U.PORTO

DOUTORAMENTO EM CIÊNCIA ANIMAL

ESPECIALIDADE EM NUTRIÇÃO

Vegetais e frutas enquanto fontes de antioxidantes em dietas para robalo (*Dicentrarchus labrax)*

Ricardo Pereira

D

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Tese para candidatura ao grau de Doutor no programa doutoral de Ciência Animal com especialização em Nutrição, submetida ao Instituto de Ciências Biomédicas Abel Salazar, enquanto instituição pertencente à Universidade do Porto

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Vegetables and fruits as antioxidant sources for European sea bass (*Dicentrarchus labrax*)

Thesis for application to the degree of Doctor in the field of Animal Sciences with specialization in Nutrition, submitted to the Institute of Biomedical Sciences Abel Salazar, as part of the University of Porto

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Financiamento

Este trabalho foi parcialmente financiado pelo Projeto MobFood - mobilização de conhecimento científico e tecnológico em resposta aos desafios do mercado agroalimentar, através do Consórcio “MobFood” e financiado pelo Fundo Europeu de Desenvolvimento Regional Europeu - FEDER (POCI-01-0247-FEDER-024524). Parte do trabalho aqui desenvolvido recebeu apoio financeiro do projeto ATLANTIDA - Plataforma de monitorização do Oceano Atlântico Norte e ferramentas para exploração sustentável dos recursos marinhos, financiado pelo Programa Operacional Regional do Norte (NORTE2020), através do Fundo Europeu de Desenvolvimento Regional Europeu - FEDER (NORTE-01-0145-FEDER-000040); O candidato ao grau de Doutor, Ricardo Jorge Silva Pereira, foi financeiramente suportado pela Bolsa de Doutoramento SFRH/BD/144631/2019 atribuída pela Fundação para a Ciência e Tecnologia (FCT). A FCT disponibilizou também suporte financeiro ao CIIMAR através do Fundo Estratégico UIDB/04423/2020 e UIDP/04423/2020.

Funding

This work was partially funded by Project MobFood - Mobilizing scientific and technological knowledge in response to the challenges of the agri-food market" (POCI-01-0247-FEDER-024524), by "MobFood" Consortium, and financed by the European Regional Development Fund (ERDF), through the Incentive System to Research and Technological development, within the Portugal2020 Competitiveness and Internationalization Operational Program. This work was also partially funded by Project ATLANTIDA - Platform for the monitoring of the North Atlantic Ocean and tools for the sustainable exploitation of the marine resources, financed by the North Portugal Regional Operational Programme (NORTE2020), through the ERDF (NORTE-01-0145-FEDER000040). Moreover, Ricardo Jorge Silva Pereira was also financially supported by a Doctoral grant (SFRH/BD/144631/2019) awarded by the Portuguese Foundation for Science and Technology (FCT). Financial support from FCT was also provided to CIIMAR within the scope of Strategic Fund UIDB/04423/2020 and UIDP/04423/2020.

Diretivas Legais

No cumprimento do disposto no Decreto-Lei no 204/2018 de 23 de Outubro, declara-se que o autor desta Tese participou na conceção e na execução do trabalho experimental que esteve na origem dos resultados apresentados, bem como na sua interpretação e na redação dos respetivos manuscritos. Nesta tese incluem-se 3 artigos científicos submetidos e/ou publicados em revistas internacionais indexadas, contendo os resultados obtidos no âmbito do trabalho experimental, e referenciados como:

Pereira R, Costa M, Velasco C, Cunha LM, Lima RC, Baião LF, Batista S, Marques A, Sá T, Campos DA, Pereira M, Jesus D, Fernández-Boo S, Costas B, Pintado M, Valente LMP. Comparative Analysis between Synthetic Vitamin E and Natural Antioxidant Sources from Tomato, Carrot and Coriander in Diets for Market-Sized *Dicentrarchus labrax*. Antioxidants. 2022; 11(4):636.
https://doi.org/10.3390/antiox11040636

Pereira R, Velasco C, Gómez-Garcia R, Dias J., Pintado M, Valente LMP. Unravelling the effects of extrusion and drying temperatures on the radical scavenging capacity of aquafeeds supplemented with mango and pineapple by-products: Chemical composition, bioactive compounds and antioxidant capacity stability. Submetido à revista Animal Feed Science and Technology (Elsevier), 2024.

Pereira R, Basto A, Pintado M, Valente LMP, Velasco C. Inclusion of pineapple by-products as natural antioxidant sources in feeds for European sea bass (*Dicentrarchus labrax*) within a circular economy context. Submetido à revista Aquaculture (Elsevier), 2024.

Legal Directives

In compliance with the provisions of Decree-Law No. 204/2018 of October 23, it is hereby declared that the author of this Thesis participated in the conception and execution of the experimental work that led to the presented results, as well as in their interpretation and the drafting of the respective manuscripts. This thesis includes 3 scientific articles submitted and/or published in international journals resulting from the entirety of the experimental work, and referenced as:

Pereira R, Costa M, Velasco C, Cunha LM, Lima RC, Baião LF, Batista S, Marques A, Sá T, Campos DA, Pereira M, Jesus D, Fernández-Boo S, Costas B, Pintado M, Valente LMP. Comparative Analysis between Synthetic Vitamin E and Natural Antioxidant Sources from Tomato, Carrot and Coriander in Diets for Market-Sized *Dicentrarchus labrax*. Antioxidants. 2022; 11(4):636.
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Acknowledgements

I would like to start by acknowledging that this PhD thesis, as well as the long path that led to it, was, at least partly, the result of a metric tonne of privilege granted to me by accident of birth. Thus, this first paragraph was written not only to honor all of those in the struggle, but also to mourn far better works that will never come to be because of people being unjustly denied of the same opportunities that I was gifted with from the very beginning. All of these would-be Sagans, Hawkings, Einsteins and Newtons devoured by the maws of systemic discrimination and late-stage capitalism remain the greatest and ever-increasing loss known to the scientific world, as well as one of the great losses of Humanity as a whole. We are quite lucky there is no God, for I doubt a just one would forgive us.

For the surely smaller number of people still reading, allow me to step into the light for a bit and recognize that none of this ugliness means that due gratitude is not in order!

Thus, I would like to thank my supervisors Cristina Velasco, Manuela Pintado and Luísa Valente, for all of the patience, support and guidance given. For all of the late-night hours reviewing manuscripts and presentations, for the good advice, and for coming through in times when understanding was paramount. This also applies to the amazing teams we have at CIIMAR, ICBAS and Universidade Católica. Thank you for teaching me how to be a scientist.

I would like to start by thanking my father José “Júnior” Pereira and my mother Ana da Silva Pereira, for being the best possible example of what parents should be – incredibly hard-working, unabashedly supportive, open-minded, kind, and sickeningly in love between themselves, with our family, and the world around them. I would also like to thank my uncle Jorge, my aunt Manuela and my cousins Anita and Joana, for always showering me with nothing but love and good times. Thank you all for teaching me how to be a man.

To Moonshade and Firemage, namely my bandmates and friends Pedro, Bruno, Luís, Daniel, Tiago, Ana, Vânia, Emanuel and Ricardo, as well as all former members, guest musicians and fans for giving me a platform to express myself, teaching me to think outside the box and be creative, to never be afraid of speaking my truth in front of a crowd, and for making not having a comfort zone my comfort zone. Thank you, so much, for letting me be a rockstar with you guys.

To Leandro Neves, Pedro F. Ribeiro, João “Joca” Ribeiro and Isac França, for always being there since the beginning. To Davina Falcão, Sílvia Ferreira, Gabriela Correia, André Oliveira, Ronaldo Costa, Bruno Lourenço, Lory Cervi, Diogo Azevedo, Daniel Laureano and Sandra Oliveira for lighting up my life with true friendship like no other. To my dear friends at Caldeirão, Luís Marques, Sofia Sebastião and Rómulo Teixeira, for giving me a hearth, times of merry and friendship, and a tall glass of mead to wash it all down. To my work-buddies Ricardo Matias, Daniela Salazar, Mariana Ferreira, Tiago Sá, Sónia Ferreira and Vera Sousa, for being there for both the hard times and the fun times. Thank you, all, for being part of my life, one way or another.

And finally, to Bia, my beloved Ziggy Stardust, for showing me what love is supposed to feel like - how it is meant to be given, received, celebrated, honoured and cherished. Thank you for shining your light upon me, and for growing flowers on these weary bones. I suspect that very light to be the beacon that allows me to find you in each lifetime throughout all possible universes. I hope we can read this again one day - in our library, by the fire, with a pitbull and two cats.

Maybe three.

In sum, thank you all, deeply, for walking down this road with me.

“*I shall be telling this with a sigh*

Somewhere ages and ages hence:

Two roads diverged in a wood, and I—

I took the one less travelled by,

And that has made all the difference.”

- Robert Frost

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List of Abbreviations and Acronyms

a*	Redness
ABTS$^{\bullet+}$	2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
ACH50	Plasma complemente activity
AEP	Antioxidant enzyme parameters of the corresponding organ(s), whether enzymatic activity or enzyme gene expression. Expressions in italic represent genes;
ADC	Apparent digestibility coefficient
ADF	Acid detergent fibre
AP	Alkaline Phosphatase
b*	Yellowness
BHA	Butylated hydroxyanisole **(**2-tert-Butyl-4-hydroxyanisole)
BHT	Butylated hydroxytoluene (2,6-Di-tert-butyl-4-methylphenol)
C*	Chroma
CAT	Catalase
CC	Carbonyl compounds
DGI	Daily growth index
DHA	Docosahexaenoic acid
DM	Dry matter
DPPH$^{\bullet}$	1,1-diphenyl-2-picrylhydrazyl
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
ER	Energy retention
EUMOFA	European Market Observatory for Fisheries and Aquaculture Products
EUR	Euros (€)
FAO	Food and Agriculture Organization
FBW	Final body weight
FCR	Feed conversion ratio
FM	Fishmeal
FO	Fish oil
g	Grams
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione (reduced)
GSSG	Glutathione disulphide (oxidized glutathione)
GST	Glutathione s-transferase

h*	Hue angle
HSI	Hepatosomatic index
H_2O	Water
H_2O_2	Hydrogen peroxide
IBW	Initial body weight
IgM	Immunoglobulin M
Immuno	Immune modulatory potential in blood and/or serum
K	Fulton's condition factor
kg	Kilograms
L*	Lightness
LPO	Lipid peroxidation
MUFA	Monounsaturated fatty acid
MF	Mango peel flour
NADPH	NADPH Nicotinamide adenine dinucleotide phosphate (reduced form
NDF	Neutral detergent fibre
NEFA	Non-esterified fatty acids
NDF	Neural detergent fibre
O_2	Oxygen
$O^{\bullet}$	Oxygen singlet
$OH^{\bullet}$	Hydroxyl radical
P	Phosphorus
PER	Protein efficiency ratio
PF	Pineapple peel flour
PTMs	Post-translational modifications
PU	Protein utilization
PUFA	Polyunsaturated fatty acid
RGR	Relative growth rate
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SGR	Specific growth rate
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TBARS	Thiobarbituric reactive substances
TG	Total glutathione
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
VFI	Voluntary Feed Intake

VSI Viscerosomatic index

WB Whole-body

WG Weight gain

Resumo

A indústria da aquacultura desempenha um papel crucial ao satisfazer a procura global de peixe, sendo responsável pelo fornecimento de mais de 53% do peixe para consumo humano. Esta relevância crescente da aquacultura na produção alimentar global é acompanhada pela necessidade de aumentar a produção e a qualidade geral dos alimentos para aquacultura, enquanto se aprimoram os processos de produção num contexto de sustentabilidade. Consequentemente, estão continuamente a ser desenvolvidas novas dietas funcionais, cada uma com características únicas, mas que por vezes se sobrepõem. Estas dietas são formuladas não apenas para melhorar o crescimento dos peixes, mas também para fortalecer as defesas imunológicas e antioxidantes, visando simultaneamente a viabilidade económica e a sustentabilidade ambiental. Os peixes cultivados enfrentam frequentemente fatores indutores de stress, em resultado da manipulação regular, transporte, doença, mudanças súbitas de temperatura, superlotação e/ou nutrição subóptima. Além disso, a escassez de farinha e óleo de peixe leva ao aumento da inclusão de fontes vegetais nos alimentos para aquacultura, o que diminui a qualidade nutricional e aumenta a suscetibilidade dos peixes a infeções bacterianas e condições de stress gerais. Isto leva a uma produção elevada de espécies reativas de oxigénio e nitrogénio intracelulares (ROS e RNS). Estes radicais livres altamente reativos causam danos estruturais em lípidos, proteínas e DNA, com consequências muito negativas para a homeostase celular, o que pode levar a stress crónico, afetando processos biológicos cruciais e, em última análise, prejudicando o crescimento. Estes efeitos negativos podem ser aliviados através de compostos antioxidantes exógenos, incluídos nos alimentos, com o propósito de inibir o início ou a progressão de reações em cadeia de radicais livres, evitando simultaneamente os efeitos negativos da oxidação nos próprios alimentos. Nos últimos anos, os antioxidantes mais comumente usados nos alimentos foram fontes sintéticas, nomeadamente o hidroxianisol butilado (BHA), o hidroxitolueno butilado (BHT) e a etoxiquina. No entanto, de acordo com estudos recentes a acumulação de BHA e BHT, ou os seus metabólitos, pode ter efeitos potenciais carcinogénicos e/ou disruptivos endócrinos, tanto para os peixes, quanto para os consumidores. Isto levou a União Europeia a proibir a etoxiquina em alimentos para animais devido a preocupações com a potencial atividade carcinogénica, sublinhando a necessidade urgente de explorar fontes antioxidantes alternativas. Simultaneamente, a indústria alimentar descarta anualmente grandes quantidades de subprodutos vegetais ricos em antioxidantes naturais, como vitaminas, carotenoides e compostos fenólicos. Estes antioxidantes naturais possuem várias propriedades benéficas, incluindo a capacidade de combater

os processos de oxidação tanto nos alimentos quanto nas células dos animais. Esta tese apresenta uma abordagem inovadora e aprofundada que se concentra na exploração do potencial de subprodutos e coprodutos ricos em antioxidantes de frutas e vegetais para inclusão em alimentos para aquacultura.

O primeiro capítulo foi focado na avaliação do impacto das várias etapas do processo de fabrico de alimentos compostos, particularmente no que diz respeito à temperatura, visando preservar a capacidade antioxidante máxima dos alimentos para aquacultura com inclusão de antioxidantes naturais. Basicamente, uma mistura de ingredientes comuns foi usada como controlo, e comparada com duas misturas funcionais com inclusão de 2% de farinha de casca de ananás ou manga. Estas misturas foram submetidas a diferentes combinações de temperaturas de extrusão (110 vs 25 °C) e secagem (60 vs 35 °C). Os resultados mostraram que a incorporação de 2% de farinha de casca de ananás ou manga aumentou o teor antioxidante e a capacidade antioxidante dos alimentos para aquacultura, sendo que as temperaturas convencionais foram mais eficazes no aumento desta capacidade antioxidante devido à libertação de compostos fenólicos das fibras antioxidantes. Os compostos fenólicos revelaram-se particularmente mais abundantes e resistentes ao processo de fabrico do que as vitaminas e carotenoides.

Tendo em conta estes resultados, a segunda parte desta tese consistiu num estudo *in vivo* onde a farinhas de subprodutos de fruta foram incluídos em alimentos para robalo-europeu (*Dicentrarchus labrax*). Procurou-se avaliar se uma inclusão de 2% de farinha de subprodutos de ananás, ou seja, casca e caule, poderia ser usada como fonte de antioxidantes naturais, estudando também o seu impacto na conservação dos alimentos em diferentes temperaturas de armazenamento. As dietas experimentais modularam as respostas enzimáticas antioxidantes, no entanto, o impacto das dietas contendo suprodutos de ananás foi insuficiente para produzir melhorias percetíveis nas defesas antioxidantes dos peixes e na resposta fisiológica a um desafio de stress. Em relação à capacidade antioxidante e oxidação lipídica da dieta, o tempo de armazenamento teve um impacto negativo nas propriedades antioxidantes da dieta, independentemente da temperatura de armazenamento. Por fim, no terceiro e último capítulo desta tese procurou-se avaliar o potencial de vários coprodutos vegetais, ricos em antioxidantes, como fontes adicionais destes compostos em dietas funcionais para robalo-europeu, adotando uma abordagem holística. Essencialmente, foram comparados os resultados relativos ao crescimento, ao sistema antioxidante e imunitário, bem como à qualidade do filete dos peixes alimentados com as dietas contendo antioxidantes naturais, com os peixes alimentados com vitamina E sintética incluída nas dietas em doses regulares ou reforçadas. Os resultados experimentais sugerem que nem uma dose aumentada de

inclusão de 500 mg kg^{-1} de vitamina E, nem uma inclusão de 2% de antioxidantes naturais forneceram proteção antioxidante adicional, em comparação com peixes alimentados com dietas que incluíam uma dose regular de 100 mg kg^{-1} de vitamina E. Em suma, esta tese revelou existirem ainda muitas lacunas de conhecimento que impedem a plena exploração das propriedades antioxidantes das fontes naturais de antioxidantes, em termos da sua inclusão em alimentos para aquacultura. No entanto, os resultados promissores em termos de capacidade antioxidante dos alimentos para aquacultura, que podem prolongar a vida útil dos alimentos e diminuir a oxidação, sugerem que a incorporação de antioxidantes naturais de determinadas subprodutos frutas e vegetais pode ser benéfica. Existe potencial para que esses antioxidantes também beneficiem o sistema antioxidante dos peixes de aquacultura, desde que os processos e níveis de inclusão em alimentos compostos sejam otimizados. Importante salientar que essa otimização deve estar alinhada com a viabilidade económica, a sustentabilidade e os princípios de uma economia circular.

Summary

The aquaculture industry plays a critical role in meeting the global demand for fish, supplying over 53% of fish for human consumption. The growing relevance of aquaculture in global food production is accompanied by a need to increase the production and overall quality aquafeeds, while simultaneously enhancing production processes within a context of sustainability. Consequently, new functional diets are continuously being developed, each with unique and sometimes overlapping features. These diets are formulated not only to enhance fish growth but also to fortify immunological and antioxidant defences, while aiming for economic feasibility and environmental sustainability. Cultured fish often experience stress-inducing factors, such as handling, transportation, disease, sudden temperature changes, overcrowding, and/or suboptimal nutrition. Moreover, scarcity of fishmeal and fish oil lead to the increased inclusion of vegetable sources in aquafeeds, which might lead to decreased nutritional quality and may increase the susceptibility of fish to bacterial infection and overall stress conditions. This leads to an elevated production of intracellular reactive oxygen and nitrogen species (ROS and RNS). These highly reactive free radicals cause structural damage to lipids, protein and DNA, with highly negative consequences for cell homeostasis, which can lead to chronic stress, subsequently impacting crucial biological processes and ultimately impairing growth. These negative effects can be alleviated via exogenous antioxidant compounds included in aquafeeds with the purpose of inhibiting the initiation or progression of free radical chain reactions, while concomitantly avoiding the negative effects of oxidation in the feeds themselves. In recent years, the most commonly used antioxidants in feeds were synthetic sources, namely butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and ethoxyquin. However, according to recent research an accumulation of BHA and BHT, or its metabolites, may have potential carcinogenic and/or endocrine disruptive effects for both fish and consumers. This prompted the European Union to ban ethoxyquin in animal feeds due to concerns of potential carcinogenic activity underscoring the urgent need to explore alternative antioxidant sources. Simultaneously, the food industry annually discards large quantities of vegetable by-products and co-products rich in natural antioxidants such as vitamins, carotenoids, and phenolic compounds. These natural antioxidants possess various beneficial properties, including the ability to combat oxidation processes within both the feed and fish cells.

This thesis introduces an innovative, in-depth approach that focuses on exploring the potential of antioxidant-rich by-products and co-products from fruits and vegetables for inclusion in aquafeeds. The first chapter targeted the evaluation of the impact of the

various steps of the manufacturing processes, particularly with regard to temperature, to preserve the maximum antioxidant capacity of aquafeeds with natural antioxidant inclusion. Essentially, a control dietary mixture, along with two dietary mixtures with 2% inclusion of either pineapple or mango peel flour, was subjected to different combinations of extrusion temperatures (110 vs 25 °C) and drying temperatures (60 vs 35 °C). Results showed that incorporating 2% of either peel flour increased the antioxidant content and antioxidant capacity of aquafeeds, while conventional temperatures were more effective at increasing feed antioxidant capacity due to the release of phenolic compounds from the antioxidant fibre. Phenolic compounds were particularly more abundant and resistant to the manufacturing process than vitamins and carotenoids.

Taking these results into account, the second part of this thesis consisted in an *in vivo* trial where fruit by-products were included in aquafeeds for European sea bass (*Dicentrarchus labrax*). This work aimed to assess if a 2% inclusion of pineapple by-product flour, i.e. peel and stem, could be used as natural antioxidant sources while also studying their impact on feed preservation at different storage temperatures. The experimental diets modulated antioxidant enzyme responses, however, the impact of diets containing pineapple stem and peel was insufficient to yield noticeable improvements in fish antioxidant defences and physiological response to a stress challenge. Regarding diet antioxidant capacity and lipid oxidation, storage time negatively impacted diet antioxidant properties, regardless the storage temperature.

Finally, the third and last chapter of this thesis consisted of evaluating the potential of vegetable antioxidants-rich co-products as additional antioxidant sources in diets for European sea bass, taking on a holistic approach. Essentially, data concerning growth, antioxidant and immune system, as well as fillet quality traits of fish fed diets containing natural antioxidants were compared to fish fed diets with synthetic vitamin E included at either a regular or reinforced dose. Experimental findings suggest that neither a heightened inclusion dose of 500 mg kg^{-1} of vitamin E, nor a 2% inclusion of natural antioxidants provided additional antioxidant protection, compared to fish fed diets including the regular dose of 100 mg kg^{-1} of vitamin E.In summary, this thesis revealed many knowledge gaps still impede the full harnessing of the antioxidant properties of natural antioxidant sources in terms of their inclusion in aquafeeds. Nevertheless, the promising results in terms of feed antioxidant capacity, which can extend feed shelf-life and lessen oxidation, suggest that incorporating natural antioxidants from select fruits and vegetables by-products can be beneficial. There is potential for these antioxidants to also benefit the antioxidant system of farmed fish, provided that the processes and inclusion levels are optimized. Importantly, this optimization must align with economic viability, sustainability, and the principles of a circular economy.

Chapter 1.

General introduction

1.1 The global state of aquaculture – main achievements and emerging threats

Aquaculture is currently the food industry sector with the highest growth rate (Figure 1.1). Although its annual growth declined to 5.8% between 2001 and 2020 compared to the previous two decades, it still resulted in the sector supplying a substantial 214 million tonnes of fish for human consumption in 2020, comprised of 178 million tonnes of aquatic animals and 36 million tonnes of algae (Bharathi et al., 2019; FAO, 2022). Indeed, in 2020, global production of aquatic animals contributed to 49% of the fish available for human consumption (FAO, 2022). Over the past twenty years, aquaculture also exhibited an increase in diversity, encompassing 40% more fish, shellfish, aquatic plant, and algal species cultivated across a wide range of marine, brackish, and freshwater systems on a global scale (Naylor et al., 2021). Moreover, recent data predicts that aquatic animal production will increase by 15% over the following decade, reaching an annual production of 202 million tonnes by 2030 (FAO, 2022). In recent years, and despite the fact that the overall growth of the sector has been negatively affected by external factors, namely the COVID-19 pandemic, total world fisheries and aquaculture production showed a 45% growth between years 2000 and 2021, peaking at 182 million tonnes and setting a new production record that represents an expansion of 56 million tonnes compared to the year 2000 (FAO, 2023). Although the growth of aquaculture production leads to associated environmental costs, the sector has been, and will remain, critical for filling the seafood demand gap, thus lowering anthropogenic pressure in marine seafood stocks while facilitating the revival of marine ecosystems (Gephart et al., 2021). Thus, given the sector’s relevance in the global economic landscape, as well as its benefits in terms of reviving fish stocks and marine biodiversity, its exponential growth is of paramount importance (Naylor et al., 2021). Indeed, there has been a growing focus on raising awareness for environmental concerns and implementing practices aimed at reducing the sector's environmental footprint, making sustainability one of its foremost challenges (Boyd et al., 2020). Moreover, as the aquaculture sector expands swiftly, it becomes increasingly dependent on its surrounding environment, making it more susceptible to environmental pressures (Naylor et al., 2021).

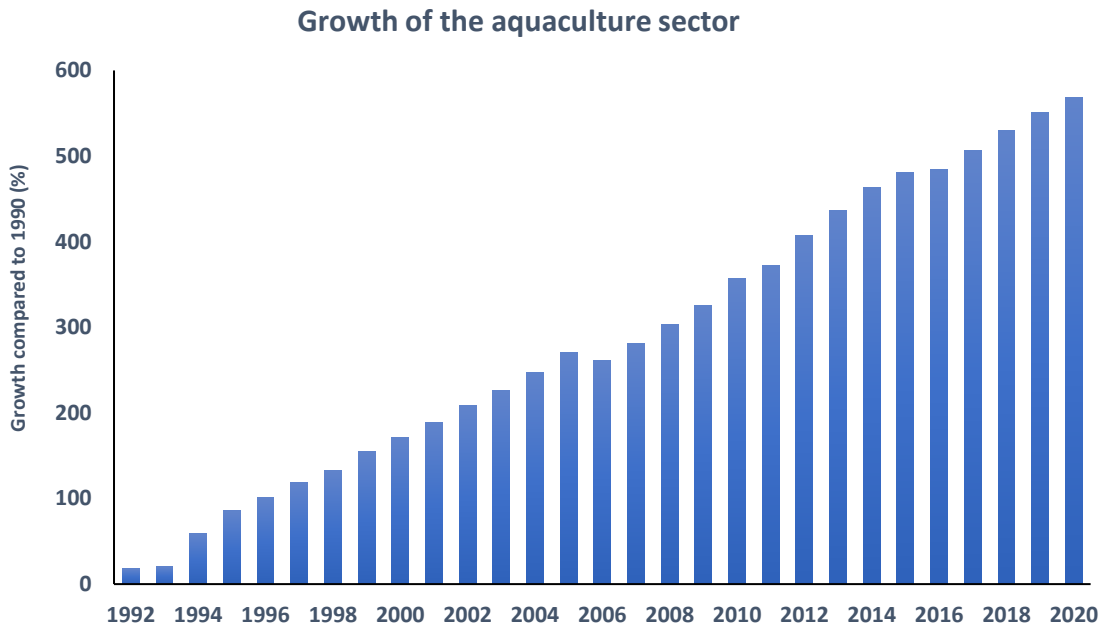

Figure 1.1 – Growth of the aquaculture sector production compared to data from 1990, compiled using data from the FAO State of World Fisheries and Aquaculture Reports from 1995 to 2022, excluding seaweed production, and provisional estimates

The aquaculture industry currently faces challenges associated with anthropogenic activities, which can lead to an increasing impact of pathogens, parasites, pests, pollution and detrimental algal blooms, among others (Naylor et al., 2021). To tackle these challenges, it is vital to prioritize the implementation of methods able to improve fish robustness, highlighting the need for innovative and enduring eco-friendly aquaculture practices. A promising strategy involves the utilization of nutraceutical functional feeds, which outperform conventional options via incorporation of specific ingredients to enhance the growth, health, and survival of cultured aquatic organisms (Leal and Calado, 2019). These functional aquafeeds have the potential to significantly contribute to enhanced sustainability, consequently reducing environmental impacts and bolstering farmers' economic gains (Leal and Calado, 2019). Moreover, with effective nutrition being a key aspect in terms of maximizing aquaculture quality and yield, aquafeed production accounts for a substantial portion, ranging from 50 – 80%, of the sector's production costs (Bharathi et al., 2019). Thus, both the success and the economic viability of aquaculture heavily rely on a nutritionally balanced diet associated with low production costs and extended feed shelf-life that can ensure high fish growth and robustness (Bharathi et al., 2019; Encarnação, 2016; Olmos-Soto, 2015).

In sum, the increasing demand for cultured fish, coupled with a rising vulnerability to environmental pressures and production costs, has underscored a need for a holistic

optimization effort of the aquaculture sector, which includes the development of novel aquafeeds to further promote fish growth and welfare while prioritizing sustainability. This objective can be achieved through the inclusion of functional feed additives (Encarnação, 2016; Olmos-Soto, 2015).

1.2 Functional aquafeeds

Functional foods are understood as those containing components that can provide health benefits beyond basic nutrition (Temple, 2022; Watts et al., 2020). In the context of aquaculture, a functional aquafeed is characterized by its ability to confer physiological advantages, enhance fish immunity and overall wellbeing, improve the culture environment, enhance the quality of the final product, and/or influence the physical and chemical characteristics of the feed (Bai et al., 2015). Indeed, the ever-increasing diversity of aquatic functional feeds allows for a wide range of options designed to optimize the ingestion, digestion, absorption and cellular transport of nutrients in farmed fish (Encarnação, 2016). This can be achieved via inclusion of additives or functional ingredients. Globally, the most commonly used additives in aquafeeds can be subdivided in six categories: attractants, prebiotics, probiotics, acidifiers, immunostimulant agents and antioxidants (Bai et al., 2022). A feeding attractant is an organoleptic additive developed according to the animal's taste and olfactory preferences with the purpose of increasing feed intake (Hancz, 2020). Prebiotics consist of indigestible fibres and other compounds that act as nourishment for beneficial gut bacteria, while probiotics are living microorganisms, namely beneficial bacteria and some beneficial yeasts, which are consumed to maintain a balanced gut microbiome (Quigley, 2019). Immunostimulants are meant to upregulate fish immune system, enhance its antimicrobial capabilities and reduce stress-related effects (Dawood et al., 2018). In contrast, antioxidants are specifically meant to upregulate both, the feed and/or the fish resistance against oxidative stress and the subsequent cellular oxidation (Aklakur, 2018).

However, the EU has specific regulations and frameworks for the use of additives in animal feeds. According to the European Food Safety Authority (EFSA, 2023), feed additives are defined as "products used in animal nutrition to achieve an effect on the feed itself, on the animals, on food products obtained from the animals consuming the feed additive, or on the environment". Specifically, Regulation (EC) Nº767/2009 states that besides providing essential macronutrients, these additives must perform micronutritional, technological, sensory or zootechnical functions, that could potentially promote health (Olmos-Soto, 2015). Subsequently, functional feed ingredients may be freely marketed without pre-market approval, provided they are safe, sound, genuine

and unaltered, and do not have a direct adverse effect on the environment or animal welfare (Olmos-Soto, 2015). Of the aforementioned categories of additives, antioxidants classify as one of the functional groups of technological additives, namely, substances that extend feed shelf-life and protect farmed animals from intracellular oxidation caused by stress.

1.3 Stress factors & Antioxidants

In the context of aquaculture, fish are commonly exposed to a myriad of stress-inducing factors, such as periodic handling, transportation, infectious diseases, abrupt temperature fluctuations, etc. (Figure 1.2). Moreover, there exists the potential for additional stressors, stemming from a certain degree of malpractice from both an ethical and technical standpoints, including overcrowding, poor rearing conditions and nutritional quality, among others (Reverter et al., 2014).

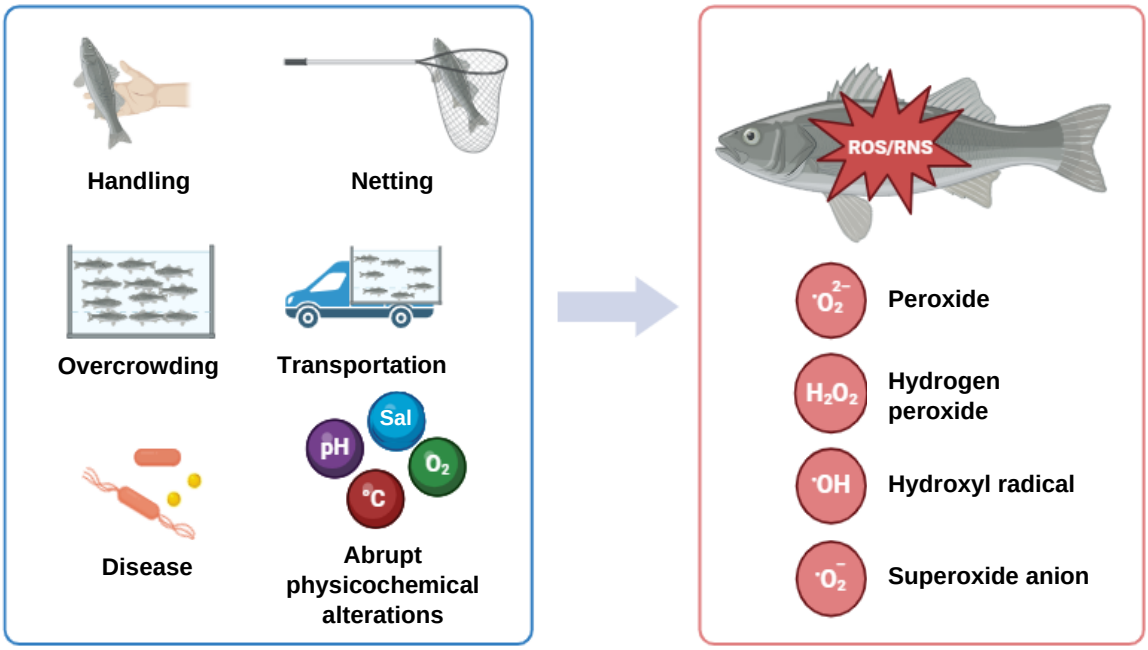

Figure 1.2 - Representative diagram of some of the root causes for ROS/RNS formation in cultured fish, as well as some of the most common free radicals formed

The practices mentioned earlier can induce stress responses in fish, manifesting as either acute or chronic stress, each with its distinct implications for physiological processes. Acute stress, triggered by various factors including handling, transportation, and environmental changes, can lead to immediate disruptions in fundamental physiological functions, which in turn are mediated by the hypothalamic-pituitary-

intrarenal axis. Furthermore, it is worth noting that prolonged activation of the hypothalamic-pituitary-intrarenal axis in fish can result in extended periods of heightened glucocorticoid production. This continuous elevation of glucocorticoids can lead to maladaptive expressions of acute stress response pathways, potentially culminating in chronic stress effects (Prentice et al., 2022). In this way, acute stress can serve as a precursor to chronic stress, further underscoring the significance of stress management in aquaculture practices. Notably, chronic stress represents a more persistent concern, as it has the potential to significantly impede the growth of cultured fish, resulting in substantial economic losses for the aquaculture sector (Guo and Dixon, 2021; Pickering et al., 1991; Van Weerd and Komen, 1998).

The common stressors in aquaculture contribute to the release of intracellular reactive oxygen and/or nitrogen species (ROS/RNS). When present in certain amounts, these highly reactive free radicals may cause oxidation of lipids, protein and DNA, as well as enzymatic inactivation, precocious cell aging and apoptosis (Guilherme et al., 2008; Poljsak et al., 2013). This oxidative damage exerts a detrimental impact on fish welfare and growth (Van Weerd and Komen, 1998), thereby affecting aquaculture's final output in terms of economic value. However, these negative effects can be mitigated through the action of either endogenous (naturally occurring within the organism) or exogenous (externally provided by feed) antioxidants, both of which mitigate oxidative damage by inhibiting the initiation or propagation of oxidative chain reactions (Baiano and Del Nobile, 2016; Halliwell and Gutteridge, 2015b). Thus, in the context of aquaculture, antioxidants are essential for preserving fish health and well-being. Simultaneously, the aquaculture feed sector is confronted with challenges related to the stability and storage of feeds. Essentially, the inclusion of fish oil in aquafeeds is of paramount importance to meet the nutritional requirements of fish, especially in terms of polyunsaturated fatty acids (PUFA), notably eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These essential fatty acids are not only vital for fish growth and welfare, but also play a crucial role in human nutrition, as they are primarily sourced from fish (NRC, 2011). However, due to their high degree of unsaturation, the PUFAs present in fish oil make aquafeeds highly vulnerable to lipid oxidation and subsequent rancidity, which is highly undesirable from a nutritional perspective and plays a crucial role in determining the feed quality and shelf-life (Aklakur, 2018; Colombo et al., 2020; Dominguez et al., 2019; Godwin and Prabhu, 2006; Lobo et al., 2010). Additionally, lipid oxidation results in elevated levels of aldehydes and ketones, thereby diminishing the palatability and overall intake of the feed (Lobo et al., 2010). Consequently, the inclusion of antioxidants in aquafeeds is of paramount importance and serves the dual purpose of enhancing fish vulnerability to

stress factors while simultaneously preventing the oxidation of both aquafeeds and refrigerated fillets (Bharathi et al., 2019).

1.3.1 Oxidative stress and antioxidant mechanisms in biological systems

Oxidation–reduction (redox) homeostasis is a fundamental process within biological systems that heavily influences almost all essential biochemical mechanisms, from bioenergetics to metabolism, subsequently permeating most life functions (Sies et al., 2017). This pivotal redox process primarily stems from the metabolization of oxygen, which subsequently leads to the formation of ROS and reactive nitrogen species (RNS), collectively referred to as reactive oxygen-based chemicals. It is important to note that the term “ROS” comprises different types of redox by-products, each exhibiting varying reactivity. Some, such as the superoxide anion radical and the hydroxyl radical, are free radicals characterized by unpaired electrons. Others, such as hydrogen peroxide (H_2O_2) or ozone (O^3), do not share this radical nature. Therefore, it is advisable to use specific chemical names when referencing ROS, whenever possible (Sies et al., 2017). Free radicals are generated through natural processes like electron transport chains (Turrens, 2003), or via leukocyte respiratory burst activity, partly as a way of combatting pathogens as a form of weaponization of these strongly oxidizing compounds by the organism (Biller, J.D.B. and Takahashi, L.S., 2018). Despite their key role in cell signalling and maintenance of cell homeostasis as initiators, transmitters and modifiers of cellular response (Kurutas, 2015), the oxidative properties of the ROS can also negatively affect biomolecules (Figure 1.3), which in turn can destabilize cell homeostasis (Novo and Parola, 2008; Sies et al., 2017). RNS share a similar dual function, exerting both detrimental and advantageous effects on living systems. For example, nitric oxide is recognized as a signalling molecule that influences blood vessel adjustments and controls vital physiological mechanisms. However, it can negatively affect homeostasis by impacting metabolic enzymes, producing peroxynitrite through reactions with superoxide, and oxidizing lipids, proteins, and DNA (Di Meo et al., 2016).

Free radicals react with biomolecules - mostly lipoproteins and unsaturated fatty acids, followed by proteins and nucleic acids - by removing electrons, which generates a new radical in the process, thus engaging a chain reaction that might lead to oxidative damage, disruption of biomolecules, cell membranes and tissue degradation, and even apoptosis (Ball et al., 2020; Novo and Parola, 2008; Sies et al., 2017). To either prevent or ameliorate the negative aspects of excessive free radical formation, antioxidants interact with these pro-oxidant compounds in order to quench these chain reactions by forming stable molecules or less reactive free radicals (Ball et al., 2020), thus preventing

oxidative stress, which occurs due to an intracellular imbalance between the production of free radicals and the continuous oxidation of these highly reactive molecules by antioxidants, maintaining them at acceptable levels (Biller, J.D.B. and Takahashi, L.S., 2018).

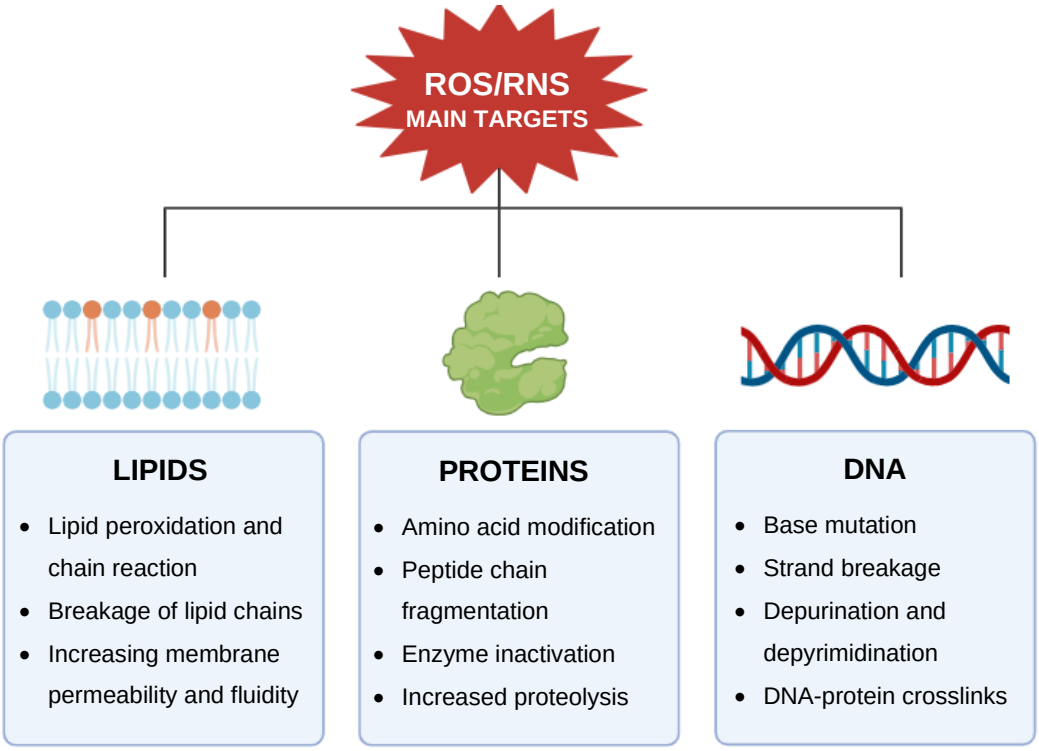

Figure 1.3 – Illustrative scheme of the main intracellular targets of ROS and RNS, as well as their main effects on these biomolecules. Adapted from Sharma et al. (2014)

One of the most detrimental effects of oxidative stress is attributed to the interactions of ROS and RNS with biological membranes. This interaction leads to the formation of lipid hydroperoxides that subsequently decompose double bonds of unsaturated fatty acids, therefore destructing said membranes (Van der Oost et al., 2003). Essentially, this phenomenon primarily targets the carbon-carbon double bonds present in fats and oils, generating harmful compounds, such as hydroperoxides, that in turn degrade fatty acids, decreasing the overall lipid energy content and potentially contributing to intracellular oxidative stress if these oxidized fats are consumed (Lobo et al., 2010). Known as lipid peroxidation (LPO), this complex process is also an endpoint oxidative stress biomarker that provides crucial information regarding the organ threshold that divides free radical imbalance from oxidative damage, something of paramount importance for interpreting

if the organism is maintaining its redox status, as well as to assess the structural damage done to lipid membranes by ROS and RNS (Kurutas, 2015).

Moreover, ROS and RNS can also affect the structural integrity of proteins via oxidation. Essentially, the biological activity of proteins demands for the creation of active sites, binding motifs and interacting domains, which heavily rely on both the alignment of secondary structure elements and their spatial organization (Mythri et al., 2013). Certain post-translational modifications (PTMs) can alter this structure-function relationship and might occur during signal transduction, protein translocation, and degradation during homeostasis, subsequently altering or inactivating specific proteins. Stress and disease are influential factors in the occurrence of abnormal and nonselective PTMs in cellular proteins (Mythri et al., 2013). Free radicals can oxidize amino acid side chains and protein backbone, as well as cross-link proteins, resulting in a variety of intracellular PTMs. Specifically, sulphur-containing amino acids (cysteine and methionine) are readily susceptible to oxidation (Mythri et al., 2013).

Antioxidant mechanisms comprise four main categories, which have been extensively studied. These include the chemical sequestration of transitional metallic ions, radical scavenging and quenching of ROS and RNS, the use of free radicals to interrupt oxidative chain reactions, and molecular repair of damage exerted by free radical imbalance (Aguilar et al., 2016). The antioxidants involved in these mechanisms can be either endogenous, e.g. antioxidant enzymes, cofactors, organic molecules with antioxidant activity naturally available in the organism, or exogenous, e.g. dietary substances with radical-scavenging properties such as vitamins, carotenoids or polyphenols that can be included in fish diets (Biller, J.D.B. and Takahashi, L.S., 2018).

1.3.3. Endogenous antioxidants

Endogenous antioxidants (Table 1.1) are an integral part of the specific mechanisms that the organism possesses to maintain an acceptable concentration of free radicals, thus achieving homeostasis. The activity of these endogenous antioxidant agents may be of an enzymatic or nonenzymatic nature (Aguilar et al., 2016). It is important to clarify that the term “endogenous antioxidants” includes the antioxidant enzymes that are produced by the organism with the specific purpose of avoiding or ameliorating the negative effects of oxidative stress. Many of these enzymes have multiple antioxidant roles, and their combined action can turn several free radicals into less reactive and more stable molecules that do not induce lipid, protein or DNA oxidation, and can be safely repurposed or excreted by the organism (Kurutas, 2015).

Table 1.1 – List and function of main antiperoxidative enzymatic and non-enzymatic endogenous antioxidants

Enzymatic	Function	Reference
Superoxide dismutase (SOD)	Catalyses the dismutation of the superoxide ion ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2).	(Peskin and Winterbourn, 2000)
Catalase (CAT)	Degrades hydrogen peroxide into water and oxygen molecules.	(Rojkind et al., 2002)
Glutathione reductase (GR)	Maintains homeostasis during oxidative stress by catalysing the oxi-reduction cycle of glutathione with the concomitant oxidation of NADPH to $NADP^+$. Serves as cofactor to glutathione-dependent enzymes and works concomitantly with glutathione S-Transferase to metabolize hydroperoxides.	(Horn, 1965)
Glutathione peroxidase (GPx)	Detoxifies organic and inorganic peroxides, being largely dependent on its interaction with glutathione in neutralizing hydroperoxides, ultimately protecting cells from peroxidative damage and thus preventing lipid peroxidation	(Ursini and Maiorino, 2013)
Glutathione S-transferase (GST)	Metabolizes hydroperoxides by conjugating electrophilic compounds with glutathione for the purpose of detoxification. Some forms of the enzyme exhibit isomerase activity toward ketosteroids and glutathione peroxidase activity toward lipid and nucleic acid hydroperoxides, and also act as binding (carrier) proteins	(Tsuchida, 2002)
Non-enzymatic		
Glutathione	Glutathione is present in both reduced (GSH) and oxidized (GSSG) forms. The proportion of reduced glutathione to oxidized glutathione inside cells serves as an indicator of cellular oxidative stress; a higher GSSG-to-GSH ratio suggests heightened oxidative stress. Protects cells directly by reducing ROS via interaction with GSSG, or by serving as cofactor for glutathione-dependent antioxidant enzymes such as glutathione S-transferase and glutathione peroxidase.	(Lu, 2013)
Alpha-lipoic acid	Its reduced form (dehydrolipoate) reacts with ROS, turning them into more stable, less reactive molecules. Also interacts with vitamin C and glutathione, thus protecting cell membranes from lipid oxidation.	(Packer et al., 1995)
Coenzyme Q	Protects cell membranes against the process of lipid oxidation initiated by ROS, via its main activated antioxidant agent responsible for the majority of its functions is the reduced form of CoQ10, known as ubiquinol. Ubiquinol, in turn, reacts with α-tocopherol and vitamin C radicals, decreasing oxidative stress. It can also regenerate alpha-tocopherol from the tocopherol radical, therefore increasing the antioxidant capacity of the organism.	(Sifuentes-Franco et al., 2022)
Ferritin	Limits the availability of Fe (II) in generating ROS. Possibly initiates genetic and protein changes that jointly mitigate the harmful effects of oxidative stress.	(Aguilar et al., 2016)
Uric Acid	A quencher of ROS, namely carbon-centred and peroxyl radicals within hydrophilic surroundings. Outside a hydrophilic environment it loses its capability to neutralize lipophilic radicals and is unable to disrupt the ongoing radical chain reaction within lipid membranes.	(Sautin and Johnson, 2008)
Bilirubin	Possesses antioxidant capabilities due to its ability to scavenge and neutralize various reactive oxygen species (ROS), such as superoxide radicals, hydrogen peroxide, and peroxynitrite.	(DiNicolantonio et al., 2018)

The main enzymes involved in oxidative stress as well as their antioxidant functions are summarised in Figure 1.4.

