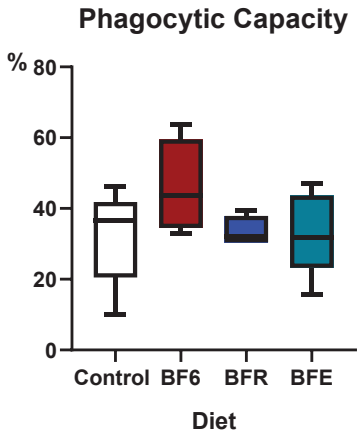

Figure 5.6. Phagocytic capacity of lymphocytes against *Listonella sp*. from experiment with banana flower extract and residue.

Plasma parameters (Experiment III)

Concerning plasma cortisol concentration in Experiment III, there was no difference between treatments at sampling points (t0, t2 and 7 days), although trends over time were noted. All diets were observed to start from higher levels at the initial time point, which was measured at 7 days. The difference was in the response at 2 hours after the stimulus, diets C and BF6E increased cortisol levels regarding t0, while diets BF6 and BF6R remained stable. At 7 days, BF6R was the diet that reached lower levels than the initial levels (**Table 5.15**).

Table 5.15. Plasm parameters obtained during confinement challenge in the experiment with banana flower extract and residue inclusion.

	Diet			
	Control	**BF6**	**BFE**	**BFR**
0 hours				
Cortisol (ug/dL)	37.14 ± 12.02	46.44 ± 3.80	34.65 ± 16.08	38.55 ± 11.86
Glucose (mg/dL)	82.08 ± 12.28	86.50 ± 9.52	84.58 ± 10.75	88.64 ± 19.17
2 hours				
Cortisol (ug/dL)	48.85 ± 1.75	46.26 ± 5.63	46.68 ± 6.40	42.46 ± 7.23
Glucose (mg/dL)	203.13 ± 52.85	185.25 ± 79.57	213.56 ± 47.86	176.67 ± 62.34
7 days				
Cortisol (ug/dL)	28.98 ± 13.96	35.10 ± 16.49	31.57 ± 11.06	21.84 ± 17.51
Glucose (mg/dL)	71.89 ± 15.62	95.33 ± 45.10	85.44 ± 29.12	70.00 ± 14.61

When we looked at the glucose response, something similar to the cortisol response happened. There were also no significant differences in sampling points and the same trends were maintained over time as in the previous case. All diets increased plasma glucose levels at 2h, but BF6 and BF6R were the diets that increased the least, remaining the most stable. At the end of the 7-day challenge, all diets have lowered glucose levels, but diets C and BF6R fall to lower levels than the initial ones.

5.4 Discussion

The present work is the first to our knowledge where not only a banana by-product was tested in juvenile seabass diets, like the whole flower, but also a secondary by-product derived from the processing of the whole pseudo-stem. The basis of this study was the inclusion of two by products from banana processing in seabass feeds which was cover by substitution in present case the dietary cornmeal in the formula feed. In the case of BP, the main difference concerning cornmeal was the carbohydrate composition, as it has a lower amount of starch (22%), compared to the 64% described in cornmeal, it also differs in the amount of cellulose (26%) compared to 2% in cornmeal, as well as in the amount of NSP (15% in BP and 10% cornmeal) (Couto *et al*., 2017; Ramírez-Bolaños *et al*., 2021). On the other hand, banana flower meal has a completely different nutritional profile from cornmeal, but this was not reflected in the experimental diets as the inclusion levels tested were lower. These composition differences may contribute to the results obtained in present work. It results also interesting to note that a similar study was carried out previously in tilapia (Ramírez-Bolaños *et al*., 2022 in progress), with the difference that in the present work with seabass the BP was not chemically pre-treated.

Contrary to what we might have expected at first, seabass responded favourably to the diets with banana by-product inclusion, even increasing growth at the end of the short feeding periods. In the case of Experiment I, the higher growth of the BP4 diet improved the result obtained in the previous study with tilapia, where it was determined that up to 5% BP could be included in the diet (Ramírez-Bolaños *et al*., 2022 in progress). This better growth may be due to including BP without pre-treatment present case with seabass, which makes it have a more interesting nutritional contribution; the minerals in the diet were not affected and the amount of NSP was probably higher than that used in the tilapia study. This higher growth provides an improvement over the inclusion of cornmeal in juvenile seabass, as this material does not provide before any growth benefit (Couto *et al*., 2017), as well as the use of pregelatinised corn starch (Enes *et al*., 2011; Machado *et al*., 2019).

Regarded the second experiment with banana flower, the higher growth obtained with 6% BF inclusion after only one month of feeding contrasts also with the experiment previously

conducted with tilapia, where there was no growth improvement with 3% BF inclusion (Ramírez-Bolaños *et al*., 2022 in progress). This result is in line with those obtained by other authors, who achieved also in an seabass an improvement in the growth with other plant materials additives like with cinnamon 1% (*Cinnamomum sp*) or thyme (*Thymus vulgaris*) (Yilmaz *et al.,* 2012; Habiba *et al*., 2021), or even with a very low amount of anise (*Pimpinella anisum)* (Ashry *et al*., 2022), although in our study a higher amount of BF up to 4% was needed to increase seabass growth.

These better results observed for the banana contribution to growth may be because carbohydrates can aid the utilisation of dietary protein (Enes *et al*., 2006), which is reflected in the 74% higher PER of the BF6 diet compared to the control diet, or even to the improved fermentation of NSPs in the gut, which have been described to generate short-chain fatty acids that would benefit fish growth through their utilization to meet energy demand (Mass *et al*., 2020).

The results of the proximal composition of muscle and liver in the experiments of this work were in agreement with what was observed by Enes *et al*. (2006), where the number of lipids present in the whole body of sea bass was also not modified by the level of carbohydrates present in the diet. In contrast to the previous work with tilapia, BP did not influence lipid digestibility, or at least not to the extent that it was reflected in the proximal composition of muscle and liver, which may indicate that no pre-treatment was more beneficial to the fish on this occasion.

The small variations observed in the fatty acid profile of the livers do not seem to be due to the fatty acid profile of the diets, as the contribution of the by-products to the composition of the diets was irrelevant, therefore, it is plausible to think that these variations in the fatty acid profile of the liver are more related with metabolic processes affected by some compound present in both by-products.

The inclusion of BP doubled the amount of linolenic acid (18:3n-3), as well as the inclusion of 6% flower in the diets, unlike what was observed in tilapia, where the inclusion of BP decreased the amount of linolenic acid, 18:3n-3 (LNA) (Ramírez-Bolaños *et al*., 2022 in progress). A similar effect was observed by Montero *et al*. (2005) with high substitution of fish meal and fish oil in seabass diets.

The observed increase in LNA may be due to an accumulation by reducing beta-oxidation in the mitochondria (Montero *et al*., 2005), thus the protection of this fatty acid against β-oxidation may be due to the polyphenols present in both BP flour and BF flour, such as protocatechuic acid (Ramírez-Bolaños *et al*., 2021). Among other properties, this polyphenol has effects described in decreasing oxidative stress, protective effect and inhibition of beta-oxidation of fatty acids by sequestering the CoA enzyme in mitochondria (Cao *et al*., 2009; Adedara *et al*., 2019; Al Olayan *et al*., 2020). In the case of Experiment II, the BF6 diet also reduced the amount

of 14:00 and 16:1n-7, which may indicate the use of these fatty acids for growth (Varga *et al*., 2020), being a primary substrate for β-oxidation (Makol *et al*., 2009), displacing the use of LNA and favouring its accumulation.

Carbohydrates also seem to influence the results obtained in the concentration of malonaldehyde in the liver of sea bass, the decrease in the concentration of MDA with the inclusion of BP in the diet was in line with what was observed by Castro *et al*. (2015), where they established that carbohydrates contribute to the reduction of oxidative stress in the liver.

Contrary to what was observed in the work with tilapia (Ramírez-Bolaños *et al*., 2022 in progress), the histopathological analysis of the liver in the first two experiments showed that the inclusion of BP at 8% and the inclusion of BF at 3% and 6% reduced the size of hepatocytes in sea bass. This may be due to a combined effect of polyphenols and polysaccharides present in both raw materials, as an effect of black tea polyphenols and polysaccharides in reducing hepatocyte size in rats has been described in other studies (Wu *et al*., 2016), this effect of reduction in hepatocyte area was also described by Torrecillas *et al*. (2007), when using MOS as an immunostimulant for sea bass. Experiment III shows that the compound responsible for this effect was found in the whole raw material and not in the extract or the residue separately, unlike in the experiment with tilapia, where the compound was found to be present in the organic extract of the flower meal (Ramírez-Bolaños *et al*., 2022 in progress). On the other hand, the degree of vacuolisation of the three experiments corresponds with the results obtained in the determination of lipids in the livers, where no differences were observed either.

In the case of the partial analysis of the intestines, the small variations that were observed with the image analysis in the size of the villi were almost imperceptible when observing the micrographs taken, this absence of effect is in line with what was observed by Couto *et al*. (2017) in juvenile sea bass, where they determined that NSPs do not affect the morphology of the intestine. In the same way, in works with supplementation with MOS no differences were observed in the structure of the anterior intestine (Torrecillas *et al*., 2007), while with the inclusion of other ingredients such as microalgae, there was an increase in the height of the villi (Messina *et al*., 2019). However, it is necessary to complete the image analysis of the other diets to be able to detect a more conclusive effect.

Regarding the influence of banana flower meal on the innate immune system of sea bass, there seems to be no effect on the basal state serum parameters. This was in agreement with what has been described by several authors using different raw materials, such as phytogenics, insect meal, date extract and a combination of carbohydrates with vegetal oils (Guardiola *et al*., 2016; Henry *et al*., 2018; Machado *et al*., 2019; Serradell *et al*., 2020). In contrast, other authors have described the influence of the inclusion of plant ingredients such as anise, which increases

lysozyme capacity (Ashry *et al*., 2022) and the inclusion of ingredients of marine origin such as microalgae, which decreases lysozyme capacity (Messina *et al*., 2019).

Poly I:C, a synthetic analogue of double-stranded RNA (dsRNA) present in some viruses, is recognized by endosomal Toll-like receptor 3 (TLR3) (Alexopoulou *et al*., 2001; Matsumoto *et al.*, 2002), triggering the synthesis of type I interferons (type I IFNs), signalling proteins that after binding to their receptors induce an antiviral state with the subsequent activation and transcription of different genes that lead the production of inflammatory cytokines, chemokines and other proteins with antiviral activity, such as Mx protein (Sadler and Williams, 2008). However, the cellular response due to Poly I:C in Experiment II of the present study was poor for all diets tested, which could be explained by a possible minor role of TLR receptors in the viral recognition in the headkidney of sea bass (Moreno *et al*., 2020).

Although the results of Experiment II showed that the inclusion of 1% of banana flowers in fish diet is insufficient to improve the immune response of animals compared with a diet without supplementation, a diet with 3% BF may have an effect on the protection of fish against potential viral infections, which does not seem to improve with the increase in the percentage of inclusion of banana flowers.

Based on the results obtained in Experiment II, the BF6 diet was selected as the basis for testing whether the effects observed in this diet could be due to compounds present in the organic extract or the residue. For this purpose, at the end of the feeding time, a confinement challenge was chosen where no deaths were recorded in the cages. This coincides with the first part of the homologous experiment in tilapia (Ramírez-Bolaños *et al*., 2022 in progress), where stress was generated in the fish by increasing salinity and no death was observed. Similarly, Serradell *et al*. (2020) recorded no deaths during confinement in juvenile sea bass. Plasma cortisol and glucose levels recorded during the challenge followed a similar trend to that recorded in the previous experiment with tilapia (Ramírez-Bolaños *et al*., 2022 in progress). In the case of plasma cortisol levels, the BF6 and BF6R diets were the most stable throughout the challenge, as was the case with tilapia. In contrast, other authors have reported decreases in plasma cortisol with diets supplemented with phytogenics and prebiotics (Serradell *et al*., 2020). On the other hand, glucose does not seem to be subject to the influence of this type of stressor, with the same type of confinement, Serradell *et al*. (2020) also found no influence on glucose, as well as with other stressors such as temperature (Islam *et al*., 2020). In addition, the inclusion of other ingredients such as microalgae did not influence plasma glucose levels in sea bass (Messina *et al*., 2019). All this seems to indicate that the compound responsible for the stability of the stress response may be from the group of polyphenols found in the extraction residue (BF6R), with

hyperglycaemia-regulating properties, such as protocatechuic acid (Talagavadi *et al*., 2016; D'Archivio *et al.,* 2018), which helps glucose metabolism and stress resistance.
When looking at the phagocytic activity of headkidney lymphocytes against *Listonella sp*, BF6 was the only diet that showed a slight increase in activity compared to the control diet, this is in agreement with what was observed when introducing date extract in the sea bass diet, where phagocytic activity was slightly increased (Guardiola *et al*., 2016). Other authors did observe an increase in phagocytic capacity when introducing plant ingredients into the diets, such as anise or MOS (Torrecillas *et al*., 2007; Ashry *et al*., 2022).

In conclusion, Banana Pseudo-stem (BP) and banana flower (BF), by-products from banana production are novel promising candidates for the aquafeed industry. BP without any chemical or enzymatic pre-treatment may be included up to 8% in seabass diets providing good performance results, and up to 6% as it was the highest level tested in the case of the BF. In both cases only slight improvement was observed; BP8 improves the liver oxidative status, reducing the MDA concentration, and BF6 enhances the cellular response by increasing the expression of the *Mx* gene during simulated infection. In addition, the stable fish stress response appears to be produced by compounds present in the BFR fraction. Better insight studies should be done on proper material pre-treatments to increase viable percentage not only as dietary ingredients as is for seabass and other marine species, but also to better preserve their functional properties mainly in the case of BF. Results obtained in the present study would highly recommend a long-term feeding trial for BP without pre-treatment to better corroborate the present results.

Chapter 6. Conclusions and recommendations

1. The *biochemical characterization* determined for the Banana Pseudo-stem (BP) and Banana Flower (BF), both by-products from banana production, indicates that they are novel promising candidates for the aquafeed industry.
2. The content of *total phenolic compounds* for BP and BF is similar to that of other plant materials already studied, which confirms present banana residues should be able as bioactive ingredients. Higher bioactivity was determined for BF concerning the BP.
3. Differences in *polyphenol profiles* were observed for BP and BF. The high content and biological response for the non-extractable polyphenols in BF (735.23 mg/100G HPP and 11200 mg/100G NEPA) with respect to other known vegetables used, may position BF as an interesting novel functional additive.
4. *BP*, chemically pre-treated, and BF may be introduced up to 5% and 3%, respectively, in diets for juvenile tilapia. Over 5% BP appears to negatively affect juvenile tilapia which could respond among others to the high mineral content contribution from this by-product, which may negatively affect the dietary mineral profile important in this species, and so digestive pH and nutrient digestibility.
5. *BP*, without any pre-treatment, may be included up to 8% in seabass diets and up to 6% in the case of BF.
6. *BP* inclusion significantly reduces the tilapia fillet lipid content, while not directly related to their respective fatty acid profile. Reduction in the lipid content for the highest BP levels (15% and 20%) gave similar values to the control fish, while lowest BP levels (2.5% and 5%) significantly increased most of the values concerning the control. So, this demonstrates the biological capacity of this specie to biosynthesise essential fatty acids when feeding high levels of banana by-products. In this sense, BP inclusions also reduce at high extent tilapia liver lipids, being the fatty acids profile closer among BP levels, and all different from the control.

   In the case of the seabass, BP could be included up to 8% without any differences in fillet and liver lipid content nor in fatty acid profile, except for a significantly higher content in the latter for the 18:3n-3, which could be a beneficial fish response besides the reduction in beta-oxidation in the mitochondria as having been reported in other studies.
7. *BF* could be included up to 3% without any differences in tilapia fillet lipid content, being the fatty acid profile improved by the BF3 diet, thus increasing DHA, n-3 and n-3 HUFA

fatty acids, and reducing n-9 and monoenoic acids. The beneficial effect on n-3 fatty acids was produced by bioactives present in the BF organic extract. Tilapia liver lipid content and fatty acid were not affected by the inclusion of BF.

Likewise, BF could be included up to 6% without any differences in seabass fillet and liver lipid content nor in fatty acid profile, except for a significantly higher content in the liver for the 18:3n-3 as shown in the fillet.

8. BP inclusion may improve seabass liver oxidation status by reducing MDA concentration.
9. 3% BF in juvenile tilapia diets results beneficial in terms of fish survival over time under osmotic stress.
10. BF significantly enhances cellular response by increasing *Mx* gene expression in seabass lymphocytes during a simulated infection.
11. The obtained results demonstrate the opportunity for the banana flower, as a primary by-product, and the banana pseudo-stem, as secondary by-products from the banana industry, towards the SDGs and the Circular Economy and sustainable fish nutrition needs.

From the global assessment of the two target by-product in present studies, BP and BF, new studies and recommendations arise, among which we would highlight the following:

1. Long-term feeding trials with BP without pre-treatment and with higher levels of BF should be performed with the two fish species, but mainly in sea bass.
2. It is of interest to determine the bioactive present in the banana flower extract that appears to stimulate the activity of fatty acid elongases and desaturases in juvenile tilapia and also provides advantages during an osmotic challenge, as does the flower.
3. The beneficial role of NSPs present in banana by-products in gut morphology requires further investigation, as they influence the absorption of macronutrients.
4. Better insight studies should be done on proper material pre-treatments, either mechanical, chemical or enzymatic, to better support increase viable percentage, not only as fish dietary ingredients but also to better preserve their functional properties.
5. Future comprehensive studies need to be performed to establish an accepted range of the main constituent of these by-products, considering the possible difference between batches of the same species due to environmental growing conditions; and thus, the real potential of the BF and BP as novel food ingredients as well as the technological aspects for their incorporation into feeds and their potential health effects.

Chapter 7. Resumen en español

1. Introducción

Materias primas

La reglamentación europea en materia de alimentación animal es amplia y está en continua renovación, ya que tiene un peso considerable, al representar el 13% de la producción global de piensos en el año 2020, siendo los principales productores europeos España, Alemania y Francia (FEFAC, 2021). Actualmente, la directiva que regula los piensos animales y las materias primas define cada uno de estos conceptos: los piensos compuestos son aquellos productos destinados a la alimentación animal formados por dos o más materias primas con presencia o no de aditivos, mientras que las materias primas vienen definidas como productos de origen vegetal o animal que satisfacen las necesidades nutritivas de los animales y los productos derivados de su transformación industrial (CE nº 767/2009). Las materias primas están a su vez catalogadas en el reglamento EC 2017/1017, en constante actualización. De más reciente introducción en la legislación y, además, más actualizado, es el concepto de subproducto, siendo éste una sustancia u objeto resultante de un proceso de producción cuya finalidad primaria no sea la producción de esa sustancia u objeto, cumpliendo lo siguiente: que se vaya a usar posteriormente, que se pueda utilizar directamente sin tener que someterse a una transformación posterior, que la sustancia se produzca como parte de un proceso productivo y que no sea dañino para la salud humana ni el medioambiente (7/2022, BOE 2022). Así mismo, podremos considerar como subproducto secundario el residuo procedente del procesado del subproducto primario en otra industria distinta a la original, ya que no hay establecida una definición legal clara.

La evolución de las distintas materias primas utilizadas en la elaboración de piensos a lo largo de los últimos 30 años, indica el aumento del uso de los cereales, con la soja, el maíz y el trigo a la cabeza, a raíz de la crisis del año 2001, y la reducción del uso de harinas animales. Con respecto a la seguridad alimentaria, desde 2001 está restringido el uso de subproductos de origen animal en piensos para ganadería por la enfermedad bovina espongiforme.

Una de las utilidades de los subproductos procedentes de cultivos agrarios ha sido el de utilizarlos para alimentación animal (Federici *et al*., 2009; Guil-Guerrero *et al*., 2016; Wang *et al*., 2016; Sivilai & Preston, 2017;), como forraje. En el caso de la platanera este uso tradicional se lleva dando en el mundo y en las Islas Canarias de forma natural (Padam *et al*., 2014). En

cambio, desde principios de los 2000, ya hay autores que señalan el potencial de los subproductos procedentes del sector agroindustrial como ingredientes funcionales para la industria de la alimentación tanto humana, como animal (Schieber *et al*., 2001). Esto se traduce en la búsqueda de nuevos usos para los subproductos procedentes de otras industrias, ya sea alimentaria, de bebidas alcohólicas o de producción de bioetanol, sobre todo en los últimos veinte años. Para el uso de estos subproductos, es fundamental la conservación y el tratamiento para mantener sus propiedades y evitar la proliferación de microorganismos (Al Khawli *et al*., 2019).

Muchos productos primarios necesitan ser a su vez procesados para obtener productos secundarios que puedan ser utilizados en otras industrias (Alao *et al*., 2017; Al Khawli *et al.,* 2019). Varios autores describen el uso en piensos animales de los productos secundarios a partir de primarios, como es el caso de la sangre y vísceras que pasan a ser concentrado de proteínas hidrolizadas después de la extracción de aceite de pescado (Stevens *et al.,* 2018), del olive cake ash a partir de la olive cake (Molina-Alcaide &Yáñez-Ruiz, 2008), el extracto de semilla de la uva después de la obtención del grape pomace (Brenes *et al*., 2016), o los fine solids procedentes del bioaqueous stream de la extracción de aceite de palma (Tan *et al.,* 2007).

Materias primas, subproductos primarios y secundarios en piensos de la industria acuícola

La acuicultura es una parte fundamental de la transformación azul impulsada por la FAO (FAO, 2022a) cuyo objetivo es la contribución máxima con la producción acuícola sostenible a la seguridad alimentaria y a los ODS relacionados con la erradicación del hambre y la pobreza de la Agenda 2030. Para ello, entre otras acciones, es necesario continuar con las líneas de investigación abiertas para hacer de la industria acuícola una industria sostenible.

El peso de la acuicultura en el mundo de los piensos es muy pequeño, tanto que ni siquiera está diferenciado en las estadísticas de los productores de pienso europeos (FEFAC, 2021). A pesar de igualar la contribución de la pesca extractiva en el año 2020 (FAO, 2022b), no ha aumentado la demanda de harinas y aceites de pescado debido a la utilización de materias primas vegetales que compiten en otros mercados, como la soja, el maíz, aceite de pescado y el trigo (Rana *et al.,* 2009). Esta circunstancia es la que obliga a la industria a innovar con nuevas materias primas que no entren en competición con otras industrias como la ganadería, la producción de bioetanol y las materias destinadas a consumo humano. Una alternativa a la harina de pescado y al concentrado de soja como fuente de proteínas en los piensos para acuicultura, son los hidrolizados de proteínas, procedentes sobre todo de subproductos acuícolas como vísceras,

piel y restos de los peces que se procesan para su salida al mercado (Martínez-Álvarez *et al*., 2015; Stevens *et al*., 2018; Hinchcliffe *et al.,* 2019). Otras fuentes de proteína alternativas que se han probado en los últimos años son: la generación de biomasa de hongos a partir de residuos de biorrefinería (Karimi *et al*., 2018) y poliquetos cultivados a partir de residuos de la extracción de agar de macroalgas (Stabili *et al*., 2019). Todavía hay pocos estudios de subproductos secundarios para alimentación acuícola, aunque está cada vez más en auge.

La tendencia a incluir ingredientes de origen terrestre en los piensos para acuicultura puede reducir la presión sobre los recursos marinos, pero puede aumentar la competición por la tierra, causando conflictos sociales y medioambientales (Malcorps *et al.,* 2019), además de quedar a merced de las consecuencias del cambio climático, que afecta en mayor medida a los cultivos terrestres (Rana *et al*., 2009).

Tilapia y lubina: especies objetivo

La tilapia y la lubina son dos especies objetivo para trabajar con estos subproductos primarios y secundarios. Ambas especies tienen una alta importancia comercial, la tilapia es la segunda especie de agua dulce más producida en el mundo (Bartley, 2022) junto con la carpa, con una producción en 2018 de 4,525 millones de toneladas (FAO, 2021b). Mientras que la lubina es la segunda especie marina en producción en Europa, después del salmón (FAO, 2021b), siendo España el segundo país en mayor producción después de Grecia (APROMAR, 2021) con una producción en 2020 de 21,709 toneladas.

La tilapia es una especie omnívora de agua dulce, aunque también puede cultivarse en agua salobre (El-Leithy *et al*., 2019). Debido a su biología, es una especie que puede aprovechar los recursos vegetales para su alimentación. Sólo en los últimos años hay varios estudios de sustitución del aceite de pescado por diferentes aceites vegetales como el aceite de palma (Peng *et al*., 2016; Larbi Ayisi *et al*., 2018; Nakharuthai *et al*., 2020), de soja (Nassef *et al*., 2019; Nakharuthai *et al*., 2020), de lino(Li *et al.,* 2016; Peng *et al*., 2016; El Asely *et al*., 2020; Nakharuthai *et al.,* 2020), de chía (Montanhes *et al.,* 2015), de maíz (El Asely *et al.,* 2020), de girasol (El Asely *et al*., 2020), de colza (Peng *et al*., 2016) y de microalga (Sarker *et al*., 2016), aunque los mejores sustitutos son el aceite de maíz, y sobre todo, el aceite de lino, que incluso mejora el crecimiento en peso al sustituir totalmente el aceite de pescado por el de lino (El Asely *et al*., 2020). Esto es así por la capacidad de sintetizar ácidos grasos esenciales como el EPA y el DHA a partir de ácidos grasos de 18 carbonos por la estimulación de las elongasas y desaturasas (Teoh *et al.,* 2011).

En el caso de la fuente de proteína, hay muchos estudios desde hace más de veinte años que exploran las vías de sustitución de la harina de pescado en piensos para tilapia (El Sayed, 1998; El Sayed, 1999). Es una especie que no depende de la harina de pescado para la obtención de aminoácidos. La principal alternativa a la proteína procedente del pescado es la soja, debido a su perfil óptimo de aminoácidos esenciales (O´Keefe, 2003), y en las dietas para tilapia se puede sustituir el 100% con concentrado de soja (Zhao *et al*., 2010). Pero la soja presenta una gran desventaja, y es que es utilizada por el mercado ganadero y en la alimentación humana, convirtiéndose así en el segundo ingrediente más caro de los piensos de acuicultura, sólo detrás de la harina de pescado (Brown et al., 2008). Debido a esto, está la necesidad de sustituir la soja a su vez por otros ingredientes más asequibles. En este sentido, hay numerosos estudios que emplean diferentes materias primas e incluso subproductos para sustituir la harina de pescado, como los subproductos de soja (Vidal *et al*., 2017)**,** la harina de judía fermentada (Valdez-González *et al.,* 2017)**,** la harina de insecto (Sánchez-Muros *et al.,* 2016; Agbohessou *et al*, 2020)**,** la harina de anacardo (Pradhan *et al*., 2020)**,** mezcla de microalgas (Sarker *et al.,* 2020), raíces de yuca (Obasa *et al.,* 2021)**,** macroalgas (Eissa *et al*., 2021) y subproducto de Sacha inchi (Khieokhajoukhet *et al*., 2022).

Por otro lado, la lubina es una especie principalmente carnívora de agua salada. Desde principios del siglo XXI se vienen desarrollando estudios sobre sustitución de harinas y aceites de pescado en los piensos de lubina en los que principalmente se utiliza rapeseed oil, linseed oil, soybean oil and palm oil (Montero *et al.,* 2005; Mourente *et al*., 2005; Mourente & Bell, 2006; Richard *et al*., 2006; Castro *et al.,* 2016), pero también se ha utilizado aceite de oliva y aceite de canola (Mourente *et al.,* 2005; Wassef *et al.,* 2016). En estos estudios se ha establecido que se puede sustituir entre un 60% y un 70% del aceite de pescado con aceites vegetales, aunque esto afecta al perfil de ácidos grasos de la lubina, ya que aumentan los niveles de ácidos grasos C18 y disminuyen los n-3 HUFA (Montero *et al*., 2005; Mourente *et al*., 2005; Machado *et al*., 2019). Esto se debe a la baja actividad enzimática de las enzimas elongasa y desaturasa (Mourente & Dick, 2002), de la misma manera que, dietas sin EPA ni DHA estimulan la expresión del gen *fads2*, pero no aumenta la actividad enzimática, lo que hace que disminuya la capacidad de convertir los PUFA en HUFA (Geay *et al*., 2010).

Para la sustitución de la harina de pescado como fuente de proteínas en la dieta de la lubina, también se utiliza la soja y el concentrado de soja, pero también se utilizan otras harinas vegetales como el gluten de trigo, la harina extrusionada de guisante, el germinado de trigo, harina de insecto o levaduras (Bonaldo *et al*., 2008; Messina *et al*., 2013; Rimoldi *et al*., 2015; Torrecillas *et al*., 2018; Pérez-Pascual *et al*., 2019; Reis *et al.,* 2019). Con las harinas de soja, y el

gluten de trigo, se han llegado a sustituciones de entre el 70% y el 100% (Messina *et al*., 2013; Rimoldi *et al.,* 2015; Pérez-Pascual *et al*., 2020), aunque en el caso de la sustitución total con harina de soja, se ha comprobado que añadir un 0.4% de MOS como aditivo, mejora los efectos negativos producidos por la soja en el intestino y en el sistema inmune de los peces (Torrecillas *et al*., 2018). Con todos estos datos, la lubina es una especie de pez marino carnívoro que tolera muy bien la inclusión de materias primas vegetales en la dieta, por lo que es una especie idónea para seguir explorando nuevas materias primas derivadas de subproductos.

Cultivo de plataneras: Aplicación de la Economía Circular

La platanera (*Musa acuminata var Cavendish*) es una planta herbácea de porte arbóreo, el pseudo-tallo está formado por capas de hojas nuevas que se quedan enrolladas y el 95% de su composición es agua. El pseudo-tallo maduro es muy robusto y puede soportar el peso de racimos de más de 50kg (Robinson *et al*., 2012). La inflorescencia se compone de un pedúnculo con flores femeninas, que son las que originan el fruto, hermafroditas y masculinas, que nunca llegan a salir de las brácteas de la inflorescencia (FIGURA XX). Los agricultores cortan el pedúnculo en la base de las flores hermafroditas y lo retiran, evitando el crecimiento del mismo y la posible rotura del pseudo-tallo por el peso del racimo de frutos (Robinson *et al*., 2012; Lau *et al*., 2020).

El plátano es la fruta más producida en el mundo y en el año 2019 alcanzó los 116 millones de toneladas (FAO, 202b). En Europa es la fruta más consumida, siendo la región con mayor importación de plátanos del mundo. La producción europea de plátano se concentra en las regiones tropicales y subtropicales de las Indias Occidentales Francesas, las Islas Canarias y Madeira (FAO, 2021a). En Canarias, la producción de plátano en el año 2021 fue de 411,732Tn (ASPROCAN) siendo el mayor productor europeo.

En las plantaciones de plataneras se genera una gran cantidad de residuos por la forma de cultivo y cosechado de la fruta: se elimina parte del pedúnculo y de las flores para permitir el desarrollo de las flores femeninas y su maduración hasta el fruto, y, una vez cosechado el racimo de plátanos, el pseudo-tallo se tala, dejando las raíces para que se desarrolle la planta hija (Lau *et al*., 2020). Estos residuos representan el 80% del total de la biomasa (Padam *et al*., 2014), la gestión de estos residuos puede generar un problema no sólo ambiental, relacionado con la toxicidad del suelo y huella de carbono elevada (Adsal *et al*., 2020), sino que también puede ser un problema de seguridad alimentaria (Campos *et al*., 2020). El manejo de estos residuos se hace fundamental, además, presentan una gran cantidad de usos potenciales como subproductos para industrias como la biorrefinería, la industria textil, sustitución de plástico en

envases, industria automóvil y refuerzo para materiales de construcción (Ortega *et al*., 2016; Campos *et al*., 2020; Pryadarshana *et al*., 2020; Rodríguez *et al*., 2020).

Tradicionalmente los restos de pseudotallo de platanera se han utilizado para alimentación de ganado (Wang *et al*., 2016), también, junto con la flor y el rizoma, se usan en poblaciones de todo el mundo en medicina tradicional para el tratamiento de enfermedades gástricas, diabetes, bronquitis, úlceras y calambres menstruales (Lau *et al*., 2020). Otros usos asociados a residuos del cultivo de plátanos es la utilización de los descartes en dietas para corderos (Menezes *et al*., 2017) y la utilización de la flor y el pseudotallo como complementos dietéticos para pacientes diabéticos (Bhaskar *et al.,* 2011)

También contienen una gran variedad de bioactivos (Lau *et al*., 2020), aunque no hay muchos estudios de caracterización del pseudotallo y de la flor de platanera. El único estudio que encontramos hasta el momento con la caracterización de estos dos subproductos en conjunto es el de Bahskar *et al*. (2012), en el que se utiliza la especia *Musa sp. Elakki bale* y concluye que son fuente de fibra dietética con polifenoles asociados. Otros estudios sobre la flor de platanera corroboran la cantidad de polifenoles presentes (Schmidt *et al*., 2015), así como la importante presencia de minerales como el magnesio, el hierro y el cobre, además de aminoácidos esenciales bien balanceados (Sheng *et al*., 2010).

En base a todo lo descrito anteriormente, tanto el pseudo-tallo como la flor de platanera son dos residuos que pueden ser utilizados como subproductos y ser asimilados en la dinámica de la Economía Circular en el marco de los SDGsODS (FAO, 2018), con el objetivo de obtener nuevos productos de valor añadido y reducir la huella ecológica de los cultivos.

Polifenoles: Bioactivos presentes en residuos vegetales

Los polifenoles son antioxidantes dietéticos presentes principalmente en vegetales, que presentan en su estructura uno o más grupos fenólicos (Pérez-Jiménez *et al*., 2013). Estos bioactivos están presentes en los residuos agrícolas, junto con otros compuestos como esteroles y triterpenos (Singh *et al*., 2016; Lau *et al*., 2020), y muestran actividad biológica de diferente tipo como secuestradores de radicales libres, modulación de la microbiota, regulación de la homeostasis de la glucosa, relacionada con la obesidad y la diabetes tipo II, el metabolismo lipídico y como conservante natural en alimentos (Cao *et al*., 2019; Fraga *et al*., 2019; Lau *et al*., 2020; Ma & Chen, 2020; Hamed & Abdel-Tawwab, 2021).

Los distintos polifenoles se clasifican en función de sus estructuras de acuerdo a las clases y subclases establecidas en la herramienta Phenol-Explorer (Neveu *et al*., 2010) que puede verse

en la tabla XX. La amplia variedad de estructuras de los compuestos fenólicos hace que todavía se sigan identificando nuevas estructuras (Nguyen *et al*., 2019).

De acuerdo a la forma de extracción, se pueden clasificar en polifenoles extraíbles (EPP), que son los que pueden ser obtenidos con solventes acuosos y orgánicos, y polifenoles no extraíbles (NEPP). Entre estos últimos se encuentran los polifenoles hidrolizables (HPP), compuestos fenólicos de bajo peso molecular asociados a polisacáridos y a proteínas, y las proantocianidinas no extraíbles (NEPA), que son estructuras de alto peso molecular. Los NEPP son metabolizados en el intestino por la microbiota intestinal y liberan los metabolitos secundarios para realizar diferentes funciones (Pérez-Jiménez & Saura-Calixto, 2015). Tradicionalmente, los polifenoles más estudiados y de más interés han sido los EPP, aunque los NEPP están tomando relevancia en estudios más recientes (Pérez-Jiménez *et al*., 2013).

Los compuestos fenólicos identificados en el plátano, en la piel, en el pseudo-tallo, en las flores y en el rizoma son: ácidos fenólicos, flavonol, epicatequinas, ácido gálico y ácido protocatéquico (Bhaskar *et al*., 2012; Kandasamy & Aradhya, 2014; Tsamo *et al*., 2015; Pico *et al*., 2019). Se han descrito efectos causados por estos polifenoles relacionados con la salud: el ácido protocatéquico mejora la respuesta ante algunos tipos de cáncer, ante la diabetes tipo I y II, regulando la hiperglicemia, y un efecto neuroprotector frente a contaminantes (Talagavadi *et al.,* 2016; Erukainure *et al*., 2017; D'Archivio *et al*., 2018; Adedara *et al*., 2019; Al Oyan *et al*., 2020; Yuliana *et al.*, 2020).

2. Objetivos

El objetivo principal de este estudio es la valorización de los subproductos de platanera como ingredientes en piensos acuícolas

Objetivos específicos:

1. Caracterización bioquímica y funcional de los subproductos de platanera Pseudo-tallo y flor.
2. Efecto en el crecimiento, histomorfología y estatus oxidativo de la inclusión del pseudo-tallo con un pretratamiento en la dieta para juveniles de tilapia.
3. Efecto en el crecimiento, histomorfología y estado oxidativo de la inclusión del pseudo-tallo sin pretratamiento en la dieta baja en harina y aceite de pescado de lubina.
4. Efecto en el crecimiento, histomorfología, estado oxidativo y resistencia al estrés de la inclusión de harina de flor de platanera en la dieta para juveniles de tilapia.

5. Efecto en el crecimiento, histomorfología, estado oxidativo y parámetros inmunes exvivo de la inclusión de harina de flor de platanera en la dieta baja en harina y aceite de pescado de lubina
6. Evaluación del extracto orgánico de la flor de platanera y del residuo generado para comprobar en qué fracción están los bioactivos responsables de los efectos de la flor de platanera en el estatus oxidativo, la resistencia al estrés y los parámetros inmunes de ambas especies.

3. Estudios realizados

El primer estudio que se realizó, y que está publicado en la revista International Journal of Food Science and Technology consistió en la caracterización de las materias primas.

La flor del plátano (DBF) y el pseudo tallo del plátano (DBPS) son subproductos de la cosecha y el procesamiento del plátano. El interés por las potenciales actividades biológicas de estos residuos del plátano se ha renovado en los últimos tiempos. Sin embargo, los estudios sobre la caracterización fitoquímica de los DBF y DBPS son todavía escasos y se centran en los polifenoles extraíbles (EPP), aunque cada vez hay más evidencias que muestran la relevancia de los polifenoles no extraíbles (NEPP) (Pérez-Jiménez *et al*., 2013).

A partir de una primera evaluación de los compuestos fenólicos totales, la DBF emergió como una fuente relevante de polifenoles (11,1-14,9% dw), con un 90% perteneciente a la clase de proantocianidinas no extraíbles (NEPA). En el caso del pseudo-tallo del plátano, todos los polifenoles (0,11-0,19% dw) estaban presentes como polifenoles no extraíbles. El análisis detallado por HPLC-ESI-QTOF MS mostró que, tanto en las fracciones EPP como HPP, los ácidos fenólicos eran los constituyentes más relevantes, aunque también se identificaron algunos flavanoles en la fracción EPP. El ácido protocatéquico se detectó en la DBF en una concentración superior a la observada en la mayoría de los alimentos comunes, según la base de datos Phenol-Explorer (Neveu et al., 2010).

El análisis detallado de los compuestos fenólicos en la DBF y la DBPS demostró que la DBF puede utilizarse como una fuente adicional de compuestos fenólicos, en particular el NEPA, aunque puede ser necesario establecer rangos de contenido aceptados entre los años de cosecha para su uso como ingrediente funcional.

En el segundo estudio, enviado a la revista Aquaculture Reports, se desarrolló la valorización de los subproductos como ingredientes en pienso de arranque para tilapias. La elevada cantidad de

residuos derivados de la cosecha del plátano es una oportunidad para crear sinergias entre la industria del plátano y la acuicultura, desde una perspectiva de economía circular.

El objetivo del trabajo fue evaluar la inclusión de subproductos del plátano, como el pseudo tallo y la flor del plátano, en la alimentación de tilapias, y explorar los extractos de la flor del plátano en el desarrollo y la salud de los juveniles de tilapia. Se realizaron tres ensayos consecutivos de alimentación para evaluar las inclusiones de subproductos de prueba: Experimento I) con 0, 2,5, 5, 15 y 20% de pseudo-tallo de plátano con un pretratamiento químico; Experimento II) con 0, 0,2, 0,5, 1 y 3% de flor de plátano; y Experimento III) con 3% de flor de plátano frente a su correspondiente extracto orgánico y residuo de la extracción.

Las pruebas de estrés por salinidad se realizaron después de los ensayos de alimentación en los experimentos II y III. A partir de los resultados obtenidos, hasta el 5% de pseudo-tallo de plátano y el 3% de inclusión de flor de plátano se consideraron adecuados para el crecimiento de los peces y la salud del hígado, así como la proporción de ácidos grasos esenciales en el músculo, a pesar de la reducción del porcentaje total de lípidos. En cuanto a los desafíos de estrés, la flor y, más concretamente, el residuo restante de su extracción orgánica, parece regular los niveles de cortisol y glucosa en plasma y reducir los parámetros de oxidación en el hígado y el músculo de los peces, lo que puede deberse a los polifenoles presentes tanto en la flor de plátano entera como en su residuo de extracción orgánica.

El tercer y último trabajo está en proceso de elaboración para ser enviado a revistas científicas. En este estudio se siguió la misma filosofía que en el anterior trabajo, pero cambiando la especie objetivo por la lubina, y probando el pseudo-tallo sin hacerle ningún pretratamiento.

La lubina es la segunda especie de pez marino más importante producido en Europa y en el Mediterráneo, y es una especie que tolera grandes inclusiones de ingredientes vegetales en sustitución de la harina y el aceite de pescado. Debido a esto, en este trabajo se realizaron tres estudios para evaluar la inclusión de los subproductos en las dietas de juveniles de lubina con bajo aceite de pescado y baja inclusión de harina de pescado: Experimento I) se probaron cuatro niveles de inclusión 0, 2, 4 y 8% de BP sin pretratar; Experimento II) cuatro niveles de inclusión de BF 0, 1, 3 y 6%; y el Experiment III) con el nivel de 6% de flor frente a su extracto orgánico y el residuo generado durante la extracción.

Los dos primeros experimentos arrojaron unos resultados óptimos, en cuanto a que la inclusión de ambos subproductos mejoró el crecimiento después de un período corto de alimentación. En los experimentos II y III se realizaron pruebas sobre el efecto de BF en el sistema inmune de

la lubina. La flor no parece tener un efecto muy pronunciado en la mejora del sistema inmune de la lubina, aunque sí hubo pequeñas mejoras en la respuesta celular de los linfocitos.

4. Conclusiones y recomendaciones

1. La caracterización bioquímica determinada para el Pseudotallo de Platanera (BP) y la Flor de Plátano (BF), ambos subproductos de la producción de plátano, indica que son candidatos novedosos y prometedores para la industria de piensos acuícolas.

2. El contenido de compuestos fenólicos totales para el BP y la BF es similar al de otros materiales vegetales ya estudiados, lo que confirma que los actuales residuos del plátano deberían ser capaces de actuar como ingredientes bioactivos. Se determinó una mayor bioactividad para la BF con respecto al BP.

3. Se observaron diferencias en los perfiles de polifenoles para el BP y el BF. El alto contenido y la respuesta biológica para los polifenoles no extraíbles en BF (735,23 mg/100G HPP y 11200 mg/100G NEPA) con respecto a otros vegetales conocidos utilizados, puede posicionar a BF como un interesante aditivo funcional novedoso.

4. BP, pretratado químicamente, y la BF pueden introducirse hasta un 5% y un 3%, respectivamente, en las dietas de juveniles de tilapia. Más del 5% de BP parece afectar negativamente a los juveniles de tilapia, que podrían responder, entre otras cosas, al elevado aporte de contenido mineral de este subproducto, que puede afectar negativamente al perfil mineral de la dieta, importante en esta especie, y por tanto al pH digestivo y a la digestibilidad de los nutrientes.

5. BP, sin ningún tipo de pretratamiento, puede incluirse hasta un 8% en las dietas de lubina y hasta un 6% en el caso de BF.

6. La inclusión de BP reduce significativamente el contenido de lípidos de los filetes de tilapia, aunque no está directamente relacionado con su perfil de ácidos grasos. La reducción del contenido de lípidos para los niveles más altos de BP (15% y 20%) dio valores similares a los de los peces de control, mientras que los niveles más bajos de BP (2,5% y 5%) aumentaron significativamente la mayoría de los valores con respecto al control. Por lo tanto, esto demuestra la capacidad biológica de esta especie para biosintetizar ácidos grasos esenciales cuando se alimenta con altos niveles de subproductos del plátano. En este sentido, las inclusiones de BP también reducen en alto grado los lípidos del hígado de la tilapia, siendo el perfil de ácidos grasos más cercano entre los niveles de BP, y todos diferentes al control.

En el caso de la lubina, se pudo incluir BP hasta un 8% sin que hubiera diferencias en el contenido de lípidos del filete y del hígado ni en el perfil de ácidos grasos, salvo un contenido significativamente mayor en este último para el 18:3n-3, lo que podría ser una respuesta beneficiosa del pez, además de la reducción de la beta-oxidación en las mitocondrias como se ha informado en otros estudios.

7. BF pudo incluirse hasta un 3% sin que hubiera diferencias en el contenido lipídico del filete de tilapia, siendo el perfil de ácidos grasos mejorado por la dieta BF3, aumentando así los ácidos grasos DHA, n-3 y n-3 HUFA, y reduciendo los n-9 y los ácidos monoenoicos. El efecto beneficioso sobre los ácidos grasos n-3 fue producido por los bioactivos presentes en el extracto orgánico de la flor. El contenido de lípidos y ácidos grasos del hígado de la tilapia no se vio afectado por la inclusión de BF.

Asimismo, se pudo incluir BF hasta un 6% sin que hubiera diferencias en el contenido de lípidos del filete y del hígado de la lubina ni en el perfil de ácidos grasos, salvo un contenido significativamente mayor en el hígado para el 18:3n-3 que se muestra en el filete.

8. La inclusión de BP puede mejorar el estado de oxidación del hígado de la lubina al reducir la concentración de MDA.

9. El 3% de BF en las dietas de los juveniles de tilapia resulta beneficioso en términos de supervivencia de los peces a lo largo del tiempo bajo estrés osmótico.

10. La BF mejora significativamente la respuesta celular al aumentar la expresión del gen *Mx* en los linfocitos de la lubina ante una simulación de infección.

11. Los resultados obtenidos demuestran la oportunidad de la flor del plátano, como subproducto primario, y el pseudotallo de platanera, como subproducto secundario de la industria del plátano, hacia los ODS y la Economía Circular y las necesidades de una nutrición sostenible de los peces.

De la evaluación global de los dos subproductos objetivo en los presentes estudios, BP y BF, surgen nuevos estudios y recomendaciones, entre los que destacamos los siguientes:

1. Los ensayos de alimentación a largo plazo con BP sin pretratamiento y con mayores niveles de BF deberían realizarse con las dos especies de pez, pero principalmente en lubina.

2. Es de interés determinar el bioactivo presente en el extracto de flor de plátano que parece estimular la actividad de las elongasas y desaturasas de ácidos grasos en los juveniles de tilapia y que además proporciona ventajas durante un estrés osmótico, como lo hace la flor.

3. El papel beneficioso de los NSP presentes en los subproductos del plátano en la morfología intestinal requiere más investigación, ya que influyen en la absorción de macronutrientes.

4. Deben realizarse estudios más profundos sobre los pretratamientos adecuados del material, ya sean mecánicos, químicos o enzimáticos, para favorecer el aumento del porcentaje de viabilidad, no sólo como ingredientes dietéticos para peces, sino también para preservar mejor sus propiedades funcionales.

5. Es necesario realizar futuros estudios exhaustivos para establecer un rango aceptado de los principales constituyentes de estos subproductos, teniendo en cuenta la posible diferencia entre lotes de la misma especie debido a las condiciones ambientales de cultivo; y, por tanto, el potencial real de los BF y BP como nuevos ingredientes alimentarios, así como los aspectos tecnológicos para su incorporación a los piensos y sus posibles efectos sobre la salud.

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