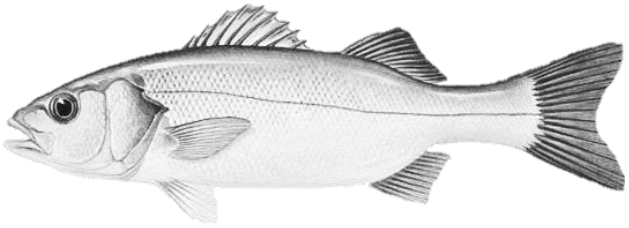

Effect of novel feed ingredients on the physiological responses of European seabass (*Dicentrarchus labrax*) subjected to different rearing temperatures and salinity oscillation

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

Sworn statement

I, Diogo Luis Filipe Amaral, enrolled in the Master's degree Biological aquatic resources at the Faculty of Sciences of the University of Porto hereby declare, in accordance with the provisions of paragraph a) of Article 14 of the Code of Ethical Conduct of the University of Porto, that the content of this dissertation reflects perspectives, research work and my own interpretations at the time of its submission.

By submitting this dissertation, I also declare that it contains the results of my own research work and contributions that have not been previously submitted to this or any other institution.

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Diogo Luís Filipe Amaral
28/09/2022

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Scientific divulgations

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Within this work and the Ocean3R project, an online scientific divulgation series entitled "DEMYSTIFYING AQUACULTURE", composed of ten e-posts regarding the importance in scientific investigation in aquaculture took place in Ocean3R's website, and CIIMAR's social networks: Facebook, Instagram and LinkedIn.

Abstract

Aquaculture is considered the fastest growing animal food-producing sector. If it continues with current practices it is destined to become unsustainable and contribute even further to the climate emergency, which is a growing challenge to the industry itself due to irregular weather patterns and abnormal environmental water parameters that may reduce fish performance, welfare and increase the risks of diseases outbreaks. Aquaculture has heavily relied on fish meal for feed production but due to ethical and sustainability concerns, this ingredient must be gradually phased out of aquafeed production. Multiple industries generate plant-based by-products with high amounts of indigestible carbohydrates, lower protein content and an unbalanced amino acid profile, which may limit their application in feed production. These potential ingredients may be upgraded by biotechnological processes such as solid-state fermentation (SSF), resulting in higher protein concentrations, higher bioavailability of nutrients and bioactive compounds and lower content of anti-nutritional factors.

This study aimed to evaluate the effects of the dietary inclusion of fermented plant-based ingredients in European seabass feed. For that purpose, four isoproteic experimental diets containing 20% and 40% inclusion levels of non-fermented plant-based ingredients mixture (20Mix and 40Mix) and two test diets containing 20% and 40% inclusion levels of the same mixture fermented by *Aspergillus niger* in SSF conditions (20Mix-SSF and 40Mix-SSF).

Two fish trials were carried out, an *in vivo* digestibility trial, followed by an environmental stress trial, where European seabass juveniles were reared at three different temperatures (17, 21 and 26 ºC) and subjected to weekly salinity oscillations. Growth performance, digestive capacity, immune and oxidative stress responses were analysed. Apparent digestibility coefficients (ADCs) of dietary protein, dry matter and energy were significantly improved by plant-based mixture fermentation when included at a 20% level, whereas at 40% inclusion level the opposite effect of fermentation on diet ADCs was observed. α-Amylase activity was significantly higher in diets where plant-based ingredients mixture was included at a 40% level, regardless of mixture treatment. Fermentation of plant-based ingredients led to significantly higher lipase activity, while the opposite was observed for trypsin activity.

Regarding the environmental stress trial, no dietary effect was observed on digestive enzymes activities, whereas rearing temperature and salinity oscillation modulated digestive enzyme activities. Oxidative stress responses were significantly affected by experimental diets, temperature and salinity conditions. Catalase and glutathione

peroxidase activities showed an interactive effect, where fish reared at 21ºC showed higher enzymatic activity when fed the Mix-SSF. Conversely, fish reared at 17ºC and 26ºC showed higher enzymatic activity when fed the control diet (Mix). Overall, no dietary effect was observed on LPO, which was significantly increased in fish reared at 17ºC. Innate immune responses analysed were affected by rearing conditions with fish fed the fermented diet showing higher ACH50 activity.

Higher rearing temperatures led to increased growth rates. A reduction in growth performance, was observed in seabass fed the fermented diet, due to an apparent reduction of dietary palatability.

Overall, the fermentation process of plant-based ingredients using *A. niger*, as carried out in this work, was unable to provide a more efficient ingredient for European seabass juveniles, leading to an overall decrease in growth performance.

Key-words: European seabass (*Dicentrarchus labrax*), Solid state fermentation, Digestibility, Innate immune system, Oxidative stress, Digestive enzymes.

Resumo

A aquicultura é considerada o setor de produção de animais para consumo com maior ritmo de crescimento. As práticas de produção atuais são insustentáveis e se este setor não adotar medidas para minimizar o seu impacto ecológico atual, contribuirá ainda mais para a emergência climática, que é, em si, um desafio crescente para a própria indústria devido aos padrões climáticos irregulares e parâmetros ambientais anormais da água que podem reduzir o desempenho e o bem-estar dos animais e aumentar o risco de surtos de doenças.

A aquicultura está extremamente dependente da farinha de peixe para a produção de rações, mas devido a questões éticas e de sustentabilidade, é necessário que este ingrediente seja gradualmente eliminado desta produção. Várias indústrias criam subprodutos de origem vegetal, caracterizados por grandes quantidades de hidratos de carbono indigeríveis, menor teor proteico e um perfil de aminoácidos desequilibrado, o que pode limitar sua aplicação na formulação de rações. Estes potenciais ingredientes podem ser melhorados através de processos biotecnológicos, como a fermentação em estado sólido (SSF), resultando num produto com um teor proteico superior, maior biodisponibilidade de nutrientes e compostos bioativos e menor teor de fatores anti-nutricionais.

Este estudo teve como objetivo avaliar os efeitos da inclusão de ingredientes de origem vegetal fermentados em ração para robalo. Para tal, foram formuladas quatro dietas: duas dietas controlo com níveis de inclusão de 20% e 40% de mistura de ingredientes de origem vegetal não fermentados (20Mix e 40Mix), e duas dietas experimentais com níveis de inclusão de 20% e 40% da mesma mistura fermentada por *Aspergillus niger* em condições SSF (20Mix-SSF e 40Mix-SSF).

Foram realizados dois ensaios, um ensaio de digestibilidade *in vivo*, seguido de um ensaio de stress ambiental, onde juvenis de robalo foram mantidos a três temperaturas diferentes (17, 21 e 26 ºC) e submetidos a oscilações semanais de salinidade. Parâmetros de crescimento, capacidade digestiva, respostas imunes e indicadores de stress oxidativo foram analisados.

Os coeficientes de digestibilidade aparente (ADCs) de proteína, matéria seca e energia foram significativamente melhorados pela fermentação dos ingredientes, quando incluídos a 20% na dieta. Quando incluídos a 40%, foi observado o efeito oposto da fermentação nos ADCs da dieta. A atividade da α-amilase foi significativamente superior nas dietas em que os ingredientes de origem vegetal foram incluídos a 40%, independentemente do tratamento da mistura. A fermentação de ingredientes levou a

uma maior atividade de lípase, enquanto o efeito oposto foi observado na atividade da tripsina.

Relativamente ao ensaio de stress ambiental, não foi observado qualquer efeito dietético nas atividades das enzimas digestivas, enquanto a temperatura e a oscilação da salinidade modularam as atividades enzimáticas. As respostas fisiológicas ao stress oxidativo foram significativamente afetadas pelas dietas, temperatura da água e salinidade. As atividades da catalase e da glutationa peroxidase revelaram um efeito interativo das dietas e temperatura, onde os peixes mantidos a 21ºC apresentaram maior atividade enzimática quando alimentados com a dieta 20Mix-SSF. Por outro lado, os peixes mantidos a 17ºC e 26ºC apresentaram maior atividade enzimática quando alimentados com a dieta controlo (20Mix). Nenhum efeito da dieta foi observado na LPO, que foi significativamente superior nos peixes mantidos a 17ºC. As respostas imunes inatas analisadas foram afetadas pelos parâmetros ambientais, com os peixes alimentados com a dieta fermentada (20Mix-SSF), apresentando uma maior atividade de ACH50.

Temperaturas da água superiores levaram a maiores taxas de crescimento, contudo, foi observada uma redução do consumo da dieta fermentada devido uma aparente redução da palatibilidade da dieta, o que terá levado a um crescimento inferior dos peixes.

De uma forma geral, o processo de fermentação de ingredientes de origem vegetal por *A. niger*, como realizado neste trabalho, não levou a um ingrediente mais eficiente para juvenis de robalo, levando a uma redução do crescimento destes animais.

Palavras-chaves: Robalo (*Dicentrarchus labrax*), Fermentação em estado sólido, Digestibilidade, Stress oxidativo, Parâmetros imunes inatos, Enzimas digestivas

Table of contents

Table list

Figure list

Figure 13 - Apparent digestion coefficient of gross energy in 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value = 0.032), Inclusion (*p*-value = 0.005), Interaction (*p*-value < 0.001)). Different lowercase letters indicate significant differences between ingredient treatment. Different uppercase letters indicate significant differences between inclusion level. Values are presented as mean ± SD (n=3) (%)

Figure 14 - Apparent digestion coefficient of dry matter in 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value > 0.05), Inclusion (*p*-value = 0.007), Interaction (*p*-value = 0.002)). Different lowercase letters indicate significant differences between ingredient treatment. Different uppercase letters indicate significant differences between inclusion level. Values are presented as mean ± SD (n=3) (%)

Figure 15 - α-Amylase enzymatic activities in *D. labrax* juveniles fed the 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value > 0.05), Inclusion (*p*-value = 0.002), Interaction (*p*-value > 0.05)). Different letters indicate significant differences. Values are presented as mean ± SD (n=9) (U.mg Protein^{-1})

Figure 16 - Lipase enzymatic activities in *D. labrax* juveniles fed the 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value < 0.001), Inclusion (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different letters indicate significant differences. Values are presented as mean ± SD (n=9) (mU.mg Protein^{-1})

Figure 17 - Trypsin enzymatic activities in *D. labrax* juveniles fed the 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value = 0.032), Inclusion (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different letters indicate significant differences. Values are presented as mean ± SD (n=9) (U.mg Protein^{-1})

Figure 18 - Chymotrypsin enzymatic activities in *D. labrax* juveniles fed the 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value > 0.05), Inclusion (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Absence of letters indicates no significant differences. Values are presented as mean ± SD (n=9) (U.mg Protein^{-1})

Figure 19 - α-Amylase - trypsin activities ratio in *D. labrax* juveniles fed the 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value > 0.05), Inclusion (*p*-value = 0.026), Interaction (*p*-value > 0.05)). Different letters indicate significant differences. Values are presented as mean ± SD (n=9)

Figure 20 - Trypsin - chymotrypsin activities ratio in *D. labrax* juveniles fed the 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value > 0.05), Inclusion (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Absence of letters indicates no significant differences. Values are presented as mean ± SD (n=9)

Figure 21 - Feed conversion ratio (FCR) of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANCOVA, Temperature (*p*-value > 0.05), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANCOVA, Salinity (*p*-value > 0.05), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Absence of letters indicates no significant differences. Values are presented as mean ± SD (n=3, except for Fixed 21ºC, n=2)

Figure 22 - Protein efficiency ratio (PER) of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANCOVA, Temperature (*p*-value > 0.05), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANCOVA, Salinity (*p*-value > 0.05), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Absence of letters indicates no significant differences. Values are presented as mean ± SD (n=3, except for Fixed 21ºC, n=2)

Figure 23 - Daily growth index (DGI) of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANCOVA, Temperature (*p*-value = 0.037), Diet (*p*-value = 0.011), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANCOVA, Salinity (*p*-value > 0.05), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and different greek letters indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=3, except for Fixed 21ºC, n=2) (%BW.day^{-1})

Figure 24 - Thermal growth coefficient (TGC) of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANCOVA, Temperature (*p*-value > 0.05), Diet (*p*-value = 0.018), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANCOVA, Salinity (*p*-value > 0.05), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different greek letters indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=3, except for Fixed 21ºC, n=2)

Figure 25 - α-Amylase enzymatic activities in *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC)

and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value < 0.001), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value < 0.001), Diet (p-value > 0.05), Interaction (p-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and salinity, respectively. Values are presented as mean ± SD (n=6) (EU.mg Protein^{-1})

Figure 26 - Lipase enzymatic activities in *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value = 0.015), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value < 0.001), Diet (p-value > 0.05), Interaction (p-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and salinity, respectively. Values are presented as mean ± SD (n=6) (mEU.mg Protein^{-1})

Figure 27 - Trypsin enzymatic activities in *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value = 0.002), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value = 0.020), Diet (p-value > 0.05), Interaction (p-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and salinity, respectively. Values are presented as mean ± SD (n=6) (EU.mg Protein^{-1})

Figure 28 - Chymotrypsin enzymatic activities in *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value = 0.014), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value = 0.002), Diet (p-value > 0.05), Interaction (p-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and salinity, respectively. Values are presented as mean ± SD (n=6) (EU.mg Protein^{-1})

Figure 29 - α-Amylase – Trypsin enzymatic activities ratio in *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value < 0.001), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA,

Salinity (*p*-value > 0.05), Diet (*p*-value = 0.004), Interaction (*p*-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and different greek letters indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=6)

Figure 30 - Trypsin - Chymotrypsin enzymatic activities ratio in *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (*p*-value < 0.001), Diet (*p*-value > 0.05), Interaction (*p*-value = 0.030)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (*p*-value > 0.05), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different uppercase letters indicate significant differences between temperature groups for each experimental diet. Values are presented as mean ± SD (n=6)~

Figure 31 - Lipid peroxidation (LPO) in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (*p*-value = 0.013), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (*p*-value > 0.05), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different letters indicate significant differences regarding effect of temperature. Values are presented as mean ± SD (n=6) (nmolTBA.g^{-1})

Figure 32 - Catalase (CAT) activity in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (*p*-value > 0.05), Diet (*p*-value = 0.003), Interaction (*p*-value < 0.001)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (*p*-value > 0.05), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different greek letters indicate significant differences regarding effect of diet and different uppercase letters indicate significant differences between temperature groups for each experimental diet. Values are presented as mean ± SD (n=6) (µmol.min^{-1}.mg prot^{-1})

Figure 33 - Glutathione peroxidase (GPx) activity in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (*p*-value > 0.05), Diet (*p*-value = 0.006), Interaction (*p*-value < 0.001)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (*p*-value > 0.05), Diet (*p*-value = 0.015), Interaction (*p*-value > 0.05)) Different

greek letters indicate significant differences regarding effect of diet and different uppercase letters indicate significant differences between temperature groups for each experimental diet. Values are presented as mean ± SD (n=6) (nmol.min^{-1}.mg prot^{-1})

Figure 34 - Glutathione reductase (GR) activity in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (*p*-value = 0.006), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (*p*-value > 0.05), Diet (*p*-value = 0.040), Interaction (*p*-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and different letters with asterisk indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=6) (nmol.min^{-1}.mg prot^{-1})

Figure 35 - Glutathione S-transferase (GST) activity in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (*p*-value < 0.001), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (*p*-value < 0.001), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and salinity, respectively. Values are presented as mean ± SD (n=6) (nmol.min^{-1}.mg prot^{-1})

Figure 36 - Total glutathione (TG) activity in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (*p*-value = 0.009), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (*p*-value = 0.002), Diet (*p*-value > 0.05), Interaction (*p*-value = 0.037)). Different lowercase letters indicate significant differences regarding effect of temperature and different uppercase letters indicate significant differences between salinity groups for each experimental diet. Values are presented as mean ± SD (n=6) (nmol.min^{-1}.mg prot^{-1})

Figure 37 - Reduced glutathione (GSH) activity in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (*p*-value = 0.006), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA,

Salinity (*p*-value < 0.001), Diet (*p*-value < 0.001), Interaction (*p*-value = 0.009)). Different letters indicate significant differences regarding effect of temperature and salinity, respectively and different greek letters indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=6) (nmol.min^{-1}.mg prot^{-1})

Figure 38 - Oxidised glutathione (GSSG) activity in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (*p*-value > 0.05), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (*p*-value > 0.05), Diet (*p*-value = 0.034), Interaction (*p*-value > 0.05)). Different greek letters indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=6) (nmol.min^{-1}.mg prot^{-1})

Figure 39 - Reduced glutathione - oxidised glutathione activities ratio (GSH – GSSG ratio) in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (*p*-value < 0.001), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (*p*-value < 0.001), Diet (*p*-value < 0.001), Interaction (*p*-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and salinity, respectively and different greek letters indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=6) (nmol.min^{-1}.mg prot^{-1})

Figure 40 - Peroxidase activity in plasma of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O) (Two-way ANOVA, Temperature (*p*-value < 0.001), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (*p*-value > 0.05), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different letters indicate significant differences regarding effect of temperature. Values are presented as mean ± SD (n=6) (EU.ml^{-1})

Figure 41 - Lysozyme activity in plasma of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (*p*-value > 0.05), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (*p*-value =

0.007), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different letters indicate significant differences regarding effect of salinity. Values are presented as mean ± SD (n=6) ($EU.min^{-1}.ml^{-1}$)

Figure 42 - Alternative complement pathway in plasma of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (*p*-value > 0.05), Diet (*p*-value < 0.001), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (*p*-value < 0.001), Diet (*p*-value = 0.006), Interaction (*p*-value > 0.05)). Different letters indicate significant differences regarding effect of salinity and different greek letters indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=6) ($ACH50\ U.ml^{-1}$)

Abbreviation list

A / T - Amylase to trypsin ratio

ACH50 - Alternative complement pathway.

ADC – Apparent digestion coefficient

AOAC - Association of Official Analytical Chemists

ANCOVA - Analysis of covariance

ANOVA - Analysis of variance

BHT - Butylated hydroxytoluene

BOGA - Bioterium of Aquatic Organisms

CAT - Catalase.

CPI - Crude protein intake

DGI - Daily growth index

DNA - Deoxyribonucleic acid

DNS - 3.5 - dinitrosalicylic acid

DPPH - 2,2-diphenyl-1-picrylhydrazyl

EC - Enzyme Commission

FAO - Food and agriculture organization

FBW - Final body weight

FCR - Feed conversion ratio.

FI - Feed intake

GPx - Glutathione peroxidase.

GR - Glutathione reductase.

GRAS - Generally regarded as safe

GSH - Reduced glutathione.

GSH / GSSG - Reduced glutathione to oxidized glutathione ratio.

GSSG - Oxidized glutathione.

GST - Glutathione s-transferase.

IBW - Initial body weight

LPO - Lipid peroxidation

MDA - Malondialdehyde

$NADP^+$ - Nicotinamide adenine dinucleotide phosphate

PER - Protein efficiency ratio

PVC - Polyvinyl chloride

RAS - Recirculation aquaculture system

ROS - Reactive oxygen species.

SmF - Submerged fermentation

SSF - Solid state fermentation

SOD - Superoxide dismutase

T / C - Trypsin to chymotrypsin ratio

TBARS - Thiobarbituric acid reactive substances

TG - Total glutathione levels

TGC - Thermal growth coefficient

TMB - Tetramethylbenzidine

TNB - Nitrobenzoic acid

US$ - United States Dollar

WG - Weight gain

1 INTRODUCTION

1.1 Current state of global aquaculture

Aquaculture is the controlled culture ("farming") of aquatic organisms (GSA, 2019). Aquaculture production has been steadily increasing, with the Food and Agriculture Organization of the united Nations estimating an average growth of 4.6% per year over the past decade, reaching a record production of 122.6 million tonnes of live weight in 2020 (Figure 1), of which 87.5 million tonnes were aquatic animals, comprised of finfish (57.2 million tonnes), mollusks (17.7 million tonnes), crustaceans (11.2 million tonnes), marine invertebrates (522 thousand tonnes), and semi-aquatic species including turtles and frogs (537 thousand tonnes), while the remaining biomass consisted of 35.1 million tonnes of marine algae (FAO, 2022). Despite the growth of the aquaculture sector and its contribution to the seafood industry, finfish aquaculture output has not yet been able to surpass capture fisheries and it accounted for only 46% of the total finfish production in 2020, while mollusc and crustacean aquaculture production exceeded their respective capture fisheries production. (FAO, 2022). Global aquaculture production is mostly comprised of inland production, accounting for 56% of total aquaculture production (Bostock et al., 2010), This global production is unevenly distributed on a regional level, with Asia accounting for 91,6% of global output of aquatic animals and algae production, this is mostly due to Chinese aquaculture production which accounts for 57.2% of total global aquaculture production, followed by Indonesia and India which account for 12.11% and 7.1% of global production, respectively (FAO, 2022).

Global per capita seafood consumption, increased from 9.0 kg in 1960 to 20.5 kg in 2019 (FAO, 2022), and capture fisheries production has stagnated over the past 30 years, due to overfishing. Aquaculture production has expanded to meet seafood demand and this expansion usually entails an intensification of production methods that requires artificial feed to be supplied to the rearing units. As a result, fed aquaculture has overtaken the non-fed subsector of the aquaculture industry, accounting for 72.2% of total farmed aquatic animal production in 2020 (FAO, 2022) which may pose a challenge to a growing industry in a world with increasingly limited resources (Imelda, 2013). In 2014, the contribution of the aquaculture sector to seafood production for human consumption, surpassed that of capture fisheries and accounted for 56% of production in 2020 (FAO, 2022), highlighting the increasing importance of this sector to the food security of a growing human population.