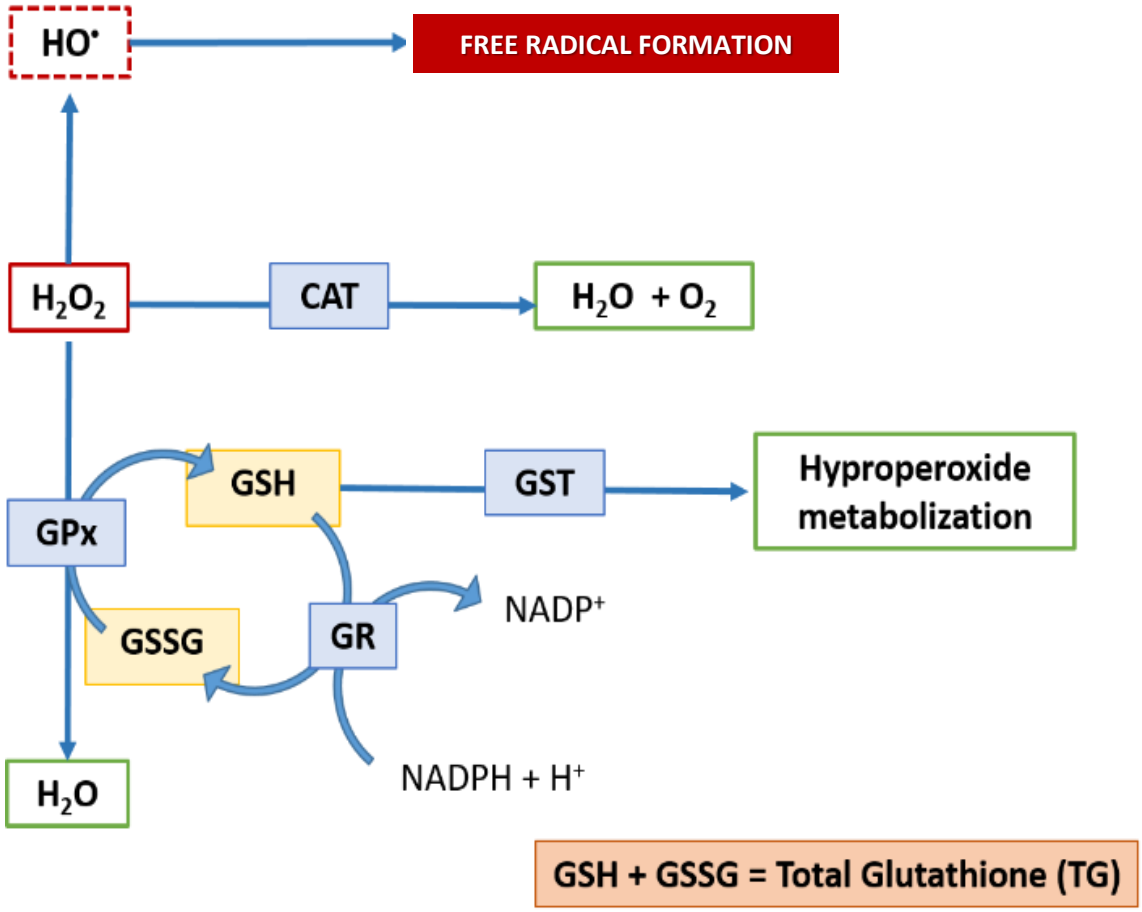

Figure 1.4 - Representative diagram of some of the interactions between the main antioxidant enzymes of the hydroperoxide redox mechanism in animals. CAT – Catalase; GPx – Glutathione Peroxidase; GSSG – Oxidized glutathione; GSH – Reduced glutathione; GR – Glutathione reductase; GST – Glutathione s-transferase; H_2O_2 – Hydrogen peroxide; $OH^{\bullet}$ – Hydroxyl radical

Total glutathione (GT) is the sum of oxidized glutathione (GSSR) with reduced glutathione (GSH), being an essential cofactor for antiperoxidative enzymes such as glutathione reductase (GR), glutathione s-transferase (GST), glutaredoxins, glutathione peroxidases (GPx) and peroxiredoxins, while also being responsible for chelation of xenobiotic molecules, rendering them chemically inert (Aguilar et al., 2016; Deponte, 2013). It is important to mention that shifting dynamics between antioxidant enzymes and cofactors tell us very little when not compared to an endpoint of either protein or lipid oxidation. Antioxidant enzyme production increases beyond basal levels when free radicals act as cellular signals to indicate an imbalance between ROS/RNS and antioxidants. While this response is vital for maintaining

redox homeostasis, excessively heightened enzyme activity can be counterproductive since it means that the organism needs to spend additional energy to generate these antioxidant enzymes (Kurutas, 2015) in order to maintain basal levels of lipid and/or protein oxidation.
Some endogenous antioxidants are also non-enzymatic (Table 1.1), exerting direct antioxidant activity through mechanisms like free radical chelation, binding and radical scavenging (e.g. polyamines, alpha-lipoic acid, uric acid, bilirubin, coenzyme Q), while others, such as glutathione, also serve as cofactors for antioxidant enzymes (Aguilar et al., 2016; Moussa et al., 2019). Specifically, glutathione is one of the most relevant cofactors for antioxidant enzymes.

1.3.3. Exogenous antioxidants

In order to avoid the negative effects of ROS/RNS on feed oxidation and nitrogenation during storage, as well upregulating fish antioxidant defences, antioxidant products are commonly incorporated into aquafeeds. In terms of exogenous antioxidants, a compound must have the capacity of donating a hydrogen atom to a radical, in order act as a primary antioxidant (Bragadóttir et al., 2001). Thus, the free radical imbalance can only be ameliorated if such molecules are available in sufficient amounts (Moussa et al., 2019). This can be ensured by the inclusion of exogenous molecules with antioxidant activity i.e. vitamins, carotenoids and polyphenols in aquafeeds (Moussa et al., 2019). But the antioxidant capacity of these external sources is largely dependent on their inclusion level, chemical composition, feed matrix, and processing technologies. Thus, inclusion of external antioxidant sources can lead to varied responses ranging from increased antioxidant response (Hamed and Abdel-Tawwab, 2021; Metwally, 2009) to negatively affecting antioxidant defences and/or increasing lipid peroxidation (Lizárraga-Velázquez et al., 2019; Lopes et al., 2020; Sallam et al., 2018). Indeed, while an overuse of antioxidants might induce pro-oxidant effects (Kurutas, 2015), the physical properties of certain antioxidant compounds might directly impact nutrient digestibility, potentially causing stress while diminishing antioxidant bioavailability (Arfaoui, 2021; Castenmiller and West, 1998; D'Archivio et al., 2010). An endpoint comparison that considers lipid and protein oxidation levels at tissue level can provide valuable insight on whether the supplementation rate is too high, inducing pro-oxidant effects, or too low to induce significant antioxidant system upregulation (Azzi et al., 2004). Consequently, lipid and protein oxidation are parameters of paramount importance in analyses that concern oxidative stress resistance, making them essential in assays that comprise exogenous antioxidant supplementation. Within the organism, the liver is a major site of metabolism and detoxification, making it especially

susceptible to oxidative stress induced by either xenobiotics and metabolic processes (Chowdhury and Saikia, 2020). This makes it a particularly good organ to measure oxidative stress response in fish. Moreover, in fish, muscle tissue is also susceptible to oxidative stress and subsequent oxidation of lipids and protein (Chowdhury and Saikia, 2020) which can negatively impact on flesh quality.

1.3.4 Feed processing technologies and aquafeed antioxidant properties

The presence of antioxidants in aquafeeds is of paramount importance in order to mitigate the negative effects of free radical imbalance in both fish organism and their feeds. These antioxidants are introduced either directly into the raw material or during the feed processing stage (Aklakur, 2018). However, the antioxidant capacity of such external antioxidants may be affected by the feed manufacturing processes such as conditioning, extrusion, pelleting, and drying; enlarged surface area cause by expansion, exposure of the feed to light, water, heat, pressure, high levels of iron, as well as the oxidation catalyst formed due to the wearing of the extrusion processing equipment are also other factors that can affect the stability of antioxidants in aquafeeds (Anderson and Sunderland, 2002; Ortak et al., 2017; Riaz and Ali, 2009; Wani and Kumar, 2015). Several studies have showed that common extrusion processes involve temperatures that commonly exceed 100 °C (up to 140 °C), potentially harming antioxidant compounds present in the feed, such as vitamins, carotenoids, and polyphenols, thus accelerating feed oxidation (Anderson and Sunderland, 2002; Liu et al., 2021; Ortak et al., 2017). For instance, a study by Anderson and Sunderland (2002) revealed significant vitamin content losses in aquafeeds during extrusion and drying processes, with extrusion being particularly detrimental, causing 54–73% loss of vitamin E content. Moreover, the high unsaturation of carotenoids makes them susceptible to degradation when exposed to high temperatures, light, or pro-oxidant molecules (Schieber and Weber, 2016). However, unlike vitamins and carotenoids, phenolic compounds seem to better endure the high-temperature extrusion-cooking process to some extent, although they remain vulnerable to oxidation (Oniszczuk et al., 2019). Moreover, a different study by Grela et al. (1999) showed that extrusion can result in a significant decrease in the content of antioxidants, namely tocopherols (α-tocopherol, β-tocopherol), polyenoic fatty acids and carotenoids (β-carotene and lutein) in grass peas *Lathyrus sativus*. Overall, these findings support that manufacturing conditions may negatively impact the antioxidant capacity of feed matrices. Consequently, there is a need to explore optimization strategies for these common practices to maximize aquafeed antioxidant properties.

1.3.5 Common antioxidants used in aquaculture feeds, EU legislation and health concerns

Synthetic antioxidants such as ethoxyquin, BHA (2,3-terc-butil-4-hidroxianisol) and BHT (2,6-Di-tert-butyl-4-methylphenol) are the most used in commercial food and feed formulations, with ethoxyquin being the most commonly used in aquafeeds (Bai et al., 2022). However, it's noteworthy that ethoxyquin was outlawed in the European Union (EU) for raw materials, feed premixes, additives and food produced and/or commercialized after 2017 (EU 2017/962). This ban occurred due to inconclusive evidence regarding the potential genotoxicity of the metabolite ethoxyquin quinone imine, as well as the carcinogenic potential of p-phenetidine, an impurity of ethoxyquin (Regulation, 2017). The European Comission (2022a) later reaffirmed this ban in 2022, further cementing ethoxyquin's status as prohibited substance in animal feeds in the EU, although its use continues worldwide. In contrast, BHA (E320) and BHT (E321) are currently authorized in the EU for all animal species, albeit the used amounts, either single or combined, cannot exceed the legal amount of 150 mg kg^{-1} (Union, 2004). Nevertheless, the International Agency for Research on Cancer classifies both BHA and BHT as possible human carcinogens (Anders et al., 1987), and the European Commission on Endocrine Disruption has listed BHA as a Category 1 priority substance based on evidence for enzymatic activity disruption (Zhang et al., 2023). In the Convention for the Protection of the Marine Environment of the North-East Atlantic, BHA is listed as a chemical of potential concern due to its toxicity to aquatic organisms and bioaccumulative potential (Zhang et al., 2023). As for BHT, it has been found to inhibit humoral immune response in animals, as well as possessing carcinogenic and tumour-promoting effects (Lanigan and Yamarik, 2002). High doses of BHT have been proven as toxic for mice and rats in the long-term, leading to negative effects on liver, thyroid, lungs and kidney function, as well as blood coagulation (Nieva-Echevarría et al., 2015). Moreover, and despite not being genotoxic, BHT may play a key role on modifying the genotoxicity of other agents (Lanigan and Yamarik, 2002). Evidence of endocrine disruption (Nieva-Echevarría et al., 2015) also raises questions regarding the effects of BHT on reproduction. Simultaneously, in recent years, consumer preferences displayed a growing trend of inclination towards natural antioxidants over synthetic sources (Sanches-Silva et al., 2014). This shift, along with the aforementioned health concerns, has prompted the feed industry to explore natural alternatives with robust antioxidant properties (Bai et al., 2015; Ehsani et al., 2018; Elseady and Zahran, 2013; Encarnação, 2016; Kousoulaki et al., 2015; Sanches-Silva et al., 2014).

1.4. Fruits and vegetables as sources of natural antioxidants

The production systems depend mostly on a complex interplay of interconnected global supply chains, the global sourcing of feed ingredients, capitalizing on affordable labour for processing, and the utilization of co-products and by-products on a worldwide scale (Gephart et al., 2021). In this context, fruits and vegetables, as well as their respective co-products and by-products have emerged as promising sources of natural antioxidants for animal feeds, with particular relevance in aquafeeds (Cieślik et al., 2006; Dawood, M. et al., 2022). This is mainly due to the inherent richness of fruits and vegetables in terms of natural antioxidant compounds such as vitamins, carotenoids and phenolic compounds.

1.4.1 Vitamins

Vitamins, with their biochemical properties, serve a multitude of critical functions in living organisms. They act as hormones and hormonal cofactors, mediators of cell growth, differentiation and signalling, and play a vital role as antioxidants (Halver, 2003). In fish, an insufficient supply of vitamins leads to reduced enzymatic activities and enzyme production, resulting in growth deficiencies, increased susceptibility to disease and reduced survival rates (NRC, 2011). Although fish possess the ability to synthesize some vitamins, this endogenous production rarely yields sufficient amounts for the proper function of biological processes, which means that remaining vitamins must be obtained from the diet (Dawood et al., 2018). Thus, vitamins represent a crucial component of aquatic animal nutrition, being some of the most expensive ingredients used in aquafeed formulation (Dawood et al., 2018).

Vitamins are subdivided in two major groups: fat-soluble vitamins, which includes vitamins A, D, E, and K, and the water-soluble vitamins, which comprise the B complex vitamins and vitamin C (Ball et al., 2020). It's important to note that the classification of vitamins may vary between organisms due to differences in biosynthesis (Ball et al., 2020). Moreover, certain carotenoids also play the role of vitamins, therefore being classified according to their biochemical activity, meaning that each “vitamin” may refer to different compounds in terms of their chemical composition (Holdt and Kraan, 2011a). In fish, two of the most important vitamins in terms of their antioxidant benefits are vitamins C and E. These vitamins, often used as major antioxidant additives in the food industry, have been shown to reduce oxidative stress in fish and other animals (Dawood et al., 2018; NRC, 2011). Vitamin E interacts with free radicals, forming more stable molecules, and is converted back to its original form through a chemical interaction with vitamin C (Ball et al., 2020).

Vitamin C is present in fruits and vegetables such as rose hips, currants, parsley, quince, cabbage varieties, broccoli, potatoes, peppers, turnips, cauliflower, strawberries, kiwi,

oranges, lemons, grapefruit, papaya, pineapple, mango, etc. (Öztürk and Yaman, 2022). It is a highly effective antioxidant and immunomodulatory substance, essential for maintaining fish health, performance and overall welfare (NRC, 2011). Particularly, vitamin C participates in steroid and collagen biosynthesis, and has been proposed as an effective immunostimulant (Dawood et al., 2018; NRC, 2011), potentially enhancing immune response (Blazer, 2002), bactericidal activity, phagocytic activity, antibody levels and lysozyme activity (Dawood et al., 2018). Moreover, Vitamin C is also a strong antioxidant with high radical-scavenging properties, reducing oxidative damage to tissues (Blazer, 2002), although its exact mechanism has not been demonstrated. Fish are unable to synthesize Vitamin C (NRC, 2011) due to the absence of L-gulonolactone oxidase, which is essential for its biosynthesis, and therefore must acquire it through their diet (Dawood et al., 2018). A critical need for vitamin C has been shown in rainbow trout *Oncorhynchus mykiss*, and a necessity for L-ascorbic acid was also demonstrated in several species of salmonids, as well as in channel catfish *Ictalurus punctatus*, common carp *Cyprinus carpio*, European sea bass *Dicentrarchus labrax*, turbot *Scophthalmus maximus*, Nile tilapia *Oreochromis niloticus,* and in three species of eel, namely *Anguilla anguilla*, *Anguilla japonica* and *Anguilla rostrata* (Halver, 2003; NRC, 2011). A deficiency in vitamin C can result in reduced growth, anorexia, scoliosis/lordosis, organ structural deficiencies in eyes, gills, kidney and fins, internal haemorrhaging, lethargy, immunodeficiency and oxidative stress (NRC, 2011).

The term “Vitamin E” encompasses all naturally occurring lipid-soluble antioxidant tocopherols, tocotrienols, and respective derivatives (Blaner, 2013). Many vegetables and fruits are rich in Vitamin E, such as mango, pineapple, orange, strawberry, carrots, tomatoes, spinach, green beans, among others (Charoensiri et al., 2009; Rickman et al., 2007). Among its eight different forms, α-tocopherol has the most significant impact on fish health (Udo and Afia, 2013). Easily oxidized due to its inherent hydroxyl moiety on carbon 6, Vitamin E has a strong reducibility, which has an important role in the maintenance of normal metabolic processes and physiological function in the body, mainly due to its radical-scavenging properties (Blaner, 2013; Sen et al., 2006; Traber and Atkinson, 2007). Among natural antioxidants, vitamin E has been proven to have an essential protective role against the adverse effects of reactive oxygen species and other free radicals (NRC, 2011). Indeed, the interaction between Vitamin E and selenium (via glutathione peroxidase), contributes to a multi-component antioxidant defence system that protects the cell from free radical- induced oxidative damage to vital components like polyunsaturated membrane phospholipids, and critical proteins (NRC, 2011). Since vitamin E is synthesized exclusively by photosynthetic organisms (Muthulakshmi et al., 2023), it must be included in aquafeeds to provide its antioxidant benefits to farmed fish. Vitamin E shows antioxidant potency in a similar manner to several synthetic antioxidants such as ethoxyquin

and BHA, whilst simultaneously providing additional protection against lipid oxidation (Gatlin et al., 1992). This is also relevant for quality preservation of fish fillet, both in its raw or cooked forms, as demonstrated in several commercially reared fish species, including rainbow trout (Kamireddy et al., 2011), turbot (Ruff et al., 2003) and European sea bass (Gatta et al., 2000). The beneficial effects of vitamin E as an antioxidant have been thoroughly researched in the liver of several teleost fish, namely gilthead sea bream *Sparus aurata* (Mourente et al., 2002), red sea bream *Pagrus major* (Gao et al., 2012) and rainbow trout (Kelestemur et al., 2012; Palace et al., 1993). However, the available literature shows that the effectiveness of vitamin E as an antioxidant is largely dependent on fish age and species, as well as dosage used in the aquafeed (NRC, 2011). Beyond its role as a vital nutrient for fish, vitamin E has a key role in reducing mortality and improving fish health, by increasing specific and nonspecific immune responses (Khara et al., 2016; Lu et al., 2016) and enhancing disease resistance (Aggarwal et al., 2010; Halver, 2003). Although highly used in aquaculture, increased demand and rising prices have been a recent burden on the industry (Muthulakshmi et al., 2023). Moreover, climate change, via alterations caused in water temperature, stand to disrupt the dietary requirements and metabolic processes of farmed fish (El-Sayed and Izquierdo, 2022). This stems from vitamin E's pivotal role as the primary defence against lipid peroxidation induced by heat stress, making it advisable to increase the intake of this micronutrient in the diet, along with other essential vitamins and minerals, particularly under conditions of heat stress (El-Sayed and Izquierdo, 2022). All of the aforementioned factors strongly highlight the need for searching cost-effective and sustainable sources of antioxidants for inclusion in aquafeeds.

1.4.2 Carotenoids

Carotenoids are isoprenoid molecules which are naturally present in vegetables, fruits and seaweed, mostly in dark green leafy vegetables, coloured fruits, root and tuber crops and unicellular microalgae (Gebregziabher et al., 2023). Their presence and quantities can vary based on factors such as species, seasons, environmental conditions, growth, and reproductive cycles (Christaki et al., 2013). Carotenoids can be classified into two main categories based on their chemical composition: carotenes (hydrocarbons) and xantophylls (containing one or more oxygen molecules) (Holdt and Kraan, 2011a). It's worth noting that an increased intake of carotenoid is known to affect fish skin and muscle coloration (NRC, 2011), which, in turn, might influence consumer acceptance of fish fillets (Barbut, 2004; Carvalho and Caramujo, 2017; Moroney et al., 2015). Moreover, carotenoids have demonstrated anti-inflammatory and anti-tumour activities, and have been shown to induce upregulating immune-regulatory effects. Some carotenoids, such as α-carotene, β-carotene and β-cryptoxanthin,

serve as precursors to vitamins, such as vitamin A (Christaki et al., 2013). Carotenoid-rich aquafeeds have been proven effective in increasing fish innate immune system defences, thus increasing fish resistance to bacterial and fungal diseases (García-Chavarría and lara-flores, 2013). In addition to their health benefits, carotenoids are also considered to be strong antioxidants, free-radical scavengers and singlet oxygen quenchers (Chakraborty and Hancz, 2011; Pezeshk and Alishahi, 2015), which makes carotenoid-rich fruits and vegetables a viable option for developing functional aquafeeds with antioxidant properties. The specific antioxidant activities of carotenoids such as lutein (Kiokias and Gordon, 2004; Sindhu et al., 2010), β-carotene (Kiokias and Gordon, 2004; Mueller and Boehm, 2011), lycopene (Kaur et al., 2012; Kiokias and Gordon, 2004), α-carotene and β-cryptoxanthin (Kiokias and Gordon, 2004) have been thoroughly evaluated using *in vitro* studies. Particularly, an *in vitro* assay by Ehsani et al. (2018) found that dietary lycopene, naturally abundant in carrots and tomatoes, effectively prevents lipid oxidation in rainbow trout, subsequently delaying rancidity in trout fillet. β-carotene, found in high doses in carrot and coriander, acted as an antioxidant and immunostimulant in Nile tilapia by mitigating the negative effects of peroxide radicals stemming from mercuric chloride toxicity (Elseady and Zahran, 2013). Moreover, natural carotenoid inclusion from marigold meal, combined with synthetic astaxanthin and crab *Portunus pelagicus* waste meal, was also found to stimulate antioxidant potential and significantly reduce lipid peroxidation in European sea bass (Goda, A. et al., 2018).

Since animals, including fish and crustaceans, cannot biosynthesize carotenoids themselves, the antioxidant properties of these compounds is only accessible when they are made bioavailable within the organism via feeding (Goda, A. et al., 2018). One of the significant challenges in utilizing carotenoids is maintaining their stability in aquafeeds. Factors like prolonged exposure to light, air and heat can lead to oxidation and degradation of carotenoids (Rodriguez-Amaya, 2003). This challenge is particularly relevant during the feed manufacturing stage and subsequent storage. Particularly, Ortak et al. (2017) demonstrated that the conventional extrusion process, inherent to feed production, is known to significantly decrease the antioxidant activity of carotenoids like β-carotene and lutein due to heat exposure. This has prompted the industry to identify best strategies able to protect these compounds during the various feed production steps.

1.4.3 Phenolic compounds

Phenolic compounds are phytochemicals synthesized as secondary metabolites through the shikimic acid and phenylpropanoid pathways (de la Rosa et al., 2019). They are present in many fruits and vegetables, particularly dark green leafy and brightly-coloured vegetables, as

well as legumes and cereals, in addition to spices and fruits such as cherries, pineapple, mango and citrus (Maria de Lourdes Reis, 2013). These compounds contain one or more hydroxyl groups and two or more phenolic rings, which may or may not be halogenated (Freile-Pelegrin and Robledo, 2014). According to Otavio et al. (2017), the attached hydroxyl groups in phenolic compound chemical structures form either phenolic acids (one aromatic ring) or polyphenols (two or more aromatic rings). Their role in biological activities differs according to their chemical composition (Freile-Pelegrin and Robledo, 2014). These organic chemicals can be categorized into four groups: flavonoids, phenolic acids, hydroxycinnamic acids and lignans (Gudipati, 2017) and they are found in various plant tissues.

Derived from two polyphenolic molecules, namely gallic and ellagic acid (Garcia-Vaquero and Hayes, 2016), phenolic compounds play various roles i.e. attracting pollinators, providing structural support, shielding against ultraviolet radiation, and safeguarding plants from microbial invasion and herbivores (Bertelli et al., 2021). They also influence the sensory and nutritional characteristics of plant-based foods, affecting attributes such as astringency, colour, and odour, which vary based on the types and amounts of polyphenolic compounds present (Bertelli et al., 2021). Additionally, certain phenolic compounds have the ability to bind and precipitate macromolecules like dietary proteins, carbohydrates, and digestive enzymes, thus modulating food digestibility (Bertelli et al., 2021). Moreover, phenolic compounds can also serve as potential additives for preventing quality deterioration or to retain the quality of fish and fish products (Maqsood et al., 2013).

Phenolic compounds exhibit a wide range of bioactive properties which translate into antioxidant and overall health-promoting effects (de la Rosa et al., 2019). Consequently, to enhance their beneficial impact, postharvest treatments have been employed to boost or maintain the levels of phenolic compounds in fruits and vegetables, aiming to enhance their beneficial effects (de la Rosa et al., 2019). It is important to consider that the antioxidant capacities of these compounds are not only attributed to the presence of hydroxyl groups and phenolic rings but also depend on various biochemical and mechanistic factors such as absorption, distribution, and metabolism of each phenolic compound within the body (Otavio et al., 2017). The antioxidant potential of phenolic compounds is thoroughly established and can be evaluated through methods such as the Trolox equivalent antioxidant capacity assays. Additionally, they exhibit scavenging abilities towards stable free radicals like 2,2-diphenyl-1-picryl-hydrazyl (commonly known as $DPPH^{\bullet}$), due its affinity with fat-soluble hydrophobic compounds, as is the case with phenolic compounds (Holdt and Kraan, 2011a; Pezeshk and Alishahi, 2015). Moreover, the antioxidant activity of some more hydrophilic phenolic compounds may be assessed via the ability to scavenge the and 2,2′-azino-di-(3-ethylbenzthiazoline) sulfonic acid radical (commonly known as $ABTS^{\bullet+}$) (Gómez-García et al.,

2021). Moreover, the Oxygen Radical Absorbance Capacity (ORAC) assay, which belongs to the hydrogen atom transfer (HAT)-based assay family, gives a measure of species competing for peroxyl radicals which are naturally formed intracellularly (Apak et al., 2007), allowing for a more realistic assessment of antioxidant response in biological systems.

The biological properties of phenolic compounds are dependent on factors such as concentration, profile, dosage, and administration, while also being heavily influenced by other physical properties of food matrix. These factors determine the bioaccessibility and bioavailability of phenolic compounds in the organism (Lizárraga-Velázquez et al., 2019). Moreover, as with all antioxidants, although phenolic-rich dietary supplements can induce antioxidant effects, there is considerable evidence supporting the hypothesis that high doses of phenolic compounds may cause adverse effects through pro-oxidative action (Oniszczuk et al., 2019). Moreover, while the feed manufacturing process may negatively affect phenolic concentration via exposure to heat, overall, phenolic compounds from fruits and vegetable sources seem to be rather resistant to deactivation via high-temperature extrusion-cooking process (Oniszczuk et al., 2019).

1.4.4 Inclusion of natural antioxidants from fruit and vegetable sources in aquafeeds

The richness of fruits and vegetables in antioxidant compounds such as vitamins, carotenoids and polyphenols, associated with an increasing interest for natural products, prompted the feed industry to consider their inclusion in aquafeeds as alternative antioxidant sources in recent years, using co-products and by-products of the agri-food industry (Garcia-Vaquero and Hayes, 2016; Lee et al., 2013; Sanches-Silva et al., 2014). These natural antioxidant sources have the potential to offer cost-effective, safe, natural, sustainable and environmentally-friendly alternatives or complements to the most commonly used synthetic antioxidants (Bai et al., 2015).

The inclusion of fruits and vegetable as natural antioxidant sources has demonstrated tangible benefits, including the reduction of stress, upregulation the antioxidant and innate immune systems, as well as appetite stimulation in omnivorous, herbivorous and carnivorous fish (Arfaoui, 2021; Harikrishnan et al., 2012; Kaur and Kapoor, 2001; Kumar et al., 2020; Lau et al., 2021; Lizarraga et al., 2018; Pavaraj et al., 2011; Shalaby et al., 2006; Takaoka et al., 2011). The bioactive antioxidant molecules present within certain fruits and vegetables (Laribi et al., 2015) may actively provide the organism with exogenous antioxidant defences, upregulate the endogenous antioxidant enzyme activity and/or upregulate fish immune system, all of which can lead to an increased disease resistance (Amar et al., 2004; Betancor et al.,

2011; Lizárraga-Velázquez et al., 2019). Specifically, fruit and vegetable inclusion managed to increase growth in African catfish *Clarias gariepinus* (Adeshina et al., 2019), gilthead sea bream (Salem et al., 2019), European sea bass (Sallam et al., 2018) and Nile tilapia (Abdel-Tawwab et al., 2010; Abdel-Tawwab et al., 2018b; Anis Mohamad Sukri et al., 2022; Attalla et al., 2022; Metwally, 2009). However, it is essential to note that the effects of fruit or vegetable inclusion on fish growth can vary between studies. In some studies, growth was unaffected when aquafeeds were supplemented with low percentages of natural antioxidant sources from plants (Lizárraga-Velázquez et al., 2019; Sahu et al., 2007; Sari and Ustuner, 2018). Additionally, fruit and vegetable inclusion in aquafeeds was also proven to provide benefits in terms of flesh quality and fillet shelf-life (Ehsani et al., 2018; Pezeshk and Alishahi, 2015).

Supplementation of aquafeeds via natural antioxidant inclusion can be achieved via liquid extracts or via dried, ground products. It is important to note that the extra processing costs of liquid extracts can raise issues of economic viability in a realistic aquaculture scenario (Shofinita et al., 2021). A summary of available literature concerning the effects of the inclusion of fruit and vegetables in aquafeeds in terms of growth, survival, immune system and antioxidant activity is depicted in Table 1.2 and 1.3.

Table 1.2 – Effects of fruit and fruit by-product inclusion in fish feeds in terms of growth, survival, immune system and antioxidant activity

Reference	Fish species	IBW[1] (g)	Extract	Inclusion (g kg $^{-1}$)	Pro-oxidant challenge	Growth[2]	Survival	Immune system modulation[3]	Organs	AEP[4]	TAC[4]	LPO[4]
(Salem et al., 2019)	Gilthead seabream	0.3	Dried orange peel	1-5.5	n.d.	• WG and SGR ▲ • FCR ▼	=	n.d.	Liver	• CAT, SOD, GPx and AP ▲	▲	▼
(Al-Khalaifah et al., 2020)	African catfish	60.5	Dried palm fruit	5-15	n.d.	• WG, SGR and PER ▲ • FCR ▼	n.d.	• Phagocytic index, Lysozyme, Nitric oxide, Myeloperoxidase and Sialyltransferases ▲	Liver	• CAT, SOD and GSH ▲	n.d.	n.d.
(Hoseinifar et al., 2020a)	Rainbow trout	2.5	Dried lemon verbena	5-20	n.d.	=	n.d.	• ACH50 ▲	Serum	• SOD, GPx and GST ▲	n.d.	n.d.
(Hamed and Abdel-Tawwab, 2021)	Nile tilapia	30	Dried pomegranate peel	30-50	Silver nanoparticles	• WG [a,b], SGR [a,b] and Intake [a,b] ▼	=	• IgM [a,b], Respiratory burst [a,b] and Lysozyme [a,b] ▲	Kidney (K) Liver (L)	• SOD [b] (K) ▲ • CAT [a,b], SOD [a,b] and GSH [a,b] (L) ▲	n.d.	▼ [a,b]
(Lopes et al., 2020)	Tambaqui	2.2	Lemon peel extract	0.25-2	n.d.	• K ▲	▲	n.d.	Liver (L) Muscle (M)	• SOD (L) ▲	(L) ▲ (M) =	(L) = (M) ▲
(Guardiola et al., 2016)	European sea bass	40.5	Date palm extract	100	n.d.	n.d	n.d	• ACH50 ▲	Head kidney (HK) Gut (G)	• Gene expression of *sod* (HK) and *fbl* (G) ▲	▲	n.d
(Lizárraga-Velázquez et al., 2019)	Zebrafish	0.2	Mango peel extract	50-200	n.d.	=	n.d	n.d	Whole body	• CAT ▲	n.d	▲
(Xavier et al., 2021)	Senegalese sole	12.3-17.7	Grapeseed extract	12	Thermal stress	• RGR ▲	=	n.d.	Whole body	• PC [a] ▲ • CAT [b], GST [a], GSH [a,b], HSP70 [a] and PC [b] ▼	= [a,b]	= [a,b]

Papers with inclusion of solid and/or liquid plant-based natural antioxidant sources. "^" signifies "increase", whereas " v" signifies decrease and "=" means "measured, no discernible effects found". "n.d." means "not defined", meaning that no parameters related to the subject were measured in the corresponding study.

[1] **IBW** – Initial body weight

[2] **WG** – weight gain; **SGR** – specific growth rate **; K** – Fulton's condition factor **; PU** - protein utilization **; FCR** – feed conversion ratio **; RGR –** relative growth rate **PER** – Protein efficiency ratio **; ER** – energy retention **; DGI** – Daily growth index

[3] **Immuno** – Immune modulatory potential in blood and/or serum **; IgM** – Immunoglobulin M

[4] **AEP** – Antioxidant enzyme parameters of the corresponding organ(s), whether enzymatic activity or enzyme gene expression. Expressions in italic represent genes; **TAC** – Total antioxidant capacity of the corresponding organ(s); **LPO** – Lipid peroxidation of the corresponding organ(s), **AP** – Alkaline Phosphatase

[a] Pre-challenge; [b] Post-challenge

Table 1.3 – Effects of vegetable and herb by-product/coproduct inclusion in fish feeds in terms of growth, survival, immune system and antioxidant activity

Reference	Fish species	IBW[1] (g)	Extract	Inclusion (g kg $^{-1}$)	Pro-oxidant challenge	Growth[2]	Survival	Immune system modulation[3]	Organs	AEP[4]	TAC[4]	LPO[4]
(Metwally, 2009)	Nile tilapia	20.5	Garlic powder	40	No	• WG, SGR, Intake, FCR and PER ▲	▲	n.d.	Serum (S) Liver (L)	• CAT, SOD and GPx (S, L) ▲	n.d.	▼
			Garlic powder tablets	32	No		▲			• CAT, SOD and GPx (S, L) ▲	n.d.	▼
(Hoseinifar et al., 2020b)	Rainbow trout	2.5	Dried olive waste	0.5-5	No	• WG, SGR and FCR ▲	n.d.	• Immunoglogulin, Lysozyme and ACH50 ▲	Liver	• SOD, GPx and GST ▲	n.d.	n.d.
(Sallam et al., 2018)	European sea bass	2.2	Dried marigold flower	0.1 – 0.3	No	• WG, DGI and ER ▲	▲	n.d.	Whole body	• Carotenoids ▲	=	▲
(Adeshina et al., 2019)	African catfish	11.7	Dried clove buds	5-15	Bacterial	• WG and SGR ▲ • Intake ▼	▲[b]	• Respiratory burst and Lisozyme ▲	Serum	• CAT and SOD ▲	n.d.	n.d
(Zhou et al., 2016)	Grass carp	43.8	Green tea extract	50	No	• WG and Feed efficiency ▼	n.d.	n.d.	Serum (S) Hepatopanchreas (HP)	=	(S) =	n.d.
			Dried green tea waste	50	No	=	n.d.	n.d.		• Ubiquitin • and HSP70 (HP) ▲	(S) ^	
(Metwally, 2009)	Nile tilapia	20.5	Garlic oil	0.25	n.d.	• WG, SGR, Intake, FCR and PER ▲	▲	n.d	Serum (S) Liver (L)	• CAT, SOD • and GPx (S, L) ▲	n.d	▼
(Xavier et al., 2021)	Senegalese sole	12.3-17.7	Green tea extract	12	Thermal stress	• RGR =	=	n.d.	Whole body (WB)	• CAT [b], GSH [a,b], GST [a] and PC [b] ▼ • PC [a] and HSP70[a] ▲	= [a,b]	=[a,b]
			Curcumin extract	46		• RGR ▲				• GST [a], HSP70 [a] and CAT [b] ▼	= [a,b]	= [a,b]
(Sari and Ustuner, 2018)	Rainbow trout	50	Oregano essential oils	0.25-1	No	=	▲	• Respiratory burst and lysozyme ▲	Liver	• CAT, SOD and GPx ▲	n.d	▼
			Oregano extract	0.25-1	No	=	▲	• Respiratory burst ▲	Liver	• CAT, SOD and GPx ▲	n.d	▼

Papers with inclusion of solid and/or liquid plant-based natural antioxidant sources. "^" signifies "increase", whereas " v " signifies decrease and "=" means "measured, no discernible effects found". "n.d." means "not defined", meaning that no parameters related to the subject were measured in the corresponding study.

[1] **IBW** – Initial body weight

[2] **WG** – weight gain; **SGR** – specific growth rate **; K** – Fulton's condition factor **; PU** - protein utilization **; FCR** – feed conversion ratio **; RGR –** relative growth rate **PER** – Protein efficiency ratio **; ER** – energy retention **; DGI** – Daily growth index

[3] **Immuno** – Immune modulatory potential in blood and/or serum

[4] **AEP** – Antioxidant enzyme parameters of the corresponding organ(s), whether enzymatic activity or enzyme gene expression. Expressions in italic represent genes; **TAC** – Total antioxidant capacity of the corresponding organ(s); **LPO** – Lipid peroxidation of the corresponding organ(s), **AP** – Alkaline Phosphatase

[a] Pre-challenge; [b] Post-challenge

For example, dried clove bud inclusion (5-15 g kg^{-1} of feed) increased the activity of antioxidant enzymes CAT and SOD in African catfish serum. Moreover, said inclusion of clove bud increased respiratory burst and lysozyme activity, while simultaneously reducing post-bacterial infection mortality (Adeshina et al., 2019). In juvenile European sea bass *(*2.2-4.2 g)*,* inclusion of marigold flower meal (0.1-0.3 g kg^{-1} of feed) increased whole body lipid oxidation with 0.1 g kg^{-1} of inclusion, but decreased with 0.2 g kg^{-1}, when compared to control. Moreover, whole body carotenoids increased with all inclusion percentages. However, whole body total antioxidant capacity was not affected (Sallam et al., 2018). A study by Metwally (2009) showed an increased activity of enzymes SOD, GPx and CAT, accompanied by a decrease in lipid peroxidation, in the liver of Nile tilapia fed with diets with inclusion of garlic powder (40 g kg^{-1} of feed) and garlic powder tablets (32 g kg^{-1} of feed). Also in Nile tilapia, inclusion of cinnamon (0.25-10 g kg^{-1} of feed) increased plasma CAT and SOD activity, but also lipid oxidation, while GPx activity decreased, possibly indicating that fish antioxidant system was combating a surge of intracellular free radicals. However, post-bacterial infection mortality also decreased with all inclusion percentages of cinnamon, hinting towards the previously mentioned notion of antioxidant responses varying between stressed and non-stressed individuals. Moreover, lysozyme activity, nitrous oxide and nitroblue tetrazolium concentration also increased in the blood of infected fish, as this upregulation of fish immune system might also have contributed towards an increased resistance to bacterial infection Abdel-Tawwab et al. (2018b). In the same species, inclusion of pomegranate peel powder (30-50 g kg^{-1}) increased immunoglobulin M (IgM), respiratory burst, and lysozyme activity both before and after a pro-oxidant challenge with silver nanoparticles. In terms of antioxidant defences, muscle SOD increased post-challenge and liver antioxidant enzyme activities of CAT and SOD, as well as GSH production, increased both before and after the challenge, while LPO lowered compared to control (Hamed and Abdel-Tawwab, 2021). However, lipid oxidation in plasma increased, as well as CAT and SOD activity, while GPx activity decreased. Moreover, when using experimental diets with mango peel inclusion (50-200 g kg^{-1} of feed) in zebrafish *Danio rerio*, Lizárraga-Velázquez et al. (2019) observed that CAT activity rose at 200 g kg^{-1}, whilst no differences in lipid peroxidation could be detected, possibly meaning that the organism needed to deploy more catalase to maintain cell homeostasis at acceptable levels, when compared to the control. Inclusion of ginger rhizome (0.5-50 g kg^{-1} of feed) in feeds for orangefin labeo *Labeo calbasu* increased GST, SOD and CAT activities in all inclusion percentages in liver, gill and muscle. These effects were observed both before and after acid stress, with stressed fish showing even higher enzyme activity. Inclusion of oregano (5-20 g kg^{-1} of feed) in rainbow trout diets had no discernible effects on antioxidant

enzyme activities, lipid oxidation, respiratory burst or lysozyme activity when compared to the control diet (Sari and Ustuner, 2018). In juvenile African catfish, inclusion of palm fruit powder (5-15 g kg^{-1} of feed) improved growth while lessening feed conversion ratio, as well as upregulated fish immune system by increasing serum phagocytic index, nitric oxide, and activities of lysozyme, myeloperoxidase and sialyltransferase activities (Al-Khalaifah et al., 2020). Moreover, enzymatic activities of CAT, SOD and GSH availability increased, although no indicator of oxidative damage was measured (Al-Khalaifah et al., 2020). A study by Xavier et al. (2021) in Senegalese sole *solea senegalensis* juveniles revealed that fish fed with diets containing green tea (12 g kg^{-1}) require less production of GST and GSH to maintain LPO at similar levels to the control, hinting towards antioxidant system upregulation, while heat shock proteins and protein oxidation increased. Moreover, results revealed that, when faced with a thermal pro-oxidant challenge, fish fed diets with curcumin (46 g kg^{-1}) and grapeseed (12 g kg^{-1}) inclusion require less CAT production in order to maintain LPO at acceptable levels when compared to the control diet. Finally, and despite not being included in the feed, the direct oral administration of tackweed *Tribulus terrestris* (0.25 g kg^{-1} of feed) to Mozambique tilapia *Oreochromis mossambicus* lowered the activities of antioxidant enzymes SOD, CAT, GPx, GR, GST, as well as GSH bioavailability in liver, gills, muscle and head kidney, when compared to control. Additionally, lipid peroxidation rates were lower in the same organs (Kavitha et al., 2011).

In some studies, both productive parameters and antioxidant defences were modulated by fruit and vegetable inclusion. Specifically, Attalla et al. (2022) managed to improve growth rate, final body weight (FBW), feeding performance and immune system status of Nile tilapia fingerlings (FBW = 5 g) fed with 2% pineapple waste. Similarly, another study by Van Doan et al. (2021) reported increased growth performance, nutrient utilization, mucus and serum lysozyme and peroxidase, alternative complement, phagocytosis, respiratory burst activities and disease resistance with the addition pineapple peel powder (10 g kg^{-1}) to Nile tilapia diets. Meanwhile, the inclusion of orange peel (1.5-5 g kg^{-1}) reduced feed conversion ratio (FCR) in juvenile gilthead sea bream (FBW = 2.4 g) and simultaneously increased liver antioxidant enzyme activities of SOD, GPx and CAT throughout all inclusion percentages, resulting in decreased lipid oxidation when compared to the control (Salem et al., 2019). In tambaqui *Colossoma macropomum* fingerlings, inclusion of lemon peel extract (0.25-2 g kg^{-1}) increased condition factor, survival and liver SOD (Lopes et al., 2020). In a different approach, 50 g kg^{-1} of green tea actually decreased growth of grass carp *Ctenopharyngodon idellus* (IBW = 43.8 g), but increased serum total antioxidant capacity; both dried tea leaves and leaf liquid extract induced a higher expression of genes associated with antioxidant

activity in hepatopancreas further highlighting the potential of vegetable inclusion to upregulate fish immune and antioxidant system (Zhou et al., 2016). Finally, Hoseifinar et al. (2020a; 2020b) observed increased enzymatic activity of SOD, GPx and GST, as well as upregulation of the immune system in rainbow trout fingerlings when fed with diets containing either lemon peel or olive waste powders; the latter diet fared better in terms of immune system upregulation and increased weight gain and specific growth rate (SGR), despite the fact that both diet increased fish FCR. Overall, supplementation of diets with natural antioxidant sources appears to a promising strategy for the aquafeed industry.

1.4.5 Global production and waste management of fruits and vegetables

The concept of “food waste” is a contentious matter, given that there is no universally accepted definition. This lack of consensus poses a significant challenge as it forms the basis for developing waste inventories. Consequently, substantial disparities in food waste production data can be found depending on the information source, while they are often plagued by considerable uncertainties (Stenmarck et al., 2016). Essentially, the term “food waste” encompasses both the portions of food and inedible parts of food that are removed from the food supply chain, as defined by Tostivint et al. (2016). This includes biomass eliminated from said supply chain with the intention of being recovered or disposed of through various methods such as composting, anaerobic digestion, bioenergy production, incineration, or disposal in landfills, sewers, or bodies of water. However, it is important to note that “food waste” does not include the portions, whether edible or inedible, that are removed from the food supply chain but are intended for animal feed or for the production of bio-based chemicals (Esparza et al., 2020).

In 2019, the FAO defined food loss and waste as a decrease in either the quantity or quality of food throughout the entire food supply chain, taking place during the stages from production to the point just before retail, while food waste happens within the realms of retail and consumption (FAO, 2019). According to this study, approximately 14% of edible food intended for human consumption is discarded and wasted globally each year, which translates to 1.6 billion tonnes in food loss annually (FAO, 2019). Specifically, 44% of this food waste consists of fruits and vegetables, regardless of their viability for consumption, as they are discarded mostly due to retailer standards (Lau et al., 2021; Porter et al., 2018). Moreover, the food industry also routinely discards millions of tons of fruit and vegetable by-products, which in turn are often rich in bioactive compounds that can be harnessed within the context of a circular economy (Lau et al., 2021; Porter

et al., 2018). All of this translates into approximately 998 million tons of agricultural wastes per year, leading to a substantial rise in livestock waste, agricultural crop residues, and agro-industrial wastes (Lau et al., 2021). Thus, food waste stands as a precious asset for the aquafeed sector, albeit contingent upon various factors such as formulation, pricing, availability, feed processing, and nutritional significance (Kari et al., 2023). However, fruits and vegetables, as well as their respective by-products from the agri-food industry, are rich in natural antioxidants such as vitamins, carotenoids and polyphenols, suggesting that these products may present a significant sustainable source of recyclable natural antioxidants (Porter et al., 2018) within the context of a circular economy, in accordance with the European Commission's Circular Economy Action Plan (European Comission, 2022b).

1.5. The antioxidant properties of selected fruit by-products and vegetable co-products

A by-product and a co-product are both secondary products that result from manufacturing or production processes, but there are differences between them. By-products are secondary products that emerge unintentionally or incidentally during the processing or refinement of the main product. Although they may or may not have some economic value, their production is not the primary goal of the process. On the contrary, co-products are secondary products intentionally produced alongside the main product. Unlike by-products, co-products are planned for and managed as part of the overall production strategy, having their own specific markets or applications, and sharing the value of the main product. In this thesis, three by-products, i.e. mango peel, pineapple peel and pineapple stem, and three vegetables co-products, i.e. tomato, carrot and coriander, were used as natural antioxidant sources for aquafeeds. This approach aims to incorporate alternative antioxidant sources into aquafeeds within a circular economy framework, thus minimizing waste (Figures 1.5 and 1.6). Mango and pineapple stand as two of the most consumed fruits worldwide (Altendorf, 2019). Their high relevancy within the context of the global food market originates a high yield of peels as a primary by-product after processing. Additionally, pineapple stems are also one of the primary by-products of pineapple processing and are mostly discarded. Previous works have highlighted mango peels as rich in bioactive compounds such as various vitamins, carotenoids such as beta-carotene and lutein, and phenolic compounds such as flavonoids and phenolic acids (Ajila et al., 2010), while the same can also be said for pineapple peels and stems (Campos et al., 2020b).

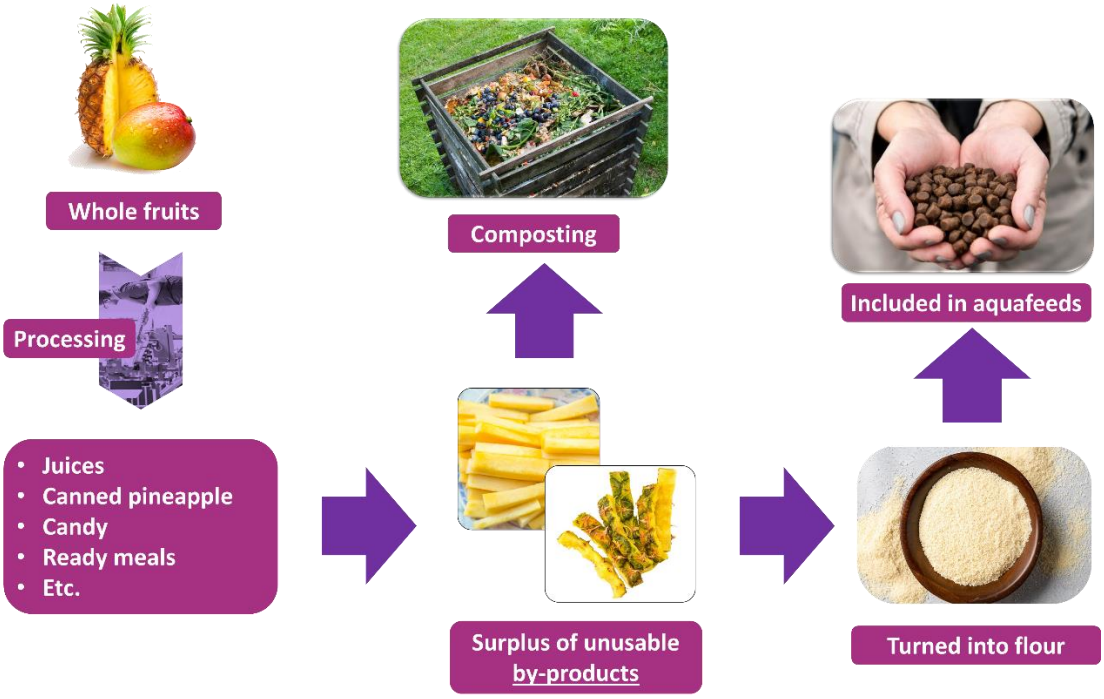

Figure 1.5 - Representative diagram of how fruit by-products are generated and how they can be incorporated in aquafeeds

Meanwhile, carrot and tomato are ranked among the top ten most economically significant vegetable crops globally, while coriander, a versatile herb used worldwide, stands as one of the most produced spices globally (FAOSTAT, 2024). Despite their importance, all three are commonly repurposed by the agri-food industry when they fail to meet cosmetic and retailers' standards (Porter et al., 2018). However, they are rich in vitamins, carotenoids and phenolic compounds (Laribi et al., 2015; Martí et al., 2016; Sun et al., 2009), making them promising sources of natural antioxidants for inclusion in aquafeeds.

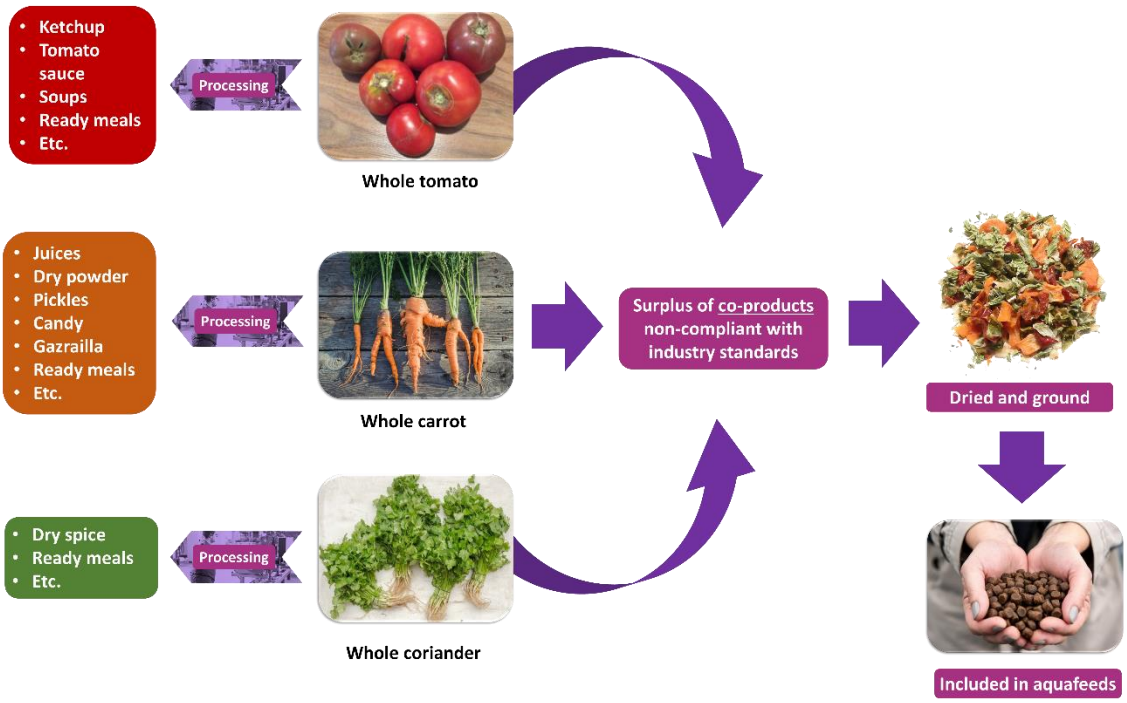

Figure 1.6 - Representative diagram of how vegetable co-products are generated and how they can be incorporated in aquafeeds

1.6. Objective of the thesis

The overarching aim of this PhD thesis is to provide a comprehensive and in-depth evaluation of the potential of fruit and vegetables as natural antioxidant sources in feeds for a relevant fish species in Mediterranean Aquaculture, the European sea bass (*Dicentrarchus labrax*). To achieve this, the thesis workplan was structured around three specific objectives, corresponding with chapters 2, 3 and 4.

The first part of this thesis, comprising chapters 2 and 3, will be dedicated to unravelling the antioxidant properties of fruit by-products as natural antioxidant sources. This will be achieved, firstly, by performing a preliminary study concerning the effect of processing technologies on the radical scavenging capacity of aquafeeds supplemented with natural antioxidants from mango and pineapple peels (chapter 2). This chapter will allow the identification of best extrusion and drying temperatures able to retain the highest antioxidant potential. In chapter 3, the potential of the best natural antioxidant sources identified in chapter 2 to modulate fish antioxidant defences and overall stress response of European sea bass juveniles will be assessed during an *in vivo* trial. Additionally, the impact of these by-products on feed preservation at different storage conditions will be evaluated.

Finally, the second part of this thesis, chapter 4, intends to assess the viability of 2% inclusion of vegetable co-products, namely carrot, tomato and coriander as natural antioxidant supplements in market-sized European sea bass; this chapter will be particularly focused on evaluating whether these natural antioxidant sources are effective in avoiding fillet oxidation during storage, and if consumer acceptance is somehow impacted. Overall, this PhD thesis seeks to advance our understanding of the potential benefits of incorporating fruit and vegetable sources as natural antioxidants in aquafeeds within the context of sustainability and a circular economy rationale. This was achieved through a multi-faceted approach encompassing pilot-scale feed technological trials for measuring antioxidant content and capacity, as well as *in vitro* trials with animals involving various methods (e.g. biochemical and physiological methodologies combined with instrumental methods for textural properties and colour, and consumer sensory analysis).

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Chapter 2.

Unravelling the effects of extrusion and drying temperatures on the radical scavenging capacity of aquafeeds supplemented with mango and pineapple by-products

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Submitted to Animal Feed Science and Technology (Elsevier)

Abstract: Antioxidant supplementation of aquafeeds with natural sources is a current research trend within the context of a circular economy. However, natural antioxidants are highly vulnerable to thermal conditions during feed manufacturing, particularly during extrusion and drying. This study examines the impact of extrusion and drying temperatures on the antioxidant properties of aquafeeds supplemented with natural antioxidants from mango and pineapple peels. A control dietary mixture and two dietary mixtures with 2% inclusion of either mango (M) or pineapple (P) peel flour were subjected to different combinations of extrusion temperatures (110 °C, -H vs 25 °C - C) and drying temperatures (60 °C vs 35 °C). Ingredients, manufacturing process intermediate stages, and final diets were analysed for their natural antioxidant composition (vitamins, carotenoids, free and fibre-bound phenolic compounds) and antioxidant capacity via the $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and ORAC assays, the latter of which is the most representative for biological models. Results show that incorporating 2% of either pineapple or mango peel flour increases the antioxidant content and capacity of aquafeeds compared to a control diet subjected to the same manufacturing conditions. Phenolic compounds were more abundant and resistant to the feed manufacturing process than vitamins and carotenoids. Specifically, ORAC results for free and fibre-bound extracts in diet PH-60 (1674.3 and 1216.2 mg TE 100g DM^{-1}, respectively) were significantly higher ($P < 0.001$) than the control CH-60 (694.8 and 422.8, respectively). Moreover, free extracts from diet PC-60 (1312.0 mg TE 100g DM^{-1}) and fibre-bound extracts from diets MH-60 and PH-35 (719.2 and 871.1 mg TE 100g DM^{-1}, respectively) were also significantly higher than the control ($P < 0.001$). A PCA analysis showed that pineapple diets with hot extrusion, as well as the mango diet with hot extrusion and hot drying, are more closely associated with higher antioxidant capacity in both free and bound extracts. Overall, fruit peel flours show promise as antioxidant supplements for mitigating oxidation in aquafeeds.

Keywords: Circular economy; Extrusion and drying temperature; Feed technology; Mango and pineapple peels; Natural antioxidants; Phenolic compounds

1. Introduction

The aquaculture sector currently provides for 53% of the worldwide production of fish for human consumption (FAO, 2018). This growth has been accompanied by an increasing demand for functional aquafeeds, which are expected to offer additional physiological benefits to the health and growth of farmed fish, going beyond what is provided by their fundamental nutritional content (Bharathi et al., 2019; Dawood et al., 2022).

Aquafeeds contain significant amounts of monounsaturated and polyunsaturated fatty acids (MUFAs and PUFAs, respectively) that are highly sensitive to oxidation via free radical chain reactions initiated by peroxides and hydroperoxides (Colombo et al., 2020). High levels of oxidation can make aquafeeds unsuitable for use due to several factors, such as the depletion of essential nutrients, lower feed palatability, diminished feed intake, and the formation of oxidation by-products that can potentially damage animal health (Gunathilake et al., 2022). Thus, there is a significant demand for diets with strong antioxidant properties, as they help boost the antioxidant defences of fish and prevent feed staling and rancidity (Encarnação, 2016; Olmos-Soto, 2015). This not only helps preserve the nutritional value of the feed but also reduces wastage and the costs associated with handling and storage (Gunathilake et al., 2022; Olmos-Soto, 2015). Moreover, farmed fish are often exposed to stress factors (Reverter et al., 2014) that induce heightened intracellular formation of reactive oxygen species (ROS) that may ultimately lead to reduced growth, higher disease susceptibility, and eventually death (Chowdhury and Saikia, 2020). However, this damage can be mitigated by continuously neutralising ROS with antioxidants, which help maintain healthy metabolic functions (Chowdhury and Saikia, 2020). Therefore, increasing the levels of endogenous and/or exogenous antioxidants can play a crucial role in mitigating oxidative damage by inhibiting the initiation or propagation of oxidative chain reactions, as demonstrated by Baiano and Del Nobile (2016).

Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and ethoxyquin (NRC, 2011) are widely used in aquafeeds. However, these compounds have raised certain health concerns. In 2017, the European Union (EU) banned ethoxyquin in feed premixes, additives, and commercialised food (EU 2017/962) due to suspicions of genotoxic and mutagenic properties (European Commission, 2017). After revaluation of potential risks, the authorisation of ethoxyquin (E 324) as an additive in animal nutrition belonging to the additive category ‘technological additives’ and to the functional group ‘antioxidants’, was denied by regulation EU 2022/1375 (European Commission, 2022). Furthermore, although the EU still permits using BHA and BHT in animal feeds (European Commission, 2004), BHT has been

linked to adverse effects, including immune system suppression and tumour development (Lanigan and Yamarik, 2002). Driven by shifts in global economic interests and consumer preferences, the rising demand for natural ingredients in animal feed and food products (Sanches-Silva et al., 2014) has prompted the feed industry to explore alternative sources of antioxidants, such as fruits and vegetables (Elseady and Zahran, 2013; Bai et al., 2015; Kousoulaki et al., 2015; Encarnação, 2016; Aklakur, 2018; Ehsani et al., 2018; Dawood et al., 2022).

In recent years, the global annual production of fresh vegetables and fruits has increased from 30 to 60 million tons (Shahbandeh, 2019). The processing of these fruits generates millions of tons of antioxidant-rich by-products, such as peels, which are mostly discarded, leading to negative environmental consequences (Kumar et al., 2020; Wu, 2016). However, the antioxidant capacity of these external sources of antioxidants relies on factors such as their chemical composition, feed matrix, and processing technologies. Indeed, the manufacturing processes of feed, including conditioning, extrusion, pelleting, and drying, expose the feed to light, water, heat, pressure, and various forms of mechanical stress. All these factors can affect the stability of natural antioxidants found in aquafeeds (Riaz and Ali, 2009). Particularly, temperatures of over 100 °C are often used (Liu et al., 2021), potentially damaging antioxidant compounds such as vitamins, carotenoids, and polyphenols, which in turn might accelerate feed oxidation (Anderson and Sunderland, 2002; Ortak et al., 2017).

According to Anderson and Sunderland (2002), significant vitamin losses occur in aquafeeds during the extrusion and drying procedures, with extrusion being the most detrimental, causing 54–73% loss of vitamin E depending on temperature and moisture. Likewise, the high unsaturation of carotenoids makes them susceptible to degradation when exposed to high temperatures, light, and pro-oxidant molecules (Schieber and Weber, 2016). Although phenolic compounds appear to withstand deactivation during the high-temperature extrusion-cooking process to some extent, they remain prone to oxidation (Oniszczuk et al., 2019).

The main objective of this study is to explore the effects of extrusion and drying temperatures on the radical scavenging capacity of aquafeeds supplemented with natural antioxidants from mango and pineapple peels. We will investigate the effects of incorporating 2% fruit peels into a commercial-like diet for European sea bass, while simultaneously assessing the impact of two different temperatures applied during the extrusion (25 °C vs 110 °C) and drying (35 °C vs 60 °C) processes. The chemical composition and radical scavenging capacity of the fruit peels, as well as the resulting diets, will be evaluated at key steps of their industrial production: homogenisation of fresh

fruit, drying fruit biomass, the mixture of feed ingredients, and extrusion and drying of the mixtures.

2. Materials and Methods

2.1. Ingredient preparation, dietary formulation and processing

The inclusion of novel ingredients, such as fruit by-products, to produce extruded diets under industrial conditions involves several key phases that may differently impact the nutritional and functional value of the resulting biomass. In this study, five key stages were defined for the feed manufacturing process: Phase 1 – Homogenisation of fresh fruit peels (mango, Mangifera indica, and pineapple, Ananas comosus, peel biomass); Phase 2 – Drying and grinding of the fruit peel biomass into flour; Phase 3 – Mixing of feed ingredients (Non-extruded dietary mixtures, NE); Phase 4 – Extrusion of the mixtures into pellets; and Phase 5 – Drying of extruded pellets. The technological processes involved in each phase (Figure 2.1) are described below.

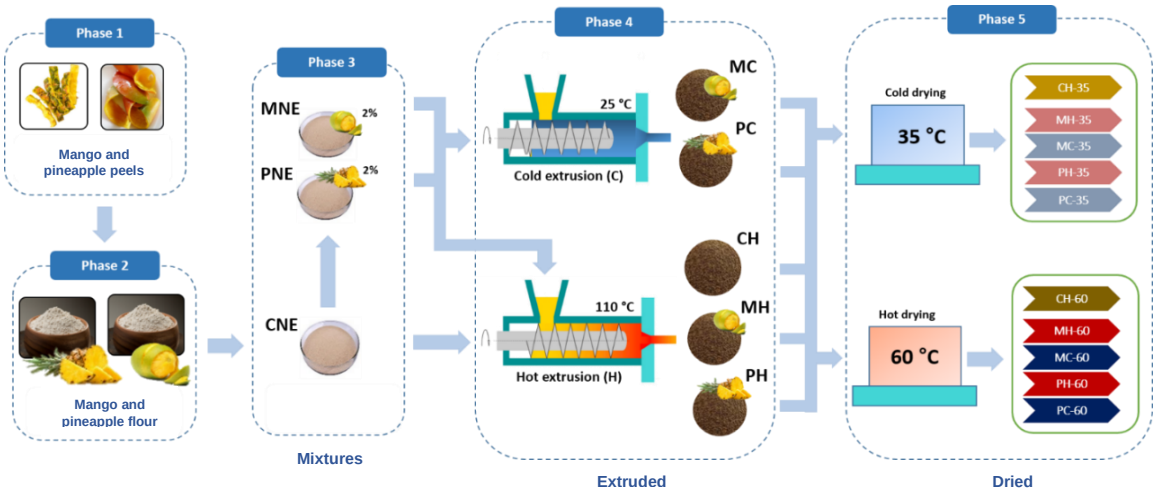

Figure 2.1 - Illustrative scheme for the five phases of experimental diet manufacturing

Phase 1: Fresh mango and pineapple peels were obtained from Nuvi Fruits, S.A., a Portuguese leading company in producing and distributing ready-to-eat fruit and dehydrated fruit products. A small portion of mango and pineapple peels was homogenised using a liquid juicer and analysed for their chemical composition and antioxidant capacity before proceeding to the next step.

Phase 2: Fresh mango and pineapple peels were processed into flour as described previously by Campos et al. (2020b), with adaptations for mango peel. Essentially, while pineapple peels were able to be homogenised in the liquid juicer for conversion into flour as described by Campos et al. (2020b), this was not possible for mango peels due to their physical properties. As such, homogenised pineapple peels and the non-processed mango peels were placed on separate metal trays and dried in an air-circulating oven at 60 °C until the moisture content was reduced to <10% (72 h for whole mango peels and 48 h for homogenised pineapple peels). The resulting dried biomasses were ground in a granite flour mill and sifted at 0.5 mm to obtain two distinct flours – mango peel flour (M) and pineapple peel flour (P). Both products were stored in separate polyethene bags at - 80 °C until they were chemically analysed.

Phase 3: A control diet was formulated to meet all the nutritional requirements of European sea bass (NRC, 2011), except for the addition of vitamins E and C with antioxidant capacity. A commercial vitamin premix without these vitamins was used to supplement the diet. All ingredients were mixed under industrial conditions by SPAROS Lda. (Portugal) to obtain a non-extruded control mixture (CNE). Two experimental diets were further formulated by including 2% of either pineapple or mango flour in the CNE at the expense of wheat meal (Table 2.2). This resulted in two distinct experimental non-extruded mixtures, pineapple (PNE) and mango mixtures (MNE).

Phase 4: Extrusion of mixtures was performed by SPAROS Lda. (Portugal) using two extrusion technologies: a) a commonly used hot extrusion employing a pilot-scale twin-screw extruder (CLEXTRAL BC45, France) with a screw diameter of 55.5 mm and a temperature of 110 °C as previously described for aquafeeds (Pereira et al., 2022; Resende et al., 2024); b) a cold extrusion process using a low-shear single screw extruder (ITALPLAST P150, Italy) operated under low-temperature conditions (c.a., 25 °C) at the extruder output to maintain the stability of natural compounds as previously suggested (Hernández et al., 2014). The PNE and MNE mixtures were extruded using either the hot standard temperature (-H, 110 °C) or a colder temperature (-C, <25 °C), generating two extruded pellets from pineapple (PH and PC) and two from mango (MH and MC). The CNE was extruded at 110 °C to simulate current commercial manufacturing processes, resulting in a control hot-extruded (CH) pellet.

Phase 5: The extruded pellets obtained from phase 4 were dried by SPAROS Lda. (Portugal) using two different methods. The first method involved drying at 60 °C, as typically used in aquafeeds (Resende et al., 2024), while the second method involved a colder drying temperature of 35 °C as suggested by Schafberg et al. (2020) until all

pellets reached an acceptable dry matter content (>90%) (Figure 2.1). This generated two control hot-extruded dried diets: the CH-60 (hot-extrusion at 110 °C and drying at 60 °C) and the CH-35 (hot-extrusion at 110 °C and drying at 35 °C), as well as four experimental dried diets containing M (MH-60, MH-35, MC-60 and MC-35), and four diets containing P (PH-60, PH-35, PC-60 and PC-35 diets). The resulting biomass from each key processing step was frozen at - 80 °C until analysed for proximate composition and determination of antioxidant properties.

2.2 Proximate composition analysis

Proximate composition of fresh peels, flours, mixtures and pellets was performed in accordance with AOAC methods (AOAC, 2006). Extruded pellets were ground and homogenised before analyses. All samples were analysed in duplicates for dry matter (DM) (105 °C for 24 h); ash through muffle furnace combustion at 500 °C for 5 h (Nabertherm L9/11/B170, Bremen, Germany); crude protein (N × 6.25) using a Leco nitrogen analyser (Model FP 528; Leco Corporation, St. Joseph, USA); and crude fat by petroleum ether extraction, using a Soxtec extractor (Model ST 2055 Soxtec™; FOSS, Hillerod, Denmark). Gross energy was determined in an adiabatic bomb calorimeter (Model Werke C2000, IKA, Staufen, Germany). Crude fibre content was analysed according to the intermediate filtration method (ISO 6865:2000); neutral-detergent fibre (NDF) was determined according to ISO 16472:2006 (Robertson and Van Soest, 1981; Van Soest and Robertson, 1985); and starch was determined as described in Thivend et al. (1972).

2.3 Carotenoids and vitamins

In all in fresh peels, flours, mixtures, extruded pellets, and dried pellets (final diets), carotenoids, retinol, and tocopherols were analysed in duplicate using extracts obtained following Slavin and Yu (2012), with slight modifications as described by Gómez-García et al. (2021). The quantification and identification of carotenoids in the resulting extract were also performed according to Gómez-García et al. (2021), through HPLC (Waters Series 600, Mildford MA, USA), using acetonitrile, methanol, dichloromethane, hexane and ammonium acetate (55:22:11.5:11.5:0.02 v/v/v/v/w) under isocratic conditions at 1 mL min^{-1} flow rate during 20 min at 30 °C. Injection volume was 50 µL and detection was performed by a 454 nm diode array detector (Waters, Massachusetts, EUA). α-carotene, β-carotene, lutein, and β-cryptoxanthin were quantified using a pure standard calibration curve and expressed as mg $100g^{-1}$ DM. Analysis of retinol and tocopherols were

performed via chromatography, using a Beckman System Gold® HPLC system (Beckman Coulter, California, U.S.A.) linked to a Waters™ 474 Scanning Fluorescence Detector (excitation wavelength of 290 nm and emission wavelength of 320 nm) with a VARIAN ProStar Model 410 AutoSampler with a normal-phase silica column (Kromasil 60-5-SIL, 250 mm, 4.6 mm ID, 5 µm particle size). The mobile phase was 1% (v v^{-1}) isopropanol in n-hexane with a flow rate of 1 mL min^{-1}. The total run time was 20 min and the injection volume was 20 µL. Standard curves of peak area vs. concentration were used for each compound quantification.

2.4 Phenolic compounds

Free phenolic compounds were determined in fresh peels, flours, mixtures, extruded pellets, and dried pellets (final diets), in triplicate, according to the method described by Xie et al. (2015) adapted by Coscueta et al. (2018), with some modifications. Briefly, 2.5 g of each sample was mixed with methanol:water (2:8) and homogeneised using an IKA T 25 digital ULTRA-TURRAX® (IKA, Germany) for 1 min, after which they were centrifuged at 15 000 × *g* for 15 min. The resulting supernatant was collected in a light-protected tube and stored at -80 °C for posterior analysis of free phenolic content. The remaining biomass was used for fibre-bound phenolic analysis, hydrolysing the fibre by mixing this pellet with 20 mL of 4 M NaOH in distilled water and placing the resulting mixture in an orbital shaker at 250 rpm for a period of 3 h. After this, acid hydrolysis was performed by adding 8 mL of HCl 32%, after which sample pH was calibrated using either NaOH or HCl 32% until the sample reached a pH of 1.5-2. The resulting mixture was washed using 25 mL of ethyl acetate and centrifuged for 5 min at 5000 × *g*. The obtained ethyl acetate fraction was drained into a new tube, and the process was then repeated, generating about 50 mL of supernatant. The ethyl acetate fraction was placed in a rotary vacuum evaporator at 50 °C (R-210 EBI 136, BÜCHI, Switzerland) until total dryness, after which it was dissolved in 5 mL of pure methanol. The resulting solution was collected in a light-protected tube and stored at - 80 °C.

Identification and quantification of phenolic compounds in both free and fibre-bound phenolic extracts were performed via high-performance liquid chromatography (HPLC, Waters Series 600, Mildford MA, USA). The stationary phase was a C18 Hypersil gold ODS2 analytical column (250 × 4.6 mm i.d.) with a particle size of 5 mm (Thermo Fisher, Waltham, MA, USA) maintained at 30 °C. The flow rate was 1 mL min^{-1}, and the absorbance was detected at 280 nm. Mobile phase A was composed of acetic acid/water (2:98, v/v), while mobile phase B was methanol. An elution gradient was implemented

as follows: 95% (A) and 5% (B) to 80% (A) and 20% (B) over 10 min; to 40% (A) and 60% (B) over 15 min (25 min, total time); to 30% (A) and 70% (B) over 5 min (30 min, total time), and re-equilibration over 10 min (40 min, total time) to the initial composition. The phenolic compounds were quantified by comparing the retention times with those of the corresponding standards using their regression equation and UV spectra obtained with an SPD-20A UV–vis Detector. Measurement of total polyphenols in both free and fibre-bound extracts was performed via spectrophotometry, according to the Folin-Ciocalteu method at 750 nm (Synergy H1 HU126, Vermont, USA), following Singleton and Joseph A. Rossi (1965), and expressed in mg of gallic acid equivalents (GAE) per 100 g of DM^{-1}.

2.5 Antioxidant capacity of samples

The antioxidant capacity of fresh peels, flours, mixtures, extruded pellets, and dried pellets (final diets) was measured through the assessment of the radical scavenging capacity of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$, as well as ORAC for superoxide anion ($O^{\bullet-}$) radical scavenging capacity. All analyses were done in triplicate using a Multidetector plate reader (Synergy H1 HU126, Vermont, USA), and final results were expressed in mg of Trolox equivalents (TE) per 100 g of DM.

The $ABTS^{\bullet+}$ radical scavenging activity was measured in the methanolic extracts, using the method described by Sánchez-Moreno (2002) and adapted by Gonçalves et al. (2009). Essentially, using a flat-bottom 96 well microplate, 180 µL of $ABTS^{\bullet+}$ working solution was added to 20 µL of sample, in triplicate. The mixture was incubated for 5 min at 30 °C, protected from light, and the absorbance measured at 734 nm. The $DPPH^{\bullet}$ assay was done according to the method of Brand-Williams et al. (1995), with some modifications. The assay was also performed in a flat-bottomed 96-well microplate; 175 µL of $DPPH^{\bullet}$ working solution was added to 25 µL of sample, in triplicate. In both analytical procedures, Trolox was used for the standard curve and methanol 80%, the solvent used for the analysed extracts, was used for the blanks. The mixture was incubated for 30 min at 25 °C, protected from light, and the absorbance was measured at 515 nm. ORAC was carried out following Dávalos et al. (2004). The reaction was achieved using a phosphate buffer (75 mM, pH 7.4) combined with 20 µL of either the free or the fibre-bound phenolic extracts for each sample, plus 120 µL of fluorescein (116.66 nM) in a black microplate (Nunc, Denmark). The solution was pre-incubated at 37 °C for 15 min, after which, 60 µL of 2,2′azobis-(2-methylpropionamidine)-dihydrochloride (AAPH) (46,6 mM) was added, followed by a second incubation period at 40 °C for 137 min. A blank was made by using a phosphate buffer instead of a sample.

2.5 Statistical analysis

All statistical analyses were performed with IBM SPSS STATISTICS, 27.0 package (IBM corporation, New York, NY, USA, 2021). Data were tested for normality and homogeneity of variances by Shapiro-Wilk and Levene's tests, respectively, and transformed whenever required before being submitted to either a two-way ANOVA with extrusion and drying temperatures as main factors (Figure 2.3), or a one-way ANOVA with diet as main effect (Table 2.5). When tests showed significance, individual means were compared using the HSD Tukey post-hoc Test. In all cases, the level of significance was set below 0.05. The principal component analysis (PCA) (Figure 2.5) was performed using the XLSTAT (Addinsoft, Inc.) software package to differentiate the samples from the different study groups.

3. Results

3.1. Phases 1 and 2 – Characterization of ingredients

The macronutrient composition of mango and pineapple peel and flour is depicted in Table 2.1. The water content was considerably lower in dried flour (90-92%) compared to fresh peels (13-19%), as expected. The protein content of pineapple peel is higher than that of mango peel, but their respective flours have similar protein content (6-7% DM). The crude lipid content of both pineapple and mango peel is very limited (<2% DM). Both mango and pineapple peel are very rich sources of carbohydrates (>88% DM). The content of complex polysaccharides, particularly measured as neutral detergent fibre, i.e., including lignin, hemicellulose, and cellulose, is substantially higher in pineapple peel than in mango peel. This also translates to pineapple peel flour, which is a much richer source of polysaccharides than mango peel flour. Starch content is low in mango peel and flour (1.8-2.5% DM), and residual in pineapple peel and flour (0.1-0.6% DM).

Table 2.1 – Proximate composition, bioactive compounds and antioxidant capacity of fruit peels from phase 1 and flours from phase 2

	Mango		Pineapple	
	Peel	**Flour**	**Peel**	**Flour**
Proximate composition [1]				
Dry matter	18.7 ± 0.2	90.2 ± 0.05	13.0 ± 0.4	92.4 ± 0.1
Ash	2.5 ± 0.2	2.6 ± 0.1	3.1 ± 0.2	3.0 ± 0.05
Protein	4.7 ± 0.1	6.2 ± 0.03	7.0 ± 0.1	6.8 ± 0.1
Lipids	1.0 ± 0.2	1.3 ± 0.1	1.8 ± 0.1	0.8 ± 0.1
Carbohydrates	91.9 ± 0.2	89.9 ± 0.1	88.1 ± 0.2	89.3 ± 0.2
Crude fibre	11.3 ± 0.1	13.4 ± 0.1	23.6 ± 0.3	22.5 ± 0.001
Neutral-detergent fibre	15.0 ± 0.02	18.3 ± 0.3	60.4 ± 0.1	56.3 ± 0.01
Starch	1.8 ± 0.03	2.5 ± 0.03	0.1 ± 0.004	0.6 ± 0.03
Energy	17.8 ± 0.1	18.4 ± 0.01	19.1 ± 0.3	18.6 ± 0.1
Vitamins [2]				
Retinol	18.8 ± 1.5	n.a	17.5 ± 0.1	5.0 ± 2.2
α-tocopherol	2732.4 ± 28.8	2580.4 ± 56.0	394.7 ± 25.4	185.0 ± 8.1
β-tocopherol	718.8 ± 59.0	209.8 ± 6.0	1715.5 ± 142.4	834.3 ± 87.8
Sum of identified vitamins	3500.8 ± 2.3	2790.1 ± 54.5	2127.6 ± 117.2	1021.5 ± 80.7
Carotenoids [2]				
Lutein	0.9 ± 0.01	0.7 ± 0.04	1.1 ± 0.1	0.5 ± 0.04
β-cryptoxanthin	0.02 ± 0.004	0.02 ± 0.001	0.02 ± 0.005	0.004 ± 0.001
α-carotene	0.3 ± 0.01	0.3 ± 0.05	0.1 ± 0.005	0.04 ± 0.001
β-carotene	5.2 ± 0.1	4.2 ± 0.1	3.4 ± 0.5	0.9 ± 0.02
Sum of identified carotenoids	6.7 ± 0.5	5.2 ± 0.2	4.6 ± 0.5	1.4 ± 0.2
Total	9.9 ± 1.0	2.5 ± 0.2	11.4 ± 1.0	3.3 ± 0.2
Free phenolic content [2]				
Gallic acid	58.3 ± 2.9	32.7 ± 6.2	2.4 ± 0.1	22.2 ± 5.2
Mangiferin	157.7 ± 35.8	53.8 ± 3.3	n.a	n.a
Rutin	16.5 ± 1.3	8.0 ± 0.3	BQL	n.a
Gentisic acid	n.a	n.a	n.a	51.1 ± 5.7
Isoferulic acid	n.a	n.a	BQL	8.6 ± 3.0
Ferulic acid	n.a	n.a	BQL	115.1 ± 0.4
Sum of identified polyphenols	202.3 ± 2.7	109.3 ± 5.8	7.4 ± 1.4	217.9 ± 12.0
Total (Folin-Cocialteau)	1155.9 ± 4.2	586.5 ± 36.1	483.5 ± 14.5	397.5 ± 24.7
Bound phenolic content [2]				
Gallic Acid	11.7 ± 0.1	285.9 ± 6.8	n.a.	n.a
Epicatechin gallate	BQL	102.6 ± 3.9	n.a	n.a.
Mangiferin	45.2 ± 7.1	14.3 ± 0.2	n.a	n.a.
Transferulic acid	n.a	n.a	444.5 ± 51.4	141.4 ± 11.8
4-coumaric acid	n.a	n.a	169.5 ± 17.1	63.8 ± 14.8
Sum of identified polyphenols	5.3 ± 1.0	404 .2 ± 3.1	614.0± 68.5	225.6 ± 50.8
Total (Folin-Cocialteau)	54.1 ± 3.3	337.5 ± 18.2	312.2 ± 21.6	547.0 ± 13.3
DPPH$^{\bullet}$ [3]				
Free phenolic extracts	2507.6 ± 34.4	729.2 ± 1.8	2600.9 ± 121.4	235.8 ± 16.2
Bound phenolic extracts	162.2 ± 14.1	91.7 ± 13.2	286.0 ± 7.1	395.6 ± 47.7
ABTS$^{\bullet+}$ [3]				
Free phenolic extracts	4003.0 ± 275.1	1235.3 ± 149.4	588.0 ± 26.1	963.0 ± 92.4
Bound phenolic extracts	140.1 ± 14.3	540.7 ± 0.2	1289.2 ± 9.2	511.8 ± 2.7
ORAC [3]				
Free phenolic extracts	7841.1 ± 53.2	2254.5 ± 114.5	5623.2 ± 47.5	2729.5 ± 59.2
Bound phenolic extracts	5174.3 ± 128.6	1495.4 ± 88.0	4194.9 ± 49.9	3655.4 ± 558.0

Values from analytical samples are presented as mean (n=2) for proximate composition, and mean ± standard deviation (n=3) for remaining variables. “n.a” stands for “non-applicable”, meaning that the chemical compound in question is inexistent in that sample. “BQL” stands for “below quantification limit”, meaning that the chemical compound in question was identified, but was not reported due to being present below the minimal quantities for quantification. [1] Proximate composition is expressed in % of dry matter (DM), except for energy, which is expressed in kJ g^{-1} DM. Carbohydrates are calculated by difference as 100- (ash+crude protein+crude lipids). DM. [2] Vitamins are expressed in µg of vitamin per 100 g of DM. Total carotenoids were measured spectrophotometrically and are expressed in mg of β-carotene equivalents per 100g of DM. Specific carotenoids were measured via HPLC are expressed in mg of carotenoid per 100g of DM. Total phenolic content was measured via the Folin-Ciocalteau method and is expressed in mg of gallic acid equivalents (GAE) per 100g of DM. Specific phenolic compounds measured via HPLC are expressed in mg of polyphenol per 100g of DM. [3] $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and ORAC, are expressed in mg of Trolox equivalents (TE) per 100g of DM.

Peels from mango and pineapple exhibited different contents of vitamins A (retinol), vitamin E (α and β-tocopherol), carotenoids, and both free and bound and phenolic compounds. When converting peels into flour, heavy losses in vitamins, carotenoids, as well as free phenolic compounds, and antioxidant capacity were perceived in both fruits (Table 2.1). While levels of retinol were similar in mango and pineapple peel (18.8 and 17.5 µg 100g DM^{-1}), this compound was completely absent in mango flour, whilst in pineapple flour, 29% of values from the peel were still retained (5 µg 100 g^{-1} DM). In terms of Vitamin E, mango peel showed higher α-tocopherol quantities (2732.4 vs 394.7 µg 100 g^{-1} DM), but lower β-tocopherol levels than pineapple (718.8 vs 1715.5 g^{-1} DM). Overall, fresh peels consistently showed higher levels of vitamins than the respective corresponding flours. Likewise, total carotenoids were found in higher amounts in mango (9.9 mg 100 g^{-1} DM) and pineapple peels (11.4 mg 100 g^{-1} DM) compared to their respective flours (2.5 and 3.3 mg 100 g^{-1} DM, respectively), possibly due to higher amounts of lutein and β-carotene. Overall, mango flour displayed a better ability to retain carotenoids compared to pineapple flour.

Concerning phenolic compounds, free forms of gallic acid, mangiferin, and rutin were identified in mango peel. However, the content of these phenolic compounds and the total free phenolic content decreased by approximately between -49.3 and -65% during the conversion process from peel to flour. In contrast, gallic acid emerged as the predominant free phenolic compound in pineapple, with its content in the flour (22.2 mg 100 g^{-1} DM) nearly ten times higher than in the peels (2.4 mg 100 g^{-1} DM). Additionally, pineapple flour contained quantifiable amounts of free gentisic acid, isoferulic acid, and ferulic acid. Overall, the values of total free polyphenols in pineapple peels were 18% higher than in flour (Figure 2.2). In terms of bound phenolic compounds, bound forms of gallic acid, epicatechin gallate, and mangiferin were found in mango peels and flour, while pineapple displayed a presence of transferulic and 4-coumaric acids. The dynamics of fibre-bound phenolic compounds measured via the Folin-Ciocalteu method showed an opposite trend compared to that observed in free phenolic compounds after the conversion from peel to flour. Essentially, in both fruits, the extraction yields of bound phenolic compounds were much higher in mango and pineapple flours (337.5 and 547.0 mg GAE 100g DM^{-1}, respectively) than in their respective peels (54.1 and 312.2 mg GAE 100g DM^{-1}, respectively). According to Figure 2.2, mango flour exhibited the highest increase, reaching 524% compared to the peel, whereas pineapple peel displayed a comparatively lower increase of 75% (Figure 2.2).

		Δ% Folin-Cocialteau		Δ% DPPH•		Δ% ABTS•+		Δ% ORAC	
		Free	Bound	Free	Bound	Free	Bound	Free	Bound
Peel »	Mango flour	-49.3	523.6	-70.9	-43.5	-69.1	286.0	-71.2	-71.1
	Pineapple flour	-17.8	75.2	-90.9	38.3	79.0	-60.3	-51.5	-12.9
CNE »	CH	-28.6	-7.3	28.9	9.7	-19.9	30.9	-7.7	139.7
	CH-60	-58.6	23.9	-31.4	-6.3	-54.6	22.1	-55.8	25.1
MNE »	MH	-22.0	-13.0	-59.5	7.9	-19.1	-16.4	-37.0	10.7
	MH-60	-29.2	72.3	-68.4	354.8	-29.4	121.9	-57.8	94.4
	MH-35	-43.7	-15.3	-53.3	3.3	-56.9	-24.1	-38.6	3.0
	MC	-19.7	-8.8	-47.3	-8.9	-54.1	-36.7	-48.3	91.7
	MC-60	-30.4	-2.7	-14.9	-4.1	6.1	-0.6	-39.9	72.6
	MC-35	-46.6	-0.3	-5.8	-8.1	-20.4	-13.0	-45.9	3.7
PNE »	PH	-28.2	-14.1	-30.6	-6.0	1.1	-59.7	63.8	22.7
	PH-60	-48.7	93.4	-11.0	356.9	3.0	42.9	-11.4	137.9
	PH-35	-43.5	152.2	29.5	379.8	-39.0	40.7	-42.7	70.4
	PC	26.2	15.0	-60.3	-15.9	8.3	-34.9	-20.7	-28.9
	PC-60	-4.1	-6.8	-85.9	-16.6	5.1	-21.9	-30.6	-14.0
	PC-35	-39.8	-3.4	-37.6	-16.1	-4.0	-25.6	-58.9	3.0

Figure 2.2 – Heatmap concerning alterations in phenolic content and antioxidant capacity of samples. All values were calculated in percentage (Δ%) concerning sample averages. “Free” refers to data from free phenolic extracts while “Bound” refers to data from bound phenolic extracts. Flours were compared to their respective peels. In both extrusions and all final diets, each percentage was calculated concerning its respective mix: CH, CH-60 and CH-35 were calculated in comparison with values from CNE; MH, MH-60, MH-35, MC, MC-60 and MC-35 were calculated in comparison with values from MNE; PH, PH-60, PH-35, PC, PC-60 and PC-35 were calculated in comparison with values from PNE

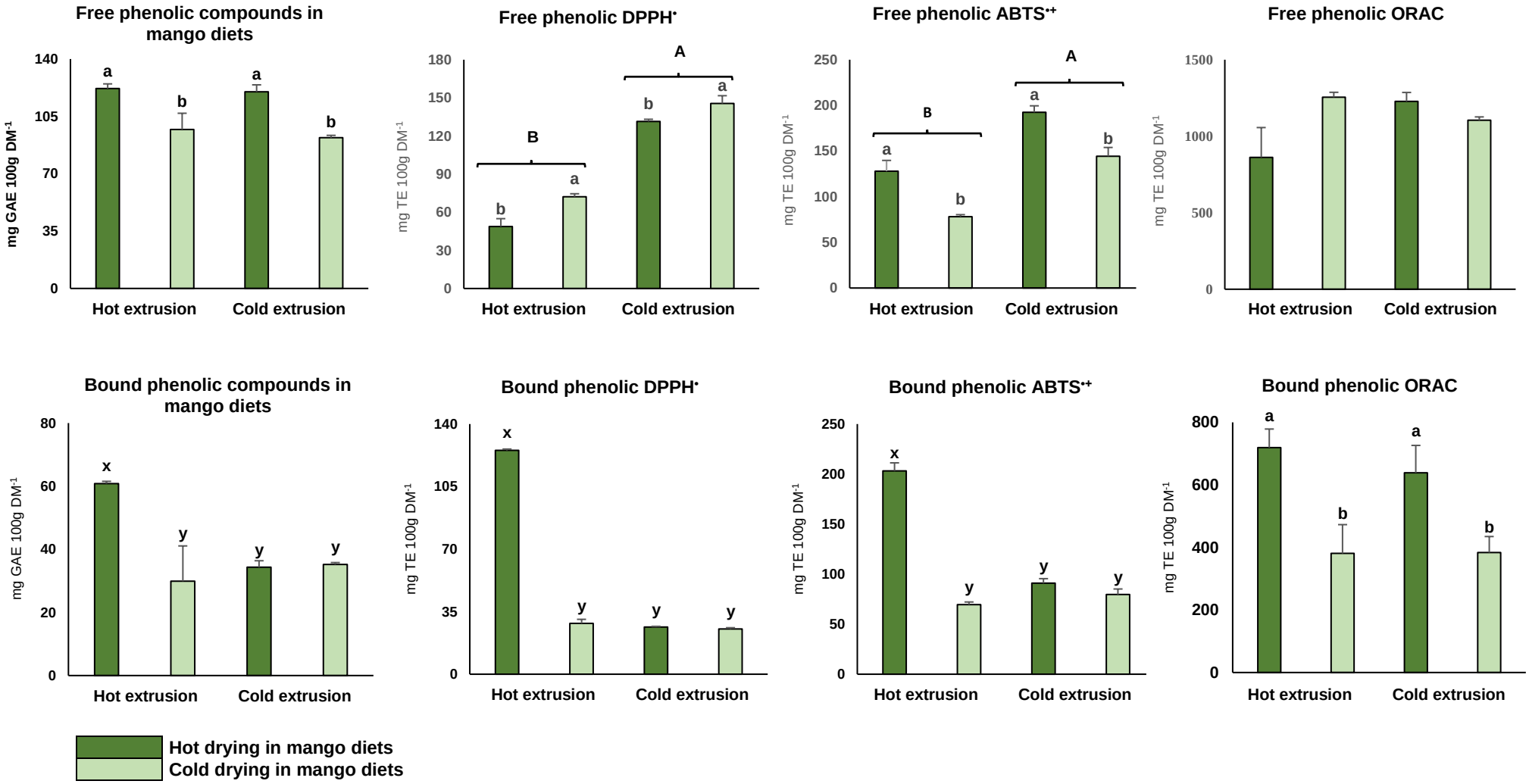

Figure 2.3 – Histograms concern phenolic compound quantification and antioxidant capacity of dried pellets from diets with 2% mango inclusion. The mixture with 2% of mango peel flour inclusion was subjected to different extrusion and drying temperatures, thus originating four experimental extruded diets: i) MH-60 (110 °C extrusion; 60 °C drying - conventional manufacturing temperatures) ii) MH-35 (110 °C; 35 °C) iii) MC-60 (25 °C; 60 °C) and iv) MC-35 (25 °C; 35 °C). Values are presented as mean ± standard deviation (n=3). A 2-way ANOVA was performed. Different superscript uppercase letters (^{A,B}) indicate significant differences between different extrusions while different superscript lowercase letters indicate significant differences between different drying temperatures(^{a,b}) or in the intersection between both variables "Extrusion" and "Drying" (^{x,y}) ($P < 0.05$).

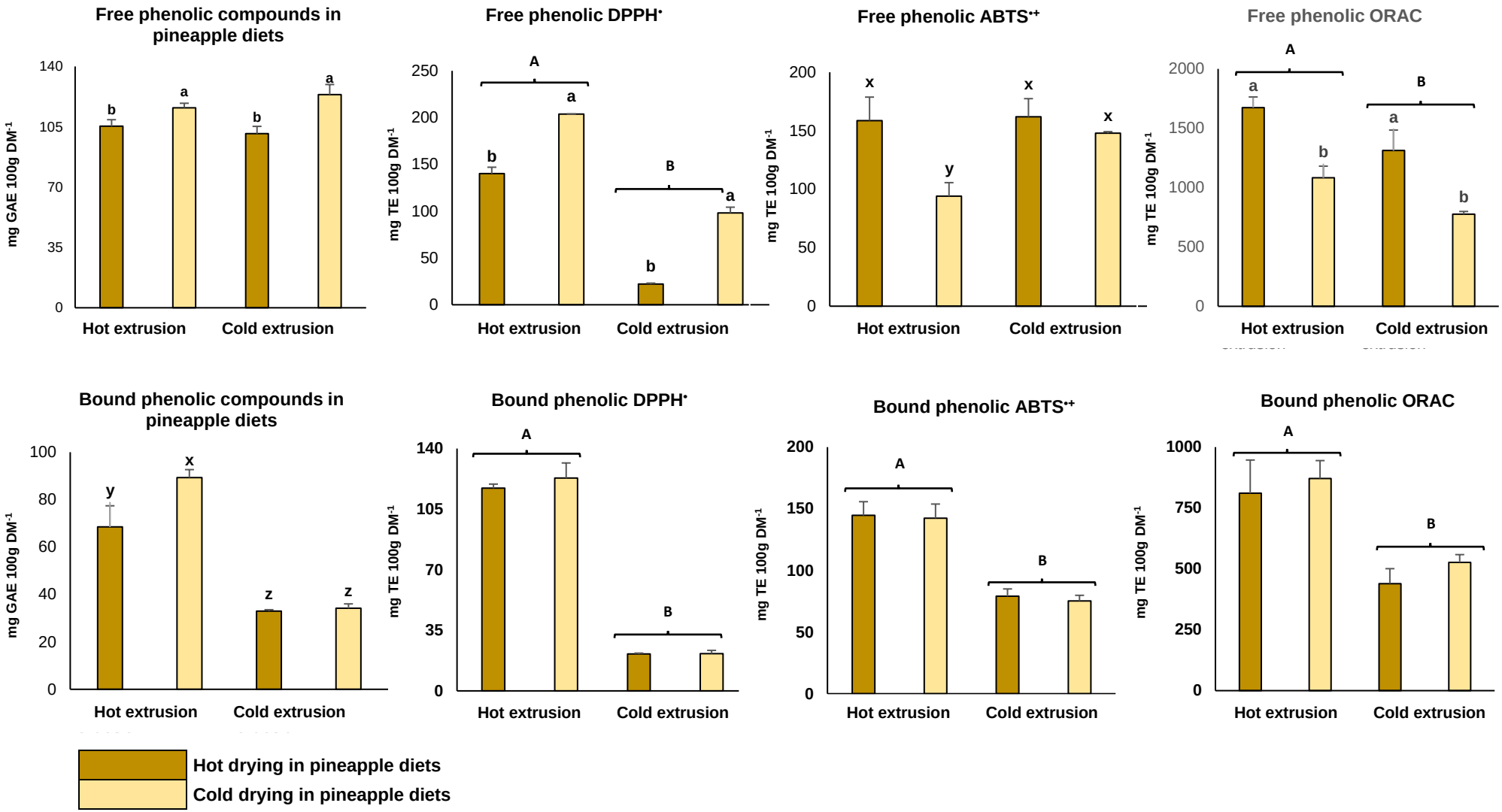

Figure 2.4 – Histograms concern phenolic compound quantification and antioxidant capacity of dried pellets from diets with 2% pineapple peel flour inclusion. The mixture with 2% of pineapple peel flour inclusion was subjected to different extrusion and drying temperatures, thus originating four experimental extruded diets: i) PH-60 (110 °C extrusion; 60 °C drying - conventional manufacturing temperatures) ii) PH-35 (110 °C; 35 °C) iii) PC-60 (25 °C; 60 °C) and iv) PC-35 (25 °C; 35 °C). Values are presented as mean ± standard deviation (n=3). A 2-way ANOVA was performed. Different superscript uppercase letters (^{A,B}) indicate significant differences between different extrusions while different superscript lowercase letters indicate significant differences between different drying temperatures(^{a,b}) or in the intersection between both variables "Extrusion" and "Drying" (^{x,y}) ($P < 0.05$).

In terms of the radical scavenging properties (Table 2.1; Figure 2.2), the free polyphenol extracts derived from mango peels showed higher values in the DPPH$^{\bullet}$ (2507.6 mg TE 100g DM^{-1}), ABTS$^{\bullet+}$ (4003.0 mg TE 100g DM^{-1}) and ORAC (7841.1 mg TE 100g DM^{-1}) assays in comparison to mango flour (729.2, 1235.3 and 2254.5 mg TE 100g DM^{-1}, respectively). The same decreasing trend was observed for the bound phenolic extracts, except for ABTS$^{\bullet+}$, which displayed higher values in mango flour (540.7 mg TE 100g DM^{-1}) when compared to peel (140.1 mg TE 100g DM^{-1}). In pineapple peel, the antioxidant capacity of both free and fibre-bound polyphenol extracts was generally higher than in the corresponding flour, except for bound DPPH$^{\bullet}$ and free ABTS$^{\bullet+}$, which exhibited higher antioxidant capacity in the flour (395.6 and 963.0 mg TE 100g DM^{-1}, respectively) when compared to the peel (286.0 and 588.0 mg TE 100g DM^{-1}, respectively).

3.2 Phase 3 - Characterization of the experimental mixtures prior to extrusion

The proximate composition analysis of the experimental mixtures before extrusion (NE mixtures) revealed that they were isolipidic, isoproteic, and isoenergetic, but they differed in terms of phenolic compounds and antioxidant capacity (Table 2.2). The quantification of free phenolic compounds using the Folin-Ciocalteu method showed that PNE had the highest levels of these compounds amongst the three mixtures (206.7 vs 163.0-172.3 mg GAE 100g DM^{-1}). However, MNE had the highest level of free gentisic, sinapic, and isoferulic acids (58.3, 4.5, and 2.8 mg 100 g^{-1} DM, respectively). Moreover, MNE was the only mixture that showed the presence of mangiferin, distinguishing it from the other two mixtures. In terms of bound polyphenolic extracts, gallic acid was only detected in MNE in quantities consistent with the expected 2% derived from mango peel flour (4.5 mg 100 g^{-1} DM). Additionally, the higher amounts of 4-coumaric (5.7 mg 100 g^{-1} DM) and transferulic (13.8 mg 100 g^{-1} DM) acids found in PNE corresponded to the addition of 2% pineapple peel flour. Overall, higher levels of free and bound polyphenols were identified and quantified by Folin-Ciocalteu method in both MNE (65.8 and 172.3 mg GAE 100g DM^{-1}, respectively) and PNE (50.2 and 206.7 mg GAE 100g DM^{-1}, respectively) when compared to CNE (57.6 and 163.0 mg GAE 100g DM^{-1}, respectively), although the increase was more evident on bound polyphenols.

Table 2.2 – Ingredients, proximate composition, phenolic content and antioxidant capacity of phase 3 (pre-extrusion)

	CNE	MNE	PNE
Ingredients			
Fishmeal Super Prime	10.0	10.0	10.0
Porcine blood meal	2.5	2.5	2.5
Poultry meal	5.0	5.0	5.0
Soy protein concentrate	16.0	16.0	16.0
Wheat gluten	9.5	9.5	9.5
Corn gluten	7.0	7.0	7.0
Soybean meal 48	10.0	10.0	10.0
Rapeseed meal	5.0	5.0	5.0
Wheat meal	16.5	14.5	14.5
Fish oil	5.1	5.1	5.1
Rapeseed oil	9.4	9.4	9.4
Soy lecithin	0.2	0.2	0.2
Vitamin Premix PV02[1]	0.2	0.2	0.2
Brewer's yeast	2.5	2.5	2.5
MAP	0.9	0.9	0.9
DL-Methionine	0.2	0.2	0.2
Mango flour	-	2.0	-
Pineapple flour	-	-	2.0
Proximate composition [2]			
Dry Matter	93.0 ± 0.1	93.1 ± 0.1	92.2 ± 0.1
Ash	6.5 ± 0.3	6.5 ± 0.3	6.7 ± 0.002
Protein	57.7 ± 0.3	57.1 ± 0.4	57.4 ± 0.2
Lipids	3.0 ± 0.03	3.2 ± 0.1	2.8 ± 0.05
Carbohydrates	32.8 ± 0.2	33.3 ± 0.1	33.1 ± 0.2
Crude fibre	3.4 ± 0.1	3.7 ± 0.0003	3.8 ± 0.05
Starch	16.1 ± 0.6	14.7 ± 0.5	15.0 ± 0.6
Energy	20.5 ± 0.2	20.6 ± 0.02	20.9 ± 0.04
Free phenolic content [3]			
Gentisic Acid	55.2 ± 0.4	58.3 ± 0.3	49.8 ± 0.9
Mangiferin	n.a	0.2 ± 0.02	n.a
Sinapic acid	3.6 ± 0.1	4.5 ± 0.1	3.9 ± 0.2
Isoferulic acid	1.7 ± 0.04	2.8 ± 0.1	0.5 ± 0.03
Sum of identified polyphenols	57.6 ± 0.5	65.8 ± 0.2	50.2 ± 1.0
Total (Folin-Ciocalteau)	163.0 ± 4.2	172.3 ± 6.4	206.7 ± 13.3
Bound phenolic content [3]			
Gallic Acid	n.a	4.5 ± 0.7	n.a
Caffeic acid	BQL	BQL	0.2 ± 0.01
Syringic acid	2.3 ± 0.1	1.1 ± 1.5	2.8 ± 0.03
Neochlorogenic acid	2.0 ± 0.1	1.5 ± 0.1	3.1 ± 0.2
4-coumaric acid	BQL	BQL	5.7 ± 0.02
Transferulic acid	5.5 ± 1.2	5.3 ± 0.4	13.8 ± 0.5
Sinapic acid	21.7 ± 1.9	18.3 ± 0.1	25.9 ± 0.4
Sum of identified polyphenols	31.5 ± 0.7	30.4 ± 0.2	50.5 ± 2.7
Total (Folin-Ciocalteau)	27.5 ± 0.1	35.3 ± 3.5	35.4 ± 0.3
DPPH$^{\bullet}$ [4]			
Free phenolic extracts	107.7 ± 16.2	154.0 ± 4.5	154.1 ± 7.7
Bound phenolic extracts	24.7 ± 0.002	27.5 ± 0.5	25.6 ± 2.0
ABTS$^{\bullet+}$ [4]			
Free phenolic extracts	183.4 ± 2.8	181.4 ± 5.4	154.1 ± 7.7
Bound phenolic extracts	66.3 ± 10.8	91.6 ± 4.5	101.8 ± 9.2
ORAC [4]			
Free phenolic extracts	1572.1 ± 40.5	2043.8 ± 47.8	1889.4 ± 26.8
Bound phenolic extracts	338.1 ± 98.6	370.0 ± 74.2	511.2 ± 29.7

Values from analytical samples are presented as mean ± standard deviation, with (n=2) for proximate composition, and (n=3) for remaining variables. “n.a” stands for “non-applicable”, i.e. the compound in question is inexistent in that sample. “BQL” stands for “below quantification limit”, meaning that the chemical compound in question was found but not quantifiable to tue its low amount. To the control mix with no extrusion (CNE) 2% of either mango peel flour (MNE) or pineapple peel flour (PNE) were added. [1]Vitamin premix (mg or IU/kg mix): vitamin D3 (DL-cholecalciferol), 2000 IU; vitamin K3 (menadione sodium bisulfite), 25 mg; vitamin B1 (thiamine hydrochloride), 30 mg; vitamin B2 (riboflavin), 30 mg; vitamin B6 (pyridoxine hydrochloride), 20 mg; vitamin B12 (cyanocobalamin), 0.1 mg; vitamin B5 (pantothenic acid), 100 mg; vitamin B3 (nicotinic acid), 200 mg; vitamin B9 (folic acid), 15 mg; vitamin H (biotin), 3 mg; betaine, 500 mg; inositol, 500 mg; choline chloride, 1000 mg; vitamin C (stay C),1000 mg.[2]Proximate composition is expressed in % of dry matter (DM), except for energy, which is expressed in kJ g^{-1} DM. [3]Total polyphenols measured via the Folin-Ciocalteau method are expressed in mg of gallic acid equivalents (GAE) per 100 g of DM. Specific phenolic compounds measured via HPLC are expressed in mg of polyphenol per 100g of DM.[4] DPPH$^{\bullet}$ ABTS$^{\bullet+}$ and ORAC, are expressed in mg of Trolox equivalents (TE) per 100 g DM.

The analysis of antioxidant capacity revealed that free phenolic extracts of both MNE (154.0 mg TE 100 g-1 DM) and PNE (154.1 mg TE 100 g-1 DM), had higher DPPH$^{\bullet}$

compared to those from CNE (107.7 mg TE 100 g-1 DM). In terms of free $ABTS^{\bullet+}$, PNE showed the lowest radical scavenging activity of all mixtures (154.1 vs 181.4-183.4 mg TE 100 g-1 DM), contrarily to bound extracts that had higher bound $ABTS^{\bullet+}$ in MNE and PNE (91.6 and 101.8 mg TE 100 g-1 DM, respectively) compared to CNE (66.3 mg TE 100 g-1 DM). Concerning ORAC, values were lowest in CNE for both free and bound phenolic extracts (1572.1 and 338.1 mg TE 100 g-1 DM, respectively). MNE displayed the highest superoxide anion (O•-) radical scavenging capacity in free phenolic extracts (2043.8 mg TE 100 g-1 DM), whereas PNE exhibited the highest capacity in bound phenolic extracts (511.2 mg TE 100 g-1 DM).

3.3 Phase 4 - Characterization of the extruded pellets

The impact of extrusion on the antioxidant capacity of the resulting pellets, before drying, was evaluated (Table 2.3). Concerning free phenolic content, the sinapic acid was exclusively detected in the cold extruded pellets MC and PC. Moreover, only the MH and MC pellets, showed the presence of mangiferin.

Table 2.3 – Phenolic profile and antioxidant capacity of the extruded pellets from phase 4, prior to drying

	CH	MH	MC	PH	PC
Free phenolic content [1]					
Gentisic Acid	28.9 ± 11.9	14.0 ± 0.2	14.7 ± 0.8	14.3 ± 0.4	14.8 ± 0.2
Mangiferin	n.a	BQL	BQL	n.a.	n.a.
Sinapic acid	n.a	n.a	7.1 ± 1.9	n.a	6.2 ± 0.6
Isoferulic acid	BQL	BQL	BQL	BQL	BQL
Sum of identified polyphenols	28.9 ± 11.9	14.0 ± 0.2	21.5 ± 0.7	14.3 ± 0.4	22.1 ± 0.2
Total (Folin-Ciocalteau)	123.5 ± 0.7	134.5 ± 2.1	138.3 ± 8.5	147.3 ± 6.8	186.0 ± 5.7
Bound phenolic content [1]					
Gallic Acid	n.a	3.4 ± 0.1	2.1 ± 0.4	n.a	n.a.
Caffeic acid	BQL	BQL	BQL	BQL	BQL
Syringic acid	2.1 ± 0.4	1.1 ± 0.1	0.7 ± 0.01	1.4 ± 0.1	0.9 ± 0.1
Neochlorogenic acid	1.1 ± 1.2	BQL	BQL	BQL	BQL
4-coumaric acid	BQL	0.1 ± 0.1	BQL	4.8 ± 0.1	3.4 ± 0.2
Transferulic acid	6.2 ± 0.1	3.8 ± 0.3	3.6 ± 0.3	8.7 ± 0.7	7.7 ± 0.9
Sinapic acid	21.2 ± 1.0	27.9 ± 1.7	7.6 ± 0.6	28.8 ± 0.6	9.2 ± 0.04
Sum of identified polyphenols	29.5 ± 0.3	33.0 ± 1.1	14.3 ± 1.5	43.6 ± 1.6	21.1 ± 1.2
Total (Folin-Ciocalteau)	25.5 ± 6.2	30.7± 4.7	32.2 ± 0.2	30.4 ± 1.1	40.5 ± 6.5
$DPPH^{\bullet}$ [2]					
Free phenolic extracts	138.9 ± 9.6	62.6 ± 12.3	81.5 ± 23.1	78.1 ± 18.9	62.4 ± 10.7
Bound phenolic extracts	27.1 ± 3.6	29.7 ± 0.1	25.1 ± 3.9	24.1 ± 0.004	21.5 ± 3.8
$ABTS^{\bullet+}$ [2]					
Free phenolic extracts	146.9 ± 24.9	146.8 ± 14.9	83.3 ± 9.3	155.8 ± 14.9	166.9 ± 15.1
Bound phenolic extracts	78.4 ± 10.9	76.6 ± 18.1	58.0 ± 4.9	40.7 ± 0.8	65.9 ± 9.7
ORAC [2]					
Free phenolic extracts	1450.8 ± 585.4	1286.9 ± 8.9	1056.4 ± 41.3	3093.9 ± 313.4	1498.5 ± 77.7
Bound phenolic extracts	810.3 ± 138.9	409.4 ± 75.1	709.2 ± 91.8	627.2 ± 41.1	363.6 ± 75.8

Values are presented as mean ± standard deviation (n=3). "n.a" stands for "non-applicable", meaning that the chemical compound in question is inexistent in that sample. "BQL" stands for "below quantification limit", meaning that the chemical compound in question was identified, but was not reported due to being present below the minimal quantities for quantification. [1]Total phenolic content was measured via the Folin-Ciocalteau method and are expressed in mg of gallic acid equivalents (GAE) per 100 g of DM. Specific phenolic compounds measured via HPLC are expressed in mg of polyphenol per 100g of DM. [2]$DPPH^{\bullet}$, $ABTS^{\bullet+}$ and ORAC, are expressed in mg of Trolox equivalents (TE) per 100 g of DM.

This phenolic compound was exclusively detected in the MNE mixture, but its amount decreased greatly after extrusion. Among all extruded pellets, the PC displayed the highest level of total free phenolics, having experienced a reduction of -9% compared to PNE, while the remaining extruded pellets displayed a decrease between -20% and -29% in free phenolic compounds compared to their corresponding mixtures (Figure 2.2). In the bound phenolic extracts, 4-coumaric acid had the highest amounts in both PH and PC pellets (4.8 and 3.4 mg 100 g-1 DM, respectively) (Table 2.3). This acid was already present in much higher amounts in PNE (5.7 mg 100 g-1 DM) compared to MNE and CNE mixtures where it was below the quantification limit (Table 2.2), but after extrusion, a lower level was detected in both PH and PC, yet mainly in the PC pellets. Moreover, despite a general decrease in bound transferulic acid content observed in all extruded pellets, compared to their respective dietary mixtures, higher levels of this polyphenol were still found in both PH and PC (8.7 and 7.7 mg 100 g-1 DM, respectively) (Table 2.3). This is consistent with the fact that PNE was the dietary mixture with higher amounts of bound transferulic acid (13.8 mg 100 g-1 DM) (Table 2.2). Finally, bound sinapic acid was found in higher quantities in hot extruded pellets compared to those extruded at a lower temperature, regardless of the diet. Concerning bound phenolics, quantified by the Folin-Ciocalteu method, PC displayed higher amounts than all other extruded pellets (40.5 mg GAE 100 g-1 DM), even showing a 15% increase in these compounds compared to its initial dietary mixture (Figure 2.2). Comparatively, mango extruded pellets, MH and MC, lost -13% and -9% of bound phenolics, respectively, when compared to the MNE mix.

In terms of the antioxidant capacity of free phenolic extracts, DPPH• values were lower in all extruded pellets (62.4-81.5 mg TE 100 g-1 DM) when compared to the CH (138.9 mg TE 100 g-1 DM) (Table 2.3). Mango and pineapple extruded pellets exhibited a -60% and -31% reduction in their DPPH• values compared to their corresponding mixtures, MNE and PNE, whilst DPPH• values increased by 29% in CH pellets compared to the CNE mixture (Figure 2.2). In bond phenolic extracts, DPPH• is quite similar among extruded pellets (21.5-29.7 mg TE 100 g-1 DM) (Table 2.3). $ABTS^{\bullet+}$ radical scavenging capacity of free extracts lowered by -20% in the CH pellets compared to CNE. The very same trend was observed in both mango (MH and MC) extruded pellets, with an $ABTS^{\bullet+}$ reduction of -19 and -54% respectively, in relation to MNE. Contrarily, pineapple (PH and PC) free extracts kept their $ABTS^{\bullet+}$ values after extrusion (Figure 2.2). In the bound extracts, the CH increased $ABTS^{\bullet+}$ scavenging capacity by 31% in relation to the respective mixture, while all remaining extruded pellets showed a decrease, particularly pronounced in MC, PH and PC pellets (reduction from -16% to -60%). Finally, the free extracts of the PH pellets showed much higher ORAC values (3093.9 mg TE 100 g^{-1} DM)

than the remaining extruded pellets (1056.4-1498.5 mg TE 100 g^{-1} DM), being the only pellets that increased ORAC values after extrusion (Table 2.2). Moreover, hot extrusion resulted in higher ORAC values for each fruit (Figure 2.2). ORAC in bound phenolic extracts generally increased after extrusion, with the exception of PC.

3.4 Phase 5 – Characterization of the dried extruded pellets

The final chemical composition of dried extruded pellets was evaluated for each diet (Table 2.4). The macronutrient composition of all diets was not altered by the extrusion and drying processes.

In free phenolic extracts, the highest amounts of gentisic acid were found in diets MC-60, MC-35, PH-60, and PC-35 (40.2, 36.5, 40.3, and 38.0 mg 100 g-1 DM, respectively) (Table 2.4). Moreover, sinapic acid was only found in the free phenolic extracts of diets with fruit flour inclusion. Additionally, mangiferin was only identified in mango diets with cold drying, namely MH-35 e MC-35, albeit in trace amounts below the quantification limit, while disappearing completely in mango diets after hot extrusion. The drying stage had a significant impact on total free phenolic compounds: hot drying led to higher levels in mango diets MH-60 and MC-60 compared to cold drying MH-35 and MC-35, but the same trend was not observed in both the controls and the pineapple diets (Figure 2.4). Moreover, pineapple diets with cold drying PH-35 and PC-35 showed the highest total free phenolic compounds (Figure 2.3). Overall, the drying process resulted in a -59% and -41% free phenolic compound decrease, in CH-60 and CH-35, respectively, when compared to the CNE mixture. Mango diets showed a decrease between -29% and -30%, and between -44% and -47% in free phenolic compounds, after hot and cold drying, respectively, when compared to the MNE mixture (Figure 2.2). In the pineapple diets, drying led to a -4% decrease in free phenolic compounds for the PC-60 diet, while all other diets exhibited reductions of between -40% and -49% compared to the PNE mixtures (Figure 2.2).

Table 2.4 – Proximate composition, phenolic profile and antioxidant capacity of all experimental diets from phase 5 (pellets after extrusion and drying processes)

	Control diets		Mango diets				Pineapple diets			
	CH-60	**CH-35**	**MH-60**	**MH-35**	**MC-60**	**MC-35**	**PH-60**	**PH-35**	**PC-60**	**PC-35**
Proximate composition [1]										
Dry matter	95.4 ± 0.1	93.8 ± 0.1	94.4 ± 0.1	94.3 ± 0.1	98.8 ± 0.1	92.3 ± 0.1	94.5 ± 0.1	93.1 ± 0.2	97.8 ± 0.04	92.2 ± 0.03
Ash	5.3 ± 0.03	5.5 ± 0.1	5.3 ± 0.4	5.3 ± 0.3	5.6 ± 0.03	5.5 ± 0.1	5.5 ± 0.04	5.6 ± 0.2	5.6 ± 0.1	5.7 ± 0.02
Protein	48.9 ± 0.8	48.5 ± 0.6	45.4 ± 0.2	47.5 ± 0.7	48.1 ± 0.04	48.5 ± 0.3	48.0 ± 0.7	49.8 ± 0.01	48.6 ± 0.2	49.0 ± 0.03
Energy	22.4 ± 0.05	22.3 ± 0.02	23.2 ± 0.2	23.7 ± 0.1	23.8 ± 0.7	24.0 ± 0.2	23.4 ± 0.05	23.8 ± 0.4	23.5 ± 0.1	23.6 ± 0.1
Lipids	16.0 ± 0.6	16.3 ± 0.1	18.3 ± 0.04	18.6 ± 0.7	18.2 ± 0.1	18.3 ± 0.2	16.8 ± 0.4	15.9 ± 0.004	18.0 ± 0.6	17.6 ± 0.2
Carbohydrates	29.8 ± 1.3	29.7 ± 0.4	31.0 ± 0.7	28.7 ± 0.3	28.1 ± 0.04	27.7 ± 0.3	29.7 ± 1.0	28.7 ± 0.15	27.8 ± 0.9	27.8 ± 0.1
Crude fibre	3.6 ± 0.2	3.2 ± 0.2	3.7 ± 0.2	3.7 ± 0.2	4.5 ± 0.02	4.0 ± 0.2	3.9 ± 0.1	3.1 ± 0.1	4.7 ± 0.1	3.8 ± 0.001
Starch	14.7 ± 0.5	14.8 ± 0.6	12.7 ± 0.1	12.7 ± 0.4	12.6 ± 0.4	13.1 ± 0.4	12.9 ± 0.4	13.6 ± 0.4	13.1 ± 0.3	13.5 ± 0.4
Free phenolic extracts [2]										
Gentisic Acid	23.7 ± 2.7	18.4 ± 0.7	26.5 ± 5.7	20.7 ± 1.3	40.2 ± 1.2	36.5 ± 1.9	40.3 ± 1.6	29.1 ± 0.1	21.0 ± 0.2	38.0 ± 1.0
Mangiferin	n.a	n.a	n.a	BQL	n.a	BQL	n.a	n.a	n.a	n.a
Sinapic acid	n.a	n.a	4.1 ± 0.1	2.0 ± 0.7	7.3 ± 0.6	2.2 ± 0.1	4.5 ± 0.1	1.8 ± 0.01	1.1 ± 0.05	5.7 ± 1.5
Isoferulic acid	BQL	BQL	BQL	BQL	0.4 ± 0.1	BQL	0.5 ± 0.1	BQL	BQL	0.2 ± 0.02
Sum of identified polyphenols	23.7 ± 2.7	18.4 ± 0.7	30.0 ± 6.7	22.7 ± 0.5	47.5 ± 0.5	39.2 ± 1.7	45.8 ± 0.8	30.8 ± 0.05	22.1 ± 0.2	43.7 ± 1.6
Bound phenolic extracts [2]										
Caffeic acid	BQL	BQL	n.a.	n.a.	BQL	BQL	BQL	0.1 ± 0.0002	BQL	BQL
Gallic acid	n.a	n.a	3.9 ± 0.5	2.1 ± 0.9	4.2 ± 0.5	3.2 ± 0.2	n.a.	n.a.	n.a.	n.a.
Syringic acid	2.1 ± 0.1	2.0 ± 0.1	3.7 ± 0.3	1.3 ± 0.7	1.7 ± 0.04	1.5 ± 0.1	3.5 ± 0.6	4.1 ± 0.5	2.0 ± 0.1	1.5 ± 0.1
Neochlorogenic acid	1.8 ± 0.1	2.3 ± 0.1	4.1 ± 0.8	n.a	1.0 ± 0.1	0.9 ± 0.2	2.5 ± 0.4	2.6 ± 0.5	1.1 ± 0.4	0.6 ± 0.1
4-coumaric acid	BQL	0.1 ± 0.01	2.8 ± 0.2	BQL	BQL	BQL	11.8 ± 1.2	12.9 ± 0.3	4.1 ± 0.005	3.4 ± 0.4
Transferulic acid	4.7 ± 0.5	5.1 ± 0.4	13.5 ± 0.6	3.6 ± 1.2	4.4 ± 0.1	4.3 ± 0.3	25.0 ± 3.1	24.1 ± 0.4	10.5 ± 0.01	9.0 ± 1.1
Sinapic acid	28.5 ± 0.4	31.0 ± 1.2	57.0 ± 9.6	19.7 ± 9.8	20.0 ± 0.2	19.6 ± 1.1	55.5 ± 2.7	65.6 ± 6.4	19.6 ± 1.3	19.2 ± 1.0
Sum of identified polyphenols	37.1 ± 1.2	40.3 ± 1.3	97.0 ± 3.3	26.8 ± 12.6	31.5 ± 0.5	29.6 ± 1.9	98.4 ± 5.0	109.3 ± 8.0	37.4 ± 1.8	32.4 ± 2.7

Values are presented as mean ± standard deviation (n=2) for proximate composition, and mean ± standard deviation (n=3) for remaining variables. "n.a" stands for "non-applicable", meaning that the chemical compound in question is inexistent in that sample. "BQL" stands for "below quantification limit", meaning that the chemical compound in question was identified, but was not reported due to being present below the minimal quantities for quantification. The feed mix with 2% of mango peel flour inclusion was subjected to different extrusion and drying temperatures, thus originating four experimental diets: i) MH-60 (110 °C extrusion; 60 °C drying - conventional manufacturing temperatures) ii) MH-35 (110 °C; 35 °C) iii) MC-60 (25 °C; 60 °C) and iv) MC-35 (25 °C; 35 °C). [1]Proximate composition, fibre and starch are expressed in % of dry matter (DM), with the exception of energy, which is expressed in kJ g^{-1} DM. Carbohydrates are calculated by difference as % of DM by adding values from protein, ash and lipids and subtracting that value from DM. Specific phenolic compounds measured via HPLC are expressed in mg of polyphenol per 100g of DM.

In extracts made from the antioxidant fibre, gallic acid was only found in diets with mango peel flour inclusion (Table 2.4), which is consistent with the high amounts of gallic acid found in mango peel flour (Table 2.1). With the sole exception of MH-35, all mango diets demonstrated a rise in bound gallic acid concentrations following the drying process in comparison to their respective extruded mixtures (Table 2.4). Fibre-bound 4-coumaric and transferulic acids were found in much higher quantities in pineapple diets compared to mango diets, with the highest values observed in diets that underwent hot extrusion (PH-60 and PH-35), regardless of the drying temperature (Table 2.4). Total bound phenolic compounds were significantly affected by both the extrusion process and drying temperature, with a significant interaction observed between these two factors (Figure 2.3). The PH-35 diet exhibited the highest final amounts of bound polyphenols, followed by PH-60 and MH-60 (Table 2.4). These diets represent an increase of 152%, 93%, and 72%, respectively, in comparison to their corresponding initial mixtures (Figure 2.2).

Figure 2.3 presents a 2-way ANOVA analysis comparing phenolic compounds and antioxidant capacity in mango and pineapple diets. Although the “Extrusion” process did not seem to have a significant impact on free phenolic extracts of the different diets, the antioxidant capacity varied significantly among diets. In mango diets, analysis of free phenolic extracts showed that cold extrusion (MC-60 and MC-35) induced increased levels DPPH• and $ABTS^{\bullet+}$ radical scavenging capacity when compared to mango diets with hot extrusion (MH-60 and MH-35). In pineapple diets, hot extrusion induced higher DPPH• radical scavenging capacity and ORAC in both free and bound phenolic extracts, as well as higher $ABTS^{\bullet+}$ radical scavenging capacity in bound phenolic extracts (Figure 2.3). Moreover, when compared to the PNE mixture, diet PH-35 increased free phenolic DPPH• radical scavenging capacity by 29% when compared to the PNE mixture. Additionally, compared to PNE, PH-60 and PH-35 increased bound phenolic DPPH• radical scavenging capacity by 357% and 380%, while also increasing bound phenolic $ABTS^{\bullet+}$ radical scavenging capacity by 43% and 41%, and bound phenolic ORAC by 138% and 70%, respectively (Figure 2.2).

Significant differences associated with the drying stage were observed among the various experimental diets. Results from mango diets show that higher drying temperatures increase total free phenolic compounds, free phenolic $ABTS^{\bullet+}$ radical scavenging capacity, and bound phenolic ORAC when compared to lower drying temperatures (Figure 2.3). Indeed, hot drying increased bound ORAC by 94.4 and 72%, respectively, when compared to the MNE mixture (Figure 2.2). However, in pineapple diets, data shows that lower drying temperatures lead to higher total free phenolic values (Figure 2.3). This was accompanied by higher $DPPH^{\bullet}$ radical scavenging

capacity in free phenolic extracts when compared to pineapple diets dried with hotter temperatures (Figure 2.3). Specifically, diet PH-35 was able to increase free phenolic DPPH• radical scavenging capacity by 29% when compared to the PNE mixture (Figure 2.2).

Some interaction effects between variables “Extrusion” and “Drying” were observed (Figure 2.3). In mango diets, the interaction between hot extrusion and hot drying (MH-60) strongly benefited both bound phenolic content and antioxidant capacity. Namely, MH-60 showed much higher amounts of bound phenolic content, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radical scavenging capacity compared to remaining mango diets, representing an increase of 72%, 355%, and 122% compared to MNE (Figure 2.2). However, in pineapple diets, the highest levels of total bound phenolic content were observed in the PH35 diet, as a result of the interaction between hot extrusion and cold drying, followed by the PH-60 diet, due to the interaction between hot extrusion and hot drying. Respectively, these diets represent an increase of 152% and 93% compared to the PNE mixture (Figure 2.2). Finally, regarding ABTS$^{\bullet+}$ of free phenolic extracts, Figure 2.3 shows that radical scavenging capacity is decreased by the combined effect of hot extrusion and cold drying (PH-35), which in turn represents a decrease of -39% compared to the PH extrusion (Figure 2.2).

Table 2.5 shows a 1-way ANOVA comparison between all diets. DPPH• radical scavenging capacity of free extracts is significantly higher in the PH-35 diet, followed by the MC-35, PH-60, and MC-60 diets (203.8, 145.6, 140.1, and 131.4 mg TE 100 g -1 DM, respectively) compared to all other diets. In terms of bound phenolic extracts, the PH-60, PH-35 and MH-60 diets presented the highest DPPH• radical scavenging capacity values (117.1, 123.0 and 125.3 mg TE 100 g -1 DM, respectively). ABTS$^{\bullet+}$ radical scavenging capacity of free phenolic extracts was significantly higher in the MC-60 diet (192.4 mg TE 100 g -1 DM) than in diets PC-60, PH-60, PC-35, MC-35, and MH-60 (161.9, 158.7, 147.9, 144.3 and 128.0 mg TE 100 g -1 DM, respectively). In bound phenolic extracts, MH-60 had significantly higher values (203.2 mg TE 100 g -1 DM) than PH-60 and PH-35 (144.6 and 142.3 mg TE 100 g -1 DM, respectively). Finally, the highest values of ORAC in both free and bound phenolic extracts belong to the PH-60 diet (1674.3 and 1216.2 mg TE 100 g -1 DM, respectively). Particularly, bound phenolic extracts in PH-60 were significantly higher than in all remaining diets.

Figure 2.5 shows a PCA plot of similarities and dissimilarities between diets, explaining 70% of the data variation. Diets were considerably dispersed, with stronger similarities between the analysed parameters and diets PH-35, PH-60, and MH-60. These three diets were more similar on the F1 axis, which explains 52% of the variation. Among these three diets, PH-60 was the diet with the highest distance from the control CH-60

on the F2 axis, which explains 18% of the variation. Pineapple diets with hot extrusion, as well as the mango diet with hot extrusion and hot drying, are more closely associated with higher antioxidant capacity in both free and bound extracts. While MH-60 and PH-35 were more associated with bound DPPH$^{\bullet}$ and ABTS$^{\bullet+}$, PH-60 and MH-60 were more closely associated with bound ORAC and free DPPH$^{\bullet}$.

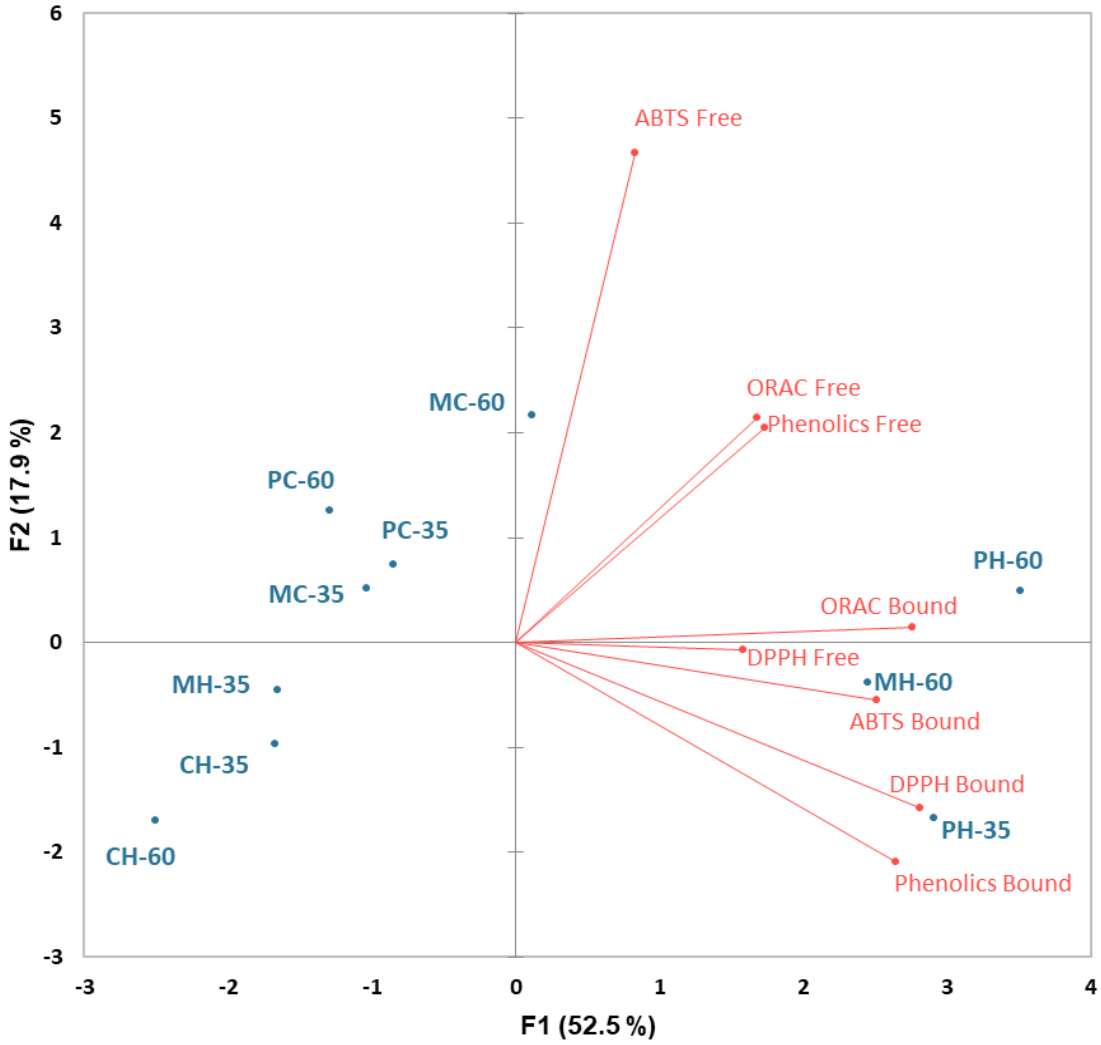

Figure 2.5 - Principal component analysis applied to phenolic content quantification and antioxidant capacity in dried pellets from control, mango and pineapple diets, at a significance level of 5. Points ascribed to diets (CH-60, CH-35, MH-60, MH-35, MC-60, MC-35, PH-60, PH-35, PC-60, PC-35) were calculated using the mean between observations (n=3). “DPPH”, “ABTS” and “ORAC” stand for the three radical scavenging assays performed in this study. The “Free” and “Bound” suffixes stand for the type of extract where the analysis was performed. “Phenolics Free/Bound” stands for phenolic compounds measured according to the Folin-Ciocalteau method.

Table 2.5 – Comparison of antioxidant capacity between all diets from phase 5 (pellets after extrusion and drying processes)

	Control diets			Mango diets			Pineapple diets				
	CH-60	**CH-35**	**MH-60**	**MH-35**	**MC-60**	**MC-35**	**PH-60**	**PH-35**	**PC-60**	**PC-35**	***p-value***
DPPH$^{\bullet+}$ [1]											
Free phenolic extracts	73.9 ± 2.1^{d}	28.2 ± 2.0^{e}	48.8 ± 6.2^{de}	72.2 ± 2.4^{d}	131.4 ± 1.7^{bc}	145.6 ± 6.1^{b}	140.1 ± 6.9^{b}	203.8 ± 0.2^{a}	62.4 ± 10.7^{e}	98.1 ± 6.1^{c}	**<0.001**
Bound phenolic extracts	23.1 ± 0.2^{b}	27.9 ± 0.4^{b}	125.3 ± 0.7^{a}	28.4 ± 2.3^{b}	26.4 ± 0.4^{b}	25.3 ± 0.7^{b}	117.1 ± 2.4^{a}	123.0 ± 8.7^{a}	21.4 ± 0.4^{b}	21.5 ± 1.9^{b}	**<0.001**
ABTS$^{\bullet+}$ [1]											
Free phenolic extracts	83.3 ± 6.3^{d}	69.9 ± 5.5^{d}	128.0 ± 11.7^{bc}	78.1 ± 2.2^{d}	192.4 ± 7.1^{a}	144.3 ± 9.6^{b}	158.7 ± 20.1^{b}	94.1 ± 11.6^{cd}	161.9 ± 15.5^{b}	147.9 ± 1.3^{b}	**<0.001**
Bound phenolic extracts	81.0 ± 8.8^{d}	75.8 ± 1.4^{d}	203.2 ± 8.0^{a}	69.5 ± 2.6^{d}	91.0 ± 4.5^{cd}	79.7 ± 5.5^{d}	144.6 ± 11.1^{b}	142.3 ± 11.5^{bc}	79.0 ± 6.0^{d}	75.2 ± 4.6^{d}	**<0.001**
ORAC [1]											
Free phenolic extracts	694.8 ± 274.4^{c}	1036.6 ± 87.0^{bc}	862.3 ± 194.2^{abc}	1255.1 ± 31.9^{ab}	1227.3 ± 58.7^{abc}	1104.7 ± 21.6^{abc}	1674.3 ± 89.3^{a}	1082.9 ± 97.8^{bc}	1312.0 ± 172.8^{ab}	776.1 ± 22.9^{bc}	**<0.001**
Bound phenolic extracts	422.8 ± 26.0^{de}	428.7 ± 13.0^{de}	719.2 ± 59.4^{bc}	381.1 ± 91.8^{e}	638.7 ± 87.5^{bcd}	383.7 ± 51.2^{e}	1216.2 ± 136.1^{a}	871.1 ± 187.5a^{b}	439.5 ± 61.3^{de}	526.6 ± 31.9^{cde}	**<0.001**

Values are presented as mean ± standard deviation (n=3). A 1-way ANOVA was performed. Different superscript uppercase letters (^{a,b,c}) indicate significant differences ($P < 0.05$). A control mix (CNE) was subjected to a conventional hot extrusion process (110 °C), after which it was subjected to two distinct drying temperatures (60 and 35 °C), generating control diets CH-60 and CH35, respectively. The feed mix with 2% of mango peel flour inclusion was subjected to different extrusion and drying temperatures, thus originating four experimental diets: i) MH-60 (110 °C extrusion; 60 °C drying - conventional manufacturing temperatures) ii) MH-35 (110 °C; 35 °C) iii) MC-60 (25 °C; 60 °C) and iv) MC-35 (25 °C; 35 °C). [1]DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and ORAC are expressed in mg of Trolox equivalents (TE) per 100 g DM.

4. Discussion

The primary goal of this research is to assess how varying extrusion and drying temperatures influence the radical scavenging capability of aquafeeds enriched with natural antioxidants (i.e., vitamins, carotenoids, and polyphenols) sourced from mango and pineapple peels.

Extensive research has demonstrated the antioxidant and immunostimulant properties of certain vitamins, namely vitamins E (tocopherols) and A (retinol) (Udo and Afia, 2013). Consequently, synthetic versions of these vitamins are now widely used as antioxidants in aquafeeds (Morin et al., 2021; NRC, 2011). The present results show that both mango and pineapple peels are natural sources of these vitamins. However, pineapple peels lost much more tocopherols and retinol than mango in the conversion process from peel to flower when compared to mango peels. Ultimately, none of the peel flours used in this study can be considered good sources of these vitamins for aquafeeds, as quantification values were substantially below minimal requirements for cultured fish – namely 10 000 µg 100 g^{-1} and 600 µg 100 g^{-1}, respectively (NRC, 2011).

The antioxidant properties of carotenoids stem from their highly unsaturated structure, making them prone to oxidation. This allows them to neutralise singlet oxygen ($O^{\bullet -}$) ROS and capture free radicals (Aklakur, 2018). However, since fish are not able to synthesize carotenoids, their bioavailability within the organism is dependent on the diet (Aklakur, 2018). Carotenoid inclusion in aquafeeds has shown benefits to the antioxidant and immune systems of cultured fish (Aklakur, 2018; Faehnrich et al., 2016; García-Chavarría and Lara-Flores, 2013; Nakano and Wiegertjes, 2020). However, these beneficial effects are heavily dependent on the carotenoid source, dose, and mode of administration (Carvalho and Caramujo, 2017; Nakano and Wiegertjes, 2020). Although the inclusion levels of carotenoids in aquafeeds as antioxidant sources are not standardized, previous studies have used levels far exceeding those provided by the peels used in this study (Bacchetta et al., 2019; Goda et al., 2018; Saleh et al., 2018; Sallam et al., 2017). Moreover, the present study´s findings demonstrate that converting fruit peels into flour results in losses of over 70% of total carotenoids, leading to a negligible increase in carotenoid quantities in the feed mixture at a 2% peel flour inclusion rate. This sensitivity of carotenoids to thermal processing is well-documented (Meléndez-Martínez et al., 2022). Indeed, their highly unsaturated structure makes carotenoids particularly susceptible to degradation when exposed to high temperatures, light, mechanical stress or other pro-oxidant conditions common in feed manufacturing (Achir et al., 2010). Thus, the already low levels of carotenoids in these flours are further reduced by the significant negative impact of common physicochemical conditions during feed manufacturing.

More than 8000 phenolic compounds have been identified from plant sources, most of which exhibit strong antioxidant capacity, i.e., the ability to scavenge free radicals and transform them into more stable molecules via the donation of a hydrogen atom from the phenolic O-H group (Ahmadifar et al., 2021; Brglez Mojzer et al., 2016; Kondo et al., 1999; Williams et al., 2004). Consequentially, natural phenolic compounds can be used as additives in aquafeeds to preserve their quality and prevent rancidity during storage. Additionally, natural phenolic compounds in aquafeeds may also confer supplemental antioxidant protection that might increase the antioxidant defences of farmed fish, as previously reported (Ahmadifar et al., 2021; Maqsood et al., 2013). However, the antioxidant properties of phenolic compounds are highly influenced by their chemical structure and their

interactions with other macromolecules, including fibre, protein, lipids, and carbohydrates. Thus, the antioxidant capacity of by-product matrices, such as fruit peels, is determined as much by their phenolic compound profile as by their total phenolic content (Ahmadifar et al., 2021). Overall, the Folin-Ciocalteu method revealed heavy losses of free phenolic compounds in both mango and pineapple peels during their conversion process into flour, with reductions of approximately 50% and 18%, respectively. The degradation of these polyphenols due to thermal stress is one of the main mechanisms proposed to explain the yield dynamics of phenolic compounds in plant-based matrices exposed to high temperatures (Antony and Farid, 2022). Alternatively, thermal degradation caused by high temperatures might also release phenolic compounds from lignin, by either breaking the chemical bonds between them or by degrading lignin itself, thus increasing the final amounts of phenolic compounds (Antony and Farid, 2022). The flour manufacturing process used in this study involved drying fruit peels at 60 °C until moisture dropped below 10%, as higher temperatures are usually considered detrimental to phenolic compounds (Campos et al., 2020b). This highlights the possibility that thermal degradation alone cannot fully explain the behaviour of phenolic compounds observed in this study, as previous studies have shown that thermal degradation of these biomolecules occurs primarily at temperatures above 80 °C (Antony and Farid, 2022). Air exposure during the drying procedure could have been one of the main causes, as oxygen induces the degradation of these biomolecules through oxidation (Tanaka et al., 2009). Moreover, the oxidation of phenolic compounds is accompanied by the reduction of an oxygen molecule, generating the superoxide anion and hydrogen peroxide (Tanaka et al., 2009). These potent ROS further promote the oxidation of macronutrients and phenolic compounds in food matrices such as fruit peels, as well as in the aquafeeds themselves (Colombo et al., 2020; Kurutas, 2015). For example, anthocyanins are known to be present in pineapple and mango peels, albeit in low amounts (Luan et al., 2023; Ranganath et al., 2018). Padayachee et al. (2012) proposed an alternative mechanism for the dynamics of anthocyanins, reporting that these phenolic compounds can significantly bind with primary components of plant cell walls, i.e. cellulose and pectin. This binding occurs through both ionic interactions with pectin and hydrophobic interactions with cellulose, leading to changes in the amounts of free and bound polyphenols, as well as their bioaccessibility (Padayachee et al., 2012).

In this study, the free phenolic extracts of both flours were found to be rich in gallic acid (3,4,5-trihydroxybenzoic acid), a potent antioxidant and free radical scavenger that can protect biological cells, tissues, and organs from damage caused by oxidative stress (Badhani et al., 2015; Gao et al., 2019). They also revealed the presence of rutin (quercetin-3-O-rutinoside), whose antioxidant and anti-inflammatory effects have been extensively studied (Afanas'eva et al., 2001). Mango flour was also found to contain mangiferin, a glucosylxanthone with specific antioxidant properties related to mango products (Mei et al., 2021). Additionally, pineapple flour showed high levels of gentisic and ferulic acids, alongside isoferulic acid. These phenolic compounds are proven to have potent anti-inflammatory, antirheumatic, and antioxidant properties, mainly exerted via their phenoxyl group (Amić et al., 2020; Joshi et al., 2012; Mei et al., 2021; Wang et al., 2011; Zduńska et al., 2018). However, further in vivo studies with the present diets will be required to fully demonstrate this potential on fish. Both flours showed distinct profiles of bound polyphenol. Mango flour contained the highest concentration of gallic acid among all the antioxidant polyphenols, followed by epicatechin gallate, which also has proven antioxidant properties (He et al., 2018), and mangiferin. The increase in gallic acid levels in mango flour compared to its levels in the peel might be related to the presence of bound epicatechin gallate in

mango peel. Specifically, thermal degradation of the catechin dimer has been suggested as the mechanism responsible for generating gallic acid from catechin derivatives during the drying process (Lee et al. (2010). Meanwhile, pineapple flour revealed high amounts of bound transferulic acid, followed by 4-coumaric acid, both of which exhibit potent radical-scavenging activity (Rezaeiroshan et al., 2022; Shen et al., 2019). Despite both fruit flours having a similar proximate composition, pineapple peel flour contains more neutral detergent fibre, potentially explaining the higher levels of fibre-bound phenolic compounds found in pineapple flour compared to mango flour (Jakobek and Matić, 2019). This increase in fibre-bound polyphenols in both flours, relative to their respective peels, may be attributed to a bioencapsulation effect, where phenolic compounds bind with lignin, pectin, and cellulose. This effect might have facilitated the extraction of bound polyphenols following the thermal stress induced during the peel-to-flour phase (Antony and Farid, 2022). Despite the vulnerability of these phenolic compounds to high extrusion and drying temperatures, several studies have demonstrated that heat treatments can enhance the antioxidant content and capacity of fruit peels, such as citrus peel (Xu et al., 2007). Furthermore, the increased bioavailability of phenolic compounds, facilitated by the weakening of chemical bounds between fibre and phenolic content in the fish digestive tract, could potentially benefit fish, as previously reported in humans (Campos et al., 2020a). Indeed, in humans, Jakobek and Matić (2019) showed that dietary fibres might function as a regulatory mechanism for modulating phenolic bioaccessibility in different parts of the digestive tract, potentially increasing the amounts that reach the lower intestinal sections, where phenolic compounds, once released, can exert beneficial effects (Barba et al., 2020; Călinoiu and Vodnar, 2019; Juániz et al., 2015).

Compared to the peels, DPPH• increased in the bound phenolic extracts of pineapple flour. Moreover, both the bound phenolic extracts of mango and the free phenolic extracts of pineapple flour increased $ABTS^{\bullet+}$ activity. However, there was an overall decrease in antioxidant capacity, measured by $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and ORAC radical scavenging capacity assays, after converting both peels into flour, indicating the need for optimization efforts to maximize this parameter. Methods for measuring the ability of compounds to act as free radical scavengers or hydrogen donors, widely used in food science, play a crucial role in assessing antioxidant content and efficacy for preservation and/or protection against oxidative damage (Kedare and Singh, 2011). All three methods used in this study to assess antioxidant capacity - $ABTS^{\bullet+}$, $DPPH^{\bullet}$, and ORAC - can be used in aqueous and nonpolar organic solvents, enabling examination of both hydrophilic and lipophilic antioxidants. However, $ABTS^{\bullet+}$ diluted in water, as employed here, has an affinity for both hydrophilic and lipophobic compounds, including hydrogen atom donors, while $DPPH^{\bullet}$ shows a higher affinity for lipophilic compounds (Floegel et al., 2011). The prevalence of hydrophilic phenolic acids across all samples throughout the manufacturing chain accounts for the generally higher $ABTS^{\bullet+}$ values compared to $DPPH^{\bullet}$ in most samples. Moreover, while the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays mimic free radicals, the ORAC assay uses a biological radical ($O2^{\bullet-}$), naturally formed within cells, making it more relevant to measure radical scavenging capacity in in vitro experiments (Ratnavathi and Komala, 2016). Therefore, the ORAC assay provides a more realistic assessment of biological models. Nonetheless, all three assays are essential for a comprehensive evaluation of a sample's antioxidant capacity. However, it is worthy of note that the antioxidant capacity of these extracts depends on the yield of phenolic compounds during extraction.

The experimental mixtures CNE, PNE, and MNE are isoproteic, isolipidic, and isoenergetic, and these properties were maintained in the corresponding diets. Although PNE and MNE

are richer in fibre compared to CNE, they contain less starch, possibly due to the replacement of wheat meal with peel flour, as wheat is mostly composed of starch (Hu et al., 2023).

In terms of phenolic content, mangiferin was detected in both free and bound forms across the entire mango manufacturing chain. Indeed, this phenolic compound was found in mango peel, in the MNE mixture, and in extruded pellets MH and MC, as well as in the dried pellets from MH-35 and MC-35. However, only trace amounts were found in all samples after the extrusion process. In addition to its strong radical-scavenging properties, mangiferin can modulate important metabolic pathways such as glycolysis, the tricarboxylic acid cycle, lipid and amino acid metabolism, and energy biosynthesis (Antony and Farid, 2022; Mei et al., 2021). However, its low solubility leads to poor transmembrane permeability and increased intestinal metabolic instability, which can directly impact its bioavailability and biological activity in farmed fish (Mei et al., 2021). On the other hand, the MNE mixture showed high amounts of bound gallic acid, consistent with the expected 2% content observed in mango peel flour. This highlights that mango extruded pellets were the only samples capable of retaining gallic acid in the bound form. Moreover, the bound phenolic content of the PNE mixture matches the expected 2% increase conferred by pineapple peel flour content, in terms of 4-coumaric and transferulic acids. Diets PH-60 and PH-35, containing pineapple, showed double the maximum amounts of these polyphenols compared to other diets. We can thus conclude that mango peel flour is an effective source of bound gallic acid when included in aquafeeds, while pineapple flour is an effective source of 4-coumaric and transferulic acids.

Extruded pellets from diets MH-60, PH-60, and PH-35 showed approximately double the amount of free phenolic compounds than the control diet CH-60. This includes significantly higher levels of gentisic acid, known for its anti-inflammatory, antimutagenic, hepatoprotective, neuroprotective, antimicrobial, and antioxidant properties (Abedi et al., 2020). Moreover, the bound phenolic compounds in MH-60, PH-60, and PH-35 diets also showed higher levels compared to both control diets. These diets demonstrated significant increases in specific fibre-bound phenolic compounds with potent antioxidant activity, namely syringic acid (Srinivasulu et al., 2018), sinapic acid (Chen, 2016), and transferulic acid (Srinivasan et al., 2007). Additionally, 4-coumaric acid, recognised for its potent antioxidant and ROS scavenger capabilities (Shen et al., 2019), was one of the most prevalent bound phenolic acids in diet PH-60, while both control diets showed only trace amounts.

The effects of thermal stress on the profile and bioavailability of phenolic compounds, as well as their ability to bond with fibre, have been extensively studied (Arfaoui, 2021; D'Archivio et al., 2010). While some studies have reported detrimental changes in the nutritional and antioxidant profiles of vegetable-based food matrices at higher temperatures (Juániz et al., 2015), others have observed beneficial effects (Xu et al., 2007). Indeed, the outcomes of these studies are highly influenced by the specific food matrix and thermal processing methods used (Arfaoui, 2021). In this study, results revealed that conventional extrusion temperatures (~100 °C) and drying temperatures (60 °C), typically used in aquafeed manufacturing, are more effective in preserving dietary antioxidants and overall feed antioxidant properties compared to colder extrusion processes (~25 °C) and significantly longer drying periods at lower temperatures (35°C). Indeed, since diets must be dried to < 12% moisture (NRC, 2011), short-term drying at high temperatures seems to be more effective than prolonged drying at lower temperatures. Lower drying temperatures require extended exposure to oxygen, light, and heat, which are influential factors in

polyphenol degradation. Higher extrusion and drying temperatures may have affected the structural stability of fibre or its chemical bonds with phenolic compounds, making them more extractable with the solvents used in this study (Arfaoui, 2021). Moreover, while higher temperatures may be damaging to free phenolic compounds, potentially disrupting the bioencapsulation effect provided by fibre and making bound phenolic compounds more vulnerable to degradation, they could also possibly increase the bioavailability of bound phenolic compounds (D'Archivio et al., 2010).

In our inter-diet comparison using principal component analysis (PCA), we found that pineapple diets subjected to hot extrusion (PH-60 and PH-35), along with the mango diet that underwent both hot extrusion and hot drying (MH-60), were more closely associated with higher $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and ORAC values. Specifically, PH-60 showed a pronounced association with ORAC values. Furthermore, a One-Way ANOVA comparison showed that the PH-60 diet had significantly higher ORAC values than all other diets containing pineapple and mango peel. These results suggest that the PH-60 diet may provide the most effective antioxidant supplement for mitigating oxidation in aquafeeds. Compared to both control diets, CH-60 and CH-35, all three diets (PH-60, PH-35, and MH-60) showed higher phenolic content and increased antioxidant capacity in terms of $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and ORAC. Overall, the present results support the incorporation of pineapple and mango flour, concomitantly with higher extrusion and drying temperatures, as biopreservatives and nutraceuticals in aquafeeds. Phenolic compounds have been extensively studied and used in the food industry to reduce the reliance on synthetic antioxidants, thereby enhancing the shelf-life of perishable products such as animal feeds, and inhibiting lipid oxidation chain reactions (Ullah et al., 2022). This approach has been recognised as viable for preserving the quality of fish and fish products over time (Maqsood et al., 2013), and it has shown resistance to deactivation during the high-temperature extrusion-cooking process (Oniszczuk et al., 2019). While the tested diets have not yet been evaluated in fish, several studies have reported positive effects associated with the inclusion of polyphenol-rich fruit by-products in aquafeeds. For instance, incorporating orange peel in diets for Gilthead seabream, *Sparus aurata*, resulted in increased antioxidant enzyme activity and decreased lipid peroxidation in the liver (Salem et al., 2019). Similar benefits were reported with the inclusion of pomegranate peel in diets for Nile tilapia, *Oreochromis niloticus* (Hamed and Abdel-Tawwab, 2021), whilst lemon peel extract increased liver total antioxidant capacity in tambaqui (*Colossoma macropomum*) (Lopes et al., 2020). Moreover, pineapple peel flour has been reported to increase bacterial infection survival rates and disease resistance in Nile tilapia, *Oreochromis niloticus* (Attalla et al., 2022; Van Doan et al., 2021). It can be inferred that the biological properties of polyphenols in organisms are heavily dependent on factors such as the phenolic concentration of the natural antioxidant source, dosage, and method of inclusion. Additionally, synergies between different antioxidants, as well as the bioaccessibility and bioavailability of phenolic compounds in the organism, can influence their effectiveness (Lizárraga-Velázquez et al., 2019). Therefore, conducting an in vivo trial with these specific diets is essential to evaluate their practical efficacy in enhancing fish antioxidant defences.

5. Conclusions

Our study demonstrated that incorporating 2% pineapple or mango peel flour in aquafeeds increases their antioxidant content and capacity compared to a control diet subjected to the same manufacturing conditions. Conventional temperatures proved more effective in

increasing feed antioxidant capacity by releasing phenolic compounds from antioxidant fibre. These compounds were more abundant and evidenced greater resistance throughout the feed manufacturing process compared to vitamins and carotenoids. These findings are a significant contribution to understanding how heat treatments impact natural antioxidant potency in aquafeeds and highlight pineapple and mango peel flours as effective sources of additional antioxidants in aquafeeds, supporting a circular economy approach. However, conducting an in vivo trial with these diets is crucial to determine whether these enhanced antioxidant properties translate into tangible benefits for farmed fish.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank NuviFruits S.A. (Portugal) for providing the mango and pineapple peels, as well as AgroGrIN Tech® (Portugal) for technical guidance in flour manufacturing. This work was funded by project "ATLANTIDA— Platform for the monitoring of the North Atlantic Ocean and tools for the sustainable exploitation of the marine resources." (NORTE-01-0145-FEDER-000040) by CCDRN, within the context of the EU Norte 2020 Operational Program. The doctoral scholarship of Ricardo Pereira was financed by Fundação para a Ciência e Tecnologia (FCT, Portugal) through the grant SFRH/BD/144631/2019.

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Chapter 3.

Inclusion of pineapple by-products as natural antioxidant sources in feeds for European sea bass (*Dicentrarchus labrax*) within a circular economy context

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Submitted to Aquaculture (Elsevier)

Abstract

The fruit industry generates a considerable annual surplus of by-products rich in natural antioxidants, like fruit peels. Their inclusion in aquafeeds may increase feed antioxidant capacity and reduce oxidative stress in farmed fish, in alignment with rising consumer demands for natural products. This study investigates the potential of pineapple by-products as antioxidants for European sea bass (*Dicentrarchus labrax*) and their impact on feed preservation at different storage temperatures. For this, a control diet (CTRL) was formulated without antioxidants. Three experimental diets were then formulated by supplementing the CTRL diet with 100 mg kg^{-1} of Vitamin E (VITE diet), or by also adding 2% of pineapple peel flour (P2) or pineapple stem flour (S2). All diets remained isoproteic, isolipidic, and isoenergetic. Triplicated groups of fish (average weight 13.5 ± 0.8 g) were reared in a recirculating aquaculture system and fed *ad libitum* for 12 weeks. After the growth trial, fish were subjected to a stress challenge consisting in 1 minute of air exposure followed by a 5-minute confinement at 100 kg m^{-3}. All the diets were well accepted by fish, with no differences in terms of growth. All fish responded to stress, but without differences among diets. However, diets did modulate antioxidant enzyme responses, with fish fed VITE showing a reduced need for antioxidant enzyme production to maintain lower levels of liver lipid oxidation in both groups. While this reinforces the importance of incorporating vitamin E in aquafeeds, the impact of diets containing pineapple stem and peel was insufficient to yield noticeable improvements in fish antioxidant defences. Regarding diet antioxidant capacity and lipid oxidation, storage time negatively impacted diet antioxidant properties, regardless of temperature. This suggests that other more effective strategies are warranted in order to improve the antioxidant properties of aquafeeds during storage. Further research is necessary to maximize antioxidant capacity, such as experimenting with different inclusion percentages, and explore variations in flour and diet manufacturing conditions to assess the effects of these natural antioxidants on fish stress resistance.

Keywords: Aquaculture; Sustainability; Functional aquafeeds; Oxidative stress

1. Introduction

Aquaculture practices have grown exponentially over the last two decades, while simultaneously becoming more integrated into global food systems (FAO, 2022). In 2020, this sector supplied 214 million tons of aquatic food for human consumption, including 88 million tons of fish, accounting for 56% of worldwide fish consumption (Bharathi et al., 2019; FAO, 2022). The rapid growth of aquaculture has been primarily driven by the depletion of wild fish stocks and the ever-increasing demand for fish from a growing global population (Naylor et al., 2009; Naylor et al., 2021). This underscores the need to continuously improve and optimize practices within the aquaculture industry to assure a sustainable value chain. Among the many research topics aimed at improving both the output quality and quantity of aquaculture production, the development of novel aquafeeds that promote fish welfare within a sustainability context is of particular interest (Encarnação, 2016). A significant aspect of feed modulation efforts concerns the inclusion of antioxidants to avoid feed oxidation and fortify fish antioxidant defences (Bharathi et al., 2019). Essentially, the aquafeed manufacturing process usually requires adding oil to provide feeds with monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) (Colombo et al., 2020). However, these fatty acids are highly susceptible to oxidation through free radical chain reactions initiated by peroxides and hydroperoxides (Colombo et al., 2020). This oxidation process can be ameliorated by antioxidant inclusion, subsequently avoiding feed staling and rancidity (Olmos-Soto, 2015). Moreover, farmed fish are commonly subjected to stress factors, mainly due to periodic handling and transportation (Reverter et al., 2014). In this context, antioxidants play a crucial role in maintaining fish cell homeostasis and preventing the intracellular formation of reactive oxygen species (ROS), which can lead to oxidative stress and, ultimately, lipid and protein oxidation, DNA damage, enzymatic inactivation, precocious cell aging and even apoptosis (Guilherme et al., 2008; Poljsak et al., 2013). Thus, exogenous antioxidant sources have been reported to boost fish antioxidant defenses and enhance disease resistance, while preventing oxidation of aquafeed (Aklakur, 2018; NRC, 2011).

Simultaneously, there is a growing trend among consumers towards natural antioxidants, as opposed to synthetic sources (Sanches-Silva et al., 2014). The most commonly used synthetic antioxidants in aquafeeds, such as ethoxyquin (6-Ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline, E324), BHT (2,6-diterc-butil-*p*-creso, E321) and BHA (2,3-terc-butil-4-hidroxianisol, E320), raise concerns regarding bioaccumulation, immune system inhibition, genotoxicity, endocrine disruption, carcinogenic potential and tumour promoting effects (Anders et al., 1987; Lanigan and Yamarik, 2002; Nieva-Echevarría et al., 2015). In 2022,

the EU definitely prohibits the use of ethoxyquin as an antioxidant feed additive (EU 2022/1375, EU2022) (European Comission, 2022a). Therefore, the feed industry is under external pressure to find safe and natural alternative compounds with strong antioxidant activity (Bai et al., 2015; Ehsani et al., 2018; Elseady and Zahran, 2013; Encarnação, 2016; Kousoulaki et al., 2015; Sanches-Silva et al., 2014).

In recent years, the global annual production of fruits and vegetables has doubled from 30 to 60 million tons (Shahbandeh, 2019). This generates a steady output of antioxidant-rich by-products, such as fruit peels and stems, which are often discarded after processing (Porter et al., 2018). This practice not only results in increased waste but also has adverse environmental consequences (Kumar et al., 2020; Wu, 2016). Specifically, fruit by-products are rich in vitamins, carotenoids and phenolic compounds, all of which are proven to be good sources of antioxidants with considerable radical scavenging properties (Dawood, M.A.O. et al., 2022; Pereira et al., 2022b).

Indeed, beyond simply acting as radical scavengers, the antioxidant compounds in fruit peels may also increase the baseline activity of antioxidant enzymes, fortifying oxidative defence mechanisms (Dawood, M.A.O. et al., 2022; Lizarraga et al., 2018). All of this contributes towards effectively decrease lipid peroxidation, a pivotal factor in preserving the nutritional value of fish flesh intended for human consumption (Lizárraga-Velázquez et al., 2019; Salem et al., 2019). In addition, when added to aquafeeds as exogenous antioxidant sources, fruit peels play a central role in modulating the fish immune system, leading to improved disease resistance and heightened resistance to xenobiotics (Hamed and Abdel-Tawwab, 2021; Lizárraga-Velázquez et al., 2019; Lopes et al., 2020; Salem et al., 2019). Thus, these bioactive compounds can be used to enhance the antioxidant capacity in aquaculture feeds, as well as boosting the immunological and antioxidant defence system of farmed fish, which may even lead to improved fish robustness (Lopes et al., 2020; Salem et al., 2019). However, the modulation of fish antioxidant defenses through natural antioxidants depends on the fish species, with factors like the antioxidant source, the feed matrix, and the method and percentage of inclusion influencing the response (Dawood, M.A.O. et al., 2022). Additionaly, physico-chemical elements linked to feed manufacturing processes such as extrusion, pelleting, drying and conditioning, namely varying levels of exposure to light, water and heat, pressure and other forms of mechanical stress, can all affect the stability and effects of natural antioxidants (Riaz and Ali, 2009). Though, literature concerning the effects of natural antioxidant inclusion on the antioxidant capacity of aquafeeds remains scarce. Specifically, pineapple is a tropical fruit known for its sweet and tangy flavor that offers several health benefits, including antioxidant properties, which are highly modulated by its richness in various phenolic compounds, including flavonoids and phenolic acids (Campos et al. (2020a). While the phenolic content of pineapple may be

potentially lower compared to other widely commercialized fruits like berries (Fu et al., 2011), it still stands out within the food industry due to its significant content of antioxidant-rich by-products, such as peels and stems (Upadhyay et al., 2010), which are mostly used for composting (Nguyen et al., 2021). This creates conditions that facilite their inclusion in aquafeeds in terms of availability and market price. Moreover, a study conducted by our research group demonstrated that incorporating 2% of either pineapple or mango peel flour in aquafeeds can increase the antioxidant content and capacity of aquafeeds compared to a control diet under the same manufacturing conditions (Pereira et al., 2022b).

Thus, the main objective of the present study is to analyze the potential of pineapple by-products, as natural antioxidant sources in feeds for European sea bass (*Dicentrarchus labrax*) juveniles. Specifically, we will assess the capacity of incorporating 2% pineapple peel flour or stem flour to modulate fish antioxidant defences and overall stress response. Additionally, the impact of these by-products on feed preservation at different storage conditions will also be evaluated.

2. Material & Methods

2.1 Ethical statement

The trial herein described, and all associated animal handling procedures were subjected to an ethical review process overseen by CIIMAR's animal welfare body (ORBEA-CIIMAR_18_2017), and approved by the Portuguese Veterinary Authority (1005/92, DGAV-Portugal) in compliance with the guidelines of the European Union (directive 2010/63/UE).

2.2 Flours and experimental diets

The pineapple (*Ananas cosmosus* L.) peel and stem flours were obtained from AgroGrIN Tech, a Portuguese start-up company specialized in the sustainable valorisation of fruit and its by-products. For the manufacturing of both flours, pineapple peels and pineapple stems were rinsed with tap water and homogenised in a liquid juicer. The resulting homogenate's solid component was collected, followed by a careful drying step in an oven at 60 °C for 48 h. The resulting dried biomass was then ground in a granite flour mill and sifted at 0.5 mm, originating two distinct flours – pineapple peel flour and pineapple stem flour. Both products were kept in separate polyethylene bags and stored at -80 ºC until further analysis and incorporation into the diets. Proximate composition and antioxidant capacity of flours are described on Table 3.1. A commercial-based diet was formulated without any antioxidants

added. This diet was then extruded by SPAROS (Lda.) and was used as a negative control (CTRL diet). Three experimental diets were then formulated by supplementing the CTRL diet with a regular dose of 100 mg kg^{-1} of vitamin E (Lutavit® E50), creating a positive control (VITE diet), or by further adding 2% of either peel or stem flour to the VITE diet (P2 and S2 diets, respectively), at the expense of wheat meal.

2.3 Feeding trial and sampling

For the feeding trial, 240 European sea bass juveniles were acquired from the commercial fish farm Aquacultura Balear S.A.U. (Culmarex group; Murcia, Spain) and transported to CIIMAR facility (Matosinhos, Portugal). After 2 weeks in quarantine, fish were anaesthetized with 2-phenoxy-1-etanol (200 µL L^{-1}), and individually weighed (g) and measured (total length, cm). Homogeneous groups of 17 fish (13.5 ± 0.1 g of initial weight; 11.1 ± 0.1 cm of initial total length) were randomly distributed by 16 tanks (50 L per tank, 4.6 kg m^{-3}), establishing quadruplicate groups for each experimental diet. Fish were fed thrice a day until apparent satiation for 81 days. The water temperature was maintained at 22.0 ± 1.0 ºC, salinity at 35.0 ± 0.5‰ and water oxygen levels at a minimum of 90% saturation. Redox potential (300 mV), pH levels (7.5) and salinity were recorded daily. Total ammonium, nitrite and nitrate were monitored twice a week and maintained at levels ≤0.05 mg L^{-1}, ≤ 0.5 mg L^{-1} and ≤ 50 mg L^{-1}, respectively, as is recommended for marine fish species (Blancheton, 2000). After a 24 h fasting period, ten fish from the initial stock and twenty fish per treatment by the end of the growth trial were sacrificed by an anesthetic overdose (2-phenoxy-1-ethanol, 500 µL L^{-1}) and stored at -80 ºC for whole-body composition analysis. After the growth trial and a 24 h fasting period, 24 fish per treatment were immediately sampled, while another subset of 24 fish per treatment was further subjected to a stress challenge involving air exposure for 1 min, and confinement at 100 kg m^{-3} during 5 min, replicating common aquaculture practices. After the stress challenge, fish were returned to their original tanks with the same density of the growth trial (4.6 kg m^{-3}) and were allowed a 2-hour recovery period prior sampling. All fish were slightly anesthetized with 2-Phenoxyethanol (200 µL L^{-1}) for blood collection from the caudal vein using with heparinized syringes, and centrifuged at 10,000 g for 5 min at 4 ºC. The collected plasma was stored at -80 ºC until analysis. Fish were then sacrificed by a sharp blow on the head, after which intestine and liver were collected and weighed for calculation of somatic indexes. Liver and left dorsal muscle samples from each fish were immediately frozen in liquid nitrogen and kept at -80 ºC until further analysis. All sampled fish were individually weighed (g) and measured (total length, cm).

2.4 Chemical Analysis

Freeze-dried whole fish, flours and diets were ground and homogenized prior to proximate composition analysis. All samples were analyzed in duplicates for ash, DM, crude protein (N × 6.25), lipids and gross energy, according to AOAC methods (AOAC, 2006) as described by Basto et al. (2020).

2.5 Radical-scavenging capacity and lipid peroxidation in flours, experimental diets and fish muscle

The antioxidant capacity of both the selected flours and the experimental diets were analyzed at two different time points: immediately after the manufacturing process and following an 81-day storage period (duration of the growth trial) at two different temperatures, i.e. 4 ºC and 25 ºC. Diets were mixed with methanol:water (80:20%) in a proportion of 1:10, blended using an IKA T 25 digital ULTRA-TURRAX® (IKA, Germany), and centrifuged at 5000 × *g*. The supernatant, i.e. the free phenolic compound extract, was collected and stored at -80 ˚C until further analysis, while the remaining pellet was used for bound phenolic compound extraction. The pellet was hydrolyzed with 4 M NaOH in distilled water at room temperature, using an orbital shaker (250 × *g* for 3 h). The mixture was acidified to pH 1.5-2.0 via the addition of HCl 32%. Then, the acid mixture was washed three times with ethyl acetate. The resulting fraction was collected and totally dried using a rotatory vacuum evaporator at 30 ºC. The resulting residues were then dissolved in pure methanol, generating the bound phenolic extracts. Quantification of phenolic compounds via the Folin-Cocialteau method was performed in accordance with Ainsworth and Gillespie (2007). Radical scavenging activity was measured by three assays: the $ABTS^{\bullet+}$ assay, performed using the method described by Sánchez-Moreno (2002) and adapted by Gonçalves et al. (2009); the $DPPH^{\bullet}$ assay, performed according to the method of Brand-Williams et al. (1995) and adapted by Gómez-García et al. (2021); and the ORAC assay, performed according to Apak et al. (2007) as applied by Gómez-García et al. (2021). These analyses were conducted for both the free and bound extracts of the diets using a multi-detection plate reader (Synergy H1 HU126, Winooski, VT, USA). Calibration curves were performed with Trolox standards and results are expressed as nmol of gallic acid equivalents (GAE) per mg of diet in the case of Folin-Cocialteau, or of Trolox equivalents per mg of diet for all remaining analysis.

Fish muscle samples from both non-stressed and stressed groups were processed for analysis according to Valente et al. (2015). Briefly, fish muscle samples were subjected to acid hydrolysis using a glycine-HCl 32% buffer (0.1 M). Pepsin solution was then added,

and samples were incubated for 6h at 37 ºC in an oscillator at 120 rpm. After this, samples were heated for 5 min at 100 ºC to inactivate the enzyme and centrifuged, following the addition of NaOH 1 M to neutralize the pH value. Radical scavenging potential in terms of $ABTS^{\bullet+}$ and ORAC was measured using the methodologies described for diets according to Valente et al. (2015) and Ribeiro et al. (2020), respectively. Calibration curves were developed with Trolox standards. Results were expressed as nmol of Trolox equivalents per mg of fresh tissue for all remaining analyses. For lipid peroxidation evaluation, muscle samples were homogenized using phosphate buffer (0.1M, pH 7.4) in a proportion of 1:10 (w:v). Lipid peroxidation (LPO) was assessed via the methods described in Bird and Draper (1984) through the quantification of thiobarbituric acid reactive substances (TBARS), which are mainly composed of malondialdehyde (MDA), a by-product of the decomposition of fatty acids (PUFAs) induced by chemically unstable hydroperoxides. Absorbance was measured at 535 nm at 25 ºC, and the rate of LPO was expressed as nmol of TBARS formed per g of muscle. All analyses were performed using a multi-detection plate reader (Synergy H1 HU126, Winooski, VT, USA).

2.6 Oxidative stress biomarkers in fish liver

Fish livers from both non-stressed and stressed groups were homogenized using phosphate buffer (0.1M, pH 7.4) in a ratio of 1:10 (w v^{-1}). To 300 µL of homogenate, 5 µL of butylated hydroxytoluene (BHT, 4%, diluted in methanol) were added. Thus, aliquots for LPO and carbonyl compounds (CC) were made and subsequently stored at -80 ºC. The remaining homogenate was centrifuged at 10 000 × *g* at 4 °C for 20 min, after which post mitochondrial supernatant (PMS) was extracted and stored at -80 ºC for analysis of antioxidant enzymes activity and glutathione quantification. Protein content of PMS was measured according to Bradford (1976) in order to standardize the antioxidant enzyme activity measurements.

The kinetics of catalase (CAT) were recorded in accordance with Greenwald (1987). Briefly, hydrogen peroxide (H_2O_2) at 30% was used as the substrate, and absorbance was registered at 240 nm at 25 °C. Enzyme activity was expressed in µmol of H_2O_2 consumed per minute per mg of protein. Glutathione peroxidase (GPx) activity was assessed as reported by Mohandas et al. (1984) via the oxi-reduction cycle of glutathione, an enzymatic cofactor used by GPx. The reaction was performed at 25 ºC, using H_2O_2 as the substrate and including sodium azide (NaN_3) to inhibit CAT. The decrease in absorbance of ß-nicotinamide adenine dinucleotide phosphate (reduced; NADPH) was measured at 340 nm at 25 ºC, as the oxidation of NADPH to $NADP^+$ is indicative of GPx activity. Units of enzyme

activity were expressed as nmol of oxidized NADPH per minute per mg of protein. Glutathione s-transferase (GST) was determined following the methods described by Habig (1974). Total GST activity, encompassing both cytosolic and microsomal fractions, was determined via measurement of the conjugation between 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH). Absorbance was recorded at 340 nm at 25 ºC for 5 min, and enzyme activity was expressed in nmol of CDNB conjugate formed per minute per mg of protein. The activity of glutathione reductase (GR) was assessed according to Cribb et al. (1989) via consumption of NADPH measured at 340 nm for 3 min at 25 ºC. Results were expressed in nmol of oxidized NADPH per minute per mg of protein. Total glutathione (GSHt) and oxidized glutathione (GSSG) were measured at 412 nm via the formation of 5-thio-2-nitrobenzoic acid (TNB), in accordance with Baker et al. (1990). Results were expressed as nmol of conjugated TNB formed per min per mg of protein. LPO was measured using the methodologies described for muscle, and the results were expressed as nmol of TBARS formed per g of liver. The total antioxidant capacity of fish liver was measured using a total antioxidant capacity assay kit (Sigma MAK187), according to the manufacturer's instructions. Results were expressed in nmol per mg of tissue.

2.7 Plasma metabolites and immune system status

Glucose, lactate, triglycerides, cholesterol, non-esterified fatty acids (NEFA) and cortisol, in fish plasma were enzymatically assessed using commercial kits (1001190, 1001330, 1001313, 1001090, Spinreact, Spain; 434-91795 NEFA-HR (2) R1 and 436-91995 NEFA-HR (2) R2, Wako Chemicals, Germany; RE52611, Tecan, Switzerland, respectively), following the manufacturer's instructions adapted to a microplate format. For immune system status analysis, peroxidase activity was measured using the procedure described by Quade and Roth (1997) adapted by Costas et al. (2011). Lysozyme activity was obtained following the protocol developed by Parry et al. (1965), with adaptation for a 96-well microplate by Hutchinson and Manning (1996). Complement activity (ACH50) was analyzed using the protocol described by Sunyer and Tort (1995), using rabbit blood.

2.8 Statistical analysis

Data were tested for normality and homogeneity of variances, considering the Kolmogorov-Smirnov and Levene's tests, respectively, and, if necessary, appropriately transformed. Both one and Two-Way ANOVA were used to analyze data, considering diet as fixed factor for the One-Way ANOVA, and diet and stress as fixed factors for the Two-Way ANOVA.

The analyses were performed using the Statistica v13.5 (TIBCO Software Inc., Palo Alto, CA, USA) software. When significant effects were found, a pairwise multiple comparison test (Tukey HSD) was carried out.

3. Results

3.1 Chemical composition and antioxidant capacity of pineapple flours and diets before and after storage

The proximate composition and antioxidant content of both peel and stem flours are depicted in Table 3.1. Both flours presented very similar protein and fat content, ash and gross energy values. The pineapple peel flour showed a higher total quantification of phenolic compounds as well as higher DPPH$^{\bullet}$ radical scavenging capacity and ORAC in the extracts of free phenolic compounds compared to the stem flour. The same trend was observed for bound phenolic extracts. Besides, pineapple peel flour also showed higher ABTS$^{\bullet+}$ radical scavenging capacity of bound phenolic compounds. Lipid peroxidation was highest in pineapple peel four than in pineapple stem flour.

Table 3.1 – Proximate composition and antioxidant capacity of pineapple by-product flours

	Peel flour	**Stem flour**
Proximate composition[1]		
Dry matter	92.7 ± 0.1	89.4 ± 0.1
Crude protein	5.2 ± 0.01	4.8 ± 0.04
Crude fat	0.5 ± 0.01	0.9 ± 0.02
Ash	3.2 ± 0.01	2.8 ± 0.01
Gross energy	16.7 ± 0.2	17.8 ± 0.01
Carbohydrates	91.2 ± 0.1	91.4 ± 0.1
Free phenolic extracts[2]		
DPPH$^{\bullet}$	322.0 ± 0.7	223.8 ± 2.6
ABTS$^{\bullet+}$	845.6 ± 10.3	941.0 ± 103.7
ORAC	3722.7 ± 65.9	1613.9 ± 74.9
Total phenolic compounds	1157.6 ± 97.8	622.6 ± 2.4
Bound phenolic extracts[2]		
DPPH$^{\bullet}$	886.3 ± 143.6	702.7 ± 19.1
ABTS$^{\bullet+}$	9232.2 ± 931.6	6089.3 ± 101.7
ORAC	9377.5 ± 957.4	6487.2 ± 529.1
Total phenolic compounds	953.8 ± 13.2	502.1 ± 35.4
Lipid peroxidation[3]		
TBARS	167.1 ± 1.5	121.0 ± 2.8

Values are presented as mean ± standard deviation (n=3). [1] Proximate composition is expressed in % of dry matter (DM), with the exception of gross energy, which is expressed in kJ g^{-1} DM. Carbohydrates are calculated by adding values from protein, ash and lipids and subtracting that value from 100. [2] Antioxidant capacity of both extracts was measured via the DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and ORAC radical scavenging capacities, and is expressed in mg of Trolox equivalents (TE) per 100 g of DM. Total quantification of phenolic compounds in both extracts was measured via the Folin-Ciocalteau method and is expressed in mg of gallic acid equivalents (GAE) per 100 g of DM. [3] Lipid peroxidation is expressed in nmol TBARS g^{-1} of DM.

Table 3.2 – Formulation and proximate composition of experimental diets

	CTRL	VITE	P2	S2
Ingredients				
Fishmeal Super Prime[1]	15.0	15.0	15.0	15.0
Poultry meal[2]	10.0	10.0	10.0	10.0
Porcine blood meal[3]	2.0	2.0	2.0	2.0
Soy protein concentrate[4]	15.0	15.0	15.0	15.0
Wheat gluten[5]	6.0	6.0	6.0	6.0
Corn gluten meal[6]	10.0	10.0	10.0	10.0
Soybean meal 44[7]	8.0	8.0	8.0	8.0
Sunflower meal 40[8]	5.0	5.0	5.0	5.0
Wheat meal[9]	10.0	10.0	7.9	7.9
Wheat bran[9]	4.1	4.1	4.1	4.1
Pineapple peel[10]			2.0	
Pineapple stem[10]				2.0
Vitamin & Mineral Premix PV02[11]	1.0	1.0	1.0	1.0
Vitamin C35[12]	0.03	0.03	0.03	0.03
Vitamin E50[12]		0.02	0.02	0.02
Yttrium (III) oxide	0.02	0.02	0.02	0.02
Monoammonium phosphate	0.97	0.97	0.97	0.97
Fish oil[13]	7.0	7.0	7.0	7.0
Soybean oil[14]	5.9	5.9	6.0	6.0
Proximate composition[15]				
Dry matter	96.2 ± 0.3	98.1 ± 0.02	94.8 ± 0.02	97.1 ± 0.1
Ash	6.3 ± 0.05	6.4 ± 0.1	6.4 ± 0.03	6.4 ± 0.0
Protein	55.8 ± 0.02	55.1 ± 0.3	55.3 ± 0.1	56.3 ± 0.2
Lipids	17.3 ± 0.4	17.0 ± 0.4	16.7 ± 0.4	17.5 ± 0.5
Gross energy	22.5 ± 0.02	21.7 ± 0.1	22.7 ± 0.1	22.7 ± 0.01
Carbohydrates	20.4 ± 0.3	21.5 ± 0.3	21.3 ± 0.6	20.7 ± 0.4

Values are presented as mean ± standard deviation (n=3). Ingredients are expressed in % of dry matter. [1] Peruvian fishmeal super prime: 66.3% crude protein (CP), 11.5% crude fat (CF), Pesquera Diamante, Peru. [2] Poultry meal: 62.4% CP, 12.5% CF (SAVINOR UTS, Portugal). [3] Porcine blood meal: 89.1% CP, 0.4% CF (SONAC BV, The Netherlands). [4] Soy protein concentrate: 62.2% CP, 0.7% (CF) (ADM, The Netherlands). [5] Wheat gluten: 80.4% CP, 5.8% CF (Roquette, France). [6] Corn gluten meal: 61.2% CP, 5.2% CF (COPAM, Portugal). [7] Soybean meal 44: 43.8% CP, 3.5% CF (Ribeiro & Sousa Lda., Portugal). [8] Sunflower meal (HiPro): 42.9% CP, 3.8% CF (AGP Slovakia, S.R.O., Slovakia). [9] Wheat meal: 11.7% CP, 1.6% CF (Molisur, Spain); Wheat bran: 15.2% CP, 4.7% CF (Ribeiro e Sousa Lda., Portugal). [10] Pineapple peel flour: 5.2% CP, 0.5 CF; Pineapple stem flour: 4.8% CP, 0.5% CF (AgroGrIN Tech, Portugal). [11] Vitamin and mineral premix: Vitamins are expressed mg or IU per kg of diet: vitamin A (retinyl acetate), 20,000 IU; vitamin D3 (DL-cholecalciferol), 2000 IU; vitamin K3 (menadione sodium bisulfite), 25 mg; vitamin B1 (thiamine hydrochloride), 30 mg; vitamin B2 (riboflavin), 30 mg; vitamin B6 (pyridoxine hydrochloride), 20 mg; vitamin B12 (cyanocobalamin), 0.1 mg; vitamin B5 (pantothenic acid), 100 mg; vitamin B3 (nicotinic acid), 200 mg; vitamin B9 (folic acid), 15 mg; vitamin H (biotin), 3 mg; betaine, 500 mg; inositol, 500 mg; choline chloride, 1000 mg; vitamin C (stay C), 1000 mg; Minerals (% or mg/kg diet): Mn (manganese oxide), 9.6 mg; I (potassium iodide), 0.5 mg; Cu (cupric sulphate), 9 mg; Co (cobalt sulphate), 0.65 mg; Zn (zinc oxide), 7.5 mg; Se (sodium selenite), 0.01 mg; Fe (iron sulphate), 6 mg; Cl (sodium chloride), 2.41%; Ca (calcium carbonate), 18.6%; NaCl (sodium), 4%; WISIUM, Premix Lda., Portugal. [12] Vitamins C (35%) and E (50%) (DSM Nutritional Products, Switzerland) [13] Fish oil: 98.1% CF, of which 16% is EPA and 12% is DHA (Sopropêche, France). [14] Soybean oil: 98.6% CF (JC Coimbra, Portugal). [15] Proximate composition is expressed in % of dry matter (DM), with the exception of gross energy, which is expressed in kJ g^{-1} DM. Carbohydrates are calculated by adding values from protein, ash and lipids and subtracting that value from 100.

The 2% inclusion of these flours in the experimental mix did not affect the diets' proximate composition (Table 3.2). Moreover, immediately after extrusion (initial sampling point), both the total free and bond phenolic compounds were highest in diet P2 (Table 3.3).

Table 3.3 – Antioxidant potential of experimental diets from before the growth trial, and after storage at 4°C and 25°C for 12 weeks

	CTRL			VITE			P2			S2		
	Initial	**4 °C**	**24 °C**	**Initial**	**4 °C**	**24 °C**	**Initial**	**4 °C**	**24 °C**	**Initial**	**4 °C**	**24 °C**
Free phenolic extracts[1]												
DPPH$^{\bullet}$	187.0 ± 2.0	178.4 ± 25.6	229.4 ± 22.4	211.9 ± 6.8	185.4 ± 8.3	210.0 ± 25.3	194.2 ± 11.4	197.6 ± 2.3	180.1 ± 9.2	186.0 ± 4.5	195.1 ± 13.8	180.1 ± 14.5
ABTS$^{\bullet+}$	596.1 ± 4.9	577.9 ± 77.3	654. ± 8.9	689.0 ± 60.5	482.8 ± 14.0	563.9 ± 83.2	701.0 ± 5.0	518.8 ± 25.0	519.7 ± 22.6	597.1 ± 25.8	513.3 ± 33.5	518.4 ± 60.5
ORAC	1828.3 ± 176.6	2076.5 ± 73.7	1655.1 ± 278.3	2046.3 ± 8.0	2314.4 ± 202.7	2709.6 ± 39.1	1911.8 ± 52.1	2974.4 ± 213.8	2142.9 ± 64.2	1746.4 ± 161.9	2061.3 ± 120.5	1794.5 ± 229.3
Total phenolic compounds	606.5 ± 11.2	585.4 ± 8.9	614.6 ± 15.4	646.0 ± 7.2	594.0 ± 8.5	592.7 ± 21.6	676.2 ± 11.7	652.3 ± 7.3	655.4 ± 29.3	645.3 ± 27.5	624.4 ± 11.3	631.8 ± 8.4
Bound phenolic extracts [1]												
DPPH$^{\bullet}$	43.9 ± 10.6	50.8 ± 7.3	48.9 ± 4.1	35.4 ± 1.2	45.3 ± 2.8	55.7 ± 7.5	45.0 ± 5.2	51.4 ± 1.2	56.1 ± 1.8	62.3 ± 7.5	44.8 ± 2.6	42.1 ± 2.0
ABTS$^{\bullet+}$	71.3 ± 6.2	74.1 ± 3.0	62.9 ± 5.9	64.4 ± 4.8	70.4 ± 8.2	77.3 ± 8.5	76.1 ± 9.6	94.4 ± 8.1	87.8 ± 6.5	94.9 ± 2.2	114.9 ± 8.2	105.6 ± 7.1
ORAC	1315.4 ± 75.3	1750.1 ± 74.5	1399.6 ± 61.2	1086.7 ± 88.3	1708.7 ± 50.2	1477.3 ± 89.6	1306.5 ± 113.5	1994.0 ± 89.4	1926.2 ± 141.4	1329.7 ± 62.2	1567.1 ± 95.0	1507.7 ± 180.1
Total phenolic compounds	265.3 ± 0.2	266.6 ± 4.3	257.9 ± 3.8	265.2 ± 1.0	269.9 ± 1.5	265.5 ± 4.5	279.5 ± 2.0	278.9 ± 0.4	282.0 ± 1.6	272.9 ± 0.8	272.7 ± 0.5	272.5 ± 0.7
Lipid peroxidation[2]												
TBARS	59.7 ± 3.1	140.8 ± 4.3	140.6 ± 7.4	58.8 ± 3.5	133.6 ± 5.6	134.5 ± 5.4	63.0 ± 2.5	131.5 ± 4.6	133.1 ± 3.4	63.7 ± 2.6	128.3 ± 5.2	136.6 ± 4.8

Values are presented as mean ± standard deviation (n=3). [1] Antioxidant capacity of both extracts was measured via the DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and ORAC radical scavenging capacities and is expressed in mg of Trolox equivalents (TE) per 100 g of dry matter (DM). Total quantification of phenolic compounds in both extracts was measured via the Folin-Ciocalteau method and is expressed in mg of gallic acid equivalents (GAE) per 100 g of DM. [2] Lipid peroxidation is expressed in nmol TBARS g $^{-1}$ of DM.

Vit E and P2 diets showed the highest DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radical scavenging capacity, as well as the highest ORAC values in free phenolic extracts. But in the bound phenolics the higher antioxidant capacity was evidenced in diet S2 followed by diet P2. VITE has the lowest antioxidant capacity.

Overall, and regardless of temperature, storage time was found to modulate free phenolic compound concentrations, having decreased in the VITE diet. However, after storage time, diets with pineapple by-product flour (P2 and S2) maintained their free phenolic compounds. Moreover, antioxidant potential was also modulated in both free and bound phenolics extracts. Specifically, DPPH$^{\bullet}$ radical scavenging capacity of bound extracts from diets stored at 25 °C decreased in S2, while increasing in VITE and P2. ABTS$^{\bullet+}$ radical scavenging capacity of free phenolic extracts was lower in P2 and S2, which in turn increased ABTS$^{\bullet+}$ radical scavenging capacity of bound phenolic extracts. In free phenolic extracts, VITE, P2 and S2 were associated with increased ORAC after storage, while in bound phenolic extracts, all diets presented increased ORAC after storage. Finally, and although lipid peroxidation increased after storage in all diets, levels were similar between storage at 4 or 25 °C. However, post-storage values for diets with added synthetic vitamin E, regardless of pineapple flour inclusion, were overall lower than those from the CTRL diet. In terms of storage temperature, diets were mostly equally affected by different storage temperatures, with a few exceptions. The ABTS$^{\bullet+}$ radical scavenging capacity of free phenolic extracts of the CTRL was increased at 25 °C storage. In sum, addition of these flours induced an increase in the DPPH$^{\bullet}$ radical scavenging capacity of bound phenolics extracts in P2 when compared to the remaining diets, while ABTS$^{\bullet+}$ radical scavenging capacity of diets across all sampling times increased in the free phenolic extracts of S2, followed by P2, when compared to CTRL and VITE. Moreover, ORAC of free and bound phenolics extracts is higher in P2 after storage.

3.2 Growth, intake, whole body composition and somatic indexes

As shown in Table 3.4, all diets were well-accepted by the fish, which almost quintupled in weight after 12 weeks. There were no significant differences in growth performance, feed intake, whole-body composition, or somatic indexes (Table 3.4).

Table 3.4 – Biometric parameters and whole body composition of fish fed with the experimental diets for 12 weeks

	CTRL	VITE	P2	S2	*p*-value
Growth performance[1]					
Initial body weight (g)	13.5 ± 0.2	13.5 ± 0.03	13.5 ± 0.1	13.5 ± 0.2	0.9
Final body weight (g)	64.5 ± 2.4	59.0 ± 3.4	59.5 ± 4.4	62.3 ± 2.2	0.1
Initial length (cm)	11.2 ± 0.1	11.1 ± 0.1	11.1 ± 0.1	11.1 ± 0.02	0.9
Final length (cm)	17.8 ± 0.1	17.6 ± 0.2	17.4 ± 0.2	17.6 ± 0.2	0.1
K_f	1.1 ± 0.03	1.1 ± 0.1	1.1 ± 0.05	1.2 ± 0.02	0.3
SGR	1.9 ± 0.04	1.8 ± 0.1	1.8 ± 0.1	1.9 ± 0.04	0.1
DGI	2.0 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	0.1
VFI	1.5 ± 0.1	1.5 ± 0.2	1.5 ± 0.1	1.5 ± 0.03	0.9
FCR	1.0 ± 0.04	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.03	0.8
PER	1.9 ± 0.1	1.8 ± 0.2	1.9 ± 0.1	1.9 ± 0.1	1.0
Intake[2]					
Dry matter	16.0 ± 0.6	15.8 ± 1.6	15.8 ± 0.6	15.5 ± 0.3	0.9
Protein	8.9 ± 0.4	8.7 ± 0.9	8.8 ± 0.4	8.7 ± 0.2	0.9
Lipids	2.8 ± 0.1	2.7 ± 0.3	2.6 ± 0.1	2.7 ± 0.1	0.8
Gross energy	358.9 ± 14.3	342.2 ± 34.9	359.0 ± 14.4	351.3 ± 7.6	0.6
Whole Body Composition[3]					
Dry matter	31.0 ± 0.3	31.2 ± 1.7	31.8 ± 0.6	30.6 ± 0.5	0.4
Ash	3.5 ± 0.5	3.9 ± 0.4	4.0 ± 0.4	3.6 ± 0.2	0.3
Protein	17.1 ± 0.3	17.3 ± 1.5	17.7 ± 0.5	16.8 ± 0.3	0.5
Lipids	11.0 ± 0.3	10.9 ± 1.1	11.1 ± 0.3	11.0 ± 0.9	1.0
Gross energy	7.7 ± 0.2	7.5 ± 0.6	7.8 ± 0.4	7.7 ± 0.3	0.8
Somatic Indexes					
Viscerosomatic index	6.6 ± 0.4	6.9 ± 0.7	6.8 ± 0.8	7.0 ± 0.5	0.1
Hepatosomatic index	1.1 ± 0.04	1.2 ± 0.2	1.1 ± 0.2	1.1 ± 0.04	0.3

Values are presented as mean ± standard deviation; n=68 for growth-related parameters and n=12 for somatic indexes. [1] Initial and final body weight is expressed in grams, and initial and final length are expressed in centimetres. "K_f" stands for Fulton's Condition Index - Final, "SGR" stands for Specific Growth Index, "DGI" stands for Daily Growth Index, "VFI" stands for Voluntary Feed Intake, "FCR" stands for Feed Conversion Ration, and "PER" stands for Protein Energy Ratio. [2] Intake is expressed in g of average body weight (ABW) kg^{-1} day, with the exception of gross energy which is expressed in kJ ABW kg^{-1} day .[3] Proximate composition is expressed in % of dry matter (DM), with the exception of energy, which is expressed in kJ g^{-1} DM.

3.3 Oxidative stress biomarkers in liver and muscle

The inclusion of pineapple by-products into the diet resulted in significant alteration in oxidative biomarkers in fish liver (Fig 3.1A, B, I, J, and K). However, the applied stress affected the parameters associated with antioxidant defences and oxidative stress in Fig 1B, E, H, and K, resulting in a downregulation of their activity. Specifically, catalase (CAT) activity displayed significant differences between diets (Figure 3.1A). Moreover, fish fed with the S2 diet had higher CAT activity when compared to the positive control (VITE) and to the P2 diet group, regardless of their stress condition. SOD activity showed differences between diets, as well as between stressed and non-stressed groups. Fish fed S2 had higher SOD activity when compared to the CTRL and to P2, despite not differing from the VITE group. Non-stressed groups showed significantly higher values of SOD activity for all diets, when compared to the corresponding stressed groups. GR activity and TG content did not differ between diets, but the non-stressed group displayed higher values when compared to the stressed groups. The results obtained for GSSG reflected these differences as the activity of this enzyme was also significantly higher in the non-stressed group when compared to

the stressed groups. However, LPO data showed that VITE and S2 had significantly less TBARS formation when compared to CTRL and P2. Protein oxidation differed between diets with fish fed S2 with significantly higher CC concentration than all other diets (Fig 1J). And finally, TAC showed significant differences both between diets and between stressed and non-stressed groups. While the non-stressed group showed higher values than the stressed group, fish fed with S2 were the only group that showed significantly higher TAC when compared to the negative control (CTRL). In muscle (Figure 2A, B and C), both $ABTS^{\bullet+}$ and LPO lowered in the stressed group. However, there were no significant differences between diets within the same stress group.

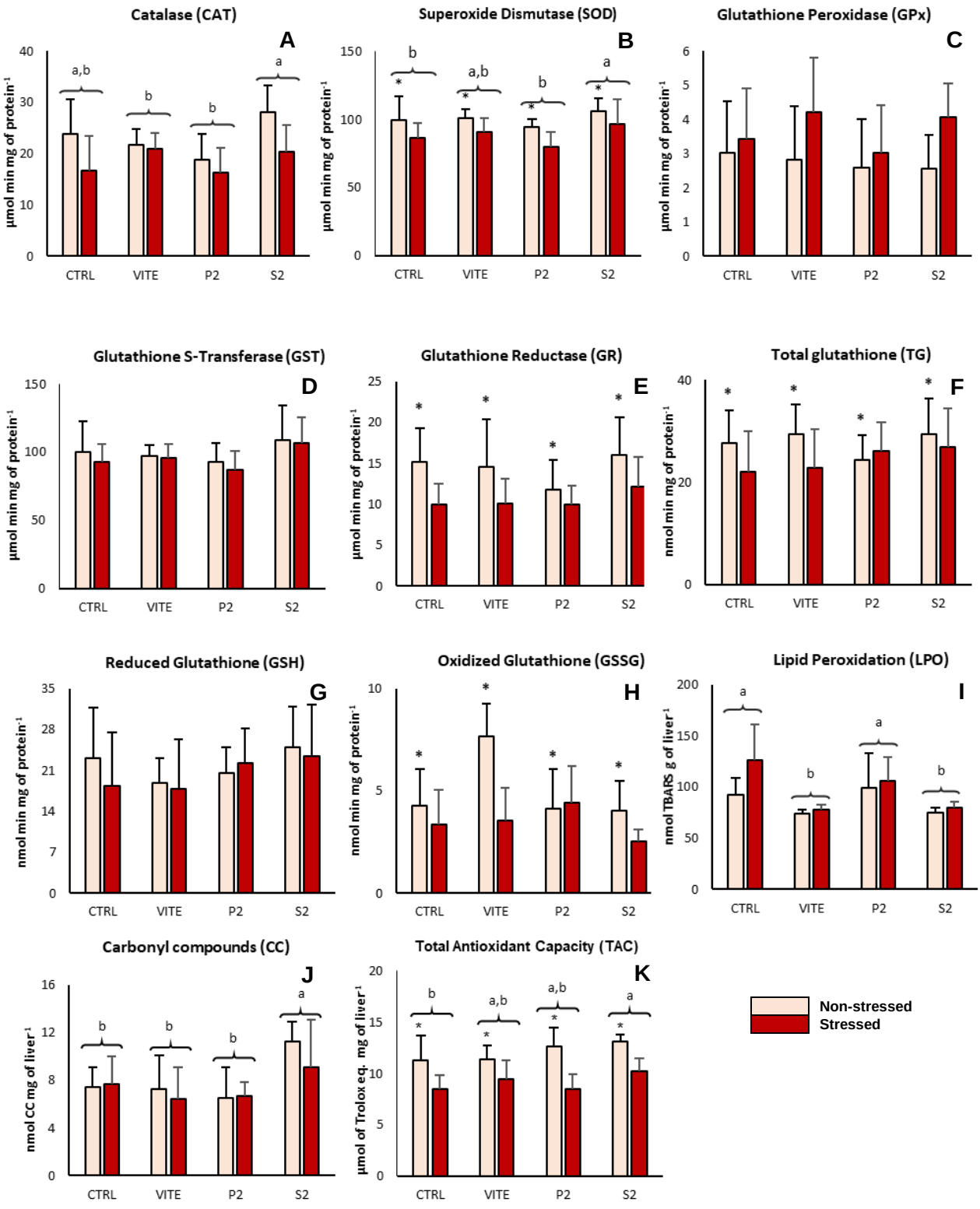

Figure 3.1. Antioxidant enzyme activity, glutathione quantification, lipid and protein oxidation and total non-enzymatic antioxidant capacity of liver from fish fed with the experimental diets. Values are pre sented as mean ± standard deviation (n = 12) per dietary treatment. A 2-Way ANOVA performed, whereas different superscript lowercase letters (a,b), indicate differences between diets, while "*" indicates differences between stressed and non-stressed groups, and different superscript uppercase letters ("A,B,C") indicate differences in the intersect between both aforementioned variables ($p < 0.05$). [1] Catalase (CAT) and glutathione peroxidase (GPx) are expressed in µmol min^{-1} mg^{-1}, while glutathione s-transferase (GST) and glutathione reductase (GR) are expressed in nmol min^{-1} mg^{-1} protein. Total glutathione (TG), reduced (GSH) and oxidized (GSSG) glutathione are expressed in mmol mg^{-1} of liver. Lipid peroxidation (TBARS) is expressed in nmol TBARS g $liver^{-1}$. Carbonyl compounds (CC) and Total antioxidant capacity (TAC) are expressed in nmol CC mg of $liver^{-1}$ and µmol Trolox equivalents mg $liver^{-1}$, respectively

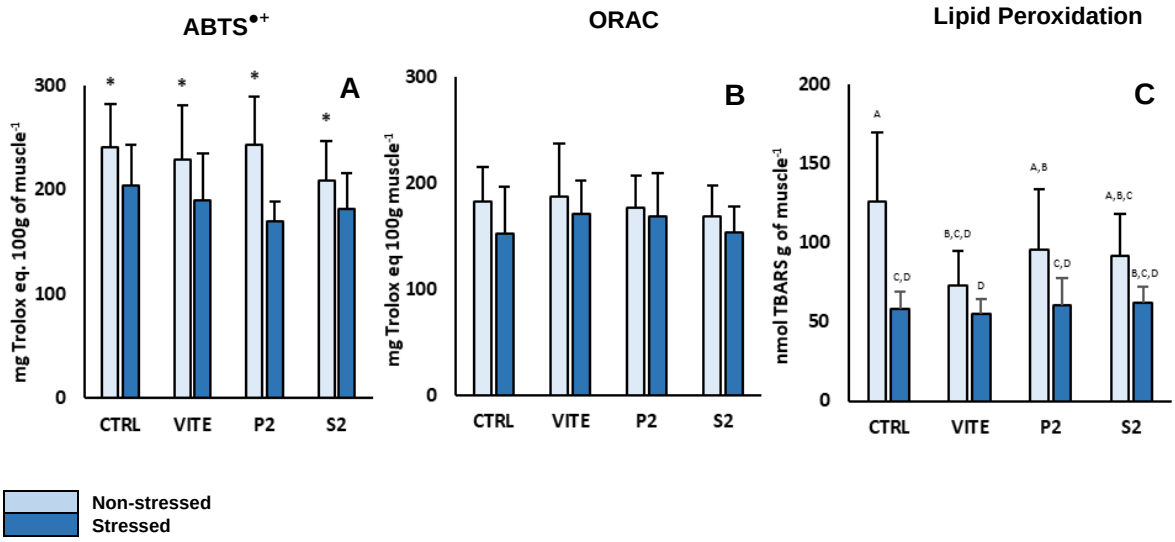

Figure 3.2. Lipid peroxidation values in fish muscle. Values are presented as mean ± standard deviation (n = 12) per dietary treatment. A 2-Way ANOVA was performed, whereas "*" indicates differences between stressed and non-stressed groups, and different superscript uppercase letters ("A,B,C,D") indicate intersect differences between variables "Stress" and "Diet" ($p < 0.05$). ABTS and ORAC values are expressed in mg of Trolox equivalents per 100g of muscle, while lipid peroxidation (LPO) is expressed in nmol TBARS mg muscle^{-1}

3.4 Plasma metabolites and innate immune system status

Levels of lactate, glucose and cortisol were shown to be significantly higher in the stressed group, but no differences could be observed between diets (Table 3.5). A significant interaction was observed between the "diet" and the "stress" factors was observed concerning the NEFA levels. In the non-stressed group, fish fed with the VITE diet had significantly lower levels of NEFA compared to those fed with P2 and S2. However, no significant differences could be perceived between diets in the stressed group. Moreover, in terms of innate immune system parameters, no differences were observed between the groups fed with the different diets (Table 3.5). But after stress, ACH50 values decreased in all fish whilst peroxidase values increased.

Table 3.5 – Plasma metabolites as bioindicators of stress in fish fed with the experimental diets for 12 weeks

	Non-stressed				Stressed					*p*-value	
	CTRL	**VITE**	**P2**	**S2**	**CTRL**	**VITE**	**P2**	**S2**	**Stress**	**Diet**	**SxD**
Plasma biomarkers											
Lactate[1]	3.3 ± 0.3	3.4 ± 0.2	4.5 ± 0.7	3.4 ± 0.2	6.6 ± 0.8*	6.6 ± 0.7*	6.0 ± 0.7*	6.6 ± 0.6*	**<0.001**	1.0	0.3
Glucose[1]	4.2 ± 0.2	4.3 ± 0.3	4.1 ± 0.3	4.6 ± 0.2	7.0 ± 0.3*	6.6 ± 0.2*	6.8 ± 0.3*	6.3 ± 0.3*	**<0.001**	0.9	0.2
Cortisol[2]	412.0 ± 43.4	394.7 ± 46.4	405.0 ± 23.3	379.8 ± 39.5	480.7 ± 44.9*	518.8 ± 32.1*	493.0 ± 57.7*	425.1 ± 31.9*	**0.01**	0.5	0.7
Cholesterol[1]	3.8 ± 0.5	2.1 ± 0.4	3.4 ± 0.5	2.7 ± 0.4	3.6 ± 0.3	3.5 ± 0.4	2.8 ± 0.5	2.8 ± 0.5	0.1	0.4	0.1
Triglycerides[1]	2.1 ± 0.3	1.3 ± 0.2	2.1 ± 0.4	1.8 ± 0.2	1.8 ± 0.1	1.6 ± 0.2	2.2 ± 0.2	1.7 ± 0.1	0.1	0.8	0.8
NEFA[1]	0.14 ± 0.01^{A,B}	0.10 ± 0.01^{B}	0.16 ± 0.02^{A}	0.15 ± 0.01^{A}	0.15 ± 0.01^{A}	0.14 ± 0.01^{A,B}	0.14 ± 0.01^{A,B}	0.13 ± 0.003^{A,B}	**0.03**	0.4	**0.01**
Immune parameters[3]											
Lysozyme	14.1 ± 4.1	13.1 ± 4.0	11.4 ± 4.0	14.7 ± 4.2	15.4 ± 4.9	13.9 ± 4.2	12.8 ± 3.7	16.1 ± 5.1	0.5	0.3	0.6
Peroxidase	55.5 ± 12.0	72.8 ± 30.4	47.0 ± 8.6	50.9 ± 10.0	64.6 ± 14.0*	77.6 ± 9.2*	62.4 ± 10.6*	68.9 ± 9.1*	**0.02**	0.7	0.9
ACH50	194.2 ± 28.3*	217.0 ± 28.8*	198.9 ± 21.7*	192.9 ± 24.1*	160.2 ± 15.0	163.8 ± 31.6	111.7 ± 29.0	108.8 ± 23.2	**0.02**	0.7	0.8

Values are presented as mean ± standard error (n = 12) per dietary treatment. A 2-Way ANOVA was performed, whereas "*" indicates differences between stressed and non-stressed groups ($p < 0.05$), and different uppercase letters "^{A,B}" represent differences in the interaction between variables "Stress" and "Diet". [1]Lactate, glucose, cholesterol, triglycerides and non-esterified fatty acids (NEFA) are expressed in mmol L^{-1}. [2] Cortisol is expressed in units mL^{-1}. [3] Peroxidase is presented in EU mL^{-1}, ACH50 is presented in units mL^{-1} and lysozyme is presented in µg mL^{-1}.

4. Discussion

There is currently a high demand for diets rich in antioxidants that contribute both to the preservation of the feed and to the enhancement of the antioxidant defenses of fish (Encarnação, 2016; Olmos-Soto, 2015). Thus, the fortification of aquafeeds with powerful antioxidant compounds serves a paramount role in preventing feed staling and rancidity, which in turn preserves the nutritional value of the feed while simultaneously reducing costs associated with feed waste, handling and storage (Gunathilake et al., 2022; Olmos-Soto, 2015). This study has shown that adding 2% of pineapple by-product flours elevated the levels of both free and bound phenolic compounds, but this was not reflected on fish antioxidant activity as a physiological response towards stress conditions.

Phenolic compounds wield a complex role as primary antioxidants (Bors and Michel, 2002; Zeb, 2020), exhibiting radical scavenging capabilities through mechanisms like hydrogen atom transfer (Zhang and Tsao, 2016) and single electron transfer via proton transfer (Lee et al., 2020). Pineapple, known for its antioxidant potency primarily derived from phenolic compounds, displays varying levels of these compounds (Hossain and Rahman, 2011). Overall, the results from the present study indicate that both types of pineapple waste flour, namely peel and stem, are good sources of phenolic compounds. Nevertheless, pineapple peel flour presented higher antioxidant properties along with a higher total content of free and fiber-bound phenolic compounds, when compared to pineapple stem flour. Indeed, these results are in line with previous studies reporting that pineapple peel has higher levels of phenolic content and greater radical scavenging activity when compared to pineapple stem (Huang et al., 2021). In addition, Huang et al. (2021) also reported that pineapple peel is richer in crude fiber than pineapple stem, which could partially explain the higher amounts of fiber-bound phenolic compounds found in pineapple peel in the present study. Indeed, pineapple is rich in fiber-bound phenolic compounds, mainly flavonoids and phenolic acids (Campos et al., 2020b; Hossain and Rahman, 2011). In this study, while the final amounts of fiber-bound phenolic compounds did not vary greatly between all diets, it is known that one of the main influencers of antioxidant activity is the antioxidant compounds composition, as certain antioxidants are more potent than others, which subsequently applies to different phenolic compounds (Rudrapal et al., 2022). In the present study, the inclusion of pineapple by-product flours (diets P2 and S2) lead to a higher extraction yield of free phenolic compounds when compared to VITE and CTRL. Moreover, while bound phenolic compounds extraction yield was similar between diets, feed radical-scavenging properties associated with bound phenolic compound extracts were higher in P2 and S2 when compared to the VITE positive control, even after storage. This is in concordance with previous study by our research team (Pereira et al., 2022b) that showed that pineapple peel

flour fiber is rich in transferulic and 4-coumaric acids, both of which are potent antioxidants that exhibit considerable radical-scavenging activity (Rezaeiroshan et al., 2022; Shen et al., 2019), and can in fact increase aquafeed radical scavenging capacity in terms of DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and ORAC. Indeed, available information shows that bound phenolic compounds are more resistant to oxidation then free forms, mainly due to the action of abiotic factors, including interactions with dietary fiber, altering their bioaccessibility and bioactivity (Rocchetti et al., 2022). Moreover, the antioxidant potential of by-product matrices like fruit peels is determined not only by their overall phenolic content, but also by the particular profile of phenolic compounds present (Ahmadifar et al., 2021), their specific chemical structure and the subsequent interactions with various macromolecules, such as fiber, protein, lipids, and carbohydrates (Leopoldini et al., 2011).

Low-moisture food matrices have been demonstrated to be less prone to oxidation over time and less affected by other abiotic factors such as temperature (Barden and Decker, 2016). However, this study revealed a notable increase in lipid oxidation after storage, indicating that storage time still seems to be a pro-oxidant factor that clearly affects the antioxidant properties of aquafeeds. Nevertheless, present results reinforce that feed antioxidant properties improve with a concomitant inclusion of vitamin E and a 2% inclusion of pineapple by-products flour, mostly due to increases in the antioxidant capacity of DPPH$^{\bullet}$ of free phenolic extracts of diets stored at 4 °C, as well as fibre-bound phenolics extracts in terms ABTS$^{\bullet+}$ and ORAC at both storage times. While it is widely acknowledged that temperature plays a pivotal role in lipid stability, surprisingly, refrigerated storage at 4 °C did not significantly hinder lipid peroxidation compared to storage at 25 °C, highlighting the fundamental role of other abiotic factors in the lipid peroxidation process in low-moisture food matrices. Overall, a refrigerated storage of feeds might present as undesirable from an economic standpoint, as a refrigerated storage of feeds would place an economic burden that might not be economically viable. It is also important to note that, in this study, the variables of air exposure and light exposure were eliminated, both of which are deciding factors in oxidation reactions that could possibly be catalyzed by temperature (Gumus and Decker, 2021). Nonetheless, data from this work did not show evidence of increased lipid peroxidation in diets after storage at both temperatures, meaning that further research is required in order to lessen the antioxidant potency of these diets during storage. However, while experiments with lower temperatures such as -20 °C or -80 °C could present improvements in the antioxidant capacity preservation of these feeds, as these temperatures are better for maintaining the biochemical structure of natural antioxidants (Hwang and Yeom, 2019), additional studies on the economic viability of this storage temperatures would be required.

Literature concerning the inclusion of fruit by-products in aquafeeds is scarce and shows differing results regarding their impact on fish growth. These differences hinge on various factors such as species, the specific fruit by-product used, method and percentage of inclusion (Al-Khalaifah et al., 2020; Hamed and Abdel-Tawwab, 2021; Lizárraga-Velázquez et al., 2019; Lopes et al., 2020; Salem et al., 2019). In the present study, the experimental diets did not significantly affect fish growth performance, feed intake or whole-body composition, demonstrating that a 2% inclusion of the pineapple flours in aquafeeds for European sea bass were well-accepted by fish. The supplementing of European sea bass diets with 100 mg kg^{-1} of vitamin E led to no differences in terms of exogenous antioxidant response, as none of the analyzed enzymes or glutathione in VITE-fed fish showed significant differences when compared to the CTRL group. However, fish fed with VITE exhibited significantly less lipid peroxidation values in both liver and muscle when compared to CTRL, meaning that the added vitamin E acted as an exogenous antioxidant source to lessen lipid peroxidation, hence the lack of necessity to produce more antioxidant enzymes and/or glutathione to reach lower levels of lipid peroxidation (Kurutas, 2015). Indeed, extensive research has demonstrated that α-tocopherol exhibits remarkable efficacy in scavenging ROS (Tucker and Townsend, 2005), since by reacting with peroxyl radicals ($LOO^{\bullet}$), α-tocopherol effectively disrupts the chain reactions that lead to lipid and protein peroxidation (Bouayed and Bohn, 2010), ultimately safeguarding against the oxidation of proteins and lipids. Consequently, synthetic vitamin E is widely used as an antioxidant in aquaculture, particularly as an alternative to ethoxyquin, which was banned in the EU (European Comission, 2022a). Recommended vitamin E doses in fish dietary formulations vary between 25 and 200 mg kg^{-1}, contingent upon the species and maturation state (NRC, 2011). Despite the demonstrated importance of vitamin E supplementation in the development of European sea bass larvae (Betancor et al., 2011), limited data exist regarding its antiperoxidative effects in commercially sized fish. Gatta et al. (2000) proposed that diets for European sea bass (IBW = 208 g) should contain α-tocopherol levels ranging from 254 mg kg^{-1} to 942 mg kg^{-1} to effectively mitigate lipid oxidation. However, previous work from our research team showed no differences in market-sized European sea bass antioxidant system when fed with either 100 or 500 mg kg^{-1} (Pereira et al., 2022a). This evidence served as a basis for the vitamin E inclusion percentage used in the present study. Indeed, based on the rich antioxidant profile and quantities present in pineapple, the concomitant inclusion of 2% of pineapple by-product flour and 100 mg kg^{-1} was performed in this work with the purpose of offering additional antioxidant and immune system protection to farmed fish beyond a standard 100 mg kg^{-1} vitamin E supplementation.

Indeed, when antioxidants are included in animal feeds, many external factors impact their antioxidant effectiveness and bioavailability within the organism (Arfaoui, 2021).

Subsequently, this impacts their capacity not only to act as exogenous antioxidant sources, but also to upregulate the innate antioxidant system defenses of farmed fish, i.e. production of antioxidant enzymes (Arfaoui, 2021). Specifically, enzymes such as CAT and SOD play a crucial role in converting harmful free radical molecules into non-damaging substances (Kurutas, 2015). Thus, the increased production of CAT and SOD observed in the liver of fish fed the with the pineapple stem diet (S2) diet suggest that S2 might induce additional intracellular oxidative stress, necessitating further production of antiperoxidative enzymes to maintain lipid oxidation levels. Additionally, the concentration of carbonyl compounds was higher in the S2 fish, indicating that the increase CAT and SOD activity was insufficient to prevent the oxidative damage of proteins promoted by ROS (Kurutas, 2015).

In this work, the activity of SOD, GR, TG and GSSG exhibited distinct changes following the stress challenge, which confirms fish physiological response towards an acute stress. Additionally, the liver's TAC was also impacted by stress, suggesting that intracellular molecules possessing antioxidant properties were likely oxidized due to increased formation of ROS (Kurutas, 2015). However, no differences in protein oxidation and lipid peroxidation were observed between the non-stressed and stressed groups, further indicating that the natural antioxidants present in these by-products were not potent enough to provide additional antioxidant protection to fish when compared to those fed the positive control diet (VITE) (Bouayed and Bohn, 2010). In terms of differences in the “Diet” variable, the liver activity of SOD in fish fed the S2 diet was higher when compared to those on the CTRL diet, while lipid peroxidation was lower in the S2 group. This is in concordance with findings from other studies, indicating the increased requirement for higher concentrations of enzymes to counter lipid peroxidation (Resende et al., 2023). However, the P2 diet yielded similar results to VITE in terms of CAT activity, suggesting that it did not provide significant benefits to the fish. GR enables the regeneration of GSH from GSSG, thereby aiding in the regulation of oxidative stress (Kurutas, 2015). The results demonstrate that differences in GSSG between non-stressed and stressed groups affected the overall levels of TG. Namely, this decrease in GSSG in the stressed group seems to be responsible for the decrease in TG in fish liver. Since GSH is a necessary cofactor for many antioxidant enzymes such as GPx and GST, while also possessing direct antioxidant and ROS-chelation properties (Hamed et al., 2004), the surge of intracellular ROS likely necessitated a higher production of GSH through the reduction of GSSG mediated by GR (Kurutas, 2015). Non-enzymatic TAC exhibited differences between diets, with the S2-fed fish displaying significantly higher TAC than CTRL fish. This tendency suggests that fish fed with the S2 diet may, to some extent, exhibit enhanced non-enzymatic antioxidant capacity in the liver. Despite this, findings from this study reveal that fish fed with different diets exhibited variations in LPO and protein oxidation (CC). Specifically, none of the experimental diets showed significantly lower levels

LPO and CC than fish fed with the positive control (VITE), indicating that, ultimately, the addition of pineapple by-products did not provide additional antioxidant protection to fish liver. Meanwhile, in fish muscle, the non-stressed group displayed higher $ABTS^{\bullet+}$ radical scavenging capacity, with lipid peroxidation following a similar trend. But the overall values were lower, implying that the stressed group experienced a depletion of exogenous antioxidants to reduce lipid peroxidation.

Overall, and similarly to data from the liver, inclusion of pineapple peel and stem flours in aquafeeds did not enhance fish liver antioxidant defenses or muscle antioxidant capacity. Indeed, given that results from this show evidence of modulation of liver antioxidant activity in the P2 and S2 diets when compared to VITE, the fact that no beneficial physiological effects were observed may be due to the percentage inclusion of pineapple by-products being too low to positively influence the antioxidant system of the fish. However, some negative effects were identified in fish fed with diets where natural antioxidants from pineapple were added to the VITE mix. The fact that the P2 group showed higher liver lipid oxidation than the VITE group, along with the fact that the S2 group showed higher rates of protein oxidation and required a higher production of CAT to achieve the same levels of lipid oxidation than VITE, implies that a higher inclusion percentage could also negatively impact the liver antioxidant system of fish fed with experimental diets containing natural antioxidants from pineapple by-products. Inversely, Guardiola et al. (2016) reported favorable results in a trial with European sea bass using diets with date palm extract inclusion, namely an increased antioxidant potential in plasma and an upregulation of genes associated with antioxidant enzymes in gills and head kidney. This was achieved using a higher inclusion level (10%, 100 g kg^{-1} of feed) when compared to the 2% of inclusion standard used in this study. In another study using zebrafish (*Danio rerio*) fed diets with mango peel inclusion (5-20%, 50-200 g kg^{-1} of feed), no differences were observed in terms of the activity of some antioxidant enzymes, as GPx and SOD remained unaffected (Lizárraga-Velázquez et al., 2019). Nonetheless, CAT activity rose at 200 g kg^{-1}, while lipid peroxidation showed no differences at that inclusion level (Lizárraga-Velázquez et al., 2019), possibly meaning that the organism required a higher CAT activity in order to maintain cell homeostasis, when compared to the control (Kurutas, 2015). Moreover, in that same study, a higher level of whole body lipid peroxidation was observed at 100 g kg^{-1} inclusion, while antioxidant enzyme activity remained unaffected (Lizárraga-Velázquez et al., 2019). In tambaqui (*Colossoma macropomum*) fingerlings, inclusion of lemon peel extract (0.025-2%, 0.25-2 g kg^{-1}) improved the condition factor, the survival and the activity of SOD in liver (Lopes et al., 2020), which is expected, since extracts are typically more concentrated than whole fruits in terms of antioxidant compounds content (Arfaoui, 2021; Brglez Mojzer et al., 2016), despite lacking economic viability in a realistic aquaculture

scenario due to processing costs. Inversely, palm oil inclusion (1.5-6%, 15-60 g kg^{-1} of feed) in diets for African catfish (*Clarias gariepinus*) lowered the lipid peroxidation in muscle (Ng et al., 2004). However, this may be caused by the very high amounts of vitamin E present in palm oil, which is one of the most abundant natural sources of vitamin E, being rich in tocotrienols and tocopherols in amounts up to 800 mg kg^{-1} (Sen et al., 2010).

When fish face acute stress, cortisol stimulates glycogenolysis and gluconeogenesis, raising glucose levels to provide sufficient energy to cope with the metabolic demands resulting from stress (Martinez-Porchas et al., 2009). In addition, during stress increased oxygen demand can lead to cellular hypoxia, causing an increase in circulating lactate levels (Fanouraki et al., 2011). Indeed, the primary source of energy for fish to withstand unfavorable conditions is blood glucose, also making it an efficient indicator of stress (Al-Khalaifah et al., 2020). This response of increased cortisol, glucose and lactate levels was verified in all treatments after 2-hours of recovery from the induced stress challenge. However, the inclusion of 2% pineapple peel and stem flour had no impact on plasma stress biomarkers, evidencing no physiological benefit in an acute stress situation.

Regarding plasma metabolites, Di Marco et al. (2008) reported that elevated plasma NEFA levels are associated with the mobilization and subsequent oxidation of lipid reserves due to increased metabolic demand. Moreover, this rise in NEFA levels, as suggested by Shearer et al. (2012), might be originated from sources like mesenteric fat or hepatic tissue, pointing toward the activation of transcription factors regulating metabolic pathways and nutrient transport. Such regulation potentially influences the plasma levels of NEFA, triglycerides, and cholesterol. Although no differences were observed in terms of triglycerides and cholesterol, our results evidenced a slight tendency to decrease post-stress NEFA levels in fish fed with the P2 and S2 diets, whereas these levels increased in both control groups. This aforementioned tendency for decreasing NEFA's in the plasma of fish fed with P2 and S2 points towards the possibility that less NEFA mobilization, and therefore less energy, is required in order to ameliorate the negative effects of acute stress (Di Marco et al., 2008). Indeed, this lipid-lowering tendency may be attributed to the effects of exogenous antioxidants, including phenolic compounds, which deter lipid peroxidation and the generation of lipo-peroxides, known contributors to atherosclerosis (Al-Khalaifah et al., 2020). Indeed, lipid lowering activities in fish metabolism via inclusion of natural antioxidant sources have also been reported via inclusion of Doum palm fruit powder (Al-Khalaifah et al., 2020), clove basil leaf extract (Abdel-Tawwab et al., 2018a) and Aloe vera polysaccharides (Gabriel et al., 2019). Further experimentation with higher inclusion percentages of pineapple by-products, or even different methods of inclusion within the context of economic viability, could possibly produce more favorable outcomes in terms of acute stress response. Finally, at the immunological level, significant differences were

observed in the activity of ACH50 and total peroxidase activity between the stressed and non-stressed groups, as expected in response to stress (Tort, 2011). However, the absence of significant differences between the diets indicates that the immune response was not reinforced by them.

5. Conclusions

The present study shows that an inclusion of 2% pineapple by-product flour (either from peels or stems) has a positive effect in the antioxidant capacity of the feed when used concomitantly with vitamin E, mainly shown by the increases in free and bound phenolic extract ORAC, as well as bound phenolic extract $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ antioxidant capacity. However, and while storage time was proven to affect the lipid oxidation levels of the diets after 81 days, no differences were observed with varying storage temperatures (4 and 25 °C), mainly due to the type of feed matrix used in this study. This proves that, in terms of avoiding feed oxidation, storage can be performed at room temperature without the need for refrigeration and its associated costs.

Concerning the *in vivo* trial with European sea bass juveniles, while all experimental diets modulated the fish antioxidant defenses to some extent when compared to CTRL, the VITE diet produced the most favorable results. This reinforces the importance of incorporating vitamin E in fish feeds, since none of the diets containing natural antioxidant showed increased benefits in terms of fish antioxidant defenses or overall stress resistance compared to the VITE diet. Further research is, however, needed to optimize the production process of flours derived from by-products to maximize the levels of antioxidants retained after processing. This could involve experiments with different inclusions percentages, different antioxidant sources and diverse food matrices. Such studies are crucial for achieving a successful upregulation of the antioxidant system of fish, in order to improve their resistance to stress, subsequently improving their disease resistance and overall health, possibly leading to benefits in terms of production quantity and quality in the aquaculture sector in a sustainable manner within the context of a circular economy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements and Funding

The authors would like to thank AgroGrIN Tech® (Portugal) for manufacturing the pineapple by-product flours. This work was funded by project “ATLANTIDA— Platform for the monitoring of the North Atlantic Ocean and tools for the sustainable exploitation of the marine resources.” (NORTE-01-0145-FEDER-000040) by CCDRN, within the context of the EU Norte 2020 Operational Program. The doctoral scholarship of Ricardo Pereira was financed by Fundação para a Ciência e Tecnologia (FCT, Portugal) through the grant SFRH/BD/144631/2019.

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Chapter 4.

Comparative Analysis between Synthetic Vitamin E and Natural Antioxidant Sources from Tomato, Carrot and Coriander in Diets for Market-Sized *Dicentrarchus labrax*

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Adapted from Antioxidants (2022) 11(4), 636

https://doi.org/10.3390/antiox11040636

Abstract: Synthetic vitamin E is commonly used in aquafeeds to prevent oxidative stress in fish and delay feed and flesh oxidation during storage, but consumers' preferences tend towards natural antioxidant sources. The potential of vegetable antioxidants-rich co-products, dried tomato (TO), carrot (CA) and coriander (CO) was compared to that of synthetic vitamin E included in diets at either a regular (CTRL; 100 mg kg^{-1}) or reinforced dose (VITE; 500 mg kg^{-1}). Natural antioxidants were added at 2% to the CTRL. Mixes were then extruded and dried, generating five experimental diets that were fed to European sea bass juveniles (114 g) over 12 weeks. Vitamin E and carotenoid content of extruded diets showed signs of degradation. The experimental diets had very limited effects on fish growth or body composition, immunomodulatory response, muscle and liver antioxidant potential, organoleptic properties or consumer acceptance. Altogether, experimental findings suggest that neither a heightened inclusion dose of 500 mg kg^{-1} of vitamin E, nor a 2% inclusion of natural antioxidants provided additional antioxidant protection, compared to fish fed diets including the regular dose of 100 mg kg^{-1} of vitamin E.

Keywords: Circular economy; European sea bass; Functional aquafeeds; Natural antioxidants; Vitamin E; Carotenoids; Polyphenols; Antioxidant activity

1. Introduction

Certain aquaculture practices, such as overcrowding, periodic handling, sudden changes in temperature and poor nutritional quality, generate stress in reared fish (Tort, 2011). These stress situations could become ameliorable through the action of exogenous antioxidants, as their intake may mitigate oxidative damage by inhibiting the initiation or propagation of oxidative chain reactions (Baiano and Del Nobile, 2016; Halliwell and Gutteridge, 2015a). Moreover, aquafeeds are usually rich in long chain polyunsaturated fatty acids (LC-PUFA), making them highly susceptible to lipid oxidation (NRC, 2011). In fish fillet, rancidity and fatty acid depletion are also promoted by the presence of highly unsaturated fatty acids, leading to a deterioration of fillet quality over time, albeit at different levels depending on species, age and diet composition (Taşbozan and Gokce, 2017). Thus, maintaining taste, colour, texture and freshness during storage is also a prime concern of the aquaculture industry (Dominguez et al., 2019).

Given the increasing importance of aquaculture production, which represents 53% of total fish supply for human consumption (FAO, 2020), the aforementioned issues associated with oxidative stress generate a rising necessity for the development of novel functional diets with physiological benefits beyond those provided by traditional feeds, particularly antioxidant benefits (Encarnação, 2016; Olmos-Soto, 2015). Due to their radical-

scavenging properties and/or capacity of antioxidant system modulation, synthetic antioxidants are often used as additives for improving fish resistance to oxidative stress, as well as avoiding oxidative rancidity of fats and oils in feed mixtures (Aklakur, 2018; Bharathi et al., 2019). Synthetic antioxidant compounds such as ethoxyquin, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are amongst the most widely used antioxidants in food and feed formulations (NRC, 2011). In 2017, the common use of ethoxyquin was banned in raw materials, feed premixes, additives and food produced and/or commercialized in the European Union (EU 2017/962). This occurred due to possible genotoxicity effects of one of its metabolites (ethoxyquin quinone imine), as well as the fact that p-phenetidine, an impurity of ethoxyquin, is recognised as a possible mutagen (European Comission, 2017). Meanwhile, BHT (E321) and BHA (E320) are both widely used as antioxidants in feed for all animal species, with a maximum limit of 150 mg kg^{-1} each, or in combination, authorised in the EU (European Comission, 2004). Nevertheless, BHT has been found to inhibit humoral immune response in animals and to possess tumour promotion effects, and despite not being genotoxic, it modifies the genotoxicity of other agents (Lanigan and Yamarik, 2002). This, together with the proven effectiveness of natural antioxidants for inhibiting rancidity in food (Kulawik et al., 2013) and in providing health advantages for consumers (Pezeshk and Alishahi, 2015; Reverter et al., 2014), prompted the scientific community and the feed industry to look for natural alternatives with strong anti-oxidant activity for inclusion in animal feeds (Bai et al., 2015; Ehsani et al., 2018; Elseady and Zahran, 2013; Encarnação, 2016; Kousoulaki et al., 2015).

Vitamins, carotenoids and phenolic compounds are antioxidant molecules commonly present in fruits, herbs and vegetables such as tomato, carrot and coriander (Laribi et al., 2015; Martí et al., 2016; Sun et al., 2009). Specifically, vitamin E is a generic term commonly used for describing eight different lipid-soluble antioxidant forms (tocopherols and tocotrienols), the form of α-tocopherol having the greatest impact on the fish antioxidant system (Udo and Afia, 2013). These compounds are easily oxidized due to their inherent hydroxyl moiety on carbon 6, which has an important role in the maintenance of normal metabolic processes and physiological functions in the body, mainly due to its radical-scavenging properties (Blaner, 2013; Sen et al., 2006; Traber and Atkinson, 2007), thus playing an important role in the protection of cell membranes from lipid peroxidation (Aggarwal et al., 2010; Halver, 2003). Vitamin E also shows a stimulatory effect on fish's immune system, while improving fish health and disease resistance (Cuesta et al., 2001; Lu et al., 2016). This antioxidant compound has been proven to have an essential protective role against the adverse effects of reactive oxygen species and other free radicals (NRC, 2011), which is important for quality preservation of fish fillet, either raw or cooked. To date, the positive influence of vitamin E on seafood quality has been studied in several

commercially reared fish species, such as rainbow trout (*Oncorhynchus mykiss*) (Kamireddy et al., 2011), turbot (*Scophthalmus maximus*) (Ruff et al., 2003) and European sea bass (*Dicentrarchus labrax*) (Gatta et al., 2000). The minimum recommended doses of vitamin E in fish dietary mixes range from 25 to 200 mg kg^{-1}, depending on the species and maturation state (NRC, 2011). Although vitamin E supplementation has been demonstrated as essential for the development of European sea bass larvae (Guerriero et al., 2004), very few data are available regarding the antiperoxidative effects of vitamin E in commercial-sized European sea bass. Gatta et al. (2000) suggested that the α-tocopherol content of diets for European sea bass (208 g) should be above 254 mg kg^{-1}, and up to 942 mg kg^{-1}, in order to reduce lipid oxidation.

Phenolic compounds and carotenoids are also associated with a wide range of biological activities, including antioxidant properties that contribute both directly and indirectly to the inhibition or suppression of oxidation processes (Chakraborty and Hancz, 2011; Pezeshk and Alishahi, 2015). Carotenoid-rich diets are effective in terms of preventing oxidative stress and enhancing innate immune system defences. Ehsani et al. (2018) found that dietary lycopene, naturally abundant in carrots and tomatoes, is effective in preventing lipid oxidation in rainbow trout, subsequently delaying fillet rancidity. β-carotene, found in high doses in carrot and coriander acted as an antioxidant and immunostimulant, mitigating the negative effects of peroxide radicals in Nile tilapia (*Oreochromis niloticus*) (Elseady and Zahran, 2013). Both synthetic and natural carotenoid dietary inclusion has been proven to stimulate antioxidant potential in European sea bass (Goda, A. et al., 2018; Sallam et al., 2018), while immunomodulatory effects were reported with the dietary inclusion of carotenoid rich sources such as seaweed (Peixoto et al., 2016).

Annual production of plants and aromatic herbs has passed 600 million tonnes per year (Leser, 2013), generating a high amount of co-products that are mostly wasted. Despite viability for consumption, about a third of total farm production in the EU is discarded, mostly due to cosmetic and retailers' standards (Porter et al., 2018). These otherwise discarded co-products can be valuable sources of nutrients, antioxidants and bioactive compounds for aquafeeds that at a reduced price may have a positive effect on weight gain, appetite stimulation and overall fish health (Harikrishnan et al., 2012; Pavaraj et al., 2011; Shalaby et al., 2006; Takaoka et al., 2011), as well as in fillet quality traits (Ehsani et al., 2018; Pezeshk and Alishahi, 2015). Adding these vegetable co-products to aquafeeds would also address current consumer trends in terms of preferring natural food sources over synthetic ones (Sanches-Silva et al., 2014), and promote a circular economy approach towards sustainability.

The novel approach of this study consisted of evaluating the potential of vegetable antioxidants-rich co-products as additional antioxidant sources in diets for European sea

bass, a carnivorous fish of high economical value, commonly reared in the Mediterranean region. The effects of such natural antioxidants on European sea bass growth, immune system and fillet quality traits will be compared to those of synthetic vitamin E included in diets at either a regular or reinforced dose (100 and 500 mg kg^{-1}, respectively).

2. Materials and Methods

2.1. Ingredients and Experimental Diets

Fresh carrot (*Daucus carota* subsp. *Sativus*), coriander (*Coriandrum sativum*) and tomato (*Solanum lycopersicum*) biomass were obtained from VITACRESS PORTUGAL, S.A., one of Europe's leading companies in the delivery of fresh, washed and ready-to-eat vegetables. Vegetables were lyophilized, packed in vacuum and stored under refrigeration at −20 °C, until use. Proximate composition, main carotenoids, vitamin E and antioxidant potential are described in Table 1. A commercial-based diet for European sea bass was formulated relying on practical ingredients (Table 2) and supplemented with a commercial vitamin premix that contained a regular dose of vitamin E (α-tocopherol, 100 mg kg^{-1}) as part of the vitamin and mineral premix. This diet was used as a negative control (CTRL) and compared with a positive control diet (VITE) containing an additional amount of added α-tocopherol (Lutavit® E50), totalling 500 mg kg^{-1}. The three experimental diets were obtained after adding 2% of each dried biomass from the three natural antioxidant sources to the CTRL mix, at the expense of wheat meal: carrot (CA), tomato (TO) and coriander (CO). Diets were grounded, extruded (110 °C, originating 4 mm pellets) and dried (60 °C until <8% of moisture) by SPAROS, LDA. (Portugal). All diets were kept isoproteic (49% of dry matter, DM), isolipidic (18% DM) and isoenergetic (23 kJ g^{-1} DM). At the beginning of the trial, 10 g of each experimental diet and ingredient were frozen at −80 °C for proximate composition analysis and antioxidant profile, including carotenoid quantification and profile, vitamin E and hydrolysable polyphenol quantification, as well as antioxidant potential through the radical scavenging potential of 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid ($ABTS^{\bullet+}$) and 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$).

Table 4.1 – Proximate composition, carotenoids, vitamin E and antioxidant potential of ingredients

	Ingredients		
	Tomato	**Carrot**	**Coriander**
Proximate composition			
Dry matter (DM)	77.3 ± 1.3	83.3 ± 1.3	93.4 ± 1.6
Crude protein	16.6 ± 0.4	12.4 ± 0.2	38.3 ± 0.1
Crude fat	3.9 ± 0.01	0.9 ± 0.11	3.2 ± 0.12
Ash	10.4 ± 0.7	7.2 ± 0.04	18.1 ± 0.7
Gross Energy	21.0 ± 0.03	18.3 ± 0.03	17.5 ± 0.01
Main carotenoids [1]			
α-carotene	BQL	4.7 ± 0.3	14.6 ± 0.6
β-carotene	BQL	BQL	57.3 ± 13.1
Lutein	1.1 ± 0.2	BQL	124.5 ± 2.2
Lycopene	18.3 ± 3.6	-	-
β -cryptoxanthin	-	-	0.6 ± 0.04
Vitamin E [2]			
α-tocopherol	32.6 ± 4 [b]	10.5 ± 3.2 [c]	48.3 ± 1.9 [a]
Phenolic content [2]			
Total polyphenols	729.6 ± 20.6 [b]	2478.7 ± 154.9	528.3 ± 27.6 [c]
Antioxidant potential [2]			
$ABTS^{\bullet+}$	62.0 ± 3.6 [c]	104.9 ± 2.8 [b]	115.2 ± 0.2 [a]
$DPPH^{\bullet}$	21.0 ± 0.9 [c]	47.2 ± 4.2 [b]	74.2 ± 0.2 [a]

Values are presented as mean ± standard deviation (n = 9). Different superscript letters within each row indicate significant differences ($p < 0.05$). [1] "BQL" stands for "below quantification limit". The quantification limit for lutein was 5.9×10^{-6} mg per 100 g of DM, α-carotene was 1.3×10^{-5} mg per 100 g of DM and β-carotene was 1.1×10^{-5} mg per 100 g of DM. [2] Vitamin E (α-tocopherol) is expressed in mg kg^{-1} DM. Total polyphenols are expressed in g of gallic acid equivalents (GAE) per 100 g DM. $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ are expressed in mg of TE per 100 g DM.

2.2. Growth Trial and Fish Rearing Conditions

The fish trial and all animal procedures were subject to an ethical review process carried out by CIIMAR animal welfare body (ORBEA-CIIMAR_18_2017) and further approved by the Portuguese Veterinary Authority (1005/92, DGAV-Portugal), in compliance with the guidelines of the European Union (directive 2010/63/UE). European sea bass juveniles obtained from a commercial fish farm (ACUINUGA, A Coruña, Spain) were transported to the Fish Culture Experimental Unit of CIIMAR (Matosinhos, Portugal). Fish were kept in

quarantine for two weeks. After acclimation, fish were fasted for 24 h, anesthetized with 2-phenoxyetanol (200 µL L^{-1}), and individually weighed (g) and measured (total length, cm). Homogeneous groups of 20 fish (initial body weight of 114 ± 0.2 g; initial length of 22 ± 0.1 cm) were randomly distributed by 250 L fibreglass tanks within a saltwater recirculation system with water flow rate adjusted to 16 L h^{-1}. Water quality parameters were continuously monitored, and temperature was maintained at 22 ± 1.0 °C, salinity at 35 ± 0.5‰ and water oxygen levels at a minimum 90% saturation. Redox potential, pH levels and salinity were measured daily. Total ammonium, nitrate and nitrite were monitored twice a week and maintained at levels ≤ 0.05 mg L^{-1}, ≤0.5 mg L^{-1} and ≤50 mg L^{-1}, respectively, as is recommended for marine species (Blancheton, 2000). A 12 h photoperiod was settled. Each experimental diet was tested in triplicate tanks, and fish were manually fed until apparent visual satiation, twice a day for 87 days.

Ten fish from the initial stock and five fish per tank by the end of the feeding trial were sacrificed by an anaesthetic overdose (2-phenoxyethanol, 500 µL L^{-1}) and stored at −80 °C for assessing whole-body composition. An intermediate weighting was performed mid-trial in order to assess fish growth and feed conversion ratio (FCR). At the end of the growth trial, after a 24 h fasting period, fish were slightly anesthetized with 2-phenoxyetanol (200 µL L^{-1}) and were individually weighed (g) and measured (total length, cm) for determination of growth rate. Blood was collected from four fish per tank, taken from the caudal vein using heparinized syringes, and centrifuged (10,000× *g* for 5 min at 4 °C); the resulting plasma was stored at −80 °C for analysing innate immune parameters. These four fish were then sacrificed by a sharp blow on the head, after which intestine and liver were weighed for determination of the viscerosomatic and hepatosomatic indexes. Afterwards, liver and left dorsal muscle samples from each fish were taken, immediately frozen in liquid nitrogen, and kept at −80 °C until analyses. Oxidative stress enzyme activity, lipid peroxidation and total antioxidant capacity, as well as chemical composition parameters, namely moisture, total lipids and ash, were performed in the liver; while left dorsal muscle was sampled for vitamin E quantification, antioxidant potential ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$), lipid peroxidation and chemical composition analyses. Additionally, the right dorsal fillet was collected for immediate instrumental colour analyses of skin and muscle. Instrumental determination of right dorsal muscle texture was also performed at this time point (Day 0).

Table 4.2 – Ingredients, proximate composition and antioxidant potential of the experimental diets

	Dietary Treatments [1]				
	CTRL	**VITE**	**TO**	**CA**	**CO**
Ingredients					
Fishmeal super prime	10.0	10.0	10.0	10.0	10.0
Porcine blood meal	2.5	2.5	2.5	2.5	2.5
Poultry meal	5.0	5.0	5.0	5.0	5.0
Soy protein concentrate	16.0	16.0	16.0	16.0	16.0
Wheat gluten	9.5	9.5	9.5	9.5	9.5
Corn gluten	7.0	7.0	7.0	7.0	7.0
Soybean meal 48	10.0	10.0	10.0	10.0	10.0
Rapeseed meal	5.0	5.0	5.0	5.0	5.0
Wheat meal	16.5	16.4	14.5	14.5	14.5
Fish oil	5.1	5.1	5.1	5.1	5.1
Rapeseed oil	9.4	9.4	9.4	9.4	9.4
Soy lecithin	0.2	0.2	0.2	0.2	0.2
Vitamin and mineral Premix [2]	0.2	0.2	0.2	0.2	0.2
Lutavit E50	0.0	0.08	0.0	0.0	0.0
Brewer's yeast	2.5	2.5	2.5	2.5	2.5
MAP	0.9	0.9	0.9	0.9	0.9
DL-Methionine	0.2	0.2	0.2	0.2	0.2
Tomato	-	-	2.0	-	-
Carrot	-	-	-	2.0	-
Coriander	-	-	-	-	2.0
Chemical composition [3]					
Dry matter	91.2	92.3	95.8	93.2	95.8
Crude protein	49.3	49.4	49.5	49.5	49.8
Crude fat	18.2	18.2	18.2	17.9	18.3
Ash	5.0	5.0	5.1	5.1	5.3
Gross Energy	23.4	23.4	23.6	23.4	23.5
Phosphorous	0.9	0.9	0.8	0.9	0.8
Main Carotenoids [3]					
Lutein	1.5 ± 0.1^{ab}	0.6 ± 0.2^{c}	1.8 ± 0.2^{a}	1.0 ± 0.1^{bc}	1.7 ± 0.05^{a}
β-cryptoxanthin	BQL	BQL	BQL	BQL	BQL
Vitamin E [4]					
α-tocopherol	23.7 ± 1.4^{b}	126.0 ± 18.3^{a}	30.5 ± 3.9^{b}	25.2 ± 0.3^{b}	25.4 ± 3.3^{b}
Phenolic content [4]					
Total polyphenols	513.6 ± 24.5^{b}	459.7 ± 31.0^{b}	537.9 ± 27.8^{b}	1179.1 ± 103.4^{a}	536.0 ± 26.1^{b}
Antioxidant potential [4]					
$DPPH^{\bullet}$	14.3 ± 0.3^{b}	13.7 ± 0.4^{b}	11.4 ± 0.6^{c}	10.6 ± 0.3^{c}	16.6 ± 1.1^{a}
$ABTS^{\bullet+}$	54.7 ± 1.5	50.3 ± 3.4	55.0 ± 1.2	54.5 ± 2.1	51.4 ± 1.8

Values are presented as mean ± standard deviation (n = 3) when necessary. Different superscript letters within each row indicate significant differences ($p < 0.05$). [1] CTRL, control, vegetable based-diet with 100 mg kg^{-1} of total vitamin E; VIT E, vitamin E, vegetable based-diet with 500 mg kg^{-1} of total vitamin E; CA, TO and CO, 2% of natural antioxidants, carrot, tomato and coriander, respectively, at the expense of wheat meal of CTRL diet. [2] Vitamins are expressed mg or IU per kg of diet: vitamin A (retinyl acetate), 20,000 IU; vitamin D3 (DL-cholecalciferol), 2000 IU; vitamin K3 (menadione sodium bisulfite), 25 mg; vitamin B1 (thiamine hydrochloride), 30 mg; vitamin B2 (riboflavin), 30 mg; vitamin B6 (pyridoxine hydrochloride), 20 mg; vitamin B12 (cyanocobalamin), 0.1 mg; vitamin B5 (pantothenic acid), 100 mg; vitamin B3 (nicotinic acid), 200 mg; vitamin B9 (folic acid), 15 mg; vitamin H (biotin), 3 mg; betaine, 500 mg; inositol, 500 mg; choline chloride, 1,000 mg; vitamin C (stay C), 1,000 mg; vitamin E, 100 mg. Minerals (% or mg/kg diet): Mn (manganese oxide), 9.6 mg; I (potassium iodide), 0.5 mg; Cu (cupric sulphate), 9 mg; Co (cobalt sulphate), 0.65 mg; Zn (zinc oxide), 7.5 mg; Se (sodium selenite), 0.01 mg; Fe (iron sulphate), 6 mg; Cl (sodium chloride), 2.41%; Ca (calcium carbonate), 18.6%; NaCl (sodium), 4%. [3] Chemical composition is expressed in % DM or kJ g^{-1} DM. For carotenoids, "BQL" stands for "below quantification limit". The quantification limit for lutein was 5.9×10^{-6} g per 100 g of DM and 1.3×10^{-12} for

β-cryptoxanthin. [4] Vitamin E (α-tocopherol) is expressed in mg kg^{-1} DM. Total polyphenols are expressed in mg of gallic acid equivalents (GAE) per 100 g DM. $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ are expressed in mg of TE per 100 g DM.

Four additional fish per tank were sacrificed by an anaesthetic overdose using 2-phenoxyethanol, and subsequently stored in Styrofoam boxes with ice and kept at 4 °C, protected from light. After 8 days on ice, the right dorsal fillet was collected for analysing instrumental colour and texture determination. Moreover, similarly to Day 0, right dorsal muscle was sampled for measuring the antioxidant potential ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$) and lipid peroxidation.

Finally, another seven fish per tank were fasted for 48 h, sacrificed by ice slurry and placed in Styrofoam boxes for 24 h before sensory analyses.

2.3. Chemical Composition

Whole fish and fish tissues were ground, homogenised and freeze dried before analyses. Proximate composition was performed in accordance with AOAC methods (AOAC, 2006). All samples were analysed in duplicates for dry matter (DM) (105 °C for 24 h); ash, through muffle furnace combustion at 500 °C (5 h) (Nabertherm L9/11/B170, Bremen, Germany); crude protein (N × 6.25), using a Leco nitrogen analyser (Model FP 528; Leco Corporation, St. Joseph, MO, USA); and crude fat (petroleum ether extraction), using a Soxtec extractor (Model ST 2055 SoxtecTM; FOSS, Hillerod, Denmark), for whole fish. Total lipids were measured in muscle and liver, using a dichloromethane: methanol (2:1 wv^{-1}) extraction followed by gravimetrical quantification (Folch et al., 1957). Total phosphorus content was determined from ashes by digestion at 150 °C in hydrochloric acid, followed by the quantification of phosphates using ammonium molybdate at 75 °C in a water bath and later determination of absorbance at 820 nm according to ISO 13730:1996 (ISO, 1996). Gross energy was determined in an adiabatic bomb calorimeter (Model Werke C2000, IKA, Staufen, Germany).

Carotenoids and α-tocopherol in ingredients and diets were analysed in duplicate, using extracts obtained in accordance with Slavin and Yu (2012), with slight modifications. Briefly, 100 mg of each sample were mixed with 3 mL of ethanol, ground in Ultra-Turrax for 2 min, and mixed with 8 mL of n-hexane, re-ground and centrifuged, after which the supernatant was collected. After this, the supernatant was mixed with 0.1% ascorbic acid (wv^{-1}), vortexed and placed in an 85 °C water bath for 5 min. Afterwards, the mixture was cooled on ice, 5 mL of NaCl 1 M and 8 mL of n-hexane were added, after which the solution was centrifuged at 1,000× *g* for 5 min at 4 °C, and the supernatant collected. After repeating the last step, the final hexane extraction volume was washed with 5 mL of 5% Na_2CO_3 (wv^{-1})

and centrifuged at 1,000× *g* for 5 min at 4 °C. The resulting supernatant was washed with 5 mL of ultrapure water and evaporated under nitrogen gas steam, after which it was dissolved in isopropanol and frozen at −20 °C. This extract was used for carotenoid quantification and identification, performed according to Gómez-García et al. (2021), through HPLC (Waters Series 600, Mildford, MA, USA), using acetonitrile, methanol, dichloromethane, hexane and ammonium acetate (55:22:11.5:11.5:0.02 *v/v/v/v/w*) under isocratic conditions at 1 mL min^{-1} flow rate during 20 min at 30 °C. Injection volume was 50 µL and detection was performed by a 454 nm diode array detector (Waters, Massachusetts, EUA). α-carotene, β-carotene, lutein, lycopene and β-cryptoxanthin were quantified using a pure standard calibration curve expressed as mg 100 g^{-1} DM. Analysis of α-tocopherol in ingredients and diets was performed via chromatography, using a Beckman System Gold® HPLC system (Beckman Coulter, Pasadena, CA, USA) linked to a Waters™ 474 Scanning Fluorescence Detector (excitation wavelength of 290 nm and emission wavelength of 320 nm) with a VARIAN ProStar Model 410 AutoSampler with a normal-phase silica column (Kromasil 60-5-SIL, 250 mm, 4.6 mm ID, 5 µm particle size). The mobile phase was 1% (*vv* $^{-1}$) isopropanol in n-hexane with a flow rate of 1 mL min^{-1}. The total run time was 20 min and the injection volume was 20 µL. Standard curves of peak area vs. concentration were used for each compound quantification.

Total phenolic compounds were determined in ingredients and diets, in duplicate, according to the method described by Xie et al. (2015), with some modifications. Briefly, 1 g of each ingredient and diet was washed with distilled water under stirring for 30 min at room temperature to remove soluble and free phenolic compounds. This extract was centrifuged at 1,000× *g* for 10 min, after which the supernatant was dissolved in pure methanol (5 mL). Measurement was performed according to the Folin–Ciocalteu method at 750 nm (Synergy H1 HU126, Winooski, VT, USA).

2.4. Innate Immune Parameters and Plasma Bactericidal Activity

In fish plasma, the activities of lysozyme, peroxidase and alternative complement pathway (ACH50) were measured using a microplate spectrophotometer (BioTek Synergy HT, Winooski,VT, USA), in triplicate. Lysozyme activity was measured using a turbidimetric assay as described by (Costas et al., 2011). A *Micrococcus lysodeikticus* solution (0.5 mg mL^{-1}, 0.05 M sodium phosphate buffer, pH 6.2) was prepared. Then, 15 µL of plasma was added, in triplicates, to a microplate and 250 µL of the above suspension were pipetted to give a final volume of 265 µL. The reaction occurred at 25 °C, after which absorbance (450 nm) was measured after 0.5 and 4.5 min. A standard curve was generated using a serial

dilution of lyophilized hen egg white lysozyme (Sigma) in sodium phosphate buffer (0.05 M, pH 6.2. The amount of lysozyme in the sample was calculated using the standard curve. Total peroxidase activity (EU mL^{-1} plasma) was measured according to Quade and Roth (1997), adapted by Costas et al. (2011), and was determined by defining that one unit of peroxidase produces an absorbance change of 1 OD. Alternative complement pathway (ACH50) activity was determined based on the lysis of rabbit red blood cells (2.8 × 108 cells mL^{-1}; Probiológica, Belas, Portugal) in the presence of ethylene glycol tetra acetic acid (EGTA; Sigma) and Mg^{2+} ($MgCl_2 \cdot {}^6H_2O$; VWR), as described by Sunyer and Tort (1995). ACH50 units were defined as the concentration of plasma giving 50% lysis of cells.

Plasma levels of IgM were analysed using an enzyme-linked immunosorbent assay (ELISA), in accordance with Costa et al. (2020). Essentially, flat-bottomed 96-well plates were coated overnight with European sea bass plasma, diluted at 1:100 using 50 mM carbonate bicarbonate buffer (pH 9.6). Samples were then blocked with 300 µL powdered low fat milk, diluted at 5% wv^{-1} in T-TBS (20 mM Tris Base, 137 mM NaCl and Tween 20 at 1% vv^{-1}) for 1 h, after which they were cleaned 3 times with T-TBS solution and subsequently incubated for 1 h with 100 µL of primary antibody (mouse anti-European sea bass IgM monoclonal antibody, 1:100 in blocking buffer; Aquatic Diagnostics Ltd., Scotland, UK). Samples were cleaned 3 times with T-TBS solution after incubation, and incubated again with 100 µL of the secondary antibody anti-mouse IgG-HRP (1:1,000 in blocking buffer, Sigma-Aldrich, Darmstadt). After this incubation, samples were cleaned with T-TBS and 100 µL of 3,30,5,50-tetramethylbenzidine hydrochloride (TMB, Sigma—Aldrich, Darmstadt, Germany) at 1 mg mL^{-1} was added. After 5 min incubation, the reaction was stopped with 100 µL of H_2SO_4 2M, and absorbance was measured at 450 nm on a Synergy HT microplate reader (BioTek Synergy HT, Winooski,VT, USA). Negative controls consisted of HBSS instead of plasma. OD values were subtracted for each sample value.

Bactericidal activity in fish plasma was measured according to Costa et al. (2020). *Vibrio anguillarum* (VA) and *Photobacterium damselae* subsp. *piscicida* (Pdp) were cultured in tryptic soy broth (TSB; Difco). For assessing bactericidal activity, 20 µL of plasma was diluted in 20 µL of the previously mentioned bacterial suspensions, in duplicate, at a concentration of 1×10^6 cfu/mL, into a U-shaped 96-well microplate. The resulting mixture was incubated for 3 h at 25 °C with shaking (100 rpm). Afterwards, 25 µL of 3-(4, 5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 1 mg mL^{-1}; Sigma) was added. The solution was then incubated for 10 min at 25 °C with shaking (100 rpm). Plates were centrifuged at 2,000× *g* for 10 min, and the resulting pellet was dissolved with 200 µL of Dimethyl Sulfoxide (DMSO, Sigma—Aldrich, Darmstadt, Germany). The solutions (100 µL) were then placed into a flat-bottomed 96-well microplate and the absorbance was measured at 490 nm using a Synergy HT microplate reader (Biotek, VT, USA). Bactericidal activity was calculated as

the difference between bacterial surviving and positive control (100%). Results are expressed as the percentage of non-viable bacteria.

2.5. Antioxidant Potential and Oxidative Stress

Antioxidant capacity of ingredients, diets, as well as fish muscle, before and after 8 days on ice, was measured through assessment of the radical scavenging potential of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$. For $ABTS^{\bullet+}$, radical scavenging activity was measured in the methanolic extracts using the method described by Sánchez-Moreno (2002) and adapted by Gonçalves et al. (2009). Essentially, using a flat-bottom 96-well microplate, 180 µL of $ABTS^{\bullet+}$ working solution was added to 20 µL of sample (in triplicate). The mixture was incubated for 5 min at 30 °C, protected from light, and the absorbance at 734 nm was measured with a multi-detection plate reader (Synergy H1 HU126, Winooski, VT, USA). The $DPPH^{\bullet}$ assay was performed according to the method of Brand-Williams et al. (1995), with some modifications. The assay was performed in a flat-bottomed 96-well microplate, to 25 µL of the sample, 175 µL of $DPPH^{\bullet}$ working solution was added, in triplicate. In both analytical procedures, Trolox was used for the standard curve and methanol 80% was used for the blanks, as it was the solvent used for the analysed extracts. The mixture was incubated for 30 min at 25 °C, protected from light, and the absorbance was measured at 515 nm with a multi-detection plate reader (Synergy H1 HU126, Vermont, USA). For both analyses, final results were expressed in Trolox equivalents (TE) per 100 g of DM, or µg of TE per g wet weight (ww^{-1}) in the case of fish muscle.

Liver samples of fish fed with the experimental diets were homogenized using phosphate buffer (0.1 M, pH 7.4), in a proportion of 1:10 (*w:v*). After this, samples were centrifuged at 10,000× *g* for 15 min at 4 °C, and the supernatant aliquoted and stored at −80 °C for determining the activity of oxidative stress enzymes. Protein content was determined in accordance with Bradford (1976) for standardizing antioxidant enzyme activity measurements. The following analysis were carried out in triplicates using a microplate reader. Catalase (CAT) activity was measured according to Greenwald (1987), using hydrogen peroxide (H_2O_2) 30% as substrate. Alterations in absorbance were registered at 240 nm, at 25 °C. CAT activity was calculated as µmol of H_2O_2 consumed per min per mg of protein. Glutathione s-transferase (GST) was determined according to Habig (1974). Essentially, total activity (cytosolic and microsomal) was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH). Changes in absorbance were recorded at 340 nm, at 25 °C for 5 min, and enzyme activity was calculated as nmol of CDNB conjugate formed per min per mg of protein. Glutathione

reductase (GR) activity was measured according to Cribb et al. (1989), assessing NADPH disappearance at 340 nm for 3 min at 25 °C, and expressing the results in nmol of oxidized NADPH per minute, per mg of protein. Total glutathione (TG) was evaluated by measuring the formation of 5-thio-2-nitrobenzoic acid (TNB) at 412 nm, as detailed in Baker et al. (1990), with results expressed as nmol of conjugated TNB formed per min per mg of protein. Glutathione peroxidase (GPx) was measured as reported by Mohandas et al. (1984), through an indirect method based on the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx. Reaction was performed at 25 °C with a pH of 8.0, using H_2O_2 as substrate and including sodium azide (NaN_3) as a catalase inhibitor. Oxidation of NADPH was recorded spectrophotometrically at 340 nm at 25 °C, after which the enzyme activity was calculated as nmol of oxidized NADPH per min per mg of protein. Lipid peroxidation (LPO) was determined in concordance with Bird and Draper (1984), by quantifying the presence of thiobarbituric acid reactive substances (TBARS), composed mainly of malondialdehyde (MDA). The decomposition of unstable peroxides derived from polyunsaturated fatty acids (PUFAs) induces the formation of MDA, subsequently quantified colorimetrically following its controlled reaction with thiobarbituric acid (TBA). The absorbance was measured at 535 nm, at 25 °C, and the rate of LPO was expressed as nmol of MDA formed per g of fresh tissue. The concentration of total antioxidant in samples was determined by using the total antioxidant capacity assay kit (Sigma MAK187), by measuring the formation of TE. Results were expressed in nmol per mg of tissue.

Muscle samples were homogenized using phosphate buffer (0.1 M, pH 7.4) in a proportion of 1:10 (w:v) and protein content determined according to Bradford (Bradford, 1976). Lipid peroxidation (LPO) was measured in muscle samples collected on both sampling days (Day 0 and Day 8), using the methodologies described for liver. LPO was expressed as nmol of TBARS formed per gram of fresh tissue, and a comparison of results was performed via two-way ANOVA between fish sampled at the end of the feeding trial and fish stored in ice for 8 days.

2.6. Instrumental Texture and Colour

Skin and muscle colour measurements were performed in two different groups of fish at Day 0 and Day 8, immediately after sampling in fish from Day 0, and after storage on ice for 8 days in fish from Day 8. Measurements were performed with a CR-400 chroma meter (Konica Minolta Inc., Osaka, Japan) with an aperture of 8 mm, with respect to CIE standard illuminant D65. The apparatus was calibrated using a white plate reference standard (Minolta Co, Ltd., Osaka, Japan). Colour parameters were measured by applying the colorimeter onto raw fillets from 12 fish per dietary treatment. Measurements were made in three points above the lateral line of each fillet. After flashing, L^*, a^* and b^* reflected light

values were recorded. From a^* and b^* values the hue angle ($h^* = \tan^{-1} b^*/a^*$) and the chroma ($C^* = (a^{*2} + b^{*2})^{1/2}$) were calculated according to Choubert et al. (1997). The same procedure was applied to fish that were stored in ice for 8 days (Day 8).

Muscle texture was also analysed in fish both before and after a storage period in ice for 8 days using a TA.XT.plus Texture Analyser (Stable Micro Systems Inc., Godalming, United Kingdom) with a 5 kg load cell and a 2.0 mm diameter probe. Texture profile parameters [hardness (N), adhesiveness (J), springiness (-), cohesiveness (-), chewiness (J) and resilience (-)] were obtained by double compression (constant speed and penetration depth of 1 mm s^{-1} 320 and 4.0 mm, respectively) on the thickest part of each raw fillet according to Batista, Sónia et al. (2020). Penetration depth was selected according to the maximum distance that did not induce fibre breakage.

2.7. Consumer Acceptance

For sensory evaluation, European sea bass were sampled and prepared for evaluation after removal of gut and scales. Heads, tails and fins were cut-off and fish were cut into three slices (anterior, middle and posterior) (Figure 4.1a). Each slice was wrapped in micro-perforated aluminium foil and steamed for 12 min at 100 °C, in a preheated industrial forced convector oven with steam (Rational Combi-Master CM61, Rational AG, Germany). Each consumer received slices from the same part of the fish (anterior, middle or posterior) across all samples (Figure 4.1b).

Figure 4.1. (**a**) Fresh sea bass descaled, gutted and sliced; (**b**) Individual slices served to participants after steam cooking wrapped in micro-perforated aluminium foil, presented in preheated white porcelain plates with a random three-digit code

The samples were served over white pre-heated (50 °C) porcelain plates, coded with a three-digit random number. Panellists were provided with cutlery, a glass of bottled natural water and unsalted crackers (Figure 1b). All panellists were asked to chew a piece of cracker and to rinse the mouth with water before testing each sample. To compensate for eventual carry-over effects, each panellist received the set of five samples following a monadic sequential presentation, with their order previously balanced, in accordance with MacFie et al. (1989). For each sample, a total of 60 naïve consumers evaluated overall liking using a 9-point hedonic scale, ranging from 1—"dislike extremely" to 9—"like extremely" (Peryam and Pilgrim, 1957). For each sample, after the overall liking evaluation, each consumer was requested to make a comment regarding the sample, considering the main negative and positive aspects. The panel was recruited from the sensory evaluation company Sense Test's consumer database (Vila Nova de Gaia, Portugal). They were mainly residents in the Oporto metropolitan area, in the North of Portugal. All participants were regular consumers of fish, at least two times per week. Besides the implementation of informed consent, the company ensures the protection and confidentiality of data through the authorization 2063/2009 of the National Data Protection Commission, and following EU Regulation (EU 2016/679), as well as a longstanding internal code of conduct. Sensory evaluation was carried in individual tasting booths in a special room equipped in accordance with ISO 8589:2007—sensory analysis—general guidance for the design of test rooms.

2.8. Calculations

2.8.1. Growth, Intake and Retention

$$ABW = (FBW + IBW)/2$$

$$K = (FBW/\text{final body length}^3) \times 100$$

$$DGI = 100 \times ((FBW)^{1/3} - (IBW)^{1/3})/days$$

$$VFI = 100 \times \text{crude feed intake}/ABW/day$$

$$FCR = \text{dry feed intake}/\text{weight gain}$$

$$(DGI) = 100 \times [(FBW)\ 1/3 - (IBW)1/3]/days$$

$$PER = \text{weight gain}/\text{crude protein intake}$$

Ɛ gain = (final carcass P, L or E content - initial carcass P, L or E content)/ABW/days;

2.8.2. Somatic Indexes

$$HSI = 100 \times \text{liver weight/FBW}$$

$$VSI = 100 \times \text{viscera weight/FBW}$$

ABW—Average body weight	K—Fulton's condition factor
FBW—Final body weight	DGI—Daily growth index
IBW—Initial body weight	VFI—Voluntary feed intake
N—Nitrogen	FCR—Feed conversion ratio
P—Phosphorous	PER—Protein efficiency ratio
L—Lipids	HSI—Hepatosomatic index
E—Energy	VSI—Viscerosomatic index

All calculations were performed according to NRC (2011).

2.9. Statistical Analysis

All statistical analyses were performed with IBM SPSS STATISTICS, 25.0 package (IBM corporation, New York, NY, USA, 2017), with the exception of the correspondence analysis that was performed with XLSTAT v. 2020 (Addinsoft, 2020).

Data were tested for normality and homogeneity of variances by Shapiro-Wilk and Levene's tests, respectively, and transformed whenever required before being submitted to a one-way ANOVA. When this test showed significance, individual means were compared using HSD Tukey Test. When data did not meet the assumptions of ANOVA, a non-parametric test, Kruskal-Wallis test, was performed. When needed, the Mann–Whitney test was carried out to identify significant differences between groups. In all cases, the level of significance was set at 0.05. A two-way ANOVA, with dietary treatment and storage time (0 or 8 days) was used to compare fish muscle antioxidant potential, colour and texture, as well as skin colour.

For overall liking, a three-way mixed effects ANOVA, with panellists as random factor, and fish part (anterior, middle and posterior) and dietary treatment as fixed factors, with no interaction effect, was used to investigate differences between treatments (Lea et al., 1997). For the open comments, a content analysis was performed counting the number of times that each attribute (positive and negative) was mentioned per sample. The frequency of mention of each attribute was determined, calculating the number of consumers who have used each attribute to describe the samples. Over this frequency matrix, a correspondence analysis (CA) was applied. Such analysis provides a sensory map of the samples, allowing

the perception of the similarities and differences between samples and their sensory characteristics (Ares et al., 2010a; Ares et al., 2010b; Ares et al., 2011).

3. Results

3.1. Characterisation of Ingredients and Experimental Diets

Dried tomato, carrot and coriander were shown to be variable sources of α-carotene, β-carotene and lutein (Table 4.1). α-carotene was below the quantification limit in tomato, while β-carotene was below the quantification limit in tomato and carrot. Coriander displayed the highest levels of α-carotene, β-carotene and lutein (14.6, 57.3 and 124.5 mg 100 g^{-1} DM, respectively). Carrot showed the second highest amount of α-carotene (4.7 mg 100 g^{-1} DM), while tomato displayed the second highest amount of lutein (1.1 mg 100 g^{-1} DM). Moreover, certain carotenoids were exclusive to specific vegetables: tomato was the only tested source of lycopene (18.3 mg 100 g^{-1} DM), while coriander was the only ingredient containing β–cryptoxanthin (0.6 mg 100 g^{-1} DM). Concerning vitamin E, coriander was the highest source (48.3 mg α-tocopherol kg^{-1} DM), followed by tomato (32.6 mg kg^{-1} DM) and carrot (10.5 mg kg^{-1} DM).

In terms of total phenolic compounds content, carrot had the highest amount (2478.7 mg GAE 100 g^{-1} DM), followed by tomato (729.6 mg GAE 100 g^{-1} DM) and coriander (528 mg GAE 100 g^{-1} DM) (Table 4.1). However, ingredients' $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ values showed coriander to have significantly higher values (62.0 and 74.2 TE 100 g^{-1} DM, respectively), followed by carrot (104.9 and 47.2 TE 100 g^{-1} DM, respectively) and tomato (62.0 and 21.0 TE 100 g^{-1} DM, respectively).

As shown in Table 4.2, TO and CO diets showed the highest amounts of lutein (1.8 and 1.7 mg 100 g^{-1} of feed, respectively) followed by the CTRL (1.5 mg 100 g^{-1} of feed), CA (1.0 mg 100 g^{-1} of feed) and VITE (0.6 mg 100 g^{-1} of feed) diets. The levels of α-tocopherol quantified in diets, after extrusion, corresponded well to the amounts of synthetic vitamin E included in each experimental diet: VITE diet had five times more α-tocopherol than any other diet. However, quantified values after extrusion were four times lower than those initially added to each diet. The CA diet contained the highest amount of total phenolic compounds (1179.1 mg GAE 100 g^{-1} of DM), followed by CO and TO (536.0 mg and 537.9 GAE 100 g^{-1} of DM, respectively). Out of all diets, the CO diet was shown to have the highest $DPPH^{\bullet}$ scavenging capacity (16.6 1.1 mg TE 100 g^{-1} of feed), in concordance with the higher $DPPH^{\bullet}$ values found in coriander when compared to the remaining ingredients.

3.2. Effects on Growth Performance, Whole Body Composition, Innate Immune Parameters and Plasma Bactericidal Activity

All diets were well accepted by fish, with no significant differences in final body weight, leading to a similar growth performance (DGI) and feed efficiency between groups (Table 4.3). Voluntary feed intake (VFI) was similar between all treatments, as well as final body composition and nutrient gain (Table 4.3). Hepatosomatic and viscerosomatic indexes and liver chemical composition also remained similar among treatments (Table 4.4).

Table 4.3 – Growth and whole-body composition of *Dicentrarchus labrax* fed with the experimental diets for 87 days

	Dietary Treatments [1]					
	CTRL	**VITE**	**TO**	**CA**	**CO**	***P*-Value**
Growth [1]						
Final body weight	239.0 ± 14.3	245.7 ± 19.0	244.8 ± 1.1	247.7 ± 6.4	236.7 ± 18.2	0.8
Final length	27.3 ± 0.1	27.4 ± 0.6	27.5 ± 0.1	27.3 ± 0.2	27.1 ± 0.4	0.7
K_f	1.2 ± 0.1	1.2 ± 0.01	1.2 ± 0.01	1.2 ± 0.01	1.2 ± 0.04	0.1
VFI	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.8
DGI	1.6 ± 0.1	1.6 ± 0.2	1.6 ± 0.02	1.6 ± 0.1	1.5 ± 0.2	0.8
FCR	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.04	1.2 ± 0.04	0.3
PER	1.7 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	0.3
Whole Body Composition [2]						
Moisture	62.3 ± 0.7	62.5 ± 0.9	63.4 ± 0.9	62.1 ± 1.6	62.4 ± 1.2	0.7
Ash	3.5 ± 0.2	3.6 ± 0.3	4.1 ± 0.6	3.5 ± 0.1	4.0 ± 0.1	0.1
Protein	19.2 ± 0.8	19.2 ± 0.1	18.3 ± 0.5	18.7 ± 0.5	18.6 ± 0.5	0.2
Lipids	15.8 ± 1.3	15.9 ± 0.9	15.0 ± 0.8	16.4 ± 2.3	15.6 ± 1.2	0.8
Energy	9.9 ± 0.3	10.1 ± 0.3	9.7 ± 0.1	10.0 ± 0.8	9.8 ± 0.4	0.8
Phosphorus	0.6 ± 0.04	0.6 ± 0.05	0.7 ± 0.1	0.6 ± 0.03	0.8 ± 0.1	0.1
Gain [2]						
Dry matter	3.2 ± 0.3	3.2 ± 0.4	3.1 ± 0.2	3.3 ± 0.4	3.1 ± 0.5	0.9
Protein	1.6 ± 0.01	1.7 ± 0.1	1.5 ± 0.1	1.6 ± 0.04	1.5 ± 0.1	0.2
Lipids	1.5 ± 0.3	1.5 ± 0.2	1.4 ± 0.1	1.6 ± 0.4	1.4 ± 0.3	0.9
Energy	88.3 ± 9.0	94.6 ± 8.8	88.3 ± 1.8	93.6 ± 15.8	86.5 ± 13.1	0.8
Phosphorus	0.03 ± 0.002	0.04 ± 0.01	0.05 ± 0.01	0.03 ± 0.003	0.05 ± 0.03	0.2

For all the items n = 3, except for initial and final body weight, which had n = 60. Values are presented as mean ± standard deviation (n = 12). Different superscript letters within each row indicate significant differences ($p < 0.05$). [1] Initial and final body weight is expressed in grams, and initial and final length are expressed in centimeters. "K_f" stands for Fulton's Condition Index - Final, "DGI" stands for Daily Growth Index, "VFI" stands for Voluntary Feed Intake, "FCR" stands for Feed Conversion Ration, and "PER" stands for Protein Energy Ratio. [2] For whole body composition, moisture, ash, protein, lipids and phosphorous are presented in % of ww^{-1}. Energy is expressed in kJ g^{-1}. For nutrient gain, values are expressed in g or kj ABW kg day^{-1}.

Table 4.4 – Somatic indexes and chemical composition of muscle and liver of *Dicentrarchus labrax* fed with the experimental diets for 87 days

	Dietary Treatments					
	CTRL	**VITE**	**TO**	**CA**	**CO**	***P*-Value**
Somatic Indexes						
Hepatosomatic index	2.2 ± 0.7	2.1 ± 0.5	2.1 ± 0.3	2.1 ± 0.3	2.4 ± 0.8	0.8
Viscerosomatic index	6.1 ± 0.8	6.5 ± 1.1	6.6 ± 0.6	6.4 ± 1.1	5.8 ± 0.7	0.2
Muscle chemical composition [1]						
Moisture	73.7 ± 1.9	72.9 ± 2.3	72.6 ± 0.5	73.5 ± 1.9	73.9 ± 1.0	0.9
Ash	1.4 ± 0.1	1.4 ± 0.01	1.5 ± 0.05	1.5 ± 0.1	1.4 ± 0.03	0.4
Lipid	4.4 ± 1.1	5.8 ± 1.8	5.1 ± 0.2	4.3 ± 1.9	4.2 ± 1.1	0.6
α-tocopherol	5.1 ± 2.2 [b]	13.7 ± 3.2 [a]	5.6 ± 1.6 [b]	4.2 ± 1.1 [b]	3.9 ± 0.7 [b]	**<0.001**
Liver chemical composition [1]						
Moisture	42.4 ± 2.3	46.3 ± 2.0	42.2 ± 1.8	42.5 ± 1.4	42.8 ± 6.3	0.5
Ash	0.8 ± 0.2	0.8 ± 0.03	0.8 ± 0.05	0.9 ± 0.1	0.9 ± 0.1	0.7
Lipid	35.1 ± 8.7	28.9 ± 4.6	33.2 ± 2.7	33.6 ± 4.7	31.2 ± 3.7	0.7

Values are presented as mean ± standard deviation (n = 12). Different superscript letters within each row indicate significant differences ($p < 0.05$). [1] Moisture, lipid and ash are presented in % of ww^{-1}. α-tocopherol is presented in mg kg ww^{-1}.

Moreover, muscle chemical composition reflected synthetic vitamin E inclusion, with muscle from fish fed VITE diet showing significantly higher α-tocopherol values than all remaining diets (13.7 mg kg w w^{-1}; Table 4.4). In fish plasma, innate immune parameters and bactericidal activity did not display any significant differences between dietary treatments (Table 4.5).

3.3. Effects on Liver Oxidative Stress Parameters

Glutathione reductase (GR) activity was significantly higher in CO and CTRL when compared to TO (Table 4.6). Lipid peroxidation (LPO) in fish fed the experimental diets was similar to CTRL; however, CO was significantly higher when compared to VITE.

Table 4.5 – Innate immune parameters of *Dicentrarchus labrax* evaluated after 87 days of feeding the experimental diets

	Dietary Treatments					
	CTRL	**VITE**	**TO**	**CA**	**CO**	***P*-Value**
Lysozyme [1]	822.7 ± 45.9	741.2 ± 48.6	893.3 ± 61.2	865.0 ± 42.9	868.5 ± 65.1	0.3
Peroxidase [1]	617.1 ± 102.0	491.3 ± 77.9	529.5 ± 76.7	523.5 ± 97.5	496.0 ± 93.5	0.9
ACH50 [1]	336.1 ± 21.9	331.7 ± 23.4	302.9 ± 18.6	345.4 ± 23.6	313.8 ± 17.2	0.6
IgM [1]	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.4
Pdp activity [2]	9.5 ± 10.5	13.6 ± 9.6	19.9 ± 13.0	17.1 ± 10.2	5.6 ± 9.6	0.1
VA activity [2]	26.8 ± 8.4	22.1 ± 9.1	26.0 ± 9.0	28.5 ± 8.6	20.8 ± 12.6	0.5

Values are presented as mean ± standard error (n = 12). [1] Lysozyme, peroxidase, ACH50 are presented in EU mL^{-1}; IgM is presented in OD 450 nm. [2] Plasma bactericidal activity of European sea bass fed different dietary treatments against *Vibrio anguillarum* (VA) and *Photobacterium damselae* subsp. *piscicida* (Pdp). Values are present in % of non-viable bacteria. One-way ANOVA was used to test differences. No significant differences observed between different diets ($p > 0.05$).

The activity of the remaining antioxidant enzymes, as well as the glutathione content and total antioxidant capacity (TAC), showed no significant differences between treatments.

Table 4.6 - Enzymatic/non-enzymatic parameters in *Dicentrarchus labrax* liver

	Dietary Treatments					
	CTRL	**VITE**	**CA**	**CO**	**TO**	***P*-Value**
Enzymatic parameters						
CAT	33.5 ± 19.4	43.9 ± 11.3	35.1 ± 17.7	41.2 ± 19.7	32.9 ± 11.4	0.4
GST	158.7 ± 65.4	155.8 ± 64.9	170.3 ± 60.3	232.9 ± 76.3	186.2 ± 82.7	0.1
GR	3.8 ± 1.6 ^{a}	3.5 ± 1.0 ^{ab}	3.6 ± 1.5 ^{ab}	4.1 ± 0.9 ^{a}	2.3 ± 1.0 ^{b}	**0.01**
TG	0.8 ± 0.4	0.8 ± 0.3	1.0 ± 0.4	1.1 ± 0.4	0.7 ± 0.2	0.05
GPx	2.1 ± 0.9	1.6 ± 0.7	1.7 ± 1.1	2.24± 0.6	1.5 ± 0.6	0.2
Non-enzymatic parameters						
LPO	96.4 ± 18.2 ^{ab}	92.1 ± 34.3 ^{b}	109.0± 36.2 ^{ab}	147.7 ± 55.6 ^{a}	114.6± 48.4 ^{ab}	**0.04**
TAC	0.03 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.8

Different superscript letters indicate within each row differences between diets ($p < 0.05$). Glutathione s-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx) and total glutathione (TG) are in nmol min^{-1} mg^{-1} protein, catalase (CAT) is in µmol min^{-1} mg^{-1} lipid peroxidation (LPO) is in nmol MDA g $liver^{-1}$. Total antioxidant capacity (TAC) is calculated in nmol mg $tissue^{-1}$.Values are presented as mean ± standard deviation (n = 12) per dietary treatment.

3.4. Effects on Antioxidant Potential and Lipid Oxidation of Muscle

Despite the fact that muscle from fish fed VITE had five times the amount of α-tocopherol compared to the remaining diets, a two-way ANOVA comparison did not show any discernible effects on muscle antioxidant potential (Table 4.7). No statistical differences between dietary treatments were found for lipid peroxidation, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ in European sea bass muscle. Likewise, no statistical differences could also be perceived in either lipid peroxidation or $ABTS^{\bullet+}$ between Day 0 (fish muscle sampled immediately after the feeding trial) and Day 8 (muscle sampled after 8 days storage on ice). However, $DPPH^{\bullet}$ showed differences between Day 0 and Day 8, with higher values after 8 days on ice.

3.5. Effects on Sea Bass Skin, Fillet Colour and Muscle Texture

The effects of the experimental diets on European sea bass skin and fillet colour and textural properties were evaluated, both before and after 8 days on ice (Table 4.7). The two-way ANOVA showed no statistical differences in skin colour between dietary treatments. However, storage time induced skin colour changes, namely a decrease in yellowness (b^*), chroma (C^*), as well as an increase in hue angle (h^*). Compared to CTRL, muscle from fish fed with the vegetable coproduct diets showed lower b^* values, while fillets from fish fed with CO showed lower C^*, whilst those fed with CA showed higher h^*. After storage time, fish muscle showed higher lightness (L^*) and h^*, as well as lower b^* and C^* values. A two-way ANOVA analysis revealed that all muscle textural parameters suffered considerable

alterations between the first and last day of storage. However, gumminess seemed to be the only parameter modulated by the experimental diets. Namely, CO displayed higher levels of gumminess when compared to TO. However, no experimental diets displayed any significant differences compared to CTRL.

Table 4.7 – Antioxidant potential, skin and muscle colour and texture of *Dicentrarchus labrax* in the first sampling day (Day 0) and after 8 days of storage (Day 8)

	Dietary Treatments [1]												
	Day 0					Day 8					*p-value*		
	CTRL	VITE	TO	CA	CO	CTRL	VITE	TO	CA	CO	Diet	Day	D×D
Muscle antioxidant potential													
DPPH$^{\bullet}$	0.2 ± 0.04	0.1 ± 0.04	0.2 ± 0.05	0.1 ± 0.04	0.1 ± 0.04	0.2 ± 0.1 *	0.2 ± 0.04 *	0.2 ± 0.1 *	0.3 ± 0.1 *	0.3 ± 0.1 *	0.9	**<0.01**	0.5
ABTS$^{\bullet+}$	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.05	0.6 ± 0.1	0.6 ± 0.1	0.9	0.9	0.5
LPO	33.8 ± 3.0	35.3 ± 5.1	35.2 ± 3.4	36.8 ± 6.3	36.5 ± 4.6	34.0 ± 2.7	32.1 ± 2.4	33.9 ± 3.4	31.5 ± 1.7	33.4 ± 2.8	0.4	0.4	0.5
Colour of skin													
*L**	53.7 ± 5.2	53.6 ± 3.4	52.6 ± 4.6	55.3 ± 4	50.2 ± 5.3	52.7 ± 4.6	51.1 ± 3.5	50.8 ± 3.3	50.3 ± 4.0	55.4 ± 2.1	0.7	0.2	0.1
*a**	−4.3 ± 0.5	−4.6 ± 0.4	−4.3 ± 0.7	−4.8 ± 0.7	−4.5 ± 0.4	−4.6 ± 0.7	−4.9 ± 0.4	−4.5 ± 0.7	−4.7 ± 0.4	−4.5 ± 0.3	0.1	0.1	0.4
*b**	7.3 ± 1.9 *	7.4 ± 0.9 *	7.0 ± 1.4 *	8.1 ± 2.8 *	6.7 ± 1.0 *	4.7 ± 2.5	5.7 ± 1.4	5.3 ± 1.8	5.5 ± 0.7	5.3 ± 0.9	0.4	**<0.01**	0.6
*C**	8.5 ± 1.7 *	8.7 ± 0.9 *	8.3 ± 1.3 *	9.5 ± 1.0 *	8.1 ± 0.9 *	6.9 ± 1.3	7.6 ± 1.1	7.1 ± 1.4	7.3 ± 0.2	7.0 ± 0.8	0.2	**<0.01**	0.6
*h**	121.5 ± 5.6 *	122 ± 3.5 *	122.2 ± 5.9 *	124.3 ± 3.5 *	124.3 ± 3.5 *	137.6 ± 20.4	132.6 ± 7.8	132.5 ± 10.3	131.6 ± 6.4	130.9 ± 5.4	0.8	**<0.01**	0.5
Colour of muscle													
*L**	42.8 ± 2.5 *	42.3 ± 2.0 *	42.2 ± 1.8 *	41.5 ± 1.3 *	41.5 ± 1.9 *	43.9 ± 2.28	44.2 ± 1.7	44.1 ± 3.4	43.9 ± 1.6	42.3 ± 2.0	0.1	**<0.01**	0.6
*a**	−2.9 ± 0.5	−3.0 ± 0.6	−2.8 ± 0.4	−2.9 ± 0.5	−2.7 ± 0.5	−2.7 ± 0.72	−3.0 ± 0.7	−2.7 ± 0.7	−2.8 ± 0.7	−2.4 ± 0.7	0.2	0.2	0.9
*b**	4.3 ± 1.1 ^{a*}	3.9 ± 0.8 ^{ab*}	3.7 ± 1.1 ^{b*}	3.9 ± 0.7 ^{b*}	3.6 ± 0.7 ^{b*}	1.4 ± 0.67 ^{a}	0.6 ± 0.6 ^{ab}	0.7 ± 1.2 ^{b}	0.5 ± 0.5 ^{b}	0.5 ± 0.8 ^{b}	**0.01**	**<0.01**	0.8
*C**	5.3 ± 0.9 ^{a*}	4.9 ± 0.6 ^{ab*}	4.7 ± 0.9 ^{ab*}	4.8 ± 0.7 ^{ab*}	4.6 ± 0.6 ^{b*}	3.2 ± 0.45 ^{a}	3.1 ± 0.5 ^{ab}	3.2 ± 0.5 ^{ab}	2.9 ± 0.6 ^{ab}	4.6 ± 0.6 ^{b}	**0.02**	**<0.01**	0.7
*h**	125.0 ± 10.3 ^{b*}	128.9 ± 8.6 ^{ab*}	128.7 ± 13.2 ^{ab*}	127.0 ± 5.4 ^{a*}	127.4 ± 7.6 ^{ab*}	151.3 ± 16.2 ^{b}	167.7 ± 13.1 ^{ab}	164.9 ± 23.9 ^{ab}	171.0± 11.3 ^{a}	170.4 ± 15.7 ^{ab}	**0.03**	**<0.01**	0.2
Muscle Texture													
Hardness	9.8 ± 2.0 *	8.0 ± 1.6 *	9.7 ± 1.8 *	9.6 ± 1.5 *	10.0 ± 1.4 *	3.3 ± 2.5	4.2 ± 2.9	2.8 ± 1.9	4.8 ± 2.7	4.7 ± 2.3	0.2	**<0.01**	0.1
Adhesiveness	−0.4 ± 0.1 *	−0.2 ± 0.1 *	−0.4 ± 0.2 *	−0.3 ± 0.1 *	−0.3 ± 0.1 *	−1.78 ± 0.8	−1.9 ± 0.6	−2.5 ± 0.6	−1.9 ± 0.9	−2.3 ± 0.6	0.05	**<0.01**	0.1
Springiness	1.1 ± 0.1 *	1.2 ± 0.2 *	1.11 ± 0.1 *	1.2 ± 0.2 *	1.2 ± 0.2 *	1.24 ± 0.25	1.2 ± 0.1	1.3 ± 0.28	1.31 ± 0.3	1.4 ± 0.3	0.5	**<0.01**	0.4
Cohesiveness	0.4 ± 0.04 *	0.4 ± 0.03 *	0.4 ± 0.1 *	0.4 ± 0.1 *	0.4 ± 0.1 *	0.3 ± 0.04	0.3 ± 0.1	0.3 ± 0.03	0.32 ± 0.03	0.31 ± 0.03	0.3	**<0.01**	0.4
Gumminess	3.5 ± 0.5 ^{ab*}	3.1 ± 0.6 ^{ab}	3.4 ± 0.5 ^{b*}	3.7 ± 0.7 ^{ab*}	4.0 ± 0.9 ^{a*}	1.0 ± 0.8 ^{ab}	1.3 ± 0.9 ^{ab}	0.8 ± 0.6 ^{b}	1.6 ± 0.9 ^{ab}	1.5 ± 0.8 ^{a}	**<0.01**	**<0.01**	0.4
Chewiness	4.2 ± 0.9 *	3.7 ± 1.2 *	3.9 ± 0.8 *	4.0 ± 0.8 *	4.2 ± 1.8 *	1.3 ± 1.0	1.6 ± 1.1	1.1 ± 0.7	1.9 ± 1.1	2.1 ± 1.1	0.1	**<0.01**	0.5
Resilience	0.6 ± 0.3 *	0.4 ± 0.1 *	0.5 ± 0.1 *	0.4 ± 0.1 *	0.4 ± 0.1 *	0.8 ± 0.5	0.8 ± 0.3	1.0 ± 0.6	0.9 ± 0.6	1.0 ± 0.5	0.8	**<0.01**	0.2

Values are presented as mean ± standard deviation (n = 12). Within a row, superscripted lowercase letters (ab) mean significant differences between diets, while (*) means differences between days ($p < 0.05$). Hardness and gumminess are in newtons, adhesiveness and chewiness are in joules. Muscle lipid peroxidation is in nmol MDA g liver^{-1}, while DPPH/ABTS assays are in µmol TE mg^{-1} ww^{-1}.

3.6. Consumer Acceptance of Sea Bass Fillets

Consumers' overall liking of fish samples was generally high (>7.5 out of 9) and without significant differences between treatments (Table 4.8).

Table 4.8 – Overall liking of fish samples with different dietary treatments

Dietary Treatments	Mean ± SD
CTRL	7.5 ± 1.2
VITE	7.5 ± 1.5
TO	7.7 ± 1.0
CA	7.5 ± 1.0
CO	7.6 ± 1.1
p-value [1]	0.651

Consumer acceptance (n = 60) of sea bass fillets using a 9-point hedonic scale. Five samples from the same part of the fish (slices from the anterior, middle or posterior part of the fish body) were presented, each corresponding to a different dietary treatment. [1] For the dietary treatment effect on a mixed-model three-way ANOVA.

Correspondence analysis applied to open comments data (positive and negative) regarding the evaluation of European sea bass (Figure 2) highlights the similarities and dissimilarities perceived by consumers for fillets from different diets, allowing a relevant and more generalized overview of the results. This biplot configuration explains 70.4% of total variance of the experimental data. Fillets from fish fed the CTRL were mainly associated with freshness, pleasant and attractive appearance, and a persistent and pleasant aftertaste, while the VITE was associated with a white colour, consistent and hard texture, and negatively correlated with dry texture. Moreover, fillets from fish fed diets CA and CO were mostly associated with terms related with odour such as pleasant, intense and typical, and also easily removable skin (more on the CO sample) and easy to chip. The TO sample was related with pleasant attributes regarding size, appearance (bright skin), texture and taste, but related with the negative attributes soft and greasy texture and low intense odour.

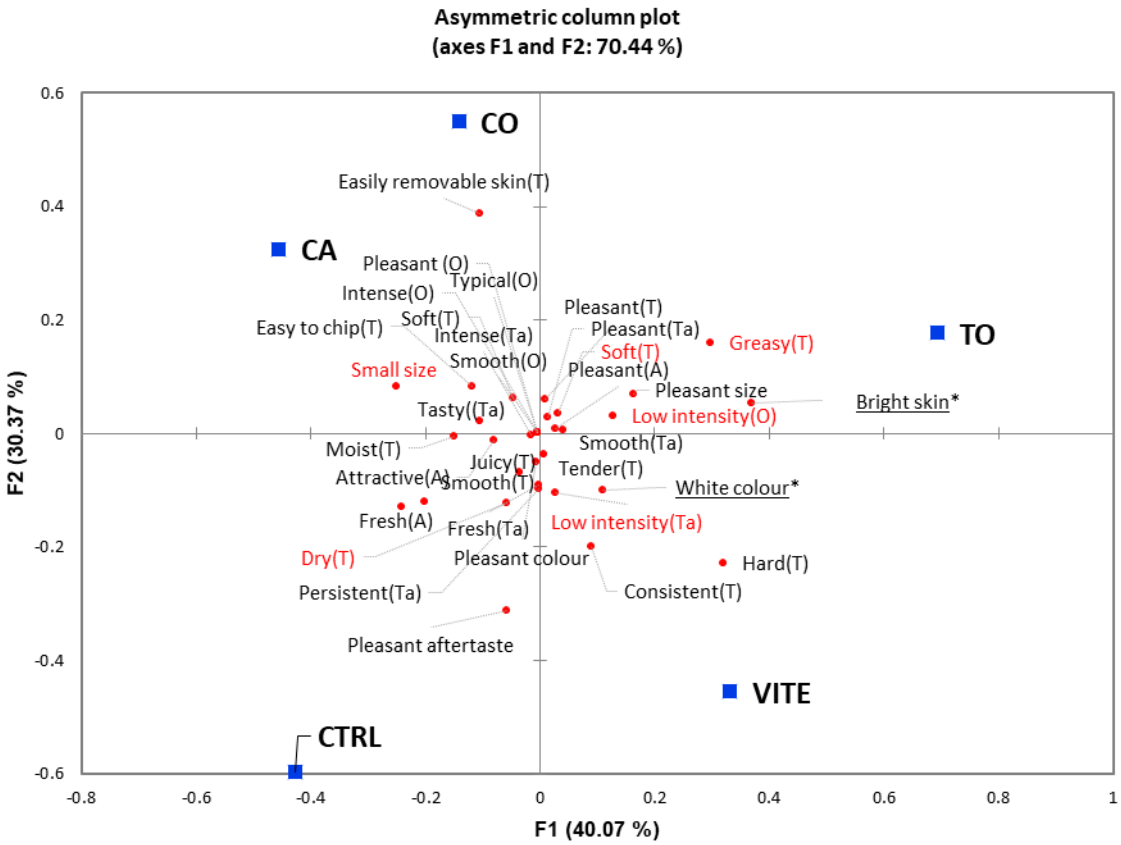

Figure 4.2. Correspondence analysis applied to open comments data regarding the evaluation of European sea bass fillet by consumers (n = 60). Samples from the same part of the fish muscle (5) were presented, each corresponding to a different dietary treatment. Attributes in red were mentioned as negative aspects; attributes in black were mentioned as positive aspects. (*) underlined attributes with a significant difference between samples, at a significance level of 5%

4. Discussion

Both synthetic and natural antioxidants are commonly included in aquafeeds in order to ameliorate the negative effects of oxidative stress, potentially increasing fish health and delaying flesh oxidation during storage (NRC, 2011), the physical and chemical conditions that occur during feed formulation, namely light exposure and high temperatures, may negatively affect antioxidant stability (Anderson and Sunderland, 2002; Ortak et al., 2017). The high vulnerability of carotenoids to high temperatures is thoroughly documented (Borsarelli and Mercadante, 2009). Even though dried carrot and coriander used in this study revealed the presence of α-carotene and β-carotene, results showed a complete absence of these

carotenoids in their respective experimental diets. In the case of lutein, although dried coriander was a very rich source, its 2% inclusion in CO diet was not reflected in the final dietary level. The measured amounts of lutein in the CO diet were 1.7 g 100 g^{-1} of DM, representing 68% of the expected value, i.e., 2.5 g 100 g^{-1} of DM. This result points towards a possible carotenoid degradation during the extrusion (110 °C) and/or drying (60 °C) stages of diet manufacture. Of the three ingredients used in this study, only coriander contained β-cryptoxanthin, but all diets just evidenced trace amounts of this pigments, again suggestion degradation during processing. The CA diet contained the highest amount of total phenolic compounds, thus significantly differing from the CTRL, possibly due to the dietary inclusion of dried carrot.

Despite synthetic vitamin E being a commonly used antioxidant in aquaculture, there is little knowledge regarding supplementation rates for commercial-sized European sea bass (*Dicentrarchus labrax*). Overall, quantification of vitamin E in experimental diets used in this study showed tocopherol amounts that reflected the different supplementation levels, since the VITE diet had five times more α-tocopherol than the remaining diets. Moreover, the dried vegetables added at a 2% inclusion rate did not provide any further significant contribution of vitamin E to the dietary formulations. However, supplementation doses did not reach targeted levels (500 mg kg^{-1} in VITE and 100 mg kg^{-1} in all other diets) as values measured after feed manufacturing procedures of extrusion and drying (126 mg kg^{-1} of DM and 25–30 mg kg^{-1} of DM, respectively) indicated a 24–30% α-tocopherol retention after feed manufacturing. During the feed formulation process, namely during the extrusion and drying stages, tocopherol suffers considerable temperature-induced degradation, an effect which is thoroughly documented (Nissiotis and Tasioula-Margari, 2002; Sabliov et al., 2009; Verleyen et al., 2001). Vitamin stability during extrusion depends on several factors, namely raw material, mixing, conditioning, temperature, pressure, moisture, energy input and extruder mechanical features (Riaz and Ali, 2009). According to Morin et al. (2021), the chemical nature of the extruded matrix, moisture and temperature levels account for a variation of 63 ± 28% of tocopherol retention. Riaz and Ali (2009) showed that the pelleting and extrusion processes alone can account for a 25% loss of vitamin E at around 80–90 °C, whilst higher extrusion temperatures, above 100 °C, increases the sensitivity of this vitamin significantly (Morin et al., 2021). According to Anderson and Sunderland (2002), most vitamin E losses occur over the course of aquafeeds extrusion process, before drying procedures, and might negatively affect the antioxidant potential of the diets. Effective technologies (e.g., colder extrusion and softer drying procedures, microencapsulation) able to protect both pigments and natural antioxidants sources prior extrusion should be envisaged.

The VITE diet exhibited less DPPH$^{\bullet}$ scavenging capacity than the CO diet, despite showing no differences when compared to control. Moreover, several synergistic mechanisms between different natural antioxidants, such as those found in coriander, are known to heighten the antioxidant potential of biological samples, generating superior antioxidant characteristics as opposed the sums of each individual one (Cano et al., 2000; Guerra et al., 2005; Liu et al., 2008). When compared to the control, the antioxidant capacity measured in diet VITE does not seem dose responsive. Previous studies suggested that the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ methods can be employed to examine lipophilic antioxidants such as tocopherols (vitamin E) (Liu et al., 2008; Prevc et al., 2015). However, the DPPH$^{\bullet}$ reaction rates are highly influenced by solvent composition (Danet, 2021), suggesting that results have to be interpreted with caution. In the present study, the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays were carried out using the same extract employed for polyphenol quantification (via the Folin–Ciocalteau method), that is, methanol and distilled water (4:1, vv^{-1}). This mixture is optimal for polyphenols due to the solvent's polarity (Do et al., 2014), but methanol is not the most appropriate solvent for tocopherol extraction, as it is a hydrophilic substance (Keramati et al., 2021; van Lith and Ameer, 2016). Thus, observed values from the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays might not reflect the totality of radical scavenging capacity resulting from the addition of synthetic vitamin E, which may explain the lack of differences between VITE and the CTRL diet.

The chemical structure of carotenoids and polyphenols allows these compounds to regulate antioxidant activity due to their radical-scavenging properties (Carvalho and Caramujo, 2017). Specifically, the antioxidant activity of lutein (Kiokias and Gordon, 2004; Sindhu et al., 2010), β-carotene (Kiokias and Gordon, 2004; Mueller and Boehm, 2011), lycopene (Kaur et al., 2012; Kiokias and Gordon, 2004), α-carotene and β-cryptoxanthin (Kiokias and Gordon, 2004) has been thoroughly evaluated in in vitro studies. Supplementation of fish feeds with carotenoids has shown health associated benefits, acting as antioxidants and immunostimulants, enhancing fish resistance to bacterial and fungal diseases (García-Chavarría and lara-flores, 2013). Additionally, polyphenols show an ability of scavenging free radicals, assisting in hindering the negative effects of ROS such as singlet oxygen, peroxynitrite and hydrogen peroxide, which must be continually removed from cells to maintain healthy metabolic function (Brglez Mojzer et al., 2016; Williams et al., 2004). Natural phenolic compounds can also serve as potential additives for preventing quality deterioration or to retain the quality of fish and fish products (Maqsood et al., 2013), and seem to be rather resistant to deactivation via high-temperature extrusion-cooking process (Oniszczuk et al., 2019). This antioxidant potential of polyphenols in terms of direct and indirect inhibition or suppression of oxidation processes is thoroughly established, mostly through trolox equivalent antioxidant capacity assays, as well as their scavenging capacity regarding the stable free radical 2,2-diphenyl-1-picryl-hydrazyl,

commonly known as DPPH•, due to its affinity with fat-soluble hydrophobic compounds, as is the case with polyphenols (Holdt and Kraan, 2011b; Pezeshk and Alishahi, 2015). In this study, the CA diet revealed higher amounts of phenolic compounds (1179.1 ± 103.4 mg GAE 100 g^{-1} DM) than all remaining diets. This could be advantageous for the conservation of feed properties, as the proven antioxidant potency of polyphenols (Bors and Michel, 2002; Maqsood et al., 2013) might provide additional resistance to feed oxidation during storage.

Data from this study show that the addition of natural antioxidants to the experimental diets did not affect any of the evaluated immune parameters in fish. Moreover, considering the results of liver antioxidant activity observed in this study, GR was significantly higher in fish fed CTRL and CO diets when compared to fish fed with the TO diet. GR is an essential enzyme for catalysing the reaction that reduces oxidized glutathione (GSSR) into reduced glutathione (GSH) (Lu, 2013), the sum of which comprises total glutathione content (TG). GSH is an essential cofactor for antiperoxidative enzymes such as glutathione peroxidase (GPx) (Lu, 2013). Therefore, a lower GR associated with the TO diet, in combination with liver lipid peroxidation data that showed no significant differences between TO and CTRL, might mean that fish fed with TO require a lower endogenous antioxidant activity in order to maintain cell homeostasis. However, TAC, which is specific for non-enzymatic antioxidants, displayed no differences between experimental treatments, meaning that we cannot directly attribute this lower production of GR to an increase in antioxidant potential stemming from exogenous antioxidants. As expected, due to the absence of differences between treatments regarding TG, glutathione-dependent antiperoxidative enzymes GPx and GST also did not show any differences when experimental diets were compared to the CTRL, as these enzymes require glutathione as a cofactor in order to perform their biological functions (Kurutas, 2015). The antioxidant function of GPx and GST is largely dependent on its interaction with TG (Bragadóttir et al., 2001), neutralizing hydroperoxides as GSH is oxidized to GSSG by GR. Considering the results obtained in this study, we can observe that a lesser production of GR did not lead to differences in glutathione dependent enzymes. Moreover, the heightened dose of polyphenols in the CA diet did not translate into additional antioxidant protection in fish liver. This raises further questions concerning the bioavailability of these polyphenols in sea bass organism. The bioaccessibility and bioavailability of natural antioxidants in the organism not only relies on the concentration of bioactive compounds in the ingredient, but also on dosage and form of administration, composition of the feed matrix, while also being heavily influenced by other factors such as pH variations, enzyme action and digestion time (Castenmiller and West, 1998; Lizárraga-Velázquez et al., 2019). Hence, further research is still warranted to clarify the full potential of natural antioxidant sources for inclusion in aquafeeds.

Overall, neither a heightened dose of vitamin E compared to standard values nor a 2% inclusion of natural antioxidant induced an upregulation of sea bass liver antioxidant system. Evaluation of the antioxidant potential present in these diets after the manufacturing process (extrusion and drying) only provides a limited perspective on their potential biological effect, since this is also greatly conditioned by their digestive bioaccessibility and bioavailability as discussed above. Dietary vitamin E and natural antioxidants at the levels used in this study had no effects on fish proximate composition and feed conversion rate, confirming data obtained by Gatta et al. (2000).

The beneficial effects of vitamin E as an antioxidant have been thoroughly evaluated in teleost fish, namely gilthead seabream (*Sparus aurata*) (Mourente et al., 2002), red sea bream (*Pragus major*) (Gao et al., 2012) and rainbow trout (*Oncorhynchus mykiss)* (Kelestemur et al., 2012; Palace et al., 1993). However, in Atlantic salmon (*Salmo salar*) (IBW = 64 g), high supplementation levels of up to 1100 mg kg^{-1} dietary vitamin E did not affect fish antioxidant defence, lipid peroxidation and overall fish muscle resistance to oxidative stress (Lygren et al., 2002). Indeed, the available literature shows that the effectiveness of vitamin E as an antioxidant is largely dependent on fish life stage and species (NRC, 2011).

Overall, studies concerning the antioxidant effects of vitamin E of commercial-sized European sea bass are rare. Silva et al. (2014) recommended a dosage of 500 mg kg^{-1} of vitamin E for adult sea bass. However, data obtained in this study suggests that a 500 mg kg^{-1} inclusion of vitamin E (VITE diet) in European sea bass feeds have no beneficial effect on muscle antioxidant potential compared to the traditional dosage of 100 mg kg^{-1} (CTRL diet). Gatta et al. (2000) also reported decreased lipid peroxidation rates in sea bass fillets when α-tocopherol supplementation was increased from 139 mg kg^{-1} feed to 493 mg kg^{-1}, but this could not be confirmed in the present study. It should be noted that all these results were obtained in optimised non-stressful rearing conditions for sea bass. Different conclusions regarding the antioxidant potential of these diets might have been reached if fish were submitted to a stress challenge, as enzymatic responses to oxidative stress are particularly promoted when fish face a pro-oxidant challenge (Abdel-Tawwab et al., 2018b). Further studies should hence be envisaged to explore fish response to stressful conditions after being fed natural antioxidant sources.

Although vegetable coproduct inclusion and different vitamin E inclusion levels failed to show any antioxidant benefits in European sea bass muscle, significant differences were identified between Day 0 and Day 8. Namely, the antioxidant potential measured through the scavenging potential for $DPPH^{\bullet}$ was higher in fish stored in ice for 8 days, irrespective of the dietary treatments. This might have contributed towards the absence of differences in muscle LPO between Day 0 and Day 8, showing that lipid oxidation levels were similar between samples

from both days. Likewise, Gatta et al. (2000) showed that vitamin E supplementations between 139 mg kg^{-1} and 942 mg kg^{-1} fed to European sea bass (IBW = 200 g) showed no differences in terms of muscle lipid peroxidation between the first and last days of a 12-day storage time. The ability to scavenge free radicals is essential for ameliorating the negative effects of oxidative stress. Due to its relative stability, the free radical $DPPH^{\bullet}$ is a prime candidate as a first approach for evaluation of radical scavenging activity (Nakajima et al., 2009). The eventual breakdown of cohesiveness between tissues, liquefaction of most organs and subsequent decomposition of proteins by hydrolysis leads to an increase in amino acid content, which might consequentially increase muscle free radical scavenging activity (Sikorski et al., 1976). Moreover, naturally-occurring ROS via interactions with co-products generated through the natural functioning processes of electron transport chains (Turrens, 2003), cease functioning after death. Thus, a post-mortem increase in amino acid content accompanied by a lesser formation of ROS might explain this increase in $DPPH^{\bullet}$ radical scavenging activity. Plant by-products have also been proven effective in delaying chemical changes and microbial growth, as well as maintaining sensory characteristics and extending the shelf-life of seafood during refrigerated storage (Pezeshk and Alishahi, 2015). Natural antioxidants, namely phenolic compounds can have positive effects in terms of upregulation of muscle antibacterial properties and retardation of bacterial growth (Pezeshk and Alishahi, 2015), which would translate into a larger product shelf-life. This was, however, not accessed in the present study and merits further evaluation.

Besides their antioxidant properties, carotenoids are also sources of pigments, and their deposition in tissues may affect skin/muscle colour and appearance in fish (Araújo et al., 2016). In this study, instrumental colour data of fish muscle was consistent with data found in the literature (Tibaldi et al., 2015). Moreover, all diets with natural antioxidant inclusion showed a significant decrease in muscle yellowness (b^*) when compared to CTRL, whilst fish fed with CA presented a significantly higher h^* than CTRL. Dietary coriander (CO) was associated with decreased muscle chroma (C^*). Despite the evidence for dietary carotenoid degradation, the different diets still seem to modulate fish muscle colour. However, these differences in raw muscle colorimetric analyses between TO, CA and CO, when compared to CTRL, could not be perceived in the cooked muscle slices that were equally well accepted by the sensory panel. Similarly, in large-sized European sea bass, significant alterations in fillet colour of fish fed with *Isochrysis* sp., could not be detected by a sensory panel (Tibaldi et al., 2015). It is important to note that consumers were only able to significantly differentiate two attributes. TO group presented brighter skin than others, while VITE samples presented a white colour significantly different from the other groups. Both differences were mentioned as positive aspects.

This is particularly important in commercial fish species, since colour and visual appearance are known to influence market value, flavour perception and acceptability of fish food products (Spence et al., 2010), thus providing a measurable parameter for flesh freshness and ultimately affecting consumer perception quality (Roth et al., 2009; Vanhonacker et al., 2013). Although differences in food colour can exert considerable influence on taste and consumers' perception (Spence et al., 2010), overall liking scores in terms of consumer acceptability, showed no significant differences between samples. Indeed, all samples had a high average score value, in line with a high mention of positive comments, revealing that consumers were unable to detect differences between dietary treatments.

One of the most important freshness quality attributes of fish muscle is texture, which is heavily dependent on several parameters such as hardness, cohesiveness, springiness, chewiness, resilience and adhesiveness, as well as fibre detachment and internal cross-linking of connective tissue (Cheng et al., 2014). In terms of muscle texture, there were no significant differences among dietary treatments. Although hardness, adhesiveness, cohesiveness, gumminess and chewiness decreased after storage time, accompanied by an increase in springiness and resilience, none of these parameters were affected by the experimental diets.

5. Conclusions

Vitamin E and carotenoid content of extruded diets showed signs of degradation during the feed manufacturing process. Dietary vitamin E and natural antioxidants at the levels used in this study had very limited effects on European sea bass growth or body composition, immunomodulatory response, muscle and liver antioxidant potential, organoleptic properties or consumer acceptance. Neither a heightened inclusion dose of 500 mg kg^{-1} of vitamin E, nor a 2% inclusion of natural antioxidants provided additional antioxidant protection, compared to fish fed diets with a regular dose of 100 mg kg^{-1} of vitamin E. It should be noted that all these results were obtained under optimised non-stressful rearing conditions for sea bass. A pro-oxidant challenge is recommended to fully ascertain the fish responsiveness towards the inclusion of antioxidants. Moreover, in order to protect pigments and natural antioxidants throughout the feed manufacturing process, further research into alternative technologies is of paramount importance to produce cost-effective functional diets.

Author Contributions: Conceptualization, L.M.P.V. and M.P. (Manuela Pintado); methodology, L.M.P.V., M.P. (Manuela Pintado), L.M.C., R.C.L. and B.C.; validation, D.A.C., C.V., L.M.P.V., M.P. (Manuela Pintado), L.M.C.; formal analyses, R.P., M.C., T.S., A.M., S.F.-B., M.P. (Miguel Pereira), D.J., L.F.B., S.B.; data curation, R.P.; writing—original draft

preparation, R.P.; writing—review and editing, all co-authors; supervision, C.V., L.M.P.V. and M.P. (Manuela Pintado); project administration and funding acquisition, L.M.P.V. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by project “MobFood—Mobilizing scientific and technological knowledge in response to the challenges of the agri-food market” (POCI-01-0247-FEDER-024524), by “MobFood” Consortium, and financed by the European Regional Development Fund (ERDF), through the Incentive System to Research and Technological development, within the Portugal2020 Competitiveness and Internationalization Operational Program. This research was also supported by national funds through FCT—Foundation for Science and Technology within the scope of UIDB/04423/2020 and UIDP/04423/2020. The doctoral scholarship of Ricardo Pereira was financed by Fundação para a Ciência e Tecnologia (FCT), Portugal, through the grant SFRH/BD/144631/2019. Authors also acknowledge financial support from National Funds from FCT, within the scope of UIDB/05748/2020 and UIDP/05748/2020 (Green-UPorto) and UIDB/04423/2020 and UIDP/04423/2020 (CIIMAR).

Institutional Review Board Statement: The present study was directed and performed by accredited scientists in laboratory animal science by the national competent authority (Direção Geral de Alimentação e Veterinária—DGAV) at a facility with permission to conduct experiments on fish, in compliance with the guidelines of the European Union (directive 2010/63/EU) and Portuguese law (Decreto-Lei nº 113/2013, de 7 de Agosto) on the protection of animals used for scientific purposes. All animal procedures were subject to an ethical review process carried out by CIIMAR animal welfare body (ORBEA-CIIMAR_18_2017) and further approved by DGAV.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 5.

General discussion, conclusions and future directions

GRAPHICAL ABSTRACT

Vegetables and fruits as antioxidant sources for European sea bass (*Dicentrarchus labrax*)

OBJECTIVES:
Assessing the impact of feed manufacturing technologies on natural antioxidants
Understanding the potential benefits of incorporating fruit and vegetable sources as natural antioxidants in aquafeeds

Chapter 2
How feed manufacturing temperatures affect natural antioxidants

Effects on feed antioxidant profile and capacity with a 2% inclusion of fruit by-products in diets with different manufacturing conditions

Extrusion (°C)	Drying (°C)	Mango peel	Pineapple peel
110	60	MHE-60	PHE-60
110	35	MHE-35	PHE-35
25	60	MCE-60	PCE-60
25	35	MCE-35	PCE-35

Antioxidant capacity
Antioxidants
Chemical composition

Main conclusions

- Ingredient processing resulted in high antioxidant losses
- 2% However, mango and pineapple peel increased feed antioxidant capacity
- 2% Conventional manufacturing temperatures were more effective at increasing feed antioxidant capacity

Chapter 3
Inclusion of 2% pineapple by-product flours concomitantly with vitamin E

Effects on feed oxidation and fish antioxidant defences

Antioxidant capacity
Phenolic content
Chemical composition

Fresh
4 ºC
25 ºC
12 weeks of storage

Plasma
- Stress and energetic state biomarkers
- Innate immune system response

Liver
- Antioxidant response
- Protein and lipid oxidation

Muscle
- Antioxidant capacity
- Lipid oxidation

Main conclusions

- 2% Pineapple increased feed antioxidant capacity regardless of storage conditions
- By-products could not enhance fish response to acute stress

Chapter 4
Inclusion of 2% dried vegetable coproducts compared with high doses of vitamin E

Effects on feed oxidation, fish antioxidant defences, fillet oxidation and consumer acceptance

Plasma
- Innate immune system response

Liver
- Antioxidant response
- Lipid oxidation

Muscle
- Oxidation over storage
- Consumer acceptance

Main conclusions

- Similar antioxidant response between 100 and 500 mg/kg of vitamin E
- Co-products were unable to enhance fish antioxidant system

FUTURE PROSPECTS INCLUDE EXPERIMENTING WITH

- Lessening antioxidant loss in ingredient processing
- Different antioxidant sources and methods of inclusion
- Different fish species

GENERAL CONCLUSIONS

- ✓ Conventional extrusion and drying temperatures proved to be the most efficient for maintaining the antioxidant capacity of aquafeeds
- ✓ A 2% Inclusion of fruit by-products increases aquafeed antioxidant capacity, but none of the used by-products or co-products increased fish antioxidant defences
- ✓ For market-sized European sea-bass, there are no significant differences in lipid oxidation or enzymatic activity between Vitamin E inclusion levels of 100 and 500 mg per kg
- ✓ Manufacturing and/or inclusion processes require optimization for the selected ingredients to benefit the antioxidant system of farmed fish

In this thesis it was hypothesized that the inclusion of natural antioxidant sources from fruits and vegetables into European sea bass aquafeeds would provide additional antioxidant protection for both the feed and/or cultured fish, thus increasing the resistance of feed to lipid oxidation and potentially enhancing the defences of farmed fish against oxidative stress. To test this hypothesis, three specific objectives were established across chapters 2-4, as stated in the graphical abstract. In Chapter 2, the effect of processing technologies on the radical scavenging capacity of aquafeeds supplemented with natural antioxidants from mango and pineapple peels was evaluated. This chapter allowed the selection of best extrusion and drying temperatures able to retain the highest antioxidant potential. Chapter 3 focused on assessing the effectiveness of top-performing natural antioxidant sources identified in chapter 2, specifically flours derived from pineapple by-products, in enhancing the antioxidant defences of European sea bass within the context of an *in vivo* trial. Additionally, the impact of these by-products on feed preservation at different storage temperatures was evaluated. Finally, in chapter 4 it was assessed if antioxidant sources from vegetable co-products (i.e. dried tomato, carrot and coriander) incorporated using low temperatures during dietary extrusion could positively impact the antioxidant system of fish, consumer acceptance and fillet oxidation during storage, all within the context of economic viability and the concept of circular economy. It is important to note that this thesis did not target the total substitution of synthetic antioxidants in aquafeeds by natural sources, but to potentially increase feed antioxidant properties and fish antioxidant defences by the dietary supplementation with natural antioxidants. This approach aligns with current consumer trends in terms of preference for natural food sources (Sanches-Silva et al., 2014) and resonates with sustainability and circular economy initiatives. In accordance with point 3.7 of the European Commission's Circular Economy Action Plan (European Comission, 2020), the strategy promotes reusing products that would otherwise be discarded, mostly due to cosmetic and retail standards (Porter et al., 2018).

Concerning the selected fish species for this thesis, namely the European sea bass (*Dicentrarchus labrax),* worldwide production lowered from 2019 to 191 thousand tonnes in 2020 - being valued at a total of 1001 million EUR - possibly due to the COVID-19 pandemic (FAO, 2022). Nonetheless, in 2020, within the EU, the production of European sea bass reached 82 thousand tonnes, valued at 510 million EUR (FAO, 2022). Around 94% of this value comes from aquaculture facilities primarily situated the Mediterranean Sea region, distributed mostly throughout Turkey, Greece, Egypt, and Spain, with Turkey being the biggest contributor (Llorente et al., 2020). Thus, the European sea bass has gained prominence due to its economic significance and high market demand, underscoring its pivotal role in aquaculture research. Moreover, this species has been thoroughly studied in terms of the dynamics of its antioxidant system, due to its high susceptibility to stress. Several strategies

have been tackled to mitigate the negative effects of said stress (Batista, Sónia et al., 2020; Batista, S. et al., 2020; Lobo et al., 2018; Peixoto et al., 2019a; Peixoto et al., 2019b; Peixoto et al., 2016; Saleh et al., 2018; Sallam et al., 2018; Vinagre et al., 2012). This extensive research background not only underlines the importance of the species, but also provides a valuable basis for further progress in aquaculture practices and nutritional strategies able to promote fish welfare.

Despite its potential benefits, incorporating natural antioxidants into aquafeeds also poses significant challenges. Vitamins, carotenoids, and phenolic compounds are widely reported as vulnerable to pro-oxidant abiotic factors, especially during feed manufacturing processes, where high temperatures are common. In Chapter 2, results highlighted that conventional extrusion and drying temperatures (110 °C and 60 °C, respectively) were more effective in preserving phenolic compounds and overall antioxidant activity of feed than cold extrusion (25 °C) and drying processes (35 °C until > 10% of moisture). Despite the degradation of vitamins and carotenoids via heat being well-documented (Arfaoui, 2021; Armstrong et al., 2019; Maiani et al., 2009), the initial values of these compounds were already relatively low in the fruit flours used in this study, especially when compared to the minimum requirements for European sea bass (NRC, 2011). It was also concluded that the flour manufacturing process led to a significant reduction of vitamins and carotenoids final amounts when compared to peels, with a 20% decrease in mango and 50% in pineapple. The interplay of antioxidants with heat is complex, and much is yet to be elucidated. However, depending on the specific compound and its interaction with the feed matrix, along with the heating method used, heat has the potential to enhance the bioavailability of carotenoids and phenolic compounds (Gunathilake et al., 2018). Additionally, the strategic application of heat, as highlighted by Shi et al. (2018), emerges as an effective approach to reduce the levels of anti-nutrients, such as oxalates and phytates. These compounds, often inherent in certain foods, are recognized for their inhibitory effects on the absorption of beneficial compounds like carotenoids and phenolic compounds (Nkhata et al., 2018), thereby enhancing their bioavailability in the consumed feed. Furthermore, the transformative potential of heat on specific compounds is evident in diverse examples. For example, the cooking process for tomatoes can convert some of the carotenoid lycopene into a more accessible form such as β-carotene (Górecka et al., 2020). Similarly, heating green tea can change the chemical structure of specific polyphenols, potentially increasing their bioavailability (Truong and Jeong, 2021). The cellular complexity of many plant-based foods presents another layer of consideration. Carotenoids and phenolic compounds are often intricately locked within cell walls. Heat application plays a pivotal role in softening or breaking down these cell walls aiding in the release of these compounds (Arfaoui, 2021; Hwang et al., 2012). This suggests that heat treatments, in specific contexts, may

facilitate the body's absorption of these beneficial compounds (Arfaoui, 2021). Additionally, carotenoids and phenolic compounds within the feed matrix are often bound to fibre, fat, proteins, or other molecules. Heat assists in releasing these compounds from their bound forms, thereby enhancing their absorption in the digestive tract (Arfaoui, 2021; van Het Hof et al., 2000). This possibly explains the variations in phenolic compounds found in diets in chapter 2, where increased phenolic compounds were observed after subsequent heat treatments, particularly in phenolic compounds bound to the antioxidant fibre. However, it is important to note that while heat can enhance the bioavailability of carotenoids and polyphenols in many cases, excessive heat and prolonged exposure can lead to the degradation of these compounds. Therefore, finding a balance between heat processing condition to enhance bioavailability and avoiding overheating is essential to preserve the nutritional benefits of these compounds. In addition to heat, it's crucial to consider other pro-oxidant factors during subsequent stages, particularly the drying phase. Factors such as air and light exposure can significantly influence the stability of these compounds by promoting oxidation of vitamins and carotenoids, as well as phenolic compounds, especially in their free form (Arfaoui, 2021; Gebregziabher et al., 2023; Morin et al., 2021). Moreover, since diets must reach a minimum level of moisture, drying at colder temperatures requires more time of exposure to air and light. Overall, results from chapter 2 showed that pineapple and mango peels are promising sources of natural antioxidants for aquafeed inclusion, especially if under high-temperature conditions, as evidenced by their resilient bound phenolic content and antioxidant activity. Notably, pineapple peel flour inclusion, when compared to mango peel flour, demonstrated greater flexibility in increasing the antioxidant properties of feeds manufactured under different extrusion/drying temperature combinations. While conventional temperatures (hot extrusion, coupled with rapid drying at elevated temperatures), proved to be the most efficient process to preserve the antioxidant potential of aquafeeds, technological improvements are required for optimizing the antioxidant capacity of fruit peel flours prior to their inclusion in aquafeeds. Moreover, exploring alternative inclusion methods (e.g. microencapsulation) able to maximize the antioxidant potential of the tested supplements could enhance the delicate balance required for optimal preservation, thus maximizing overall antioxidant capacity.

Considering the antioxidant potential of pineapple by-products demonstrated in Chapter 2, the first *in vivo* trial of this thesis (chapter 3) was performed using pineapple peel flour and pineapple stem flour in diets for European sea bass. This strategic choice aimed at embracing a more comprehensive approach by harnessing different, typically discarded parts of the same fruit known for their inherent antioxidant potential (Campos et al., 2020b). Details regarding the chemical and antioxidant properties of the natural antioxidants used in this thesis are summarized in table 5.1. Although the pineapple peel flours tested in chapters 2 and 3 were

manufactured using the same method, the latest exhibited higher amounts of free and fiber-bound phenolic compounds. Additionally, it displayed a much greater antioxidant capacity across all measured parameters, except for the $ABTS^{\bullet+}$ radical scavenging capacity of free phenolic extracts, which was higher in the pineapple peel flour from chapter 2. This enhanced antioxidant capacity likely stems from the notably higher yield of phenolic compound extraction, as a myriad of studies have reported strong correlation between elevated antioxidant activities and high phenolic contents in plant foods (Ahmadifar et al., 2021; Arfaoui, 2021; Dawood, M.A.O. et al., 2022). The antioxidant activity varies among phenolic acids depending on their chemical properties. Indeed, studies have reported differences in the antioxidant activities of free, esterified, glycosylated, and non-glycosylated phenolics (Kumar et al., 2020). Phenolic acids, notably abundant in pineapple peel flour (Table 2.1) play a crucial role as antioxidants due to the reactivity of the phenol moiety, particularly the hydroxyl substituent on the aromatic ring (Kumar et al., 2020). While several mechanisms contribute to the antioxidant activity of phenolic acids, radical scavenging through hydrogen atom donation is considered the primary method, as the presence of substituents on the aromatic ring in phenolic acids influences the stabilization of their structure, subsequently affecting their radical-quenching ability. Given the consistent processing, storage and extraction methodologies applied to both pineapple peel flours discussed in chapters 2 and 3, the higher antioxidant content and capacity of peel flour from chapter 3 may be attributed to various factors. These factors include cultivation methods, timing of harvesting, transportation, storage conditions and weather patterns during growth, all of which can affect the final antioxidant capacity of fruits (Kalt, 2005). Additionally, it is well-documented that the phenolic content of fruits, and subsequently their antioxidant potential, may substantially change among different cultivars (Kalt, 2005; Yapo et al., 2011). This underscores the importance of utilising a consistent biomass when evaluating fruit-derived ingredients in aquafeed formulations, as these nuances can profoundly influence the resulting nutritional and antioxidant profiles. Overall, all selected natural antioxidant sources tested in this thesis (fruit by-products and vegetable co-products), demonstrated notable antioxidant potency (Table 5.1).

Table 5.1. – Proximate composition, carotenoids, vitamin E, phenolic compounds and antioxidant capacity of all natural antioxidant sources used

	Mango peel	Pineapple peel (Chapter 2)	Pineapple peel (Chapter 3)	Pineapple stem	Tomato	Carrot	Coriander
Proximate composition[1]							
Dry matter (%)	90.2 ± 0.05	92.4 ± 0.1	92.7 ± 0.1	89.4 ± 0.1	77.3 ± 1.3	83.3 ± 1.3	93.4 ± 1.6
Crude protein	6.2 ± 0.03	6.8 ± 0.1	5.2 ± 0.01	4.8 ± 0.04	16.6 ± 0.4	12.4 ± 0.2	38.3 ± 0.1
Crude fat	1.3 ± 0.1	0.8 ± 0.1	0.5 ± 0.01	0.9 ± 0.02	3.9 ± 0.01	0.9 ± 0.1	3.2 ± 0.1
Ash	2.6 ± 0.1	3.0 ± 0.05	3.2 ± 0.01	2.8 ± 0.01	10.4 ± 0.7	7.2 ± 0.04	18.1 ± 0.7
Gross Energy	18.4 ± 0.01	18.6 ± 0.1	16.7 ± 0.2	17.8 ± 0.01	21.0 ± 0.03	18.3 ± 0.03	17.5 ± 0.01
Carbohydrates	89.9 ± 0.1	89.3 ± 0.2	91.2 ± 0.1	91.4 ± 0.1	69.1 ± 1.1	79.5 ± 0.3	40.4 ± 0.9
Main carotenoids[2]							
α-carotene	0.3 ± 0.05	0.3 ± 0.05	-	-	BQL	4.7 ± 0.3	14.6 ± 0.6
β-carotene	4.2 ± 0.1	4.2 ± 0.1	-	-	BQL	BQL	57.3 ± 13.1
Lutein	0.7 ± 0.04	0.5 ± 0.04	-	-	1.1 ± 0.2	BQL	124.5 ± 2.2
Lycopene	n.a.	n.a.	-	-	18.3 ± 3.6	n.a.	n.a.
β -cryptoxanthin	0.02 ± 0.001	0.004 ± 0.001	-	-	n.a.	n.a.	0.6 ± 0.04
Vitamin E[2]							
α-tocopherol	258.0 ± 5.6	18.5 ± 0.8	-	-	32.6 ± 4	10.5 ± 3.2	48.3 ± 1.9
Free phenolic extracts[3]							
$ABTS^{\bullet+}$	1235.3 ± 149.4	963.0 ± 92.4	322.0 ± 0.7	941.0 ± 103.7	62.0 ± 3.6	104.9 ± 2.8	115.2 ± 0.2
$DPPH^{\bullet}$	729.2 ± 1.8	235.8 ± 16.2	845.6 ± 10.3	223.8 ± 2.6	21.0 ± 0.9	47.2 ± 4.2	74.2 ± 0.2
ORAC	2254.5 ± 114.5	2729.5 ± 59.2	3722.7 ± 65.9	1613.9 ± 74.9	-	-	-
Total	586.5 ± 36.1	397.5 ± 24.7	1157.6 ± 97.8	622.6 ± 2.4	729.6 ± 20.6	2478.7 ± 154.9	528.3 ± 27.6
Bound phenolic extracts[3]							
$ABTS^{\bullet+}$	540.7 ± 0.2	511.8 ± 2.7	9232.2 ± 931.6	6089.3 ± 101.7	-	-	-
$DPPH^{\bullet}$	91.7 ± 13.2	395.6 ± 47.7	886.3 ± 143.6	702.7 ± 19.1	-	-	-
ORAC	1495.4 ± 88.0	3655.4 ± 558.0	9377.5 ± 957.4	6487.2 ± 529.1	-	-	-
Total	337.5 ± 18.2	547.0 ± 13.3	953.8 ± 13.2	502.1 ± 35.4	-	-	-

Values are presented as mean ± standard deviation (n=3). Different superscript letters within each row indicate significant differences ($p < 0.05$). [1] "BQL" stands for "below quantification limit". [1] Proximate composition is expressed in % of dry matter (DM), with the exception of gross energy, which is expressed in kJ g^{-1} DM. Carbohydrates are calculated by adding values from protein, ash and lipids and subtracting that value from 100. [2]The quantification limit for α-carotene was 1.3×10^{-5} mg per 100 g of DM and β-carotene was 1.1×10^{-5} mg per 100 g of DM, while lutein was 5.9×10^{-6} mg per 100 g of DM, [3] Antioxidant capacity of both extracts was measured via the $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and ORAC radical scavenging capacities, and is expressed in mg of Trolox equivalents (TE) per 100 g of DM. Total quantification of phenolic compounds in both extracts was measured via the Folin-Ciocalteau method and is expressed in mg of gallic acid equivalents (GAE) per 100 g of DM.

However, the main challenge lay in determining the optimal inclusion percentage of each source. Establishing a common baseline for inclusion percentages across all tested diets presented inherent challenges due to the diverse array of antioxidant compounds present in various matrices. Additionally, the numerous possible interactions, whether synergistic or antagonistic, among the different antioxidant sources present in the feed (Kurutas, 2015) posed further complexity. While the total antioxidant capacity of each ingredient could serve as a deciding factor in diet formulation, determining the specific response of each source post-processing within a determined mixture would present considerable challenges. Economic viability is a major variable that must be taken into consideration in order for these alternative sources to be viable in a realistic aquaculture scenario. Alas, no data is available concerning the retail price of pineapple peels and stems, as they are not usually utilized for commercial purposes (Lourenço et al., 2021), often being discarded or used for composting (Nguyen et al., 2021). This scarcity of market valuation implies that, if the aquaculture industry generates a commercial demand for these by-products within the context of a circular economy, their retail price would be relatively low (Springer and Schmitt, 2018). Concerning the selected vegetable co-products, in the EU, the retail price of fresh tomato and carrot averaged at around

0.8 EUR and 0.4 EUR per kg, respectively, in 2020 (European Comission, 2024). Comparatively, coriander the most expensive of all three, had an export price of about 2 EUR per kg in the same year. Considering these prices, a 2% dietary inclusion level of tomato, carrot or coriander would result in an additional cost of about 0.02-0.04 EUR per kg of feed, respectively. This estimate excludes processing expenses associated with drying, grinding, storage, and eventual inclusion in aquafeeds. It's worth noting that these retail prices pertain to fresh products that meet market standards and are fit for sale. However, the retail price would be significantly lower for vegetables which are to be "discarded", mostly due to cosmetic and retailers' standards (Porter et al., 2018). Nonetheless, this price must remain economically viable and able to justify the additional antioxidant benefits conferred. In the context of this thesis, an industrial rationale was adopted, based on economic factors that often restrict the inclusion level of costly ingredients in practical terms. Consequentially, a conservative inclusion level of 2% was selected for all experiments. This standardized approach facilitates practical application and economic feasibility in industrial settings, ensuring a balanced consideration of both efficacy and cost-effectiveness.

In the *in vivo* trials described in chapters 3 and 4, Vitamin E was included in the control diet at a level usually used under standard commercial conditions. The selected natural antioxidant sources were added on the top of that to evaluate their ability to confer additional protection. While the health and legal concerns associated with synthetic antioxidant sources (i.e. BHT, BHA and ethoxyquin) remain true, as mentioned in chapters 2-4, alternative commercial sources such as synthetic Vitamin E are scarce for the current high demand, which inflates prices due to an imbalance between supply and demand (Muthulakshmi et al., 2023). This poses a great challenge for an industry that still relies on fish meal and fish oil, as these raw materials are highly prone to oxidation due to inherent high levels of polyunsaturated fatty acids, thus requiring the addition of antioxidants for minimizing economic loss (Naylor et al., 2021). Vitamin premixes, which include vitamins with antioxidant potential such as Vitamin E, are amongst the most expensive components of a feed formulation, hence having a major impact of diets final cost. Thus, the inclusion level of each vitamin needs to be carefully addressed. For instance, the retail price of Lutavit E 50®, a synthetic Vitamin E product commonly used in aquaculture, stands around 22 EUR per kg. Gatta et al. (2000) proposed that diets for European sea bass (IBW = 208 g) should contain α-tocopherol levels ranging from 254 mg kg^{-1} to 942 mg kg^{-1} to effectively mitigate liver lipid oxidation. The approximate average of this (600 mg kg^{-1}) equals an inclusion percentage of 0.06%, averaging about 0.01 EUR per kg of diet in added feed cost. Considering the large scale operations in aquaculture, where 1-2.35 tons of feed produce one ton of European sea bass (Kousoulaki et al., 2015), the cost of Vitamin E can reach 10-23.5 EUR, approximately, per ton of fish produced. This

also highlights a dire need for filling the considerable knowledge gap that consists in optimizing vitamin E inclusion rates for European sea bass at different developmental stages in order to improve both fish antioxidant response and aquafeed economic viability. In this thesis, chapter 4 was proposed as a study designed to lessen the knowledge gaps regarding this topic. Results from this chapter revealed that, for market-sized European sea bass, there were no differences in lipid oxidation or enzymatic activity between a Vitamin E inclusion level of 100 mg kg^{-1} and an inclusion level of 500 mg kg^{-1}. This could be pertinent in mitigating the economic burden associated with the inclusion of synthetic Vitamin E in aquafeeds. Furthermore, the incorporation of natural antioxidants into aquafeed formulations could play a significant impact on promoting fish growth. By reducing the effects of oxidative stress on physiological processes, natural antioxidants can help fish in coping with environmental and/or rearing-induced stressors such as fluctuations in water quality, temperature, oxygen levels, periodic handling and transportation (Ahmadifar et al., 2021). Moreover, the potential enhancement of fish immune system via natural antioxidant inclusion can bolster disease resistance (Adeshina et al., 2019). These benefits collectively lead to improvements in nutrient utilization and absorption, while also minimizing growth disruptions. Consequently, this contributes to improved growth rates and more efficient conversion of feed into biomass (Bacchetta et al., 2019; Li et al., 2019; Nakano and Wiegertjes, 2020; Salem et al., 2019). Alas, the results from chapters 3 and 4 of this thesis showed that, at a 2% inclusion level, the selected natural antioxidant sources had no discernible effects on European sea bass growth or key growth performance parameters. Although available literature suggests that natural antioxidant inclusion could potentially increase fish growth and productivity, this seems to be highly dependent on various factors such as the natural antioxidant source used, the method and percentage of inclusion, the fish species, and the fish developmental stage. Indeed, inclusion of natural antioxidants in aquafeeds increased productive parameters in nine of the sixteen articles referenced in tables 1.3 and 1.4 (Adeshina et al., 2019; Al-Khalaifah et al., 2020; Hoseinifar et al., 2020b; Lopes et al., 2020; Metwally, 2009; Salem et al., 2019; Sallam et al., 2018; Xavier et al., 2021). Most of these studies focused on either herbivorous or omnivorous species during early developmental stages (IBW = 0.3 - 20.5 g), employing inclusion percentages lower than those used in the present study. Notably, both liquid and dry extracts yield positive results across these works. Meanwhile, the two other analysed studies reported impairments in weight gain and/or productive parameters (Hamed and Abdel-Tawwab, 2021; Zhou et al., 2016), utilizing inclusion percentages higher (3 – 5%) than those used in this thesis (2%). On the other hand, four of these works showed no discernible differences in growth (Hoseinifar et al., 2020a; Lizárraga-Velázquez et al., 2019; Sari and Ustuner, 2018; Zhou et al., 2016). However, despite the positive outcomes in most studies regrading fish growth and/or

other productive parameters, the wide variability in terms fish species and developmental stage, natural antioxidant source, as well as percentage and method of inclusion, hinders a robust comparison with the results obtained in this thesis. The fact that the European sea bass is a carnivorous species might influence the antioxidant bioavailability of vegetable feed matrices, potentially impacting the efficacy of these natural plant additives within the organism (Vázquez and Muñoz-Cueto, 2019). However, works specifically addressing the inclusion of plant-based natural antioxidants in aquafeeds for European sea bass are scarce. Sallam et al. (2018) approached the topic of growth with natural antioxidant inclusion in European sea bass fingerlings (IBW = 2.2g) using a 0.01-0.03% inclusion of dried marigold flour. Although this led to enhanced growth, increased levels of whole-body carotenoids, and improved survival of fish, it's noteworthy that whole body lipid peroxidation also increased, which might point towards a pro-oxidant effect. However, works with other carnivore species demonstrated distinct outcomes. In rainbow trout fingerlings (IBW = 2.5g), Hoseinifar et al. (2020b) successfully increased weight gain and SGR via a 0.05-0.5% inclusion of dried olive waste. Conversely, in a different experiment by Sari and Ustuner (2018) with rainbow trout (IBW = 50g), growth parameters remained unaffected by a 0.025-0.1% inclusion of oregano extracts, aligning with the results found in this thesis. Finally, Xavier et al. (2021) achieved an increase in relative fish growth rate in Senegalese sole (IBW= 12.3-17.7g) with a 1.2% inclusion of grapeseed extract and a 4.2% inclusion of curcumin extract, while growth remained unaffected by a 1.2% inclusion of oregano extract. These varied outcomes underscore the intricacies of the interaction between natural antioxidants and growth performance in different fish species, emphasizing the need for species-specific considerations when formulating diets enriched with these bioactive compounds.

Overall, one of the most influential variables for increasing growth via plant-based natural antioxidant inclusion seems to be fish size and developmental stage. Indeed, among the available literature, studies demonstrating improvements to productive parameters were typically conducted in fish with IBW between 0.3 and 20.5 g, with the exception of Al-Khalaifah et al. (2020), which focused on African catfish with an IBW of 60.5 g. However, it is worth mentioning that unlike the European sea bass, the African catfish is an omnivorous species, which might positively affect the bioavailability of plant-based natural antioxidants within the organism. Regardless, Al-Khalaifah et al. (2020) demonstrated growth benefits with an inclusion rate of 5%, significantly higher than the one used in this study. This raises the question of whether it would be feasible to positively impact European sea bass antioxidant defences with natural antioxidant inclusion rates higher than 2%, particularly market-sized sea bass as utilised in chapter 4. However, it's essential to consider the significant variability of antioxidant capacity between different natural antioxidant sources.

The by-products and co-products used in the three chapters of this thesis were proven rich in phenolic compounds. These compounds have been also proven to exhibit indirect antioxidant activity by promoting the induction of endogenous protective enzymes and exerting positive regulatory effects on signalling pathways (Kumar et al., 2020). While the initial study in chapter 2 proved that including natural antioxidants enhanced feed antioxidant capacity, subsequent chapters (3 and 4) revealed an overall lack of notable benefits to fish antioxidant defences at a 2% inclusion level of fruit by-products and vegetable co-products. However, evidence from most articles referenced in tables 1.3 and 1.4 suggests a modulation of fish antioxidant enzymes or general antioxidant potential in several different organs stemming from natural by-products (Abdel-Tawwab et al., 2018b; Adeshina et al., 2019; Al-Khalaifah et al., 2020; Catap et al., 2015; Guardiola et al., 2016; Hamed and Abdel-Tawwab, 2021; Hoseinifar et al., 2020a; Hoseinifar et al., 2020b; Kavitha et al., 2011; Lizárraga-Velázquez et al., 2019; Lopes et al., 2020; Metwally, 2009; Salem et al., 2019; Sari and Ustuner, 2018; Xavier et al., 2021; Zhou et al., 2016). Moreover, two studies (Guardiola et al., 2016; Zhou et al., 2016) indicated higher expression of genes associated with antioxidant potential, hinting towards the possibility of an increased antioxidant response.

One of the key innovative aspects of this thesis is its thorough analysis of antiperoxidative intracellular mechanisms for combatting oxidative stress within the context of the reported dynamics between endogenous/exogenous antioxidants, as well as free radicals. Indeed, concerning the literature on antioxidant dynamics and enzymatic kinetics in fish fed diets with natural antioxidant inclusion, tables 1.3 and 1.4 show that many studies often lack an analysis of either lipid of protein oxidation, a gap that was duly addressed in chapters 3 and 4 of this thesis. The absence of comprehensive data regarding oxidative and/or nitrosative stress raises questions about the mechanisms driving increased activity and antioxidant enzyme gene expression.

A critical aspect highlighted is the need to compare lipid and/or protein oxidation data from fish fed functional feeds versus control groups. This comparison helps assessing whether the organism synthesizes more antioxidant enzymes to maintain homeostasis, representing an increase in lipid or protein oxidation due to exacerbated ROS/RNS formation, or if the functional components of the feed are contributing towards an increased antioxidant defence system (Batista, S. et al., 2020; Kurutas, 2015). Therefore, analysing the antioxidant system of fish under ideal conditions without comparing them to a stressed group may provide incomplete results regarding the organisms' response to stress. Indeed, in the experiment of chapter 4 where no pro-oxidant challenge was applied, no significant differences were observed. This suggests consistent enzymatic activity and antioxidant capacity in the liver and muscle. It is also important to point out that the results in chapter 3, namely associated with

the effects of pineapple stem inclusion on the antioxidant defences of European sea bass, indicated that the liver of non-stressed fish fed with the 2% pineapple stem diets (S2) had to produce more CAT to achieve the same levels of lipid and protein oxidation as the positive control (VITE). Antioxidants can have varying effects on the organism, ranging from protective to innocuous or even potentially harmful, depending on the context. Therefore, their use should always be approached with a thorough understanding of the situation.

Future prospects for this research require an optimization effort that maximizes the antioxidant capacity of the natural antioxidant sources to prevent losses during processing prior their inclusion in the feed mix. Moreover, future research efforts could explore not only different fish species and natural antioxidant matrices, but also alternative inclusion methods, such as microencapsulation. Maximizing the antioxidant capacity during the processing these vegetable matrices for inclusion and experimenting with distinct feed production methods to minimize air and light exposure could also be avenues worth exploring. These efforts will contribute to a more comprehensive understanding of the efficacy and potential applications of natural antioxidants in aquafeed formulations.

Certain natural antioxidant sources can have positive effects on the immune system of fish. Tables 1.3 and 1.4 highlight studies showing that including natural antioxidants in aquafeeds can enhance the immune function and disease resistance of fish (Adeshina et al., 2019; Al-Khalaifah et al., 2020; Guardiola et al., 2016; Hamed and Abdel-Tawwab, 2021; Hoseinifar et al., 2020b; Sari and Ustuner, 2018). These effects can be achieved by enhancing the production and activity of immune cells such as phagocytes and lymphocytes (Biller, J.D. and Takahashi, L.S., 2018). Moreover, antioxidants may support the immune system's ability to recognize and combat pathogens, thereby reducing the susceptibility of fish to infections (Biller, J.D. and Takahashi, L.S., 2018). Additionally, it's worth noting that the immune system is particularly sensitive to oxidative stress, and excessive ROS/RNS production can compromise the efficiency of the immune response (Biller, J.D. and Takahashi, L.S., 2018). Thus, the inclusion of natural antioxidants is considered a valuable nutritional strategy for enhancing the immune system of farmed fish. However, in the two growth trials performed in this thesis, neither the inclusion of pineapple by-products (chapter 3) nor vegetable co-products (chapter 4) were able to induce differences in the fish immune system. Particularly, lysozyme, peroxidase and ACH50 activities, which were measurements transversal to both studies, remained unaffected by the diets. This is in concordance with results concerning fish antioxidant status, as the positive effects of natural antioxidant inclusion often rely on the upregulation of the fish antioxidant system (Biller, J.D. and Takahashi, L.S., 2018), and differences in fish antioxidant system were virtually non-existent in both works. Tables 1.3 and 1.4 illustrate that in all analysed studies, modulation of the immune system is consistently

accompanied by modulation of the antioxidant system (Adeshina et al., 2019; Al-Khalaifah et al., 2020; Guardiola et al., 2016; Hamed and Abdel-Tawwab, 2021; Hoseinifar et al., 2020a; Hoseinifar et al., 2020b; Sari and Ustuner, 2018). Moreover, in chapter 3, fish plasma metabolites associated with acute stress response remained unaffected by the inclusion of natural antioxidant sources, mirroring the findings of antioxidant enzyme activity. Examples where natural plant-based antioxidant sources modulate fish acute stress response are virtually non-existent in the available literature. However, Al-Khalaifah et al. (2020) reported that doum palm fruit powder inclusion can lower the glucose, cholesterol and triglycerides in the serum of African catfish at an inclusion rate of 1.5%. Additionally, Qader and Omar (2024) reported a significant decrease in triglycerides levels with 1% of coriander seed powder supplementation in feeds for common carp fingerlings (IBW = 25.2g). These effects are indeed dependent on the same variables as antioxidant stress response, including the fish species used, body weight, method and percentage of inclusion, as well as the chemical composition of the natural antioxidant source used. Indeed, given that the production of antioxidant stress enzymes increased in the aforementioned studies supports the hypothesis that modulation of acute stress response may be accompanied by antioxidant enzyme stress response. However, unlike chapter 3 of this thesis, the studies by Al-Khalaifah et al. (2020) and Qader and Omar (2024) lacked an endpoint analysis of the oxidation of intracellular compounds (lipids, protein and/or DNA), while also failing to analyse samples from before and after stress was applied. This leaves open the question of whether the obtained results represent an increase in basal levels of antioxidant activity or if the inclusion of doum palm fruit triggered an antioxidant response due to increased generation of ROS/RNS. Moreover, in chapter 4, the newly selected antioxidant sources also showed virtually no benefits to fish antioxidant and immune system, following the same trend as chapter 3. Thus, acute stress response was not measured in this chapter.

Overall, our results indicate that there is a considerable knowledge gap regarding the potential utilisation of the antioxidant capacity of natural antioxidants from vegetable sources for inclusion in aquafeeds. While minimizing waste and enhancing the utilization of by-products and co-products is widely recognised as favourable achievements in terms of sustainability, a deeper and more multifaceted approach is imperative to shed further light on this topic. It is crucial to assess whether incorporating natural antioxidants into aquafeeds has the potential to become a viable alternative for more sustainable management of fish rearing practices. This would require thorough investigation into various aspects such as antioxidant efficacy, impact on fish health and performance, economic feasibility, and environmental implications.

Main conclusions and future directions

The main conclusions of this thesis can be summarised as follows:

1. **Manufacturing temperatures on feed antioxidant content**: Conventional extrusion and drying temperatures proved to be the most efficient for maintaining the antioxidant capacity of aquafeeds and preserving the chemical integrity of natural antioxidants. Additionally, the 2% inclusion of flours made from fruit by-products, i.e. pineapple and mango, increases the antioxidant capacity of aquafeeds. However, technological improvements and alternative inclusion methods are still needed to optimize the antioxidant capacity of fruit peel flours and to maximize the full antioxidant potential of the tested supplements.

2. **Effects of Pineapple By-Products on European Sea Bass**: A 2% inclusion of selected pineapple by-products did not benefit European sea bass antioxidant defences or fish muscle oxidation over storage. Although higher inclusion rates might offer desired effects, economic viability must be considered. Experimentation during the conversion process from peel to flour, namely via experimenting with different drying techniques and temperatures, may enhance pineapple by-product flour antioxidant capacity. Additionally, testing different inclusion techniques or selecting pineapple cultivars might help achieve the desired effect on fish antioxidant system.

3. **Vitamin E Inclusion and Vegetable Co-Products**: For market-sized European sea bass, there are no significant differences in lipid oxidation or enzymatic activity between Vitamin E inclusion level of 100 mg kg^{-1} and 500 mg kg^{-1}. This finding could help reduce the economic burden of synthetic Vitamin E inclusion in aquafeeds. Moreover, a 2% inclusion of selected whole-dried vegetable co-products did not benefit European sea bass antioxidant defences or fish muscle oxidation over storage. While higher inclusion rates could provide the desired effects, economic viability must be considered.

Overall, these conclusions highlight the complexity and variability in utilizing fruit and vegetables as antioxidant sources in diets for European sea bass. Further research is warranted to optimize processing techniques prior extrusion, inclusion methods and rates, and cultivar selection to maximize the potential benefits of natural antioxidant sources in aquafeeds while ensuring economic feasibility.

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