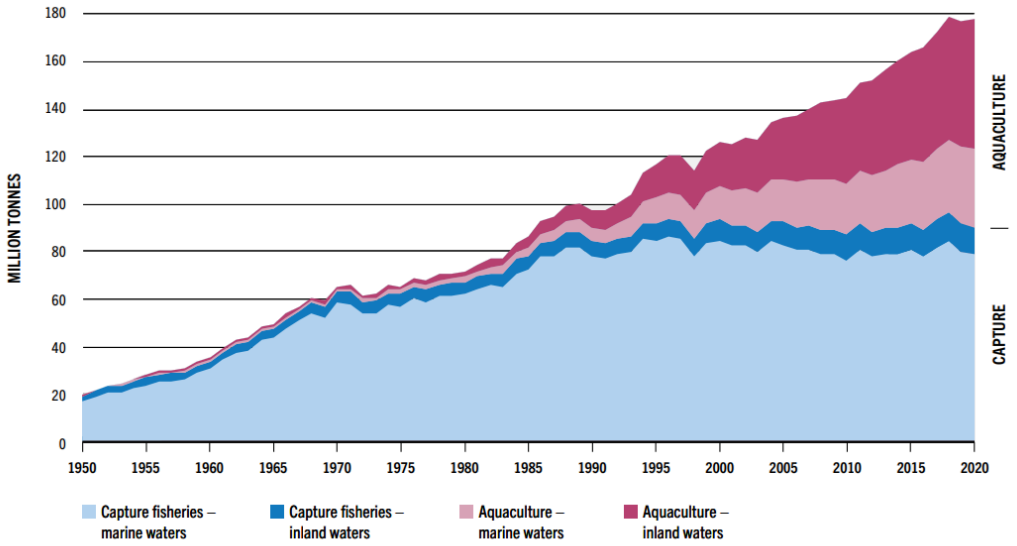

Figure 1 - World capture fisheries and aquaculture production (FAO, 2022)

1.1.1 Current state of aquaculture in Europe and Portugal

European aquaculture production has increased by 29.6% over the last decade (2010 - 2020), totalling 3.29 million tonnes of live weight in 2020 (FAO, 2022). Despite Its growth, the European share of global aquaculture production has decreased from 3.26% in 2010 to 2.69% in 2020. European aquaculture focuses on finfish and shellfish, while production of algae and crustaceans is very low or even negligible. In 2018, diadromous fish species accounted for 60% of production (2 million tonnes), mainly Atlantic salmon (*Salmo salar*) (1.57 million tonnes) and rainbow trout (*Oncorhynchus mykiss*) (400 thousand tonnes). Marine species provided 11%, led by European seabass (*Dicentrarchus labrax*) (200 thousand tonnes) and gilthead seabream (*Sparus aurata*) (170 thousand tonnes) which together account 96% of European finfish aquaculture production (Hough, 2022) Despite the growth observed over the last decade, Europoean aquaculture accounted for only 16-18% of total European seafood production in 2018 (Hough, 2022). The Portuguese aquaculture sector produced a total of 14300 tonnes (€118.5 million) of live weight in 2019, a growth of 2.5% compared to the previous year. The vast majority of production (93.4%) occurs in saltwater or transitional waters systems, where turbot (*Psetta maxima*), European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) are the most produced finfish species. Mollusc aquaculture also plays an important role, accounting for as much as 46.6% of total aquaculture production. Inland fresh water aquaculture accounts for 6% of total production, and consists almost exclusively of trout species (INE, 2021). The Portuguese

capture fisheries produced a total of 188 500 tonnes of seafood, representing 93% of total seafood production in 2019 (INE, 2021; OECD, 2021).The inability of capture fisheries to sustainably increase production and a relatively low aquaculture output in a country where the annual per capita seafood consumption is the third highest in the world, averaging 60 kg (Fernandes, 2017) may play a role in Portugal being a net importer of fish and fish products (OCDE, 2021), importing two-thirds of the seafood consumed (Rocha et al., 2022).

1.2 European Seabass

(*Dicentrarchus labrax*, Linnaeus, 1758)

Dicentrarchus labrax, commonly known as European seabass (Figure 2) was chosen as the model species. It is characterized by an elongated body that can reach a maximum length of 1m and rarely exceeding 50 cm (Collares-Pereira et al., 2021). Adults are bright silver in colour and may even have a blue or green tinge over the entire body. Juveniles are usually slightly darker and have black spots over the back (Collares-Pereira et al., 2021). It is found in shallow coastal waters (<100m), in the Atlantic Ocean, from southern Norway (60°N) to Western Sahara (30°N) and throughout the Mediterranean and the Black Sea (FAO, 2005). Since the European seabass is euryhaline (5–50‰ salinity) (Yılmaz et al., 2020). They are often found foraging in brackish estuaries, lagoons and even in the lower extents of rivers. It is also eurythermal (5–32 °C) (Vázquez; & Muñoz-Cueto, 2014), during winter it migrates from the coast to deeper waters, where the temperature is more stable, preferring temperatures above 9–10 °C (Vandeputte et al., 2019). The European seabass is a gonochoristic species (Moreira da Costa et al. 2021) that reaches sexual maturity when males and females reach a weight of 2 Kg and 3 Kg respectively, usually between 2 and 4 years of age and living up to 30 years (Collares-Pereira et al., 2021; Vandeputte et al., 2019). The annual breeding season starts when environmental temperatures reach 10-14 °C, usually in winter. Seabass spawn in waters with salinities lower than 35 ppt, near river mouths and estuaries or in coastal areas where the salinity is higher (≥30 ppt) (FAO, 2005) European seabass are predators and their diet includes small fish, shrimps, crabs and cuttlefish. (FAO, 2005) Juveniles tend to form schooling groups in search of small crustaceans and molluscs. Adults tend to roam alone, hunting fish (Collares-Pereira et al., 2021)

Figure 2 - European Seabass (*Dicentrarchus labrax*)

1.2.1 State of European Seabass aquaculture

The European seabass is the third most produce fish species in Europe and is currently the most important commercially exploited fish in Mediterranean areas (FAO, 2005; Hough, 2022). Aquaculture production exceeded fisheries output in 1992, accounting for 96% of total production in 2016 (aquaculture 165 915 tonnes vs. fisheries 6919 tonnes) (FAO, 2005). The European seabass aquaculture sector has experienced a stable growth, from 71000 thousand in 2000 to 236 thousand tonnes in 2019 (FAO, 2022; FB, 2021). The majors producers (Figure 3) are Turkey (137 000 tonnes), Greece (41 000 tonnes), Egypt (30 000 tonnes) Spain (25 000 tonnes), Croatia (6 000 tonnes) and Italy (5 000 tonnes) (FEAP, 2020; Hough, 2022) with Turkey and Greece accounting for nearly 75% of total annual production. The Portuguese seabass aquacultures had a total output of 882 tonnes in 2020, a relatively small amount compared with other producers (INE, 2021). As shown, the aquaculture industry is crucial for supplying European seabass to markets and plays an increasingly important role in the European economy (US$ 475 million in exports) (FB, 2021) and sea food accessibility. Improving feeding and production efficiency is necessary for the sustainable development of aquaculture production of this species.

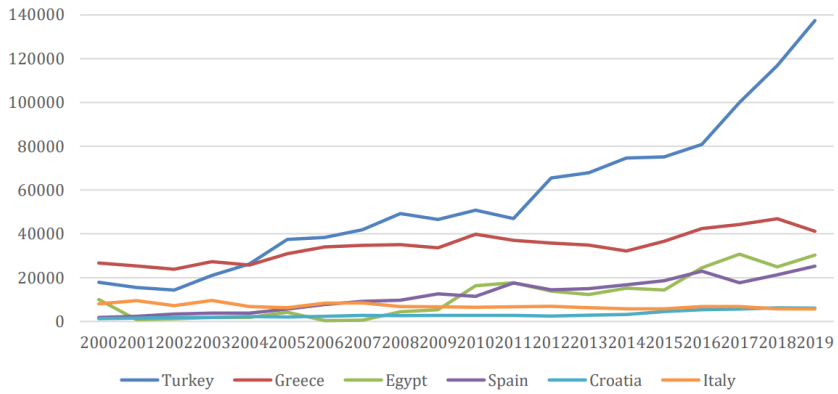

Figure 3 - Production of European seabass (tonnes) 2000-2019 of the six top producer countries in 2019 (FB, 2021)

1.3 Climate change impacts on aquaculture

Climate change is a global emergency that can affect most aspects of society and industry (IPCC, 2022). Aquaculture is an industry heavily dependent and exposed to external environmental parameters which can lead to many challenges and tribulations. Changes and difficulties in aquaculture may be caused, either temporarily or permanently, by the direct and indirect effects of climate change. Suboptimal farming conditions are one of the shot-term effects.

One of the production parameters affected by climate change is water temperature. Global warming will affect temperature patterns, leading to higher water temperatures and more frequent and severe extreme weather events, such as heat and cold waves (Islam et al., 2021). Another impact on farming conditions is water salinity, a warming climate and disrupted water cycles may lead to greater water evaporation and erratic precipitation over the oceans, which could increase the impact of salinity fluctuations on open cage production units (Cheng et al., 2020). Other short-term impacts include disease and parasites outbreaks (Reid et al., 2019), toxic algal blooms (Gobler, 2020) or infrastructure losses due to flooding and other violent weather events. Long-term impacts include scarcity of wild larvae or juvenile fish, limited access to freshwater for aquaculture production, limited access to marine and terrestrial feed ingredients, eutrophication and other disturbances (Barange et al., 2018; Brander et al., 2017). These problems can impact different aspects of the aquaculture industry, and their magnitude may vary due to regional and/or production-specific variables. However, single and combined impacts result in an overall loss of productivity and reduced animal welfare.

1.3.1 Temperature

Most fish are classified as ectothermic, meaning that fish do not use metabolic heat to maintain a stable body temperature (Prosser & Nelson, 1981). This leads to most fish species also being classified as poikilothermic, meaning that body temperature is dependent on environment temperature. This strict dependence makes water temperature one of the most influential environmental factors in ectothermic fish physiology, as it profoundly affects all biochemical reactions and thus the rate of physiological mechanisms (Reynolds & Casterlin, 2015), such as enzymatic reactions, cellular respiration and metabolic rates which influence feed intake, growth and behaviour (Kamunde et al., 2019). Because temperature is such a crucial factor in fish biology, the water temperature has been considered the abiotic ecological master factor (Brett, 1971). Ectothermic animals have a species-specific optimal body temperature range, adapted to their native environment and ensures maximum efficiency of

physiological processes and greater overall fitness (Huey & Stevenson, 2015). For example, the optimal temperature range of European seabass is between 20 and 24 ºC (Claireaux & Lagardère, 1999), and if the temperature rises or falls outside the optimal range, animal performance declines. If the temperature exceeds the upper critical temperature or falls below the lower critical temperature, the animal is at serious risk of mortality. These critical temperatures define the species' thermal range (Angilletta et al., 2010). Thermal tolerance to suboptimal temperatures, while species-specific, also depends on other factors such as genetic strain, developmental stage, age and physiological fitness (Li et al., 2015). It is known that fish respond to changes in the environment temperature, seeking more favourable temperature zones (Larsson, 2005), but in aquaculture this response is not possible due to the limitation of confinement. Since fish cannot avoid temperature changes, their physiology must adapt through a thermal stress response to restore homeostasis. This stress response may result in the production of reactive oxygen species (ROS), which can cause molecular damage to lipids, proteins and DNA (Lushchak & Bagnyukova, 2006). This stress response consists in the mobilization and relocation of energy to facilitate physiological adaptation as well as a behavioural response in a bid to adapt to the new temperature (Fabbri & Moon, 2016). If the stress becomes chronic or recurrent, the redirection of energy may impair the physiological fitness and compromise the performance of the organism (Figure 4). In such cases, long-term negative effects can develop on the immune system, growth or reproductive success (Donaldson et al., 2008; Tort, 2011). As water temperature is such a key factor in fish physiology, it also modulates the organism´s response to other abiotic factors (Vargas-Chacoff et al., 2020).

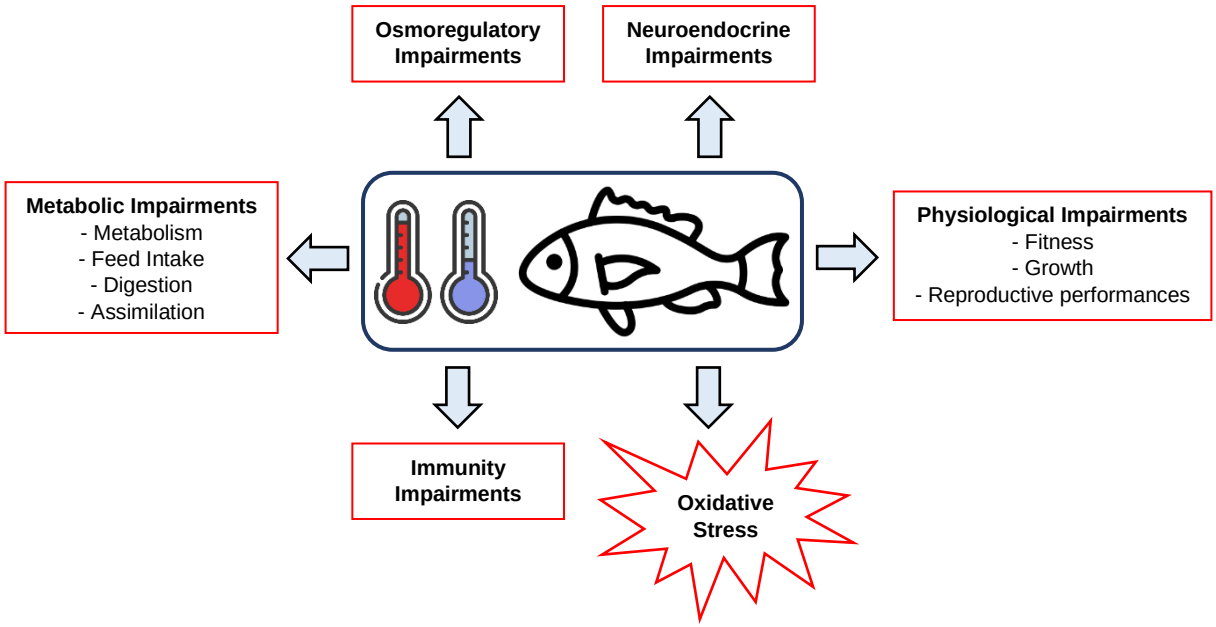

Figure 4 – Temperature stress impacts in fish (based in scheme presented in (Islam et al., 2021)

1.3.2 Salinity

Environmental salinity is one of the most important abiotic factors affecting the distribution, standard metabolic rate, feed intake and feed efficiency (Conides et al., 2004; Lambert et al., 2011) of fish and other aquatic organisms. This is because the vast majority of fish species are restricted to habitats with relatively stable salinity, defined as marine, salinity at 30 to 40 parts per thousand (ppt) or freshwater < 0.5 ppt. Some fish species can also be considered euryhaline species, meaning they are able tolerate a wide range of salinities and some species may even prefer brackish water (0.5 – 30 ppt) as juveniles, as in the case of the European seabass (Collares-Pereira et al., 2021) Although euryhaline fish species are able to tolerate and thrive in a wide range of salinities, due to an osmoregulatory system able to adapt to different salinity conditions (Grosell, 2005), this does not mean that all salinities are optimal for biological efficiency. In fact, the opposite has been observed. Different salinity levels during rearing affect growth performance (Boeuf & Payan, 2001), reproduction (Eskandari et al., 2013), digestive enzymes activity (Moutou et al., 2004), antioxidant capacity and immune efficiency (Mozanzadeh et al., 2021). The alteration of water salinity itself induces salinity stress, which can lead to the production of reactive oxygen species (ROS), and then cause oxidative stress and negatively impact innate humoral immunity (e.g. natural antibodies, lysozyme, peroxidases and complement activity) (Dominguez et al., 2005; Kim et al., 2017). Changes in physiological mechanisms, such as changes/activation of

ion transport mechanisms also occur as a direct response to salinity change (Claireaux & Lagardère, 1999). These responses are usually associated with a change in oxygen consumption (Sangiao-Alvarellos et al., 2003), suggesting an effect on metabolism which may indicate that osmoregulatory mechanisms, in response to salinity stress, having an effect on energy utilization (Figure 5) (Sangiao-Alvarellos et al., 2003). This shift in metabolic energy to maintain homeostasis has been described to have implications for overall organism function (Boeuf & Payan, 2001). In fact, Boeuf and Payan, 2001 have estimated that 10 to 50% of total energy budget may be devoted to osmoregulation, depending on salinity conditions.

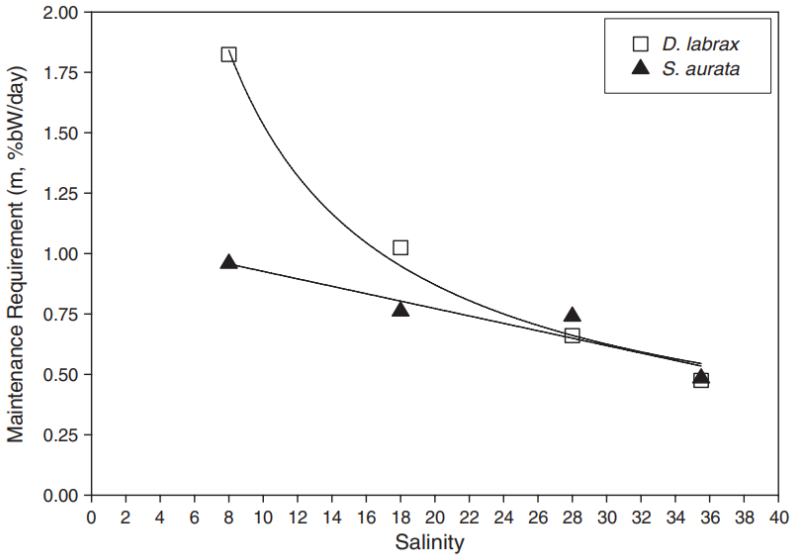

Figure 5 – Relationship between maintenance requirement (m) and salinity for European sea bass (*D. labrax*) and Gilthead sea bream (*S. aurata*), juveniles. (Conides et al., 2004)

1.4 Digestion and digestive enzymes

Understanding how feeds and in particular novel feed ingredients are digested and how their different composition may affect the nutrient digestion is essential to ensure adequate fish nutrition, thus a successful aquaculture production. Feed digestibility depends mainly on feed ingredient composition, with feeds containing high concentrations of indigestible ingredients. Depending on the fish species' feed habits, high dietary inclusion of indigestible ingredients may affect digestive physiology, modulating the digestive enzyme activities present along the digestive tract and resulting in lower digestibility and utilization of the nutrients (Gaylord et al., 2008) and on the nature of enzyme activities present along the digestive tract, which depends on how the species natural feeding habits affected the evolution of the digestive system. Digestion consists of the coordinated action of physical, chemical and enzymatic processes that begin with

the ingestion of food and consists in a progressive process that takes place in the stomach, continues along the intestine and ends with the ejection of unabsorbed matter. The action of pancreatic digestive enzymes plays an essential role in digestion, with trypsin and chymotrypsin being the main pancreatic proteases, lipase being the main pancreatic lipolytic enzyme, and amylase being known as the main pancreatic digestive enzyme for starch (Murashita et al., 2015). The activity of these enzymes, hydrolyses macronutrients into smaller molecules, such as amino acids or short peptide chains, simple sugars and fatty acids formed by the hydrolysis of proteins, polysaccharides (starch) and lipids, respectively (Caruso et al., 2009). These soluble subunits are then transported across the intestinal wall and into the circulatory system, where they eventually reach the various tissues of the organism, and are used in the maintenance, growth, and reproduction physiological processes organism (Borlongan et al., 2002; Grosell et al., 2010)

The secretion of pancreatic enzymes is controlled by both neuronal and hormonal factors (Konturek et al., 2003) and is influenced by several parameters, such as: ambient temperature (Pereira et al., 2018) and salinity (Moutou et al., 2004), age (Kuz'mina, 1996) and dietary ingredient composition but the relationships between enzyme expression and these factors have not yet been clearly established (Caruso et al., 2009; Silva et al., 2010). The analysis of digestive enzymes activity can further our understanding on how adverse environmental conditions affect digestive processes, essential for maintaining fish welfare and whether novel feed ingredients/additives are able to modulate these digestive responses.

1.4.1 α-Amylase

Fish feed contains a variety of different types of carbohydrates, ranging from highly soluble and digestible mono-, di-, and oligosaccharides, glycogen, and starch, to only slightly soluble and poorly digestible cellulose, hemicellulose and chitin. Carbohydrates play an important role in metabolism, e.g. as a protein and lipid energy sparing for other metabolic purposes, and also serve as precursors of endogenous nucleic acids and polysaccharides.

Digestion of carbohydrates in fish is carried out by several enzymes, the most important of which is amylase (Lovell, 1988) whose activity varies significantly between fish species, especially between herbivorous and carnivorous species, affecting the digestibility of carbohydrate (Chakrabarti et al., 1995). The natural feeding habits of species appear to affect not only basal amylase activity but also the response to different levels of carbohydrate intake, with higher carbohydrate intake appearing to increase

enzymatic activity in herbivorous and omnivorous species (Krogdahl et al., 2005). In carnivorous species, the responses are less clear.

α-Amylase is secreted by the exocrine pancreas and acts in the intestinal lumen and pyloric caeca, where it hydrolyses a(1-4) glucoside-linkages into complex polysaccharides and breaks them down into glucose, maltose, maltotriose and a combination of branched (1:6) oligosaccharides (Papoutsoglou & Lyndon, 2003).

1.4.2 Lipase

Lipids make food more palatable, serve as vehicles for the absorption of fatty acids and other lipid-soluble compounds, like lipo-soluble vitamins and pigments, and are the main energy source for animals, especially carnivorous fish, whose ability to use carbohydrates as an energy source is relatively limited (Chakrabarti et al., 1995). In addition, lipids provide the organism with long-chain n-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) as essential fatty acids, which are important for good overall development and growth, especially in marine fish (Chou et al., 2001).

The digestion of lipids differs from the digestion of other nutrients in that lipids are not water-soluble and therefore must be emulsified by the peristaltic movements of the intestine and by bile salts secreted by the gallbladder (Moraes & de Almeida, 2020). This emulsification enables the lipase activity of the pancreas, which consists of the hydrolysis of the ester bonds of the triacylglycerols, transforming them into diglycerides, then into monoacylglycerides and finally into glycerol and fatty acids. These are then absorbed in the form of micelles, a mixture of cholesterol, lecithin and bile salts (Moraes & de Almeida, 2020).

Lipase is the major lipolytic enzyme and enzymatic activity appears to be modulated depending on dietary lipid sources and lipid content (Bogevik et al., 2009)

1.4.3 – Trypsin

Trypsin is an endopeptidase and is considered the main protease in the fish gut, acting specifically in the hydrolysis of peptide bonds involving the carboxyl groups of lysine and arginine residues (Moraes & de Almeida, 2020). It is estimated that, in carnivorous fish, trypsin is responsible for the digestion of as much as 50% of ingested proteins (Eshel et al., 1993). Trypsin is synthesized by the pancreas and secreted in an inactive form (trypsinogen) and transported to the intestine and pyloric caeca. Its activation occurs through the action of enteropeptidase, secreted by the intestinal mucosa, on trypsinogen (Moraes & de Almeida, 2020). Trypsin is the only pancreatic protease that can activate its own inactive form (zymogen) as well as the inactive forms of other proteases secreted

by the pancreas, and thus, plays an essential role in regulating protein digestion (Hjelmeland et al., 1984). Studies have shown that proteolytic activity is generally less dependent on dietary habits (Hidalgo et al., 1999).

1.4.4 Chymotrypsin

Like trypsin, chymotrypsin is an endopeptidase synthetized in the pancreas and released into the intestinal lumen in an inactive form (chymotrypsinogen), which is then activated by the action of trypsin (Figure 6) (Moraes & de Almeida, 2020). This enzyme specifically hydrolyses peptide bonds on the carboxyl side of the aromatic chains of tyrosine, tryptophan, and phenylalanine, as well as large hydrophobic residues such as methionine. Compared to trypsin, chymotrypsin can be seen to have a much wider range of proteolytic activity (Lundstedt et al., 2004)

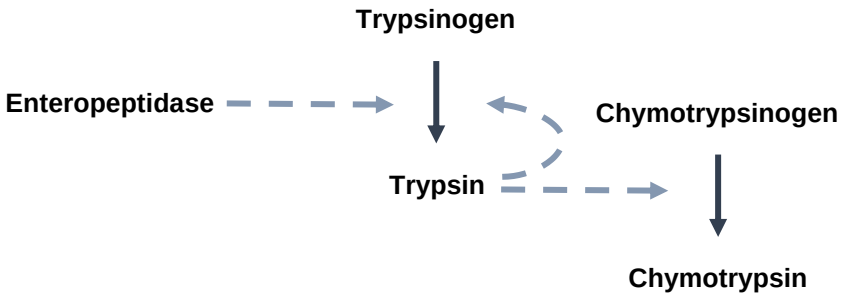

Figure 6 – Trypsin and chymotrypsin activation path. (Adapted from (Maine University, 2014)

1.4.5 Amylase to Trypsin ratio

The ratio between α-amylase (A) and trypsin (T) activities is considered an indicator of the flexibility of energy metabolism and shows the ability of fish to use carbohydrates instead of proteins or lipids as energy sources (Aragón-Axomulco et al., 2012). Thus, this ratio may be interpreted as an indicator of protein sparing in fish. However, the correlation between the A/T ratio and better growth parameters or more efficient protein utilisation is still not entirely clear (Thongprajukaew et al., 2011)

1.4.6 Trypsin to Chymotrypsin ratio

The secretion rate of trypsin (T) and chymotrypsin (C) is associated with feed intake and may be an indicator of satiety status (Rungruangsak-Torrissen et al., 2006) The ratio of their enzymatic activity has been interpreted as an indicator of the nutritional status of fish, where a higher T/C ratio may correlate with a higher absorption rate of essential amino acids, thus stimulating protein synthesis (Rungruangsak-Torrissen & Sundby, 2000)

1.5 Oxidative stress

All aerobic life generates of potentially dangerous, partially reduced species of molecular oxygen radicals due to regular oxygen metabolism (Davies, 1995). These species of molecular oxygen radicals are called reactive oxygen species (ROS).

Examples of ROS include molecular and singlet oxygen, superoxide anion, hydroxyl radical and hydrogen peroxide, a non-radical compound (Lushchak, 2011; Mishra et al., 2015). Although ROS plays a variety of roles in the normal function of the body, such as innate humoral protection against pathogens, cell cycle regulation and many cellular signalling pathways (Martínez-Álvarez et al., 2005). When the generation of ROS is greater than its neutralisation by antioxidant mechanisms, there is an accumulation of ROS, resulting in an imbalanced redox status and oxidative stress that can lead to molecular damage in cellular components, such as protein denaturation, unsaturated lipid peroxidation, DNA hydroxylation, cell damage, and eventually cell apoptosis, while prolonged extreme oxidative damage can even cause tissue necrosis (Figure 7) (Martínez-Álvarez et al., 2005; Zong & Thompson, 2006). Lipid peroxidation produces unstable lipid hydroperoxides, the products of which are highly reactive and further compromise cell integrity (Martínez-Álvarez et al., 2005). Specific stressors under aquaculture conditions include: overcrowding, hyper or hypothermia, hypoxic conditions, hyper or hypo salinity, malnutrition and pollutants of various types (Bagni et al., 2007; Lushchak, 2011; Lushchak & Bagnyukova, 2006; Pascual et al., 2003). These stressors trigger responses that affect the normal physiology of fish (Harper & Wolf, 2009). To avoid the adverse effects of unbalanced redox status, the organism has developed an antioxidant defence system with two different classes: enzymatic and non-enzymatic antioxidants. The enzymatic antioxidant system includes various enzymes such as catalase (CAT) and glutathione peroxidase (GPx) and the non-enzymatic antioxidants such as glutathione, thioredoxin, carotenoids, vitamin C and E (Mishra et al., 2015).

The actions of the different enzymes can be succinctly described as:
The first ROS formed is usually the superoxide radical (O_2^-) and the enzyme responsible for its removal is the superoxide dismutase (SOD), which catalyses the dismutation of two O_2^- molecules into oxygen (O_2) and hydrogen peroxide (H_2O_2). Without SOD activity, this radical leads to hydroxyl radicals (HO) formation. The hydroxyl radicals have a very short half-life and are considered the most reactive and harmful ROS. Since no antioxidant is capable to neutralise hydroxyl radicals, it is crucial to inhibit its formation or repair the damage caused by its action (Schieber & Chandel, 2014). Differently, the non-radical ROS, hydrogen peroxide (H_2O_2) is abundantly produced in the mitochondrial matrix during the oxygen reduction process. Catalase (CAT) (EC 1.11.1.6.) catalyses the

decomposition of H_2O_2 into H_2O and O_2 (Devi et al., 2019). Hydrogen peroxide can also be neutralized by Glutathione peroxidase (GPx) (EC 1.11.1.9.), in the presence of glutathione (GSH). This neutralization is crucial given the fact that H_2O_2 can easily pass through membranes and be released into the cell cytoplasm, where in the presence of metal ions, can be converted to the highly reactive hydroxyl radical. Glutathione peroxidase is also able reduce lipid hydroperoxides to their corresponding alcohols. Glutathione S-transferase (GST) (EC 2.5.1.18) catalyses the conjugation of reduced glutathione to several substrates and is also able to neutralize xenobiotics (Eroglu et al., 2015; Martínez-Álvarez et al., 2005). Glutathione reductase (GR) (EC 1.8.1.7) activity is required for the reduction of oxidized glutathione (GSSG) (previously oxidized by the action of glutathione dependent enzymes) leading to the regeneration of reduced glutathione (GSH) (Parolini et al., 2019). Studies suggest that the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) may indicate a more appropriate antioxidant state (Eroglu et al., 2015).

Increasing the efficiency and level of antioxidant defence mechanisms plays an effective role in mitigating oxidative stress (Lee et al., 2005). This has led to extensive research trials on increasing fish antioxidant enzymatic and non-enzymatic activities.

The liver has been shown to play a prominent role in energy storage and metabolism (Wolf & Wolfe, 2005) leading to this organ being very sensitive to the presence of oxidants (Ben Ameur et al., 2012). Therefore, it is believed that monitoring the activities of antioxidant enzymes in the liver can provide a good understanding of the antioxidant status in fish.

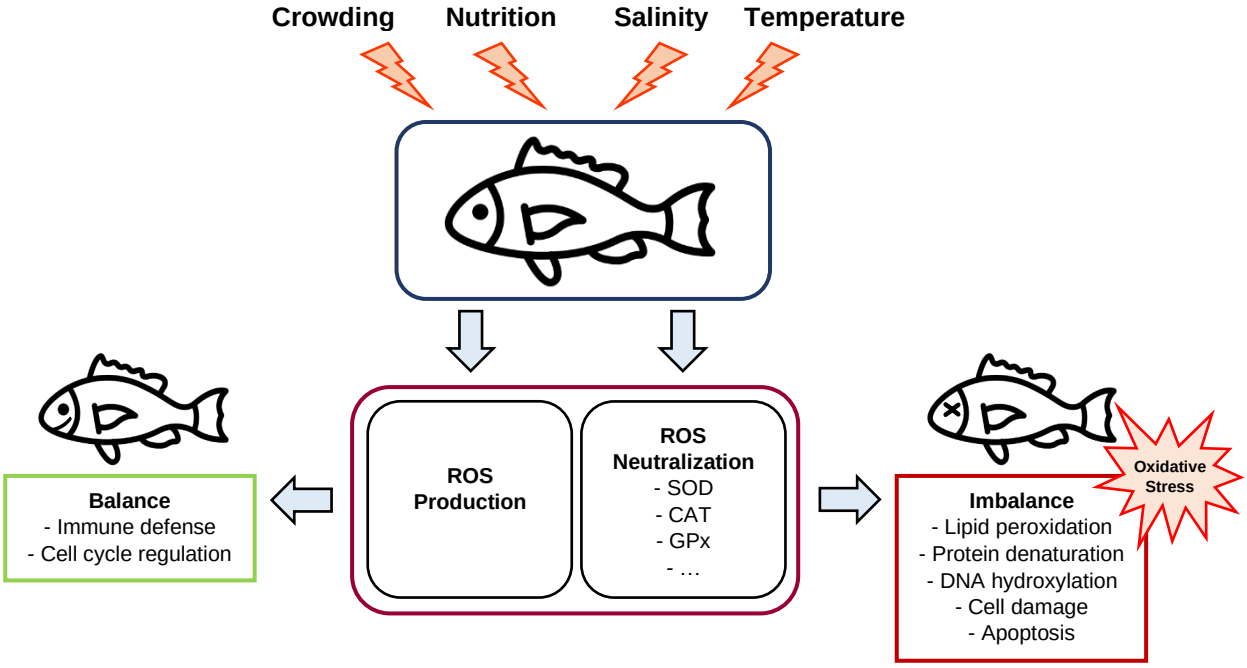

Figure 7 – Effects of ROS production and neutralization balance/imbalance, based on (Hoseinifar et al., 2021).

1.6 Humoral Innate Immunity

The frequency of disease outbreaks has increased as a consequence of aquaculture intensification. It is well known that the balance between the host, pathogen, and environment determines the disease occurrence in fish (Roberts, 2012).

The immune system of fish is a large, complex network that includes a variety of tissues, organs, cells and molecules focused on host defence (Firdaus-Nawi & Saad, 2016). These cells and molecules are able to recognise and destroy foreign substances, especially pathogens, thus protecting the organism from disease and maintaining homeostasis (Chaplin, 2010).

The immune system is able to perform two different types of responses, the innate responses, which are non-specific, and the adaptive immune responses, which target specific pathogens. This system, in fish, is generally divided into: physical, humoral and cellular factors (Kordon et al., 2019).

Physical factors include physical barriers such as fish scales, a layer of mucus and epithelial cells that line the skin, gills and digestive tract and play a vital role in preventing infections (Ellis, 2001). Humoral innate components include bacterial growth inhibitory factors such as transferrin, antiproteases, lysozyme, C-reactive protein, antibacterial peptides and complement system proteins activated via alternative and lectin pathways (Cordero et al., 2016). Among the cellular factors, many types of leukocytes are involved in the innate, non-specific cellular immunity of fish. These include Toll-like receptors, granulocytes, macrophages and non-specific cytotoxic cells (Uribe et al., 2011). These factors play a significant role in the innate cellular immune response to invaders or tissue injury.

Environmental stress and stress induced by aquaculture practices can have a suppressive effect on this type of response, while various food additives and immunostimulants can increase their efficiency (Magnadottir, 2010).

1.6.1 Peroxidase

Neutrophils and macrophages are crucial elements of the innate immune system that defend the organism from microbial infections. through phagocytosis, chemotaxis and bactericidal activity (Katzenback et al., 2012). Neutrophils, when activated, release hydrogen peroxide and myeloperoxidase (EC 1.11.2.2), a haem-containing lysosomal glycoprotein found mainly in the cytoplasmic granules of neutrophils and in much smaller quantities in monocytes and some tissue macrophages (Klebanoff, 1968). Myeloperoxidase then catalyses the chloride ions and hydrogen peroxide reaction to form hypochlorous acid, which halogenates the bacterial cell wall and kills the bacteria

(Klebanoff, 1968). Similar to the myeloperoxidase of neutrophils, eosinophils produce an eosinophil peroxidase (EC 1.11.1.7). This enzyme converts bromide into hypobromite, which is toxic to pathogens (Bielek, 1981). Peroxidase levels are used as an indicator of the immunological activity of circulating leucocytes, which increases in response to infection (Alvarez-Pellitero, 2008).

1.6.2 Lysozyme

Lysozyme (EC 3.2.1.17), a glycoside hydrolase enzyme, catalyses the hydrolysis of glycoside-β-(1,4)-bonds in peptidoglycans of the bacterial cell wall, leading to it´s lysis. Therefore, lysozyme is important in neutralizing pathogens, especially gram-positive bacteria, as they have an outer peptidoglycan layer (Itami et al., 1992). In fish, neutrophils play a major role in lysozyme synthesis (Ellis, 2001) but monocytes are also able to produce this enzyme (Fletcher & White, 1973). Therefore, fish lysozyme is distributed mainly in the leucocyte-rich organs and in a variety of animal secretions such as mucus and saliva (Fletcher & White, 1973). Lysozyme is also known as opsonin, which stimulates the activity of the complement system and phagocytes (Grinde, 1989).

1.6.3 Complement system

The complement system is one of the most important mechanisms of the humoral component of the immune system and is composed of several glycoproteins in an inactive form that are triggered by successive pathways, as the first stimulus contributes to the proteolysis of the next component (Gasque, 2004). Complement system activation can be triggered by three pathways: the classical pathway, which is triggered by the binding of antibodies to the cell surface (Holland & Lambris, 2002); The alternative pathway which is independent of antibodies and directly activated by foreign microorganisms and the lectin pathway triggered by bacterial surface carbohydrates (Sakai, 1992). The role of the complement system includes the direct elimination of infectious organisms through lysis activity, neutralization of pathogenic exotoxins (Uribe et al., 2011) and signalling to the potential presence of pathogens in the host, thus indirectly contributing to the elimination of pathogens by recruiting immune cells and through opsonisation (Boshra et al., 2006). It has been shown that the complement system of fish is influenced by light exposure (Esteban et al., 2006), ambient temperature (Tort et al., 1998) and diet (Geay et al., 2011).

1.7 The need for novel ingredients in aquaculture

The aquaculture industry has experienced a steady growth in recent decades and future global demand for fisheries products can only be met by increasing aquaculture

production, as many of the world's fish stocks are classified as fully or over-exploited (FAO, 2022). To sustain this growth, the supply of feed must also increase at a similar rate to meet demand. Fishmeal and fish oil have traditionally been the most important components of aqua feed for carnivorous and omnivorous species, with aquaculture's share of global fishmeal and fish oil consumption more than doubling in recent decades (Tacon & Metian, 2008). Fishmeal, obtained mainly from grinding whole small pelagic fish into a concentrated, protein-rich form, is the most important source of protein in the aqua feed industry due to its balanced amino acid profile and high protein content, high digestibility and good source of micronutrients and essential fatty acids (Abdelghany, 2003). Due to the increasing global demand for this finite resource, its high cost, variable quality and ethical concerns about the use of fish for fish feeding, a consensus has been reached that there is an urgent need to develop low-cost, high-quality alternative protein sources from cereals (wheat, maize, rice, etc.), oilseeds (soybean, sunflower, rapeseed, cottonseed, etc.) and legumes (beans, lupins, peas, etc.) as partial or full substitutes for fishmeal.

The ingredients considered for alternative protein and lipid sources must have suitable nutritional properties, such as low fibre, starch and antinutritional factor content, relatively high protein content, favourable amino acid profile, high nutrient digestibility and adequate palatability (Naylor et al., 2009). Certain plant ingredients, such as agricultural by-products, are abundantly available and at a relatively low cost, but despite these factors, most plant alternatives present challenges for further use, such as large amounts of indigestible organic matter in the form of insoluble carbohydrates, lower protein content and an unbalanced amino acid profile (Hardy, 2010). Alternative ingredients may undergo biochemical processing, e.g. heat and/or enzyme treatment, or biological processing, such as fermentation, to reduce some of the disadvantages and produce a more attractive ingredient (Bandara, 2018).

1.8 Solid state fermentation

Solid-state fermentation (SSF) consists of microbial growth on insoluble substrates that can be used as a source of carbon and energy, in the absence (or near absence) of water, which is similar to the natural environment to which certain microorganisms are adapted (Pandey et al., 2000). In contrast, in submerged fermentation (SmF), both the substrate and the microorganisms are immersed in water (Graminha et al., 2008).

This biotransformation process aims to cultivate microorganisms such as fungi, yeasts or bacteria in close contact with the substrate and thus achieve the highest substrate concentrations for fermentation (Hölker et al., 2004). In recent years, interest in the reuse of by-products from agriculture and the food industry has increased, as the inadequate

collection and improper disposal of these wastes can lead to serious pollution problems and a significant loss of biomass that other industries could potentially repurpose (Correia et al., 2004). Therefore, in an effort to find a potential solution for waste management and increase sustainability in the feed industry, SSF is increasingly used to improve the nutritional value of agricultural by-products and to produce enzymes and bioactive compounds (Romo Sánchez et al., 2015). The increasing adoption of SSF is also due to several advantages, such as lower operating costs, smaller fermenters, fewer downstream processes, agitation and lower sterilisation costs compared to submerged fermentation (SmF) (dos Santos et al., 2012). However, there are also some disadvantages of SSF, mainly due to uneven temperature, pH, humidity, substrate concentration or pO2 during cultivation, which are difficult to control when water availability is limited (Hölker et al., 2004).

1.8.1 *Aspergillus niger* 2088

Filamentous fungi are considered great producers of bioactive compounds by SSF, therefore, these microorganisms are the most commonly used in this process (Aguilar et al., 2008; Nigam & Pandey, 2009). In the present study, *Aspergillus niger*, a filamentous fungus belonging to the group of "Black Aspergilli", was chosen as a model microorganism because it is easy to handle and has a versatile metabolism that has made it one of the most important production organisms for industrial fermentation (Nielsen et al., 2009), being able to ferment a variety of cheap raw materials and synthesise over 19 types of enzymes, such as cellulase, pectinase, protease, etc. (Hölker et al., 2004). *A. niger* has been classified by the World Health Organisation as suitable for daily consumption and has been given the status "generally regarded as safe" (GRAS) by the US Food and Drug Administration (Max et al., 2010). However, the potential synthesis of mycotoxins (ochratoxin A and fumonisins) (Nielsen et al., 2009) may underline the need for a renewed and optimised screening procedure for mycotoxin production by these microorganisms.

1.8.2 Increased bioavailability of nutrients

Structural compounds from plant cell walls, including cellulose, hemicellulose, pectin and lignin, can account for over 50% of the dry weight of plant ingredients (Zeng et al., 2017). These fibres are not digested by the enzymes of the animal's digestive system and therefore cannot provide calories. Fibre can be converted into soluble sugars by acid hydrolysis or by depolymerizing enzymes (Cara et al., 2008), the use of which in animal feed production is recognised for the partial degradation of agro-industrial by-products used as feed ingredients (Jannathulla et al., 2018; Sousa et al., 2022). Lignocellulose-

degrading microorganisms can be selected as producers of such enzymes under specific growth conditions. One of these microorganisms is the fungus *Aspergillus niger*, which is considered a good producer of lignocellulose-degrading enzymes, which include cellulases (Raj et al., 2010), xylanases (Membrillo Venegas et al., 2013), ligninases (Filipe et al., 2019), among other enzymes. Lignocellulosic enzymes are able to break down the structure of cellulose, hemicellulose, and lignin in plant materials, increasing the digestibility and consequently the bioavailability of nutrients in plant-based ingredients. Studies have also shown that SSF can increase protein content and bioavailability in low-value plant material and decrease anti-nutritional factors (Chebaibi et al., 2019). Thus, two nutritionally important contributions can be obtained from the fermentation process: Protein enrichment and higher degradability of the feed material.

1.8.3 Increased bioavailability of bioactive compounds

Feed quality is not only a question of nutrient utilization efficiency but also of the presence and bioavailability of bioactive compounds that improve animal health and wellbeing. These compounds are extra nutritional factors that naturally occur in a wide array of ingredients (Kris-Etherton et al., 2002) and include secondary metabolites such as antibiotics, alkaloids and phenolic compounds. Phenolic compounds include flavonoids, phenolic acids and tannins (Kris-Etherton et al., 2002). Over the last few decades, these compounds have become sought after feed additives due to their potential to prevent various health problems associated with oxidative cellular processes by scavenging free radicals and chelating metals that can catalyse lipid peroxidation (Correia et al., 2004). Multiple plant-based ingredients are a good source of bioactive phenolic compounds (Dimitrios, 2006). The bioavailability of the phenolic components in these ingredients is low because most of these compounds are present in the plant cell walls in insoluble bound forms as conjugates with sugars, fatty acids or amino acids. These bounded forms of the compounds reduce their ability to act as good antioxidants (Vattem et al., 2004). The phenolic compounds can be released from the conjugated form by enzymatic hydrolysis with various carbohydrases produced by fungi during SSF of lignocellulosic wastes, thereby converting the bound phenols to the soluble form and improving the functionality of these phytochemicals (Bhanja et al., 2009).

1.8.4 Fungal immunostimulants

An immunostimulant is a compound that modulates the activity of the components of the immune system, thereby increasing the host's resistance to disease, which in most cases is caused by pathogens (Sakai, 1999). These compounds can derive from a variety of sources, such as chemical substances, animal and plant extracts, bacterial components,

yeasts and fungi (Sakai, 1999). Natural compounds are of particular interest because their use is less harmful to the environment, the relatively low cost of their source ingredients and consumer preference for antibiotic-free products (Bricknell & Dalmo, 2005).

It has already been observed that fungi added to feed can act as immune stimulants and improve the resistance of the fish to diseases (Ruiz-González et al., 2018). This is because these organisms contain a wide range of compounds with immunostimulatory properties such as lectins, terpenoids and polysaccharides (El Enshasy & Hatti-Kaul, 2013),

Fungal polysaccharides can regulate growth and immune response in fish and are considered potent immunological stimulators, directly activating defence mechanisms such as leukocytes and further stimulating their phagocytic, cytotoxic and antimicrobial activities or enhancing the growth of the gut microbiota, thus acting as prebiotic substances (Uluköy et al., 2016).

These polysaccharides are usually present in the form of glucans, the main structural polysaccharides of the fungal cell wall, accounting for 50-60% of the dry weight of this structure (Garcia-Rubio et al., 2019). Glucans, can be β-glucans or α-glucans, the former being associated with a stronger immunostimulatory effect (Wasser, 2002). β-Glucans can be extracted from the cell wall of plants, yeasts, bacteria and fungi and are directly recognised by immune cells via specific receptors on plasma membranes, initiating the downstream signalling process that leads to the enhancement of non-specific immune factos (Kiron et al., 2016) such as lysozyme and complement activities in a wide range of fish species (Aramli et al., 2015; Kumari & Sahoo, 2006; Lauridsen & Buchmann, 2010; Yamamoto et al., 2018).

In addition, fungi may be rich in vitamins (vitamin C, E, B1, B2, B3 …) and minerals (Ca, Cr, Fe, K, Na, P and Zn) (Ruiz-González et al., 2018; Sadler, 2003). Among these, some have been associated with an increase in the immune response, such as vitamin B, vitamin E and zinc (Sakai, 1999).

Immunostimulants used as dietary supplements in aquafeed are of great importance as they have the potential to improve innate immune mechanisms that are usually weakened during periods of increased stress, which usually leads to disease outbreaks in aquaculture (Bagni et al., 2000).

2. OBJECTIVES

This study aimed to evaluate the effects of dietary incorporation of solid-sate fermented plant-based ingredients on diet digestibility and physiological responses of European seabass subjected to environmental stress. To this end, an in vivo digestibility trial was conducted, where apparent digestion coefficients and digestive enzymes activities were analysed, followed by an environmental stress trial, where growth performance, digestive enzyme activities, innate immune parameters and oxidative stress indicators were analysed.

3. MATERIALS AND METHODS

All trials were conducted under the supervision of an expert in laboratory animal science accredited by the Portuguese Veterinary Authority (1005/92, DGV-Portugal) in accordance with the guidelines for the protection of animals used for scientific purposes from European Directive 2010/63/UE.

3.1 Experimental diets

The experimental diets consisted of two control diets with 20% and 40% inclusion levels of non-fermented plant-based ingredients mixture (20Mix / 40Mix), and two experimental diets with 20% and 40% inclusion levels of plant-based ingredients mixture fermented by *A. niger* 2088 CECT (20SSF-Mix / 40SSF-Mix). Plant-based ingredients mixture is composed of soybean meal (25%), rapeseed meal (25%), sunflower seed (25%) and rice bran (25%). Diets are isolipidic (18%), isoproteic (42%) (Table 1)**.** Solid state fermentation was performed by mixing 400g of the plant-based ingredients with 10^6 spores/ml, at 75% humidity for 7 days at 25 °C; the process was repeated to obtain enough fermented plant-based mixture for feed formulation (Table 1).

Feed production consisted of drying, grounding, and mixing all dietary ingredients. The mixture was then dry-pelleted through a 2 mm die using a pellet mill (California Peter mill, CPM Crawforddsville, IN, USA). After drying for 48h at 40°C, pellets were sieved and stored at -18°C until utilized and between feedings.

Table 1 - Control and experimental diets composition, proximate composition and bioactive compounds composition

Feedstuff (% dry weight)	**20Mix**	**20SSF-Mix**	**40Mix**	**40SSF-Mix**
Fish meal[1]	17.5	17.5	17.5	17.5
Fish Protein Concentrate[2]	2.5	2.5	2.5	2.5
Plant-based ingredient mix[3]	20.0	0.0	40.0	0.0
Fermented plant-based ingredient mix[4]	0.0	20.0	0.0	40.0
Wheat gluten[5]	7.5	7.5	7.5	7.5
Corn gluten[6]	13.4	11.4	5.1	1.2
Hemoglobin[7]	5.0	5.0	5.0	5.0
Wheat meal[8]	14.1	15.5	4.0	6.7
Fish oil	13.6	14.2	12.9	14.1
Hydrolysed Shrimp[9]	0.5	0.5	0.5	0.5
Chromium oxide	0.5	0.5	0.5	0.5
Vitamin premix[10]	1.0	1.0	1.0	1.0
Choline chloride	0.5	0.5	0.5	0.5
Minerals premix[11]	1.0	1.0	1.0	1.0
Binder[12]	1.0	1.0	1.0	1.0
Dicalcium phosphate	1.2	1.2	0.3	0.3
Lysine[13]	0.2	0.2	0.2	0.2
Methionine[13]	0.2	0.2	0.2	0.2
Taurine[13]	0.3	0.3	0.3	0.3
Proximate analysis (dry matter basis)				
Dry matter (%)	96.8	90.8	96.3	90.4
Crude protein (%)	42.3	41.4	41.0	41.4
Crude lipids (%)	17.3	17.4	17.4	17.0
Ash (%)	8.1	9.3	9.4	9.8
Gross energy (MJ.kg^{-1})	22.9	22.0	23.4	22.9
Cellulase (U.g^{-1})	nd	8.1	nd	12.5
Xylanase (U.g^{-1})	nd	nd	nd	nd
β-glucosidase (U.g^{-1})	nd	3.8	nd	2.3
DPPH (µmol trolox equivalents.g^{-1})	16.6	13.0	17.5	13.2
Total phenols (mg gallic acid equivalents.g^{-1})	9.4	8.9	8.6	9.6

[1] Pesquera Centinela, Stean Dried LT, Chile (CP: 68.45%; CL: 12.56%). Sorgal, S.A. Ovar, Portugal.
[2] (CP: 80.2%; CL: 15.39%). Sorgal, S.A. Ovar, Portugal.
[3] Mix (%DM) (CP: 33.0%; CL: 5.5%): 25.0, rapeseed meal (CP: 40.0%; CL: 5.8%); 25.0, soybean meal (CP: 51.9%; CL: 3.7%); 20.5, rice bran (CP: 14.2%; CL: 13.2%); 25.0, sunflower seed (CP: 40.2%; CL: 2.86%). Sorgal, S.A. Ovar, Portugal.
[4] SSF-Mix (%DM) (CP: 38.03%; CL: 2.5%): 25.0, rapeseed meal (CP: 40.0%; CL: 5.8%); 25.0, soybean meal (CP: 51.9%; CL: 3.7%); 20.5, rice bran (CP: 14.2%; CL: 13.2%); 25.0, sunflower seed (CP: 40.2%; CL: 2.86%). Sorgal, S.A. Ovar, Portugal.
[5] Wheat gluten (CP: 80.5%; CL: 1.0%). Sorgal, S.A. Ovar, Portugal.
[6] Corn gluten (CP: 62.0%; CL: 2.8%). Sorgal, S.A. Ovar, Portugal.
[7] Haemoglobin (CP: 91.5%; CL: 0.4%). Sorgal, S.A. Ovar, Portugal.
[8] Wheat (CP: 14.33%; CL: 2.09%). Sorgal, S.A. Ovar, Portugal.
[9] Hydrolysed Shrimp (CP: 69.8%; CL: 2.1%). Sorgal, S.A. Ovar.
[10] Vitamin premix (mg kg^{-1} diet): retinol, 18,000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[11] Mineral premix (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).
[12] Binder; Aquacube, Agil, UK.
[13] Feed grade-amino acids. Sorgal, S.A. Ovar, Portugal.

All four diets were tested in the *in vivo* digestibility trial, where chromium oxide (0.5% feed dry weight) was used as a digestibility marker. Only two diets were tested in the environmental stress trial, 20Mix and 20SSF-Mix diets.

3.2 Fish and Experimental facilities

Both trials took place in the Aquatic Organisms Bioterium (BOGA) facilities of the Interdisciplinary Centre for Marine and Environmental Research (CIIMAR) in Matosinhos, Portugal. The fish trials were conducted in accordance with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) Guidelines for Reporting Animal Research. European seabass (*Dicentrarchus labrax*) juveniles were acquired from a certified hatchery (SONRIONANSA; Cantabria, Spain). After transport, the fish were quarantined for 15 days in a recirculation aquaculture system (RAS) with a 2000-litter tank and fed a commercial diet (NEO diet, Aquasoja; Sorgal, S.A., Portugal).

3.3 Experimental design and experimental procedure

3.3.1 *In vivo* Digestibility trial

The assay was performed in a recirculating aquaculture system (RAS). A total of 60 *D. labrax* juveniles (70.9 ± 0.33g) were distributed by twelve 60 L fiberglass tanks with individual faeces sedimentation columns (Guelph system), designed according to (Cho et al., 1982). All diets were tested in triplicate. Fish were adapted to the experimental conditions and experimental diets for 7 days. After the adaptation period, fish were hand-fed the experimental diets *ad libitum* twice a day, for 6 days a week. Before the first daily meal, faeces were collected from the sedimentation column, and then centrifuged (4000g; 10 min) to eliminate excess water before freezing at − 18 °C. About 30 min after the last daily meal, the tanks, pipes, and settling columns were cleaned to remove remaining faeces and uneaten feed. Daily collection of the faeces was performed until the necessary amount of faeces to perform all required analysis was collected (14 days). Throughout the trial, continuous aeration in each tank and biofilter was established, the water temperature was maintained at 20-22 °C, salinity ranged from 33-35 ppt, and a water flow rate at 5 $L.min^{-1}$ with and photoperiod of 12h light/12h dark was established.

3.3.1.1 Sampling

For sampling purposes, after the faeces collection phase, fish were fed the experimental diets for another 7 days to complete 30 days of feeding with the experimental diets. To ensure a full intention, during the sampling day, fish were fed *ad libitum* 4h prior to sampling. Three fish per tank, 36 fish in total, were collected, anesthetized with ethylene glycol monophenyl ether (0.25 $ml.L^{-1}$), weighed and then euthanized by cervical dislocation. The entire intestine of fish was sampled and stored at -80°C until analysis of digestive enzymes activity.

3.3.2 Fish environmental stress trial

A total of 202 European seabass *(D. labrax*) juveniles (22.6 ± 4 g) were distributed by 24 experimental units. The trial took place simultaneously in two recirculating aquaculture systems (RAS) (RAS1 and RAS2). RAS1 and RAS2 composed of 9 and 3 tanks of 200L, respectively. To obtain 24 experimental units each tank was divided in two equal parts by implementing cages designed specifically for the 200L tanks (length: 96cm; width: 56cm; heigh: 50cm; water column height: 38cm). Each cage (length: 40cm; width: 54cm; height: 50cm) was composed of high-density polyethylene plastic netting (1cm mash) and PVC tubing (20 mm diameter) thus guaranteeing structural integrity and preventing any animal escape. Between each pair of cages, a fibre-glass mash (2mm) was installed to prevent any passage of feed between cages. The cages were built and installed in each tank according to figure 9.

In RAS1, 18 experimental units were divided into three temperature (17°C; 21°C and 26°C) groups, six units per temperature, this was achieved by the implementation of a system of chillers and thermal resistances During a six-week period, salinity was altered weekly (Table 3) in RAS1, based on data of annual salinity variation in an European seabass aquaculture production unit (Materaqua Lda, Ílhavo, Portugal) (Figure 10). RAS2 had a fixed temperature (21°C) and fixed salinity (33 ppt) through-out the trial, acting as the control group.

For both RAS1 and RAS2, the water temperature was checked, two times a day, at each tank (Table 2) and RAS water salinity was checked once a day to guarantee the environmental conditions followed the experimental design (Table 3), flow rate at 1.5 $L.min^{-1}$ with continuous aeration and natural photoperiod corresponding to 12h daylight length. The remaining water parameters were also monitored daily. In RAS1, oxygen levels (7.6 ± 0.2$mg.L^{-1}$), pH (7.5 ± 0.2), ammonia (NH_4^+) (<0.4$mg.L^{-1}$) and nitrite (<0.4$mg.L^{-1}$). In RAS2, oxygen levels (7.9 ± 0.1$mg.L^{-1}$), pH (7.7 ± 0.1), ammonia (NH_4^+) (<0.4$mg.L^{-1}$) and nitrite (<0.4$mg.L^{-1}$).

Before the environmental trial, fish were adapted to the experimental temperatures and fed the control diet (20Mix) for 7 days. Two experimental diets (20Mix and 20Mix-SSF) were tested, in triplicate. Fish were hand-fed *ad libitum* twice a day (with a 6h interval between feedings), 6 days a week for 6 weeks (42 days).

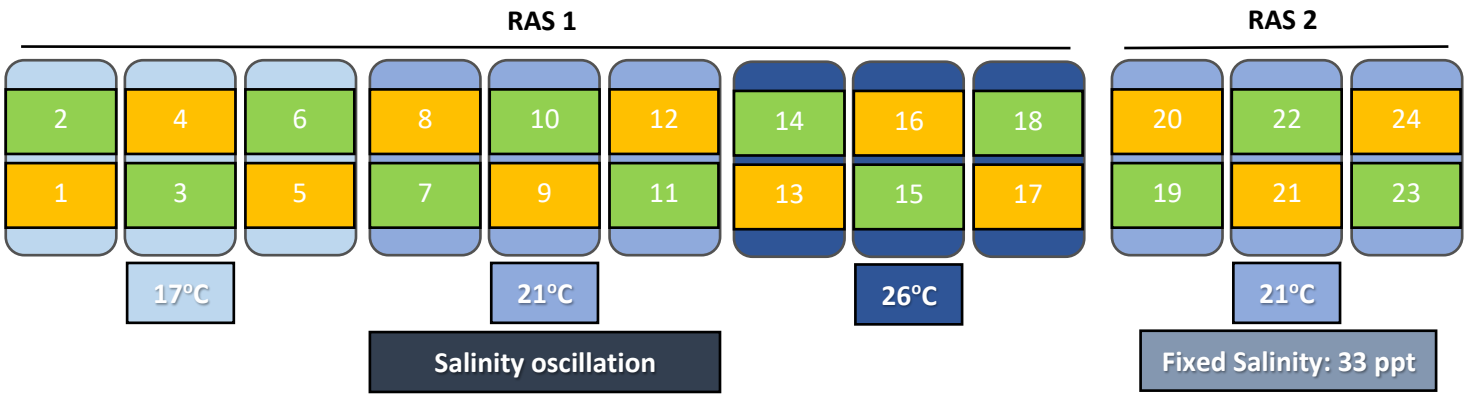

Figure 8 - Experimental trial schematic design. Six experimental units at each temperature (17, 21 and 26 ºC) and salinity treatment (oscillatory and fixed) With three experimental units of each environmental treatment for each diet (20Mix ■ and 20Mix-SSF ■), in a total of 24 experimental units.

Table 2 - Temperature (mean ± deviation) through the 42 day trial, measured 2 times a day (ºC)

RAS	Experimental design	**Real Temperature**
1	17ºC	17.2 ± 0.3
	21ºC	21.4 ± 0.4
	26ºC	25.9 ± 0.3
2	21ºC	20.9 ± 0.3

Table 3 – RAS1 and RAS2 salinity (mean ± deviation) throughout the 42 day trial, measured once a day (ppt)

RAS	Duration	Experimental design	**Real Salinity**
1	1st Week	15 ppt	15.3 ± 0.2
	2nd Week	38 ppt	38.0 ± 0.3
	3rd Week	21 ppt	21.1 ± 0.2
	4th Week	33 ppt	33.1 ± 0.2
	5th Week	27 ppt	27.2 ± 0.2
	6th Week	45 ppt	45.3 ± 0.1
2	42 days	33 ppt	33.0 ± 0.3

Figure 9 – Photo of a pair of cages in BOGA facilities and photo of a pair of cages installed in one of the 200L tanks in RAS1

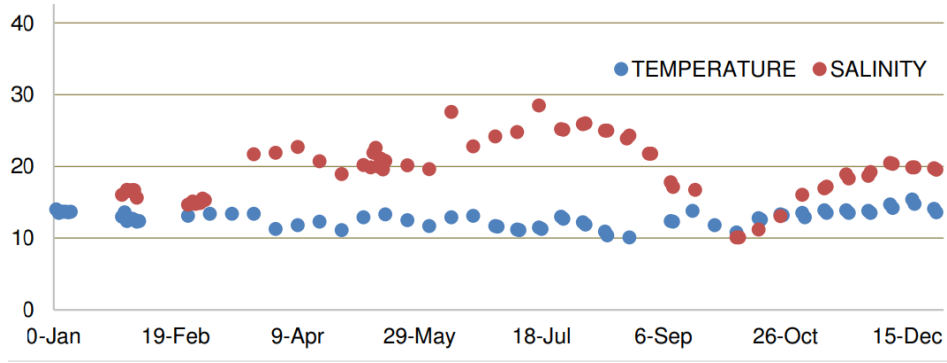

Figure 10 – Seawater salinity (ppt) and temperature (ºC) variation in Materaqua Lda. fish farm over the year of 2014 (Lobo, 2016)

3.3.2.1 Sampling

In both RAS, fish were fed ad libitum 90 minutes prior to sampling. In RAS1, 60 minutes prior to sampling the rearing salinity was changed (from 45 to 33 ppt) to induce an osmotic stress response in the fish. Two fish per unit, totalling 48 fish, were collected and anaesthetised with ethylene glycol monophenyl ether (0.25 ml.L-1). Weight and length were then recorded to calculate growth performance parameters and blood samples were taken from the caudal region using heparinised syringes before euthanasia by cervical dislocation. Fish tissues (entire intestine and liver) were collected for biochemical analyses. The blood was centrifuged (10000g; 5 min) to isolate the plasma. All samples were stored at -80°C until further analyses.

3.4 Growth Parameters

Growth performance was determined using the following parameters:

Feed conversion ratio (FCR) = $\frac{\text{Feed intake (g)}}{\text{Weight gain (g)}}$

Protein efficiency ratio (PER) = $\frac{\text{Weight gain (g)}}{\text{Crude protein intake (g)}}$

Daily growth index (DGI) = $\frac{\text{FBW}^{1/3} - \text{IBW}^{1/3}}{\text{Trial duration (days)}} \times 100$

Thermal growth coefficient (TGC) = $\frac{\text{FBW}^{1/3} - \text{IBW}^{1/3}}{\text{Temperature (°C)} \times \text{Trial duration (days)}} \times 1000$

IBW (g) and FBW (g) are the initial body weight and final body weight, respectively

3.5 Chemical analysis

Before analyses, faeces were dried (60°C for 48hr), mixed, and ground. Proximate composition analyses were performed in duplicate and according to the Association of Official Analytical Chemists (AOAC) methods (AOAC, 2000) .

Crude protein was determined (N × 6.25) according to Janssen et al. (2017) by the Kjeldahl method following acid digestion, using the Kjeltec digestion and distillation units (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively)

Crude lipids content was quantified by extraction with petroleum ether using a Soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046)

Gross energy was determined by direct combustion of the samples in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261)

Chromic oxide content in feeds and faeces was quantified by acid digestion according to Bolin et al. (1952).

3.6 Apparent Digestion coefficient

The Apparent digestibility coefficients (ADCs) of the experimental diets were calculated according to Maynard et al. (1979) :

$$\text{ADC (\%)} = \left(1 - \frac{\text{dietary } Cr_2O_3 \text{ level}}{\text{faeces } Cr_2O_3 \text{ level}} \times \frac{\text{faeces nutrient / energy level}}{\text{dietary nutrient / energy level}} \right) \times 100$$

$$\text{ADC of dry matter (\%)} = \left(1 - \frac{\text{dietary } Cr_2O_3 \text{ level}}{\text{faeces } Cr_2O_3 \text{ level}} \right) \times 100$$

3.7 Digestive enzymes activity

Enzyme extraction and protein quantification

Digestive enzymes activities were measured in samples from individual fish. The whole intestinal tract was homogenized using a Potter Elvehjem homogeniser (Thomas Scientific) in a buffer solution (1:5, w/v), consisting of 50 mM Tris-HCl and 200 mM NaCl at pH 8.0 (Sigma-Aldrich). The homogenates were centrifuged at 13,500g at 4°C for 30 min to separate the supernatant from the lipidic fraction. Total protein concentration in the supernatants was quantified by the Folin phenol method (Lowry et al., 1951) and the value obtained per sample was used to calculate enzymatic activity. Protein concentration was determined by reference to a standard curve of bovine γ-globulin (Sigma-Aldrich). Final unit is presented as protein $mg.ml^{-1}$.

α-Amylase

α-Amylase activity was measured from the increase in maltose by hydrolysis of the α-D(1,4)-glycoside bond in polysaccharides, and stained with 3.5- dinitrosalicylic acid (DNS) as described by Bernfeld (1951). Final unit is expressed as enzymatic unit per protein mg (U.mg protein^{-1})

Lipase

Lipase activity was measured using ρ-nitrophenyl substrate as described by Winkler and Stuckmann (1979). The formation of p-nitrophenol is then quantified by reading at 410 nm. Final unit is expressed as milli enzymatic units per protein mg (mU.mg protein^{-1})

Trypsin

Trypsin activity was determined as described by Torrissen (1984), using benzoyl-L-arginine-p-nitroanilide as substrate. Final product (nitroaniline) was measured spectrophotometrically at 410 nm. Final unit is expressed as enzymatic units per protein mg (U.mg protein^{-1}).

Chymotrypsin

Chymotrypsin activity was determined as described by Rungruangsak-Torrissen and Sundby (2000), using succinyl-Ala-Ala-Ala-Pro-Phep-nitroanilide as substrate. Similar to the trypsin assay, nitroaniline was measured spectrophotometrically at 410 nm. Final unit is expressed as enzymatic units per protein mg (U.mg protein^{-1}).

3.8 Oxidative Stress: Enzymatic and Non-enzymatic parameters

Livers were homogenized with a K-phosphate buffer (pH 7.4, 0.1 M) in a 1:15 (p/v) ratio. For the lipid peroxidation analysis, 2.5 µL of BHT 4% (2.6-Di-tert-butyl-4-methylphenol in methanol) was added to 150 µl of liver homogenate, for preservation, and stored at -80°C until quantification. For the remaining parameters (CAT, GPX, GR, TG and GSSG), samples were prepared by centrifuging liver homogenate (10000g, 20 min, 4°C) and the supernatant stored at -80°C. The soluble protein content was required to be close to 0.7 mg.mL^{-1} for CAT and GST quantification. For this calibration, soluble protein was quantified following the Coomassie binding principle of Bradford (1976). Final unit is protein mg.ml^{-1}.

Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) are formed as a by-product of lipid peroxidation, which can be detected by the TBARS essay using thiobarbituric acid as a reagent (Ohkawa et al., 1979). TBARS essay then measures malondialdehyde (MDA)

present in the sample which is the major product of lipid peroxidation. The final unit is expressed as nmoles of MDA formed.g^{-1}

Catalase

At a soluble protein content of about 0.7 mg.ml-1, the quantification of activity follows the principle of the action of catalase on hydrogen peroxide described by Claiborne (1985). The final unit is given as hydrogen peroxide µmol.min^{-1}.protein mg^{-1}

Glutathione peroxidase

Glutathione peroxidase was quantified by the method of Mohandas et al. (1984). The reaction was measured by the formation of oxidized NADPH ($NADP^+$), and given in the final unit of $NADP^+$ pmol.min^{-1}.protein mg^{-1}.

Glutathione s-transferase

This analysis was performed following Habig et al. (1974). Here, CDNB (1-chloro-2,4-dinitrobenzene) is conjugated with GSH to form the measurable conjugate of GSH-CDNB (1-chloro-2, 4-dinitrobenzene). Final unit is expressed in pmol GSH-CDNB conjugate formed.min^{-1}.protein mg^{-1}

Glutathione reductase

The assay for measuring glutathione reductase is based on the reduction of 5,5'- dithiobis (2-nitrobenzoic acid) to reduced glutathione (GSH) generated from an excess of oxidized glutathione (GSSG), as described by Cribb et al. (1989). This activity was quantified by the formation of $NADP^+$ and final unit is expressed in $NADP^+$ nmol.min^{-1}.protein mg^{-1}

Total and Oxidized Glutathione

Total glutathione is quantified by reaction of GSH with DTNB (5.5'-dithio-bis-2-nitrobenzoic acid), yielding a yellow coloured 5-thio-2-nitrobenzoic acid (TNB) quantified at 412 nm, as described by Baker et al. (1990). During the reaction, the GSSG is simultaneously reduced to GSH, allowing measurement of total glutathione. In the GSSG assay, 2- vinylpyridine was used to remove all free thiols present in the sample leaving only GSSG (Griffith, 1980). The GSH level was calculated as the result of subtracting the amount of GSSG from TG. Final unit is given as TNB µmol.protein mg^{-1}.

3.9 Humoral Immune Parameters

Peroxidase

Total plasma peroxidase content was measured according to Quade and Roth (1997). Using TMB (3.3′.5.5′-tetramethylbenzidine) as substrate, in the presence of hydrogen peroxide. The final unit is expressed in enzymatic units, where one enzymatic unit (U) is defined as the amount producing an absorbance change of 1.

Lysozyme

Concentration was determined by the turbidimetric test according to the Ellis method (Ellis, 1990). By measuring its effect on Micrococus lysodeikticusa bacteria. The final unit is expressed as enzymatic units, which according to the convention 1 U = 0.001 absorption units.minute^{-1}.

Haemolytic alternative complement pathway

The ACH50 was determined according to Sunyer and Tort (1995). The main principle of this procedure is to evaluate the plasma sample required to achieve 50% haemolysis in the added rabbit red blood cells. The units for the alternative complement pathway (ACH50) were defined as the serum concentration that causes 50% haemolysis of rabbit blood cells.

All analyses were performed in duplicate.

3.10 Statistical Analysis

For the digestibility trial data, a two-way analysis of variance (ANOVA) (inclusion level of plant ingredients (20% and 40%) × ingredients pre-treatment (fermented and non-fermented)) analysis was performed

Two separate two-way analysis of variance (ANOVA) were performed for the environmental stress trial data:

Analysis 1: Diet (20Mix and 20Mix-SSF) x Temperature (17, 21, 26ºC).

Analysis 2: Diet (20Mix and 20Mix-SSF) x Salinity (oscillatory and fixed).

Two-way analysis of covariance (ANCOVA) was performed for growth parameters with initial body weight as a covariate. Bonferroni's test was used for pairwise comparisons between rearing temperatures.

All analyses were performed using the software package IBM SPSS statistics 27 - Windows 10. Data transformation was applied when normality of samples (Grubbs test) is not achieved. Data was checked for homogeneity of variances (Levene's test). Tukey's test was used for pairwise comparisons between rearing temperatures (except for growth parameters analysis). A confidence level of 95% was considered in all statistical analyses

4. RESULTS

4.1 *In vivo* digestibility trial

4.1.1 Diets apparent digestibility coefficients

The apparent digestion coefficients (ADCs) of crude protein, crude lipids, gross energy, and dry matter of the experimental diets are shown in Figure 11, Figure 12, Figure 13, and Figure 14, respectively.

Inclusion level of plant-based ingredients (20% and 40%) and the treatment of plant-based ingredients (non-fermented and fermented) had an interactive effect on the apparent digestion coefficients of crude protein, gross energy and dry matter. The digestibility of protein, energy and dry matter was significantly higher in 20Mix SSF diet compared to 20Mix diet. When ingredients were included on a 40% level, fermentation had an opposite effect. Regarding the apparent coefficient digestibility of crude lipids, inclusion level and treatment of plant-based ingredients had no significant effect on lipid digestibility.

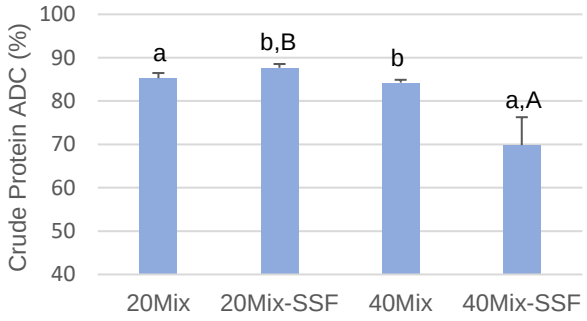

Figure 11: Apparent digestion coefficient of crude protein in 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value = 0.014), Inclusion (*p*-value < 0.001), Interaction (*p*-value = 0.002)). Different lowercase letters indicate significant differences between ingredient treatment. Different uppercase letters indicate significant differences between inclusion level. Values are presented as mean ± SD (n=3) (%)

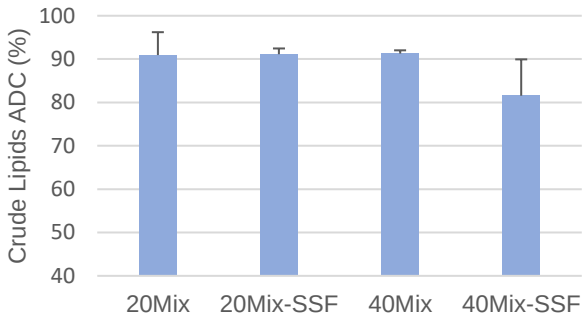

Figure 12: Apparent digestion coefficient of crude lipids in 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value > 0.05), Inclusion (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Absence of letters indicates no significant differences. Values are presented as mean ± SD (n=3) (%)

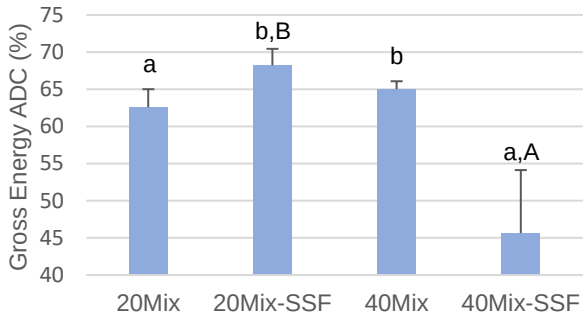

Figure 13: Apparent digestion coefficient of gross energy in 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value = 0.032), Inclusion (*p*-value = 0.005), Interaction (*p*-value < 0.001)). Different lowercase letters indicate significant differences between ingredient treatment. Different uppercase letters indicate significant differences between inclusion level. Values are presented as mean ± SD (n=3) (%)

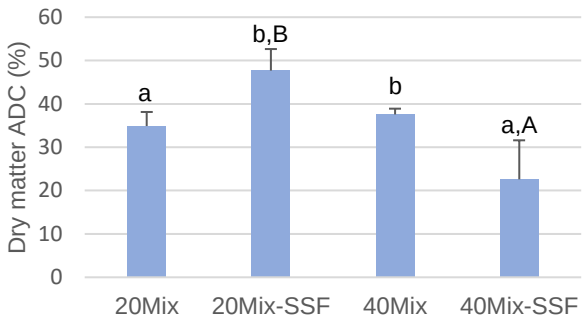

Figure 14: Apparent digestion coefficient of dry matter in 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value > 0.05), Inclusion (*p*-value = 0.007), Interaction (*p*-value = 0.002)). Different lowercase letters indicate significant differences between ingredient treatment. Different uppercase letters indicate significant differences between inclusion level. Values are presented as mean ± SD (n=3) (%)

4.1.2 Digestive enzymes activities

The α-amylase activities (Figure 15) showed a significant effect of inclusion level of plant-based ingredients in experimental diets, with α-amylase activity being significantly higher in fish fed the 40% inclusion level diets (40Mix and 40Mis-SSF diets).

Lipase activities (Figure 16) showed a significant effect of treatment of plant-based ingredients in the experimental diet, with lipase activity significantly higher in fish fed the SSF diets (20Mix-SSF and 40Mix-SSF)

Trypsin activities (Figure 17) showed a significant effect of treatment of plant-based ingredients in the experimental diets, with trypsin activity being significantly lower in fish fed SSF diets (20Mix-SSF and 40Mix-SSF).

No significant effect of treatment or inclusion level of plant-based ingredients on chymotrypsin activity (Figure 18) was observed in fish fed the experimental diets.

The ratio of α-amylase and trypsin activities (Figure 19) showed a significant effect of inclusion level of plant-based ingredients in experimental diets, where A/T was significantly higher in fish fed 40% inclusion level diets (40Mix and 40Mis-SSF diets).

The ratio between trypsin and chymotrypsin activities (Figure 20) showed no significant effect of treatment or inclusion level of plant-based ingredients.

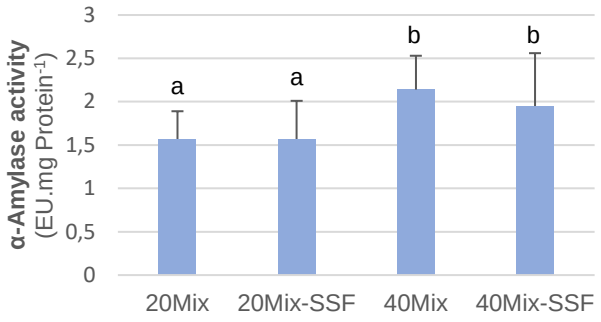

Figure 15: α-Amylase enzymatic activities in *D. labrax* juveniles fed the 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value > 0.05), Inclusion (*p*-value = 0.002), Interaction (*p*-value > 0.05)). Different letters indicate significant differences. Values are presented as mean ± SD (n=9) (U.mg Protein^{-1})

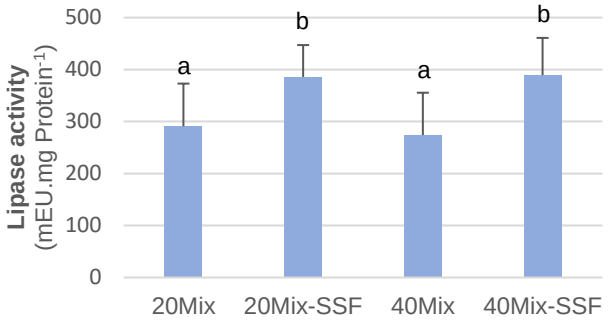

Figure 16: Lipase enzymatic activities in *D. labrax* juveniles fed the 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value < 0.001), Inclusion (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different letters indicate significant differences. Values are presented as mean ± SD (n=9) (mU.mg Protein^{-1})

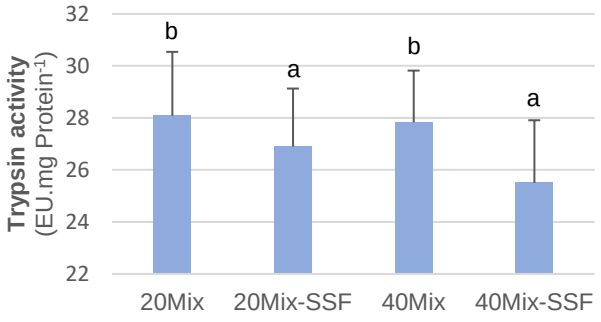

Figure 17: Trypsin enzymatic activities in *D. labrax* juveniles fed the 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value = 0.032), Inclusion (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different letters indicate significant differences. Values are presented as mean ± SD (n=9) (U.mg Protein^{-1})

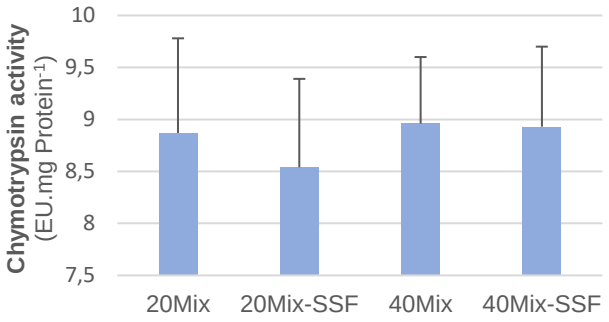

Figure 18: Chymotrypsin enzymatic activities in *D. labrax* juveniles fed the 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value > 0.05), Inclusion (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Absence of letters indicates no significant differences. Values are presented as mean ± SD (n=9) (U.mg Protein^{-1})

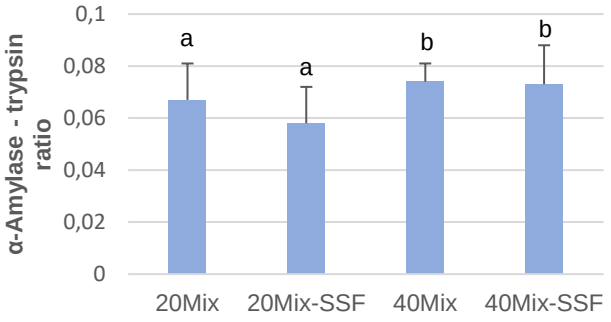

Figure 19: α-Amylase - trypsin activities ratio in *D. labrax* juveniles fed the 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value > 0.05), Inclusion (*p*-value = 0.026), Interaction (*p*-value > 0.05)). Different letters indicate significant differences. Values are presented as mean ± SD (n=9)

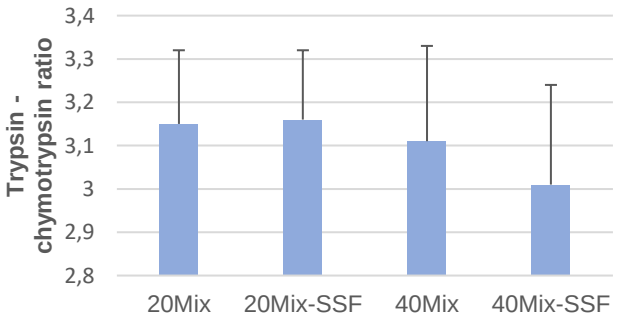

Figure 20: Trypsin - chymotrypsin activities ratio in *D. labrax* juveniles fed the 20Mix, 20Mix-SSF, 40Mix and 40Mix-SSF diets (Two-way ANOVA, Treatment (*p*-value > 0.05), Inclusion (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Absence of letters indicates no significant differences. Values are presented as mean ± SD (n=9)

4.2 Environmental stress trial

4.2.1 Growth performance

Mortality was observed on the 28th, 40th, and 42nd days of the trial in unit 16 (1 fish), unit 12 (1 fish) and unit 10 (1 fish) respectively.

On the 3rd day of the trial a localised system failure in RAS 2 prevented the trial to continue in unit 23 and unit 24. From that day until the end of the trial, RAS2 was solely composed of 4 experimental units (unit 19, unit 20, unit 21 and unit 22), meaning the "21ºC fixed salinity" condition was solely tested in duplicate

Fish grew from an initial mean body weight of 22.6 ± 4 g to a final mean body weight of 36.4 ± 6.7 g (exact values per treatment represented in table 5). The experimental diet had a significant effect on feed intake (FI), crude protein intake (CPI), weight gain (WG) and final body weight (FBW). Fish, reared at different temperatures (17, 21, 26ºC) subjected to salinity oscillation and fed the 20Mix diet showed significantly higher feed intake, crude protein intake, weight gain and final body weight than those fed the 20Mix-SSF diet. The rearing temperature had a significant effect on feed intake and crude protein intake. Fish, reared at 26ºC had a significantly higher FI and CPI than fish reared at 17ºC and 21ºC.

Table 4: Initial body weight, final body weight, weight gain, feed intake and crude protein intake of European seabass juveniles at the end of the environmental stress trial.

Salinity	Oscillatory						Fixed	
Temperature	17ºC		21ºC		26ºC		21ºC	
Diet	20Mix	20Mix-SSF	20Mix	20Mix-SSF	20Mix	20Mix-SSF	20Mix	20Mix-SSF
IBW (g)	27.18 ± 1.6	27.32 ± 0.2	20.53 ± 2.7	20.19 ± 3.9	21.09 ± 4.6	22.13 ± 4.5	20.25 ± 1.9	20.89 ± 4.5
FBW (g)	$42.98 \pm 3.0^{\beta}$	$40.47 \pm 3.6^{\alpha}$	$35.46 \pm 6.2^{\beta}$	$30.62 \pm 6.5^{\alpha}$	$40.83 \pm 6.5^{\beta}$	$37.07 \pm 7.2^{\alpha}$	30.52 ± 1.3	28.60 ± 5.7
WG (g)	$15.80 \pm 1.5^{\beta}$	$13.15 \pm 3.5^{\alpha}$	$14.93 \pm 3.90^{\beta}$	$10.43 \pm 3.8^{\alpha}$	$19.73 \pm 4.0^{\beta}$	$14.94 \pm 3.1^{\alpha}$	18.33 ± 1.7	15.73 ± 1.6
FI (g.fish^{-1})	$22.40 \pm 2.0^{a,\beta}$	$19.97 \pm 2.9^{a,\alpha}$	$22.54 \pm 4.1^{ab,\beta}$	$17.67 \pm 3.3^{ab,\alpha}$	$26.26 \pm 3.3^{b,\beta}$	$22.89 \pm 3.3^{b,\alpha}$	7.76 ± 0.7	6.36 ± 0.9
CPI (g.fish^{-1})	$9.48 \pm 0.9^{a,\beta}$	$8.11 \pm 1.0^{a,\alpha}$	$9.60 \pm 1.1^{ab,\beta}$	$7.32 \pm 1.4^{ab,\alpha}$	$11.13 \pm 1.4^{b,\beta}$	$9.41 \pm 1.5^{b,\alpha}$	7.32 ± 1.4	7.76 ± 0.7

Initial body weight (IBW, g), final body weight (FBW, g), Weigh gain (WH, g), Feed intake (FI, g.fish^{-1}) and Crude protein intake (CPI, g.fish^{-1}) of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) (FBW (Two-way ANCOVA, Temperature (p-value > 0.05), Diet (p-value = 0.018), Interaction (p-value > 0.05)); WG (Two-way ANCOVA, Temperature (p-value > 0.05), Diet (p-value = 0.019), Interaction (p-value > 0.05)); FI (Two-way ANCOVA, Temperature (p-value = 0.006), Diet (p-value = 0.007), Interaction (p-value > 0.05)); CPI (Two-way ANCOVA, Temperature (p-value = 0.004), Diet (p-value = 0.001), Interaction (p-value > 0.05))) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (FBW, WG, FI, CPI (Two-way ANCOVA, Salinity (p-value > 0.05), Diet (p-value > 0.05), Interaction (p-value > 0.05))). Different letters in the same row indicate significant differences regarding effect of temperature and Different greek letters indicate significant differences regarding effect of diet. Values presented as mean (n = 3; except for Fixed 21ºC, n=2)

Feed conversion ratio (FCR)

Feed conversion ratio (FCR; Figure 21) in fish reared at different temperatures (17, 21, 26ºC) subjected to salinity oscillation showed no significant differences, regardless of experimental diet. When comparing PER of fish reared at 21ºC and exposed to different salinity conditions, no significant differences were also observed.

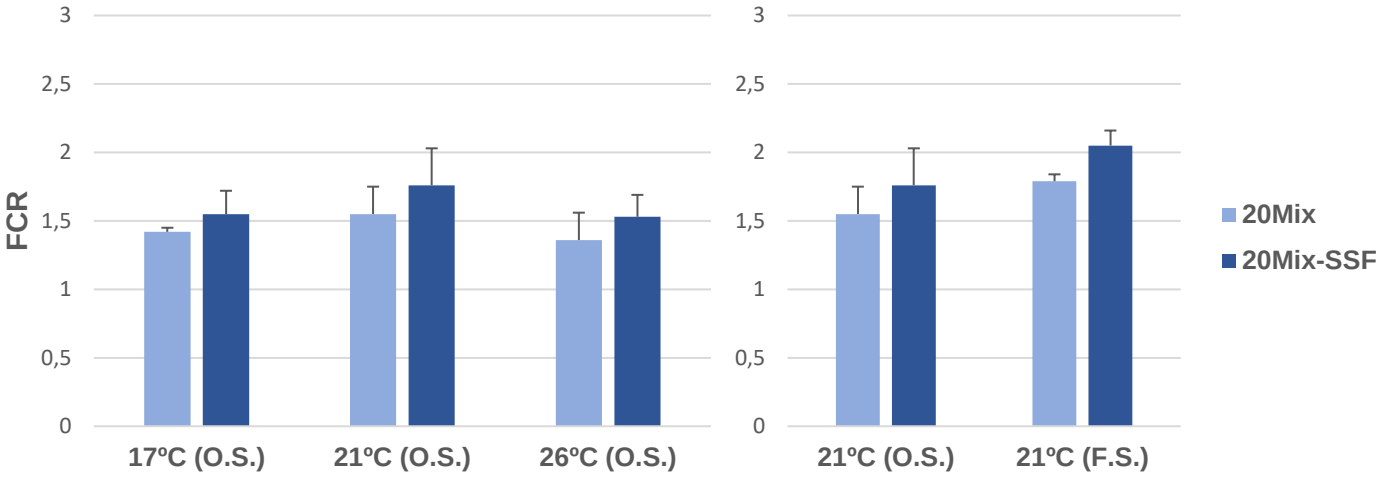

Figure 21: Feed conversion ratio (FCR) of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANCOVA, Temperature (p-value > 0.05), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANCOVA, Salinity (p-value > 0.05), Diet (p-value > 0.05), Interaction (p-value > 0.05)). Absence of letters indicates no significant differences. Values are presented as mean ± SD (n=3, except for Fixed 21ºC, n=2)

Protein efficiency ratio (PER)

Protein efficiency ratio (PER, Figure 22) in fish reared at different temperatures (17, 21, 26ºC) subjected to salinity oscillation showed no significant differences in protein efficiency ratio, regardless of experimental diet. When comparing PER of fish reared at 21ºC and exposed to different salinity conditions, no significant differences were also observed.

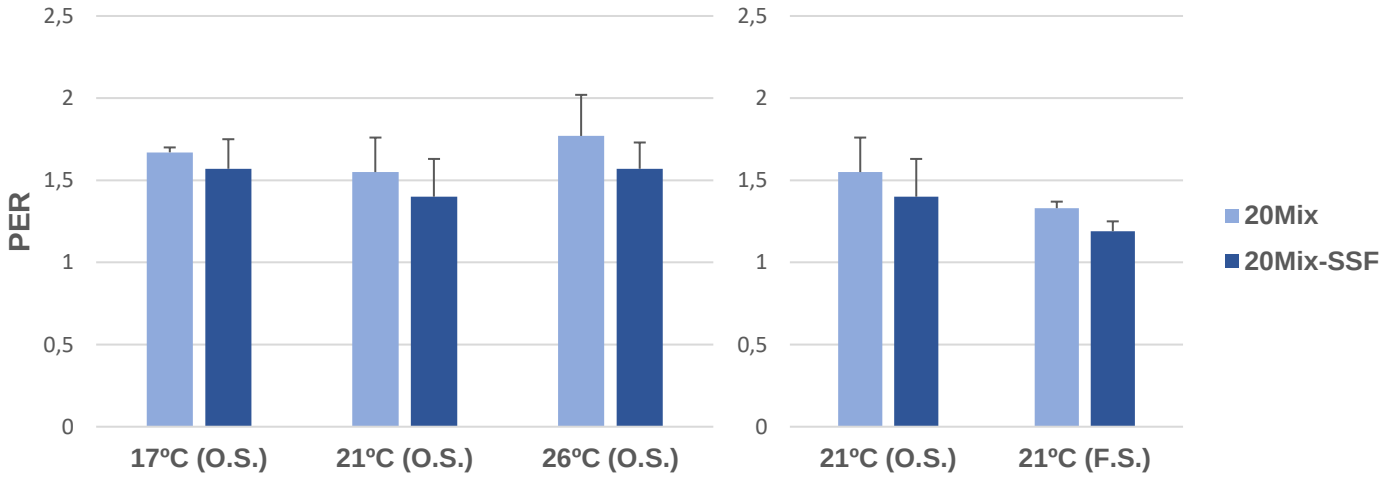

Figure 22: Protein efficiency ratio (PER) of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANCOVA, Temperature (p-value > 0.05), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANCOVA, Salinity (p-value > 0.05), Diet (p-value > 0.05), Interaction (p-value > 0.05)). Absence of letters indicates no significant differences. Values are presented as mean ± SD (n=3, except for Fixed 21ºC, n=2)

Daily growth index (DGI)

Rearing temperature had a significant effect on the daily growth index (DGI, Figure 23) of fish subjected to salinity oscillation, where fished reared at 26ºC grew at a significantly higher rate than those reared at 17ºC. Experimental diet also had a significant effect on growth rate of fish reared at different temperatures, where fish fed 20Mix diet grew significantly at a higher rate than those fed the 20Mix-SSF diet.

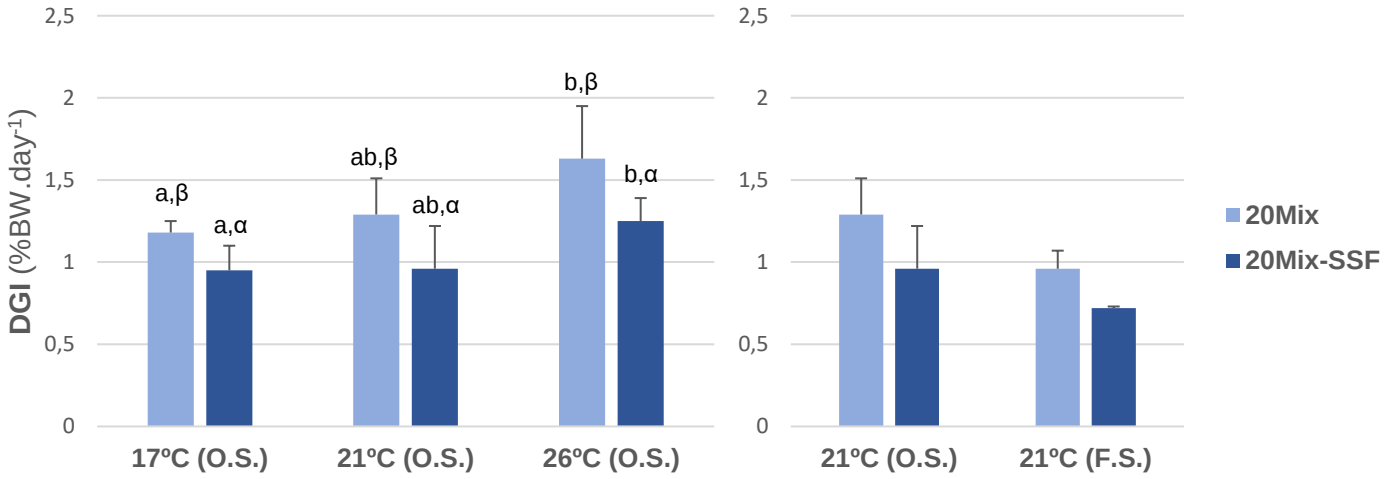

Figure 23: Daily growth index (DGI) of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANCOVA, Temperature (*p*-value = 0.037), Diet (*p*-value = 0.011), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANCOVA, Salinity (*p*-value > 0.05), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and different greek letters indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=3, except for Fixed 21ºC, n=2) (%BW.day^{-1})

Thermal growth coefficient (TGC)

Experimental diet had a significant effect on growth coefficient (Figure 24) of fish reared at different temperatures fish and subjected to salinity oscillation, where fish fed 20Mix diet showed a significantly higher coefficient than those fed the 20Mix-SSF diet.

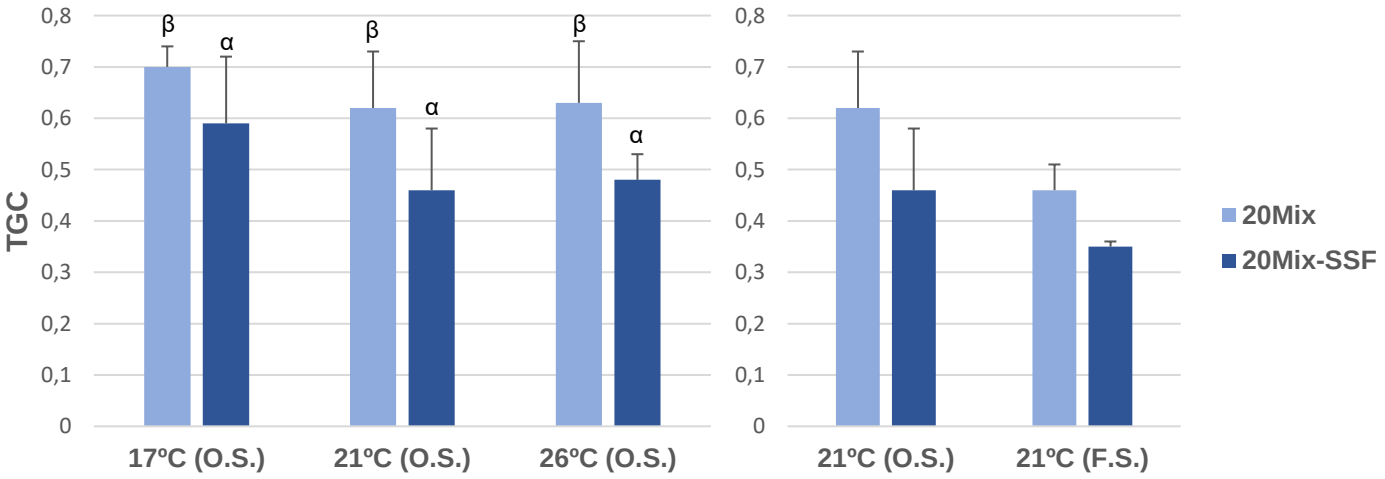

Figure 24: Thermal growth coefficient (TGC) of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANCOVA, Temperature (*p*-value > 0.05), Diet (*p*-value = 0.018), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANCOVA, Salinity (*p*-value > 0.05), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different greek letters indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=3, except for Fixed 21ºC, n=2)

4.2.2 Digestive enzymes activities

α-Amylase

The α-amylase activity (Figure 25) in fish subjected to salinity oscillation showed that temperature had a significant effect on α-amylase activity, where fish reared at 21ºC group had lower α-amylase activity than fish reared at 17ºC and 26ºC. Within the fish groups reared at 21ºC, fish subjected to salinity oscillation showed a significantly lower α-amylase activity than those reared in fixed salinity condition.

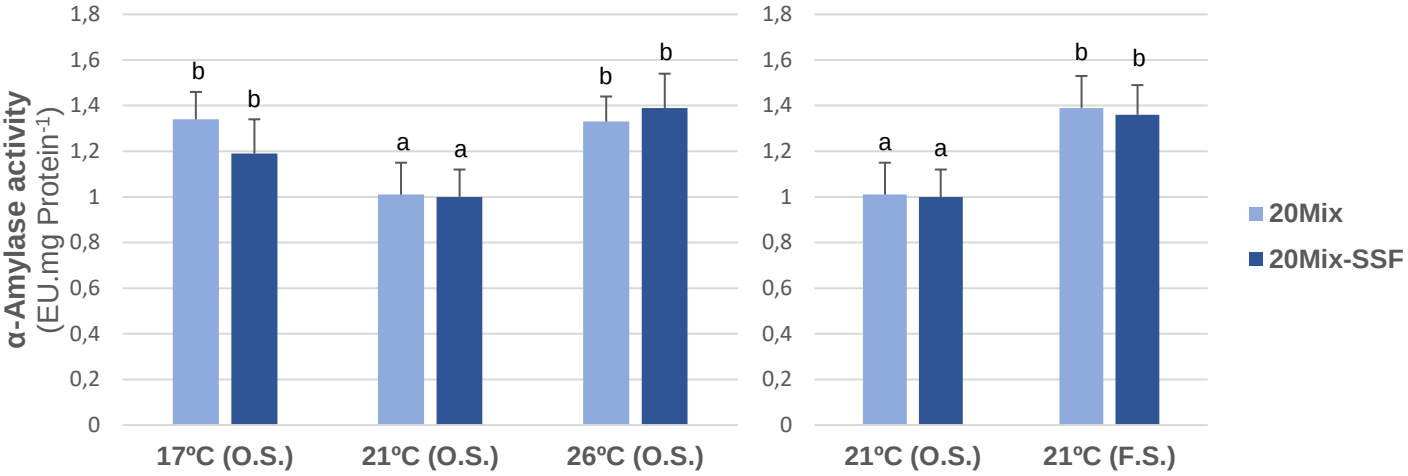

Figure 25: α-Amylase enzymatic activities in *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (*p*-value < 0.001), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (*p*-value < 0.001), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and salinity, respectively. Values are presented as mean ± SD (n=6) (EU.mg Protein^{-1})

Lipase

Lipase activity (Figure 26) in fish subjected to salinity oscillation showed that temperature significantly affected lipase activity, where fish reared at 26ºC had significantly higher lipase activity than fish reared at 21ºC. Within the fish groups reared at 21ºC, fish subjected to salinity oscillation showed a significantly lower lipase activity than those reared in fixed salinity condition.

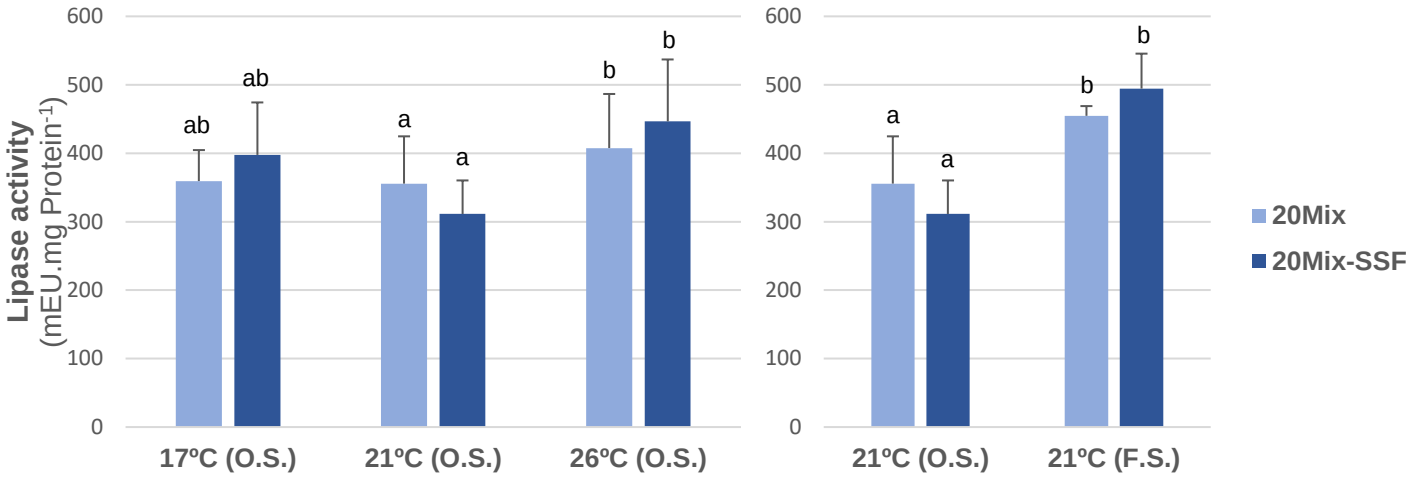

Figure 26: Lipase enzymatic activities in *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value = 0.015), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value < 0.001), Diet (p-value > 0.05), Interaction (p-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and salinity, respectively. Values are presented as mean ± SD (n=6) (mEU.mg Protein^{-1})

Trypsin

Trypsin activity (Figure 27) in fish reared at different temperatures (17, 21, 26ºC) subjected to salinity oscillation showed that temperature had a signficant effect on enzymatic activity, where fish reared at 21ºC had signficant higher trypsin activity than fish reared at 26ºC. Within the fish groups reared at 21ºC, fish subjected to salinity oscillation showed a significantly lower trypsin activity than those reared in fixed salinity condition.

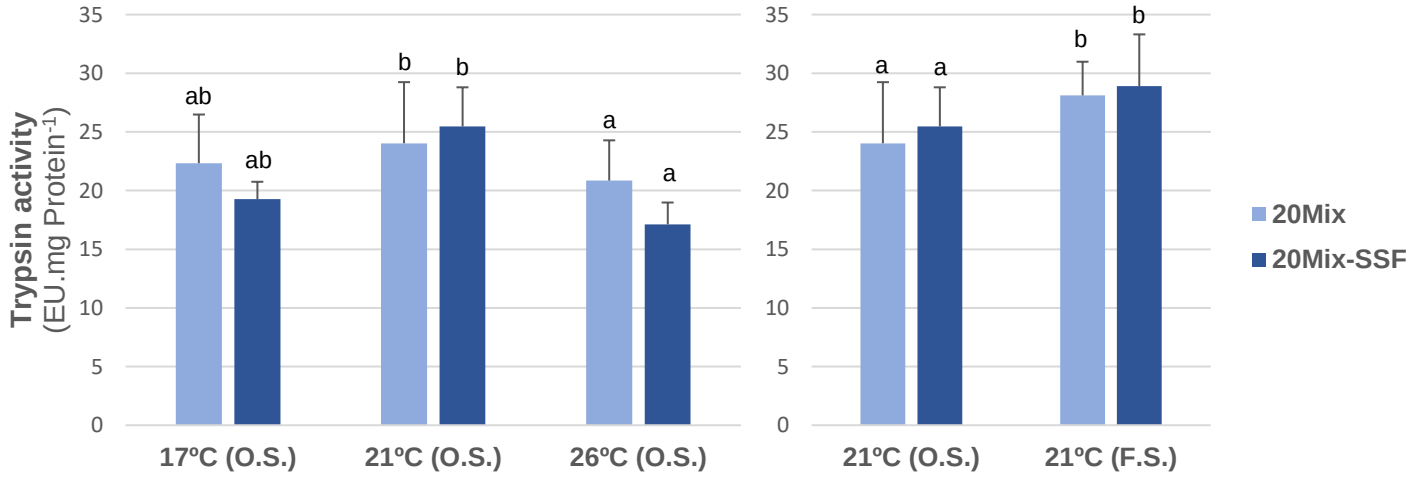

Figure 27: Trypsin enzymatic activities in *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value = 0.002), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value = 0.020), Diet (p-value > 0.05), Interaction (p-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and salinity, respectively. Values are presented as mean ± SD (n=6) (EU.mg Protein^{-1})

Chymotrypsin

Chymotrypsin activity (Figure 28) in fish reared at different temperatures (17, 21, 26ºC) subjected to salinity oscillation showed that temperature had a signficant effect on enzymatic activity, where fish reared at 17ºC had signficant higher chymotrypsin activity than fish reared at 26ºC. Within the fish groups reared at 21ºC, fish subjected to salinity oscillation showed a significantly lower trypsin activity than those reared in fixed salinity condition.

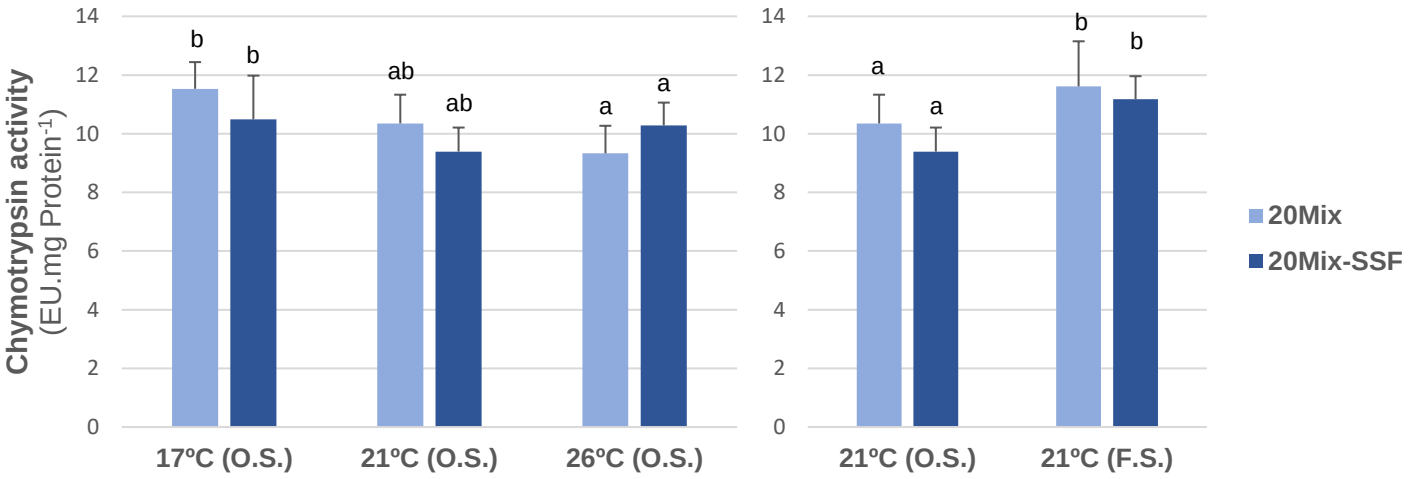

Figure 28: Chymotrypsin enzymatic activities in *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value = 0.014), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value = 0.002), Diet (p-value > 0.05), Interaction (p-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and salinity, respectively. Values are presented as mean ± SD (n=6) (EU.mg Protein^{-1})

Amylase – Trypsin ratio (A/T)

Amylase – Trypsin activities ratio (A/T, Figure 29) in fish reared at different temperatures (17, 21, 26ºC) subjected to salinity oscillation showed that temperature had a significant effect on enzymatic activity, where fish reared at 21ºC had a significant lower amylase - trypsin activity ratio than fish reared at 17ºC and 26ºC. When comparing A/T of fish reared at 21ºC and exposed to different salinity conditions, a significant effect of diet was observed, where fish fed the 20Mix diet showed significantly higher A/T than those fed the 20Mix-SSF diet.

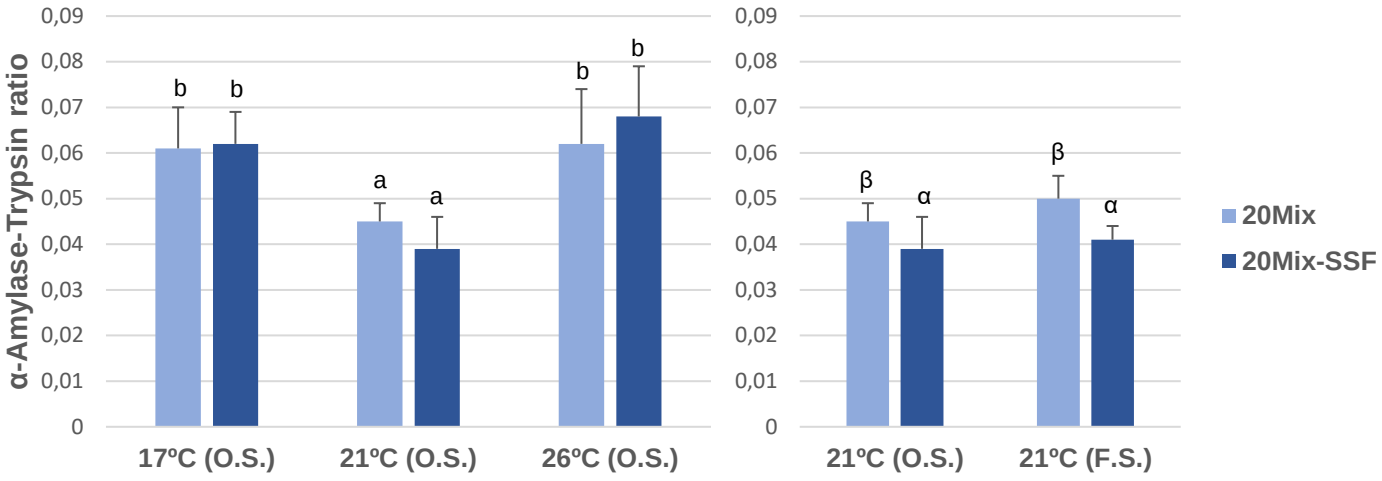

Figure 29: α-Amylase – Trypsin enzymatic activities ratio in *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value < 0.001), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value > 0.05), Diet (p-value = 0.004), Interaction (p-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and different greek letters indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=6)

Trypsin - chymotrypsin ratio (T/C)

Trypsin - chymotrypsin activities ratio (T/C, Figure 30) in fish reared at different temperatures (17, 21, 26ºC) subjected to salinity oscillation showed that temperature and diet had a significant interactive effect on T/C, where fish fed the 20Mix diet showed no significant changes in T/C when reared at different temperatures. Fish fed the 20Mix-SSF diet when reared at 21ºC had a significantly higher trypsin - chymotrypsin activity ratio than those reared at 17ºC and 26ºC.

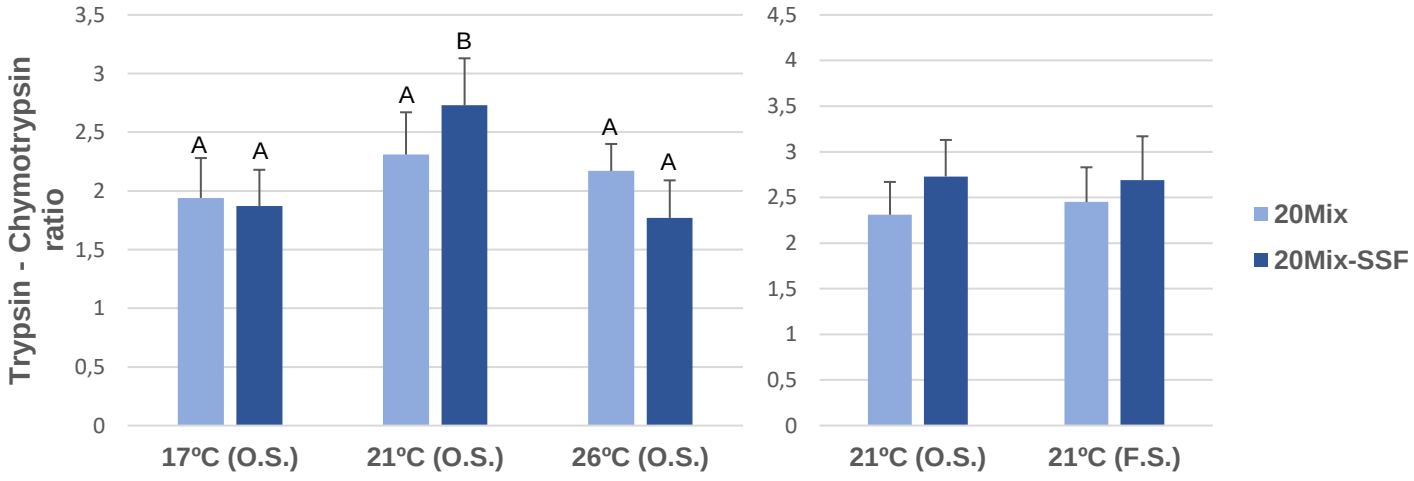

Figure 30: Trypsin - Chymotrypsin enzymatic activities ratio in *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value < 0.001), Diet (p-value > 0.05), Interaction (p-value = 0.030)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value > 0.05), Diet (p-value > 0.05), Interaction (p-value > 0.05)). Different uppercase letters indicate significant differences between temperature groups for each experimental diet. Values are presented as mean ± SD (n=6)

4.2.3 Oxidative stress

Lipid peroxidation (LPO)

Lipid peroxidation (LPO; Figure 31) in liver of fish reared at different temperatures (17, 21, 26ºC) subjected to salinity oscillation showed that temperature had a significant effect, whereas regardless of experimental diet, LPO was significantly higher in fish reared at 17ºC than those reared at 21ºC.

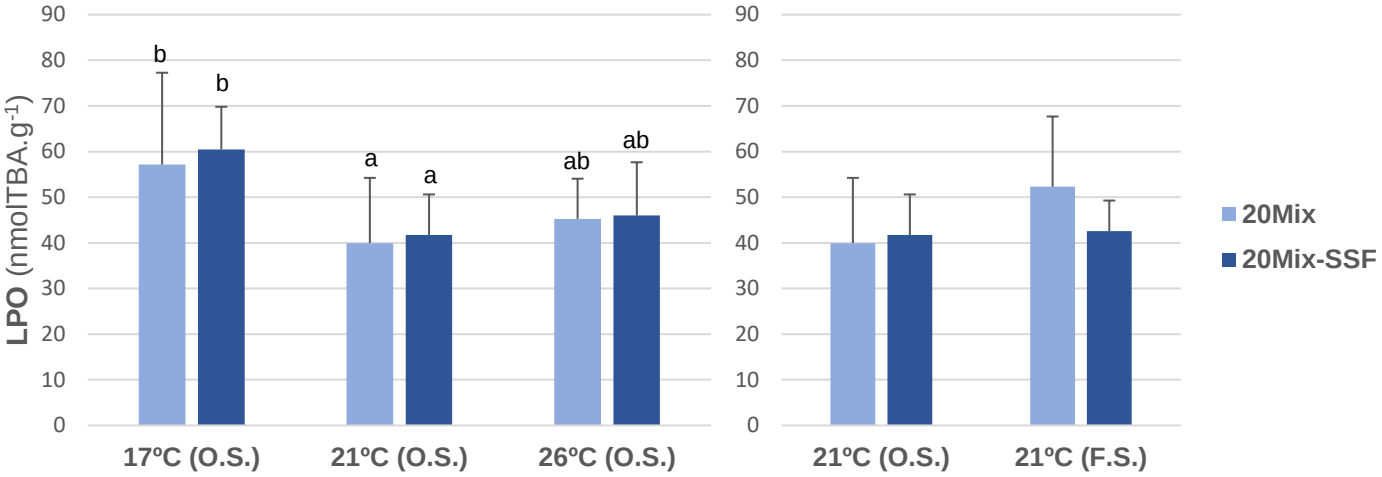

Figure 31: Lipid peroxidation (LPO) in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value = 0.013), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value > 0.05), Diet (p-value > 0.05), Interaction (p-value > 0.05)). Different letters indicate significant differences regarding effect of temperature. Values are presented as mean ± SD (n=6) (nmolTBA.g^{-1})

Catalase (CAT)

Catalase activities (CAT; Figure 32) in liver of fish reared at different temperatures (17, 21, 26ºC) subjected to salinity oscillation showed that temperature and experiemtal diet had a significant interactive effect, where fish fed the 20Mix diet showed no significant enzyme activity changes when reared in different temperatures. In contrast, fish fed the 20Mix-SSF diet showed to have higher enzymatic activity when reared at 21ºC than those reared at 17ºC and 26ºC.

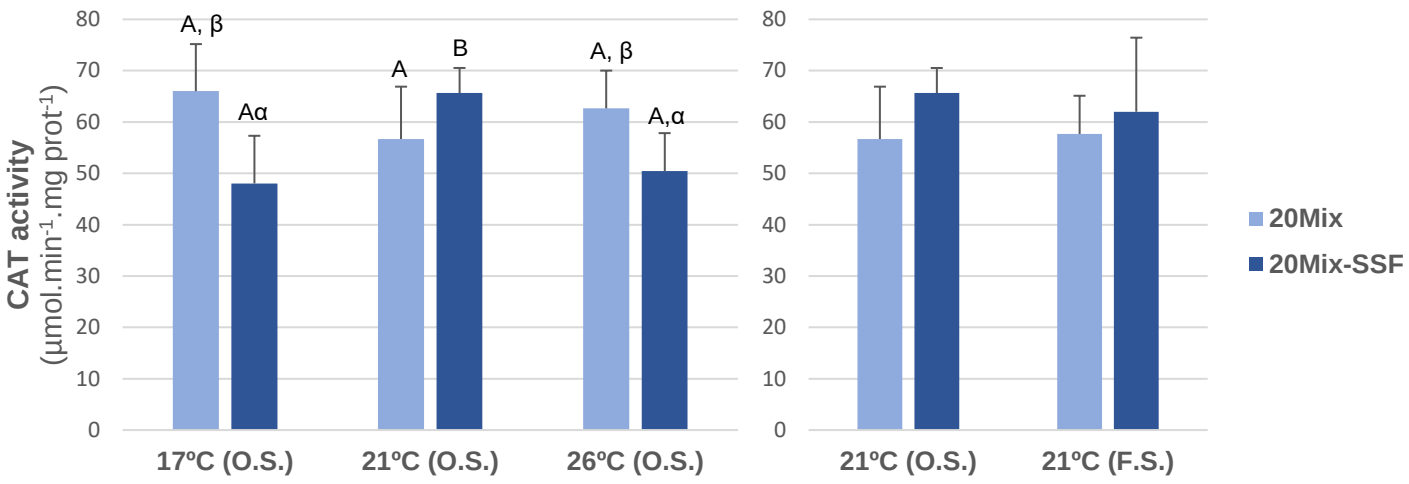

Figure 32: Catalase (CAT) activity in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (*p*-value > 0.05), Diet (*p*-value = 0.003), Interaction (*p*-value < 0.001)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (*p*-value > 0.05), Diet (*p*-value > 0.05), Interaction (*p*-value > 0.05)). Different greek letters indicate significant differences regarding effect of diet and different uppercase letters indicate significant differences between temperature groups for each experimental diet. Values are presented as mean ± SD (n=6) (µmol.min^{-1}.mg prot^{-1})

Glutathione Peroxidase (GPx)

Glutathione peroxidase activities (GPx; Figure 33) in liver of fish reared at different temperatures (17, 21, 26ºC) subjected to salinity oscillation showed that temperature and experiemtal diet had a significant interactive effect, where fish fed the 20Mix diet showed to have a significantly higher enzyme activity when reared at 17ºC and 26ºC, than those fed the same diet when reared at 21ºC. Conversely, fish fed the 20Mix-SSF diet showed to have higher enzymatic activity when reared at 21ºC than those fed the same diet when reared at 17ºC and 26ºC. When comparing GPx activity of fish reared at 21ºC and exposed to different salinity conditions, a significant effect of diet was observed, where in both salinity conditions, fish fed the 20Mix-SSF diet showed significantly higher GPx activities than those fed the 20Mix diet.

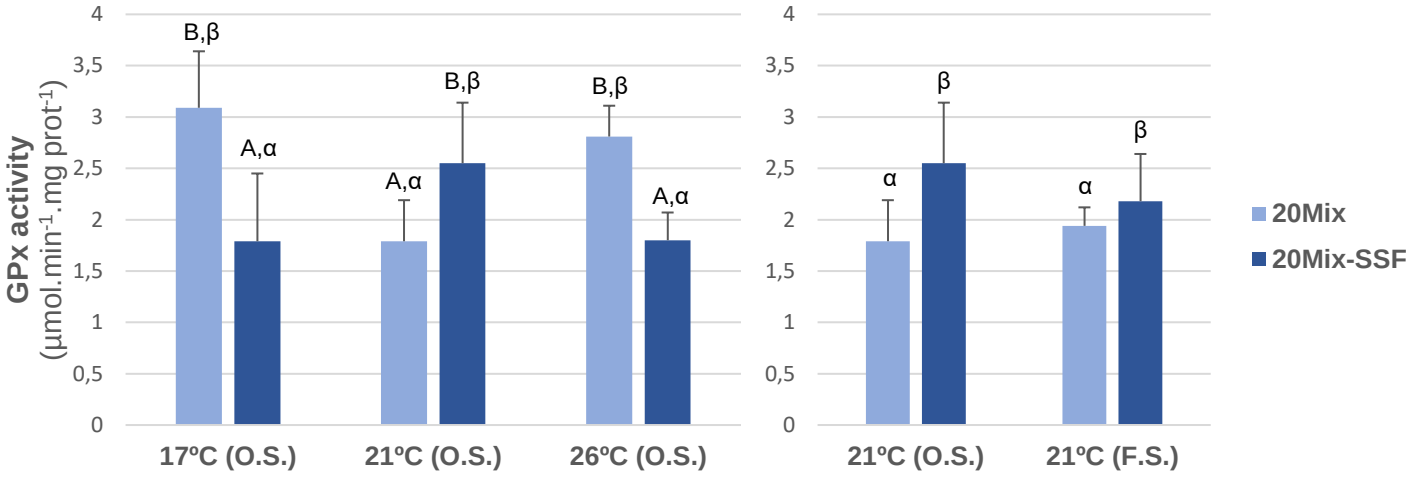

Figure 33: Glutathione peroxidase (GPx) activity in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value > 0.05), Diet (p-value = 0.006), Interaction (p-value < 0.001)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value > 0.05), Diet (p-value = 0.015), Interaction (p-value > 0.05)) Different greek letters indicate significant differences regarding effect of diet and different uppercase letters indicate significant differences between temperature groups for each experimental diet. Values are presented as mean ± SD (n=6) (nmol.min^{-1}.mg prot^{-1})

Glutathione Reductase

Glutathione Reductase activity (GR; Figure 34) in liver of fish reared at different temperatures (17, 21, 26ºC) subjected to salinity oscillation showed that temperature had a significant effect, where fish reared at 21ºC showed a significantly higher GR activity than those reared at 26ºC. When comparing GR activity of fish reared at 21ºC and exposed to different salinity conditions, a significant effect of diet was observed, where in both salinity conditions, fish fed the 20Mix-SSF diet showed significantly higher GR activities than those fed the 20Mix diet.

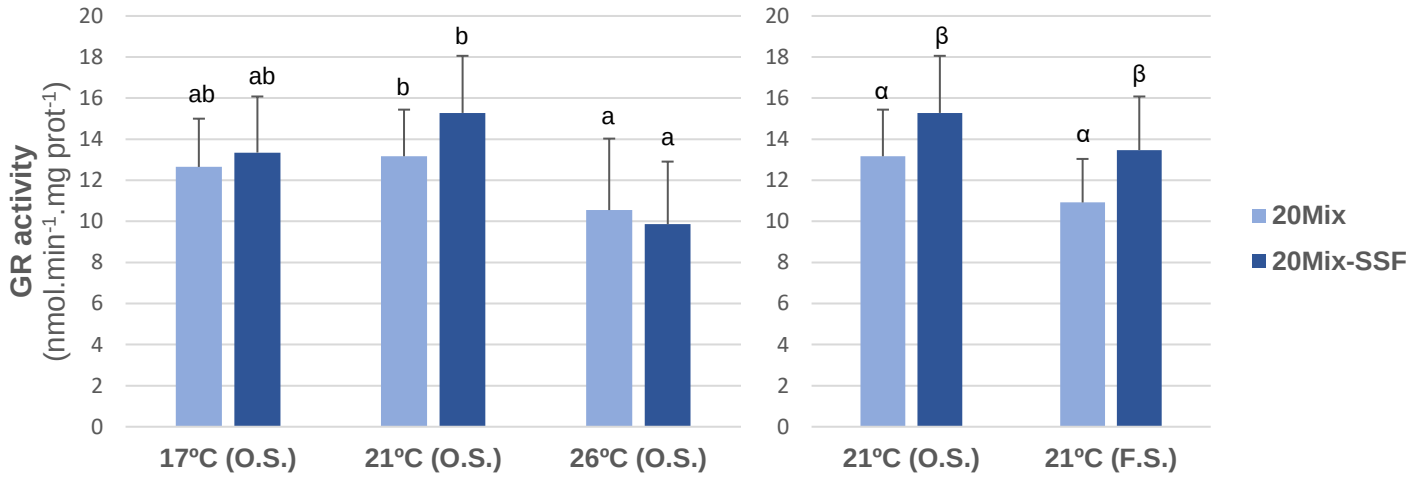

Figure 34: Glutathione reductase (GR) activity in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value = 0.006), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value > 0.05), Diet (p-value = 0.040), Interaction (p-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and different greek letters indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=6) (nmol.min^{-1}.mg prot^{-1})

Glutathione S-Transferase

Glutathione S-Transferase (GST; Figure 35) in liver of fish reared at different temperatures (17, 21, 26ºC) subjected to salinity oscillation showed that temperature had a significant effect, where fish reared at 21ºC showed a significantly higher enzymatic activity than those reared at 17ºC and 26ºC, and fish reared at 17ºC showed significantly higher enzymatic activity than those reared at 26ºC. When comparing GST activity within groups reared at 21ºC, fish subjected to salinity oscillation showed significantly higher GST activity than those reared at fixed salinity condition.

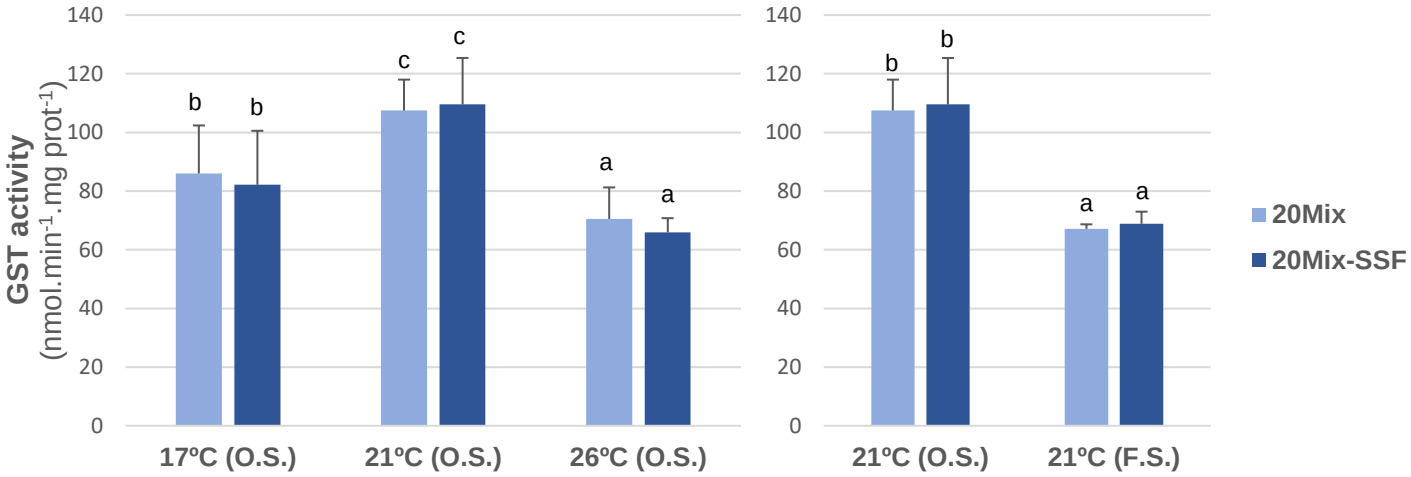

Figure 35: Glutathione S-transferase (GST) activity in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value < 0.001), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value < 0.001), Diet (p-value > 0.05), Interaction (p-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and salinity, respectively. Values are presented as mean ± SD (n=6) (nmol.min^{-1}.mg prot^{-1})

Total glutathione (TG); Reduced glutathione (GSH); Oxidised glutathione (GSSG) and Reduced glutathione – oxidised glutathione ratio (GSH/GSSG)

Fish reared at different temperatures (17, 21, 26ºC) subjected to salinity oscillation showed that temperature had no significant effect on oxidized glutathione (GSSG, Figure 38). In contrast, total glutathione (TG, Figure 36), reduced glutathione (GSH, Figure 37) and reduce glutathione – oxidized glutathione ratio (GSH/GSSG, Figure 39) were significantly higher in fish reared at 26ºC than those reared at 17ºC. GSH levels and GSH/GSSG in fish reared at 26ºC were also significantly higher than those reared at 21ºC.

When comparing both fish groups reared at 21ºC, a significant interactive effect of salinity and diet was observed, where fish fed the 20Mix-SSF diet showed significantly lower TG levels when subjected to salinity oscillation, while no significant differences were observed between salinity groups in fish fed the 20Mix diet.

The GSH levels was significantly affected by salinity and by the experimental diet, where GSH were higher in fish fed the 20Mix-SSF diet and in fish reared in fixed salinity condition.

Regardless of salinity conditions, GSSG levels were significantly higher in fish fed the 20Mix diets.

The GSH/GSSG was significantly affected by salinity and diet, where GSH/GSSG were higher in fish fed the 20Mix-SSF diet and when reared in fixed salinity condition.

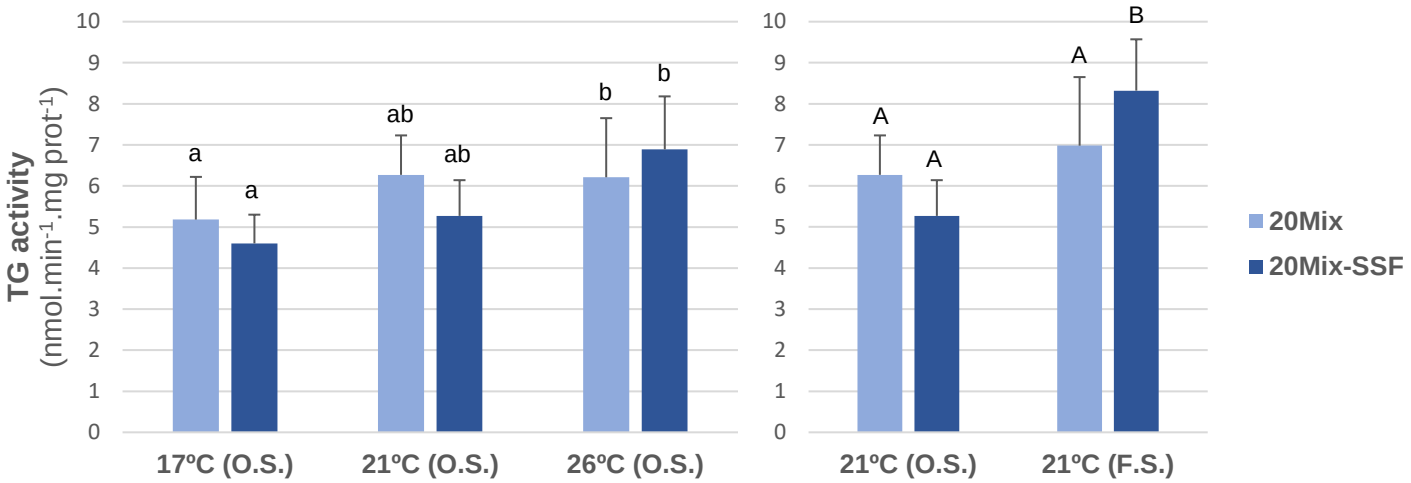

Figure 36: Total glutathione (TG) activity in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value = 0.009), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value = 0.002), Diet (p-value > 0.05), Interaction (p-value = 0.037)). Different lowercase letters indicate significant differences regarding effect of temperature and different uppercase letters indicate significant differences between salinity groups for each experimental diet. Values are presented as mean ± SD (n=6) (nmol.min^{-1}.mg prot^{-1})

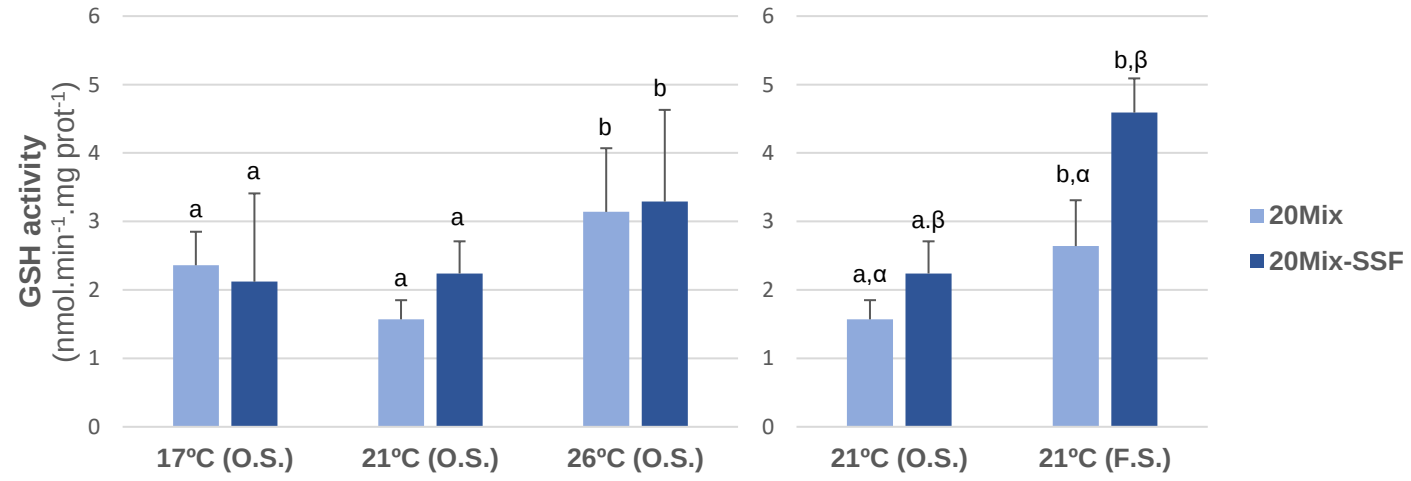

Figure 37: Reduced glutathione (GSH) activity in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value = 0.006), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value < 0.001), Diet (p-value < 0.001), Interaction (p-value = 0.009)). Different letters indicate significant differences regarding effect of temperature and salinity, respectively and different greek letters indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=6) (nmol.min^{-1}.mg prot^{-1})

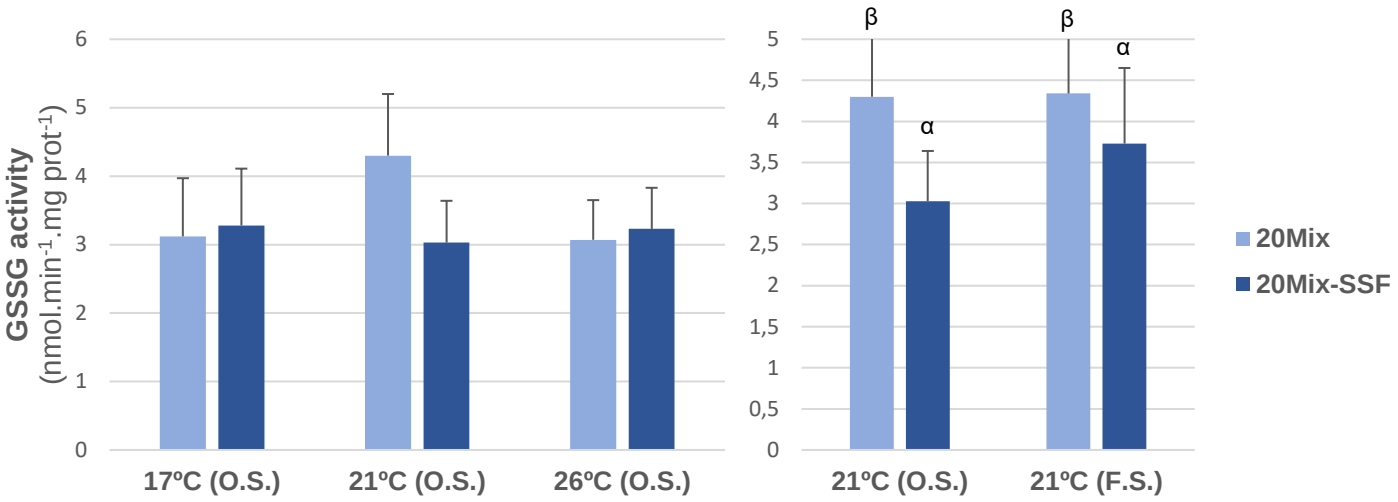

Figure 38: Oxidised glutathione (GSSG) activity in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value > 0.05), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value > 0.05), Diet (p-value = 0.034), Interaction (p-value > 0.05)). Different greek letters indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=6) (nmol.min^{-1}.mg prot^{-1})

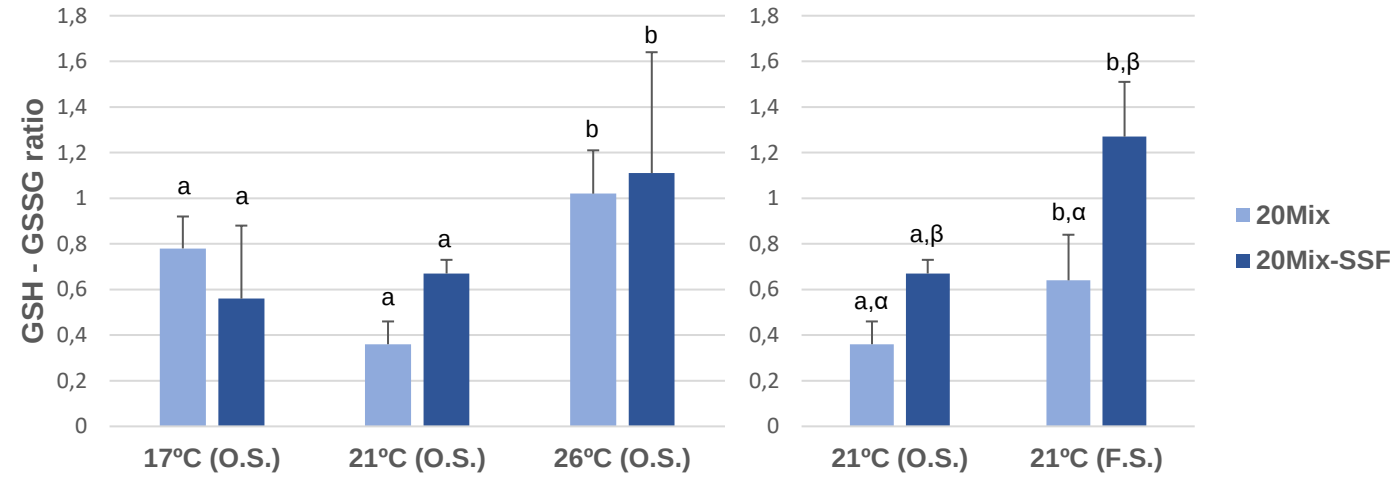

Figure 39: Reduced glutathione - oxidised glutathione activities ratio (GSH – GSSG ratio) in liver of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value < 0.001), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value < 0.001), Diet (p-value < 0.001), Interaction (p-value > 0.05)). Different letters indicate significant differences regarding effect of temperature and salinity, respectively and different greek letters indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=6) (nmol.min^{-1}.mg prot^{-1})

4.2.4 Humoral innate immune parameters

Peroxidase

Peroxidase activity (Figure 40) in plasma was significantly affected by temperature in fish subjected to salinity oscillation, where peroxidase activity decreased with increasing temperature.

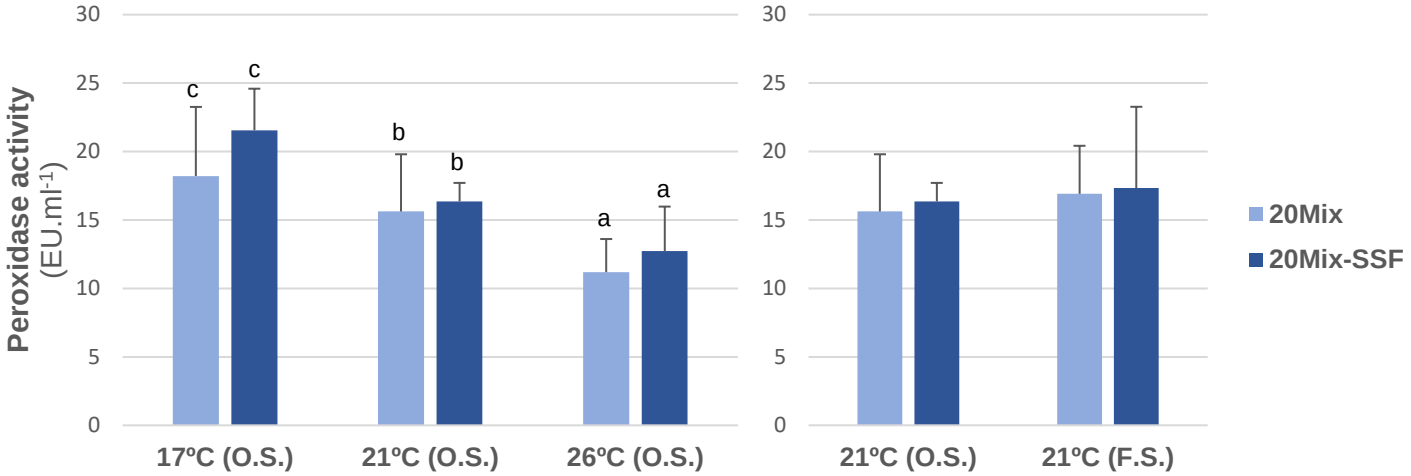

Figure 40 Peroxidase activity in plasma of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O) (Two-way ANOVA, Temperature (p-value < 0.001), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value > 0.05), Diet (p-value > 0.05), Interaction (p-value > 0.05)). Different letters indicate significant differences regarding effect of temperature. Values are presented as mean ± SD (n=6) (EU.ml^{-1})

Lysozyme

Within the fish groups reared at 21°C, lysozyme activity (Figure 41) in plasma was significantly higher in fish subjected to salinity oscillation.

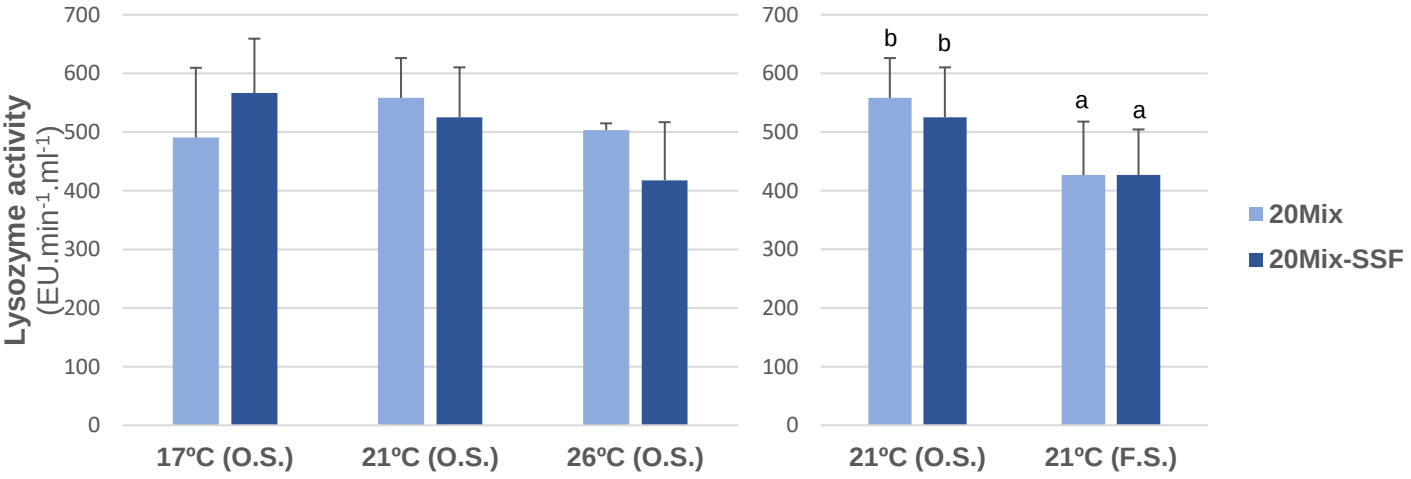

Figure 41: Lysozyme activity in plasma of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value > 0.05), Diet (p-value > 0.05), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value = 0.007), Diet (p-value > 0.05), Interaction (p-value > 0.05)). Different letters indicate significant differences regarding effect of salinity. Values are presented as mean ± SD (n=6) (EU.min^{-1}.ml^{-1})

Alternative complement pathway

Alternative complement pathway activity (ACH50; Figure 42) in plasma was significantly affected by diet in fish reared at different temperatures (17, 21, 26ºC) and subjected to salinity oscillation, where fish fed the 20Mix-SSF diet showed higher ACH50 activity. Within the fish groups reared at 21ºC, ACH50 activity was significantly affected by salinity condition and diet, where fish subjected to salinity oscillation showed lower ACH50 activity than those reared in fixed salinity conditions. In both salinity conditions, fish fed 20Mix-SSF diet showed higher ACH50 activities than those fed 20Mix diet.

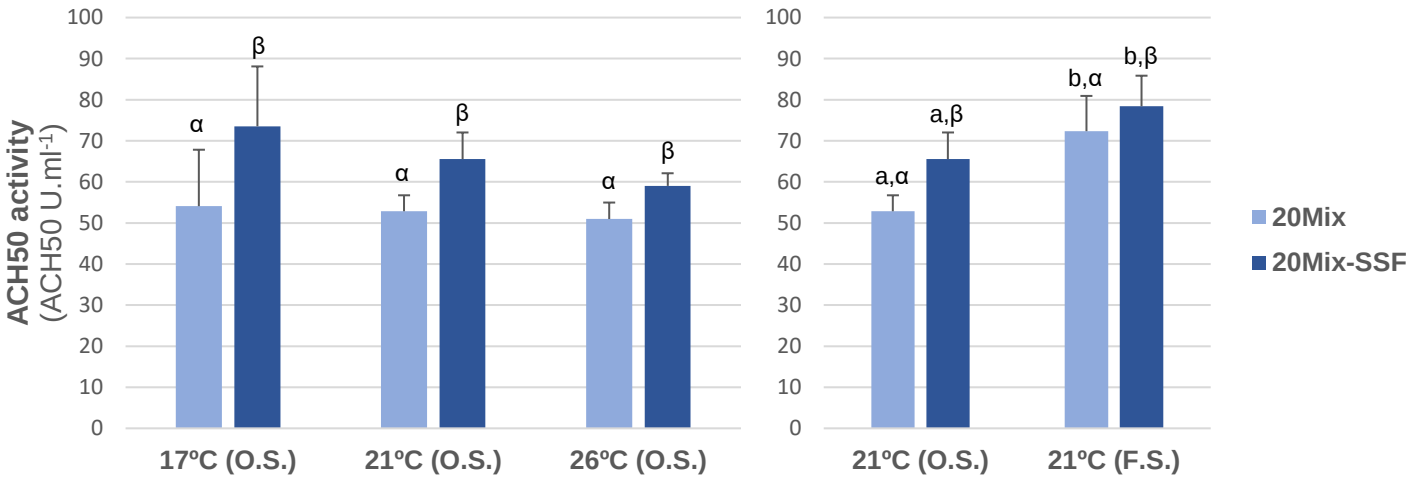

Figure 42: Alternative complement pathway in plasma of *D. labrax* juveniles, fed two experimental diets (20Mix and 20Mix-SSF) reared in different temperatures (17ºC, 21ºC and 26ºC) and subjected to oscillatory salinity (S.O.) (Two-way ANOVA, Temperature (p-value > 0.05), Diet (p-value < 0.001), Interaction (p-value > 0.05)) and groups reared at 21ºC subjected to different salinity conditions (O.S. and F.S.) (Two-way ANOVA, Salinity (p-value < 0.001), Diet (p-value = 0.006), Interaction (p-value > 0.05)). Different letters indicate significant differences regarding effect of salinity and different greek letters indicate significant differences regarding effect of diet. Values are presented as mean ± SD (n=6) (ACH50 U.ml^{-1})

5. DISCUSSION

In vivo digestibility trial

The nutritional value of a feed for a particular fish species depends on its composition, physic-chemical characteristics, and the bioavailability of nutrients and energy, which can be assessed by evaluating apparent digestibility coefficients.

Apparent digestibility

In the current study, dietary protein digestibility was generally high, with ADC ranging from 84 to 87%, except for 40Mix-SSF, whose protein ADC was significantly lower (70%). In diets where plant-based mix was included at a 20%, fermentation improved protein digestibility. To date, there is limited information on the effects of plant-based feeds fermented by filamentous fungi on nutrient digestibility in fish. Nevertheless, it is plausible to infer that such improvement may reflect the production of exogenous proteases by *A. niger* and the possible reduction of anti-nutritional factors such as phytic acid and saponin in fermenting the plant-based ingredients (de Castro et al., 2015; Freitas et al., 2013; Kumar et al., 2012), thus increasing the bioavailability and digestibility of protein as observed in recent studies (Jannathulla et al., 2018). It is considered that the apparent digestibility of energy correlates positively with the total digestibility of nutrients (Campos et al., 2018), especially with nutrients traditionally considered as energy sources in animal feed, such as lipids and carbohydrates (H. Li et al., 2019). Lipids were well digested and apparent digestibility was mostly unaffected by ingredient treatment. Dry matter digestibility of feeds reflects the digestible portions of both its organic and inorganic matter (Campos et al., 2018). Dry matter digestibility increased in fish fed 20% of the fermented mix, possibly due to the production of exogenous lignocellulosic enzymes, by the fungus, which hydrolyse the fibre-rich cell walls of the plant-based ingredients, increasing the bioavailability and consequently the digestibility of the carbohydrates and other nutrients such as protein, as reported in Fernandes et al. (2021) and Castillo and Gatlin (2015)

A distinct pattern was observed in the ADCs of the experimental diets. The dietary inclusion of 20% of plant-based ingredients significantly increased the ADC of protein, energy, and dry matter However, with the dietary inclusion of 40% of plant-based ingredients, fermentation significantly decreased the ADC of protein, energy, and dry matter. The addition of fermented plant-based ingredients included the end-product of fermentation and fungus *A. niger*. This could pose a significant problem, as both the fungal mycelium and spores released during the fermentation process have been shown

to be coated with a chitin-glucan complex that can account for 20-25% of the dry weight of the fungal cell wall (Feofilova et al., 2006). Dietary chitin has been shown to reduce feed digestibility in Atlantic salmon (*Salmo salar*) (Olsen et al., 2006), Nile tilapia (*Oreochromis niloticus*) (Sánchez-Muros et al., 2016) and meagre (*Argyrosomus regius*) (Coutinho et al., 2021) because carnivorous fish are mostly unable to digest chitin (Henry et al., 2015). Coutinho et al., (2021) noted, in meagre, that chitin inclusion does not affect feed digestibility if its content does not exceed 0.6% of dry feed weight. In European seabass, Gasco et al. (2016) reported that a dietary chitin content of 1.7% did not affect apparent digestibility. From the results of this study, it is plausible to infer that the chitin level in the 20% fermented mix was not high enough to negatively affect nutrient digestibility. However, at a 40% inclusion level of fermented mix, the dietary chitin content doubled, thus negatively impacting the overall digestibility of the 40Mix-SSF diet.

Digestive enzymes

The activity of digestive enzymes plays an essential part in the ability of fish to metabolise food, and absorb nutrients from the diet and is, thus, a crucial factor in the digestibility of nutrients (Fountoulaki et al., 2005).

The availability of amylases and other carbohydrases is crucial for carbohydrate digestion, but surprisingly little is known about the regulation of enzyme activity (Krogdahl et al., 2005). Higher carbohydrate intake in herbivorous and omnivorous species appears to increase enzymatic activity. However, in carnivorous species the response is less clear (Krogdahl et al., 2005). In the current study, α-amylase activity increased with plant-based content, most likely due to the overall higher carbohydrate content of the 40Mix and 40Mix-SSF diets. A similar effect on α-amylase activity in response to increased dietary starch content was observed in Artic charr (*Salvelinus alpinus*) (Abro et al., 2014) Lipase activity increased in fish fed fermented diets, possibly due to *A. niger*, producing exogenous lipase under solid-state fermentation conditions (Putri et al., 2020). Chen et al. (2021) reported a similar response in Russian sturgeon (*Acipenser gueldenstaedtii*) fed diets supplemented with exogenous lipase, it is plausible that the properties and optimal conditions of exogenous lipase from microorganisms are different from endogenous fish lipase and thus do not trigger a downregulation mechanism of endogenous lipase secretion. Therefore, there might have been a synergistic action of exogenous and endogenous lipase activity (Chen et al., 2021)

Solid-state fermentation of plant-based ingredients by *A. niger* has been reported to reduce the trypsin inhibitor content in soybean meal and rapeseed meal (Jannathulla et al., 2018). Yet trypsin activity was significantly lower in fish fed the fermented diets, which

may be related to the production of exogenous proteases by *A. niger* during fermentation (Freitas et al., 2013). This prior hydrolysis of dietary protein most likely increased the bioavailability of soluble peptides and thus reduced the need for endogenous fish proteases for adequate protein digestion. Robaina et al. (1995) reported that Gilthead seabream (*Sparus aurata*) fed lupin supplemented diets showed a reduction in trypsin activity with no effect on apparent protein digestibility which may be explained by the presence of an unidentified antitrypsin factor. Coutinho et al. (2021) also reported a decrease in trypsin activity in meagre fed chitin supplemented diets, but protein digestibility was also negatively affected which only partially correlates with our results.
Digestive enzymes activities and patterns of nutrient digestibility do not appear to coincide, which may indicate that exogenous fungal enzymes and overall diet composition may have had a more crucial role in determining nutrient digestibility.
The ratio between α-amylase and trypsin activities (A/T) is often interpreted as an indicator of the flexibility of energy metabolism and indicates that fish are able to use carbohydrates as an energy source and thus spare protein for growth. In the current study, fish fed the 40Mix and 40Mix-SSF diets had a significant higher A/T ratio, which can be attributed to higher α-amylase activity. Given the short trial duration and the equipment utilized in the digestibility trial, the dietary efficiency parameters, such as protein efficiency, were not calculated, so the effects of the diet on metabolic flexibility could be better understood in a dedicated growth trial.

Environmental stress trial

Growth performance

Rearing conditions and feed composition play an important role in the growth performance of fish, as both regulate feed intake, metabolism, and digestibility.

The diet including non-fermented ingredients (20Mix) was well accepted by juvenile seabass, while the diet including fermented ingredients (20Mix-SSF) was poorly accepted, which could be due to the lower palatability of the diet, which has been shown to reduce feed intake by the fish (Walker & Berlinsky, 2011). Indeed, feed intake was significantly reduced by the inclusion of fermented plant-based ingredients, resulting in a significantly lower weight gain, daily growth index and thermal growth coefficient.

Feed conversion and protein efficiency showed a non-significant tendency to increase and decrease, respectively, in fish fed the 20Mix SSF diet. This doesn't correlate with the improved digestibility observed in the digestibility trial. The initial weight of the seabass used in the digestibility trial (70.9 g ± 0.3) had three times the initial weight of the seabass used in the growth trial (22.6 g ± 4.0). The difference in fish sizes has been

reported to affect digestibility (Ferraris et al., 1986), and might partly explain the different levels of feed efficiency observed.

Water temperature has a major influence on fish growth performance (Volkoff & Rønnestad, 2020) and in this trial, feed intake and growth rate positively correlated with rearing temperature as previously reported in other seabass trials (Pereira et al., 2018; Zhou et al., 2021). However the optimal rearing temperature for European seabass is between 20 and 24 °C (Claireaux & Lagardère, 1999). Conides et al. (2004) reported that the temperature for optimal growth performance might be between 25 and 28 ºC. Although this temperature interval is considered a possible stressor, the improved growth performance could outweigh any adverse effects caused by heat stress.

Digestive enzymes

The combined effect of temperature and osmotic stress on the activity of digestive enzymes in fish has not been studied in detail in euryhaline fish, and despite the results observed in the current digestibility study, no significant effect of diet on digestive enzyme activities was observed in the environmental stress study.

Amylolytic and lipolytic enzyme activities increased in fish reared at temperatures outside the optimal range. Although it has been previously reported that α-amylase and lipase activities increased in fish reared at higher temperatures (Alexander et al., 2011; Kurtovic et al., 2009; Pereira et al., 2018), a higher α-amylase activity was also observed at a lower rearing temperature in this study. Similarly, Hani et al. (2018) reported that yellowtail kingfish (*Seriola lalandi*) showed higher intestinal proteolytic and lipolytic activity in colder water temperatures, as a possible compensation for slower gut motility.

Trypsin activity was highest in fish reared within the optimal range of the species, and it has already been shown that the activity of this enzyme correlates positively with temperature, but only within the optimal range of the species, so any increase in water temperature may lead to a reduction in enzyme activity. Indeed, Ahmad et al. (2014) showed that walking catfish (*Clarias batrachus*) reared at water temperatures between 10 and 35ºC showed higher trypsin and chymotrypsin activity when reared at 25ºC. However, in this study, chymotrypsin activity was found to be negatively correlated with temperature, with the highest enzymatic activity measured at 17ºC as observed by Pereira et al. (2018). Rungruangsak-Torrissen et al. (2006) concluded that chymotrypsin activity increased in response to reduced growth, which is consistent with the results observed in this study, as chymotrypsin activity is negatively correlated with observed growth rate.

In this study, fish reared at temperatures outside the optimal range had significantly higher amylase-trypsin ratios (A/T) than fish reared at 21ºC. Although the protein-sparing effect of dietary lipids is well documented in several fish species (Watanabe, 1982), the protein-sparing effect of carbohydrates in European seabass is controversial (Hidalgo & Alliot, 1988; Moreira et al., 2008). When comparing the different groups reared at 21ºC (21ºC O.S. and 21ºC F.S.), the amylase-trypsin ratio was significantly higher in fish fed the control diet. The A/T ratio was less than 0.1 in all groups studied, which is to be expected in a carnivorous species (Abro et al., 2014) such as European seabass. Furthermore, it would be difficult to conclude that environmental conditions and feed enabled protein sparing, as no significant effect on protein efficiency was observed. The ratio between trypsin and chymotrypsin activities (T/C) has been interpreted as an indicator of stimulation of growth performance and satiation status. In this study, fish fed the fermented diet and reared at temperatures outside the optimal range for seabass had significantly lower T/C than fish reared at 21 ºC, mainly because of reduced trypsin activity in fish reared at 17 ºC and 26 ºC

Osmotic stress significantly and clearly reduced overall digestive enzyme activity. This effect has been previously reported in yellowtail kingfish (*Seriola aureovittata*) (Shi et al., 2020) and gilthead sea bream (*Sparus aurata*) (Moutou et al., 2004). The reduction in enzymatic activity may be explained by the different water salinities, altering water drinking rates and the pH and ion concentrations and/or composition of the gut physicochemical properties, possibly affecting the activation of enzymes' zymogens and gut transit times (Moutou et al., 2004; Usher et al., 1990).

Oxidative stress: Enzymatic and non-enzymatic parameters

Environmental conditions such as water temperature and salinity are known to affect oxidative stress indicators in fish (Vinagre et al., 2012) and diet composition has been widely demonstrated to influence the ability of fish to mitigate the effects of oxidative stress (Hoseinifar et al., 2021). The addition of compounds derived from solid-state fermentation has also been shown to be an effective strategy for improving the oxidative status of fish, including European seabass (Fernandes et al., 2022; C. Li et al., 2019). However, in this study, the fermentation process was optimised for the production of lignocellulosic enzymes which resulted in a significantly reduced availability of bioactive antioxidant compounds in the fermented diets.

In this study, water temperature had a significant effect on the oxidative status of juvenile European seabass, resulting in increased lipid peroxidation (LPO) in the liver of fish reared at temperatures that differed from the optimal range for the species, which several

authors reported to be between 20 ºC and 24 ºC (Claireaux & Lagardère, 1999; Vinagre et al., 2012). Peroxidation of lipid layers increased significantly at 17 ºC, indicating a considerably reduced oxidative status at lower rearing temperatures, as also observed in seabass by Pereira et al. (2018) and in gilthead seabream (*Sparus Aurata*) by Ibarz et al. (2010). The rearing temperature had the opposite effect on total glutathione levels (TG), increasing significantly in fish reared at 26ºC, possibly caused by the increase of reduced glutathione (GSH). Consequently, a higher GSH/GSSG,, an indicator of the cellular redox status (Parolini et al., 2019), was observed.

Higher glutathione levels are correlated to improved antioxidant defences (Sitjà-Bobadilla et al., 2005), as glutathione plays an important role in lipid peroxide detoxification, reducing peroxides to their corresponding alcohols (Mourente et al., 2007). When comparing the TG and GSH levels with the LPO results in fish reared in temperatures outside the optimal range, it seems reasonable to infer that the higher glutathione levels may have prevented more significant lipid damage in livers of fish reared at 26ºC (Sitjà-Bobadilla et al., 2005).

Glutathione reductase (GR) activity was significantly higher in fish reared at 21 °C, possibly due to the need to regenerate GSH and rebalance cellular redox status (Parolini et al., 2019; Sánchez-Nuño et al., 2018). Higher oxidation of GSH may be an effect of higher activity of glutathione-dependent antioxidant enzymes such as glutathione S-transferase (GST) observed at 21 °C.

Catalase (CAT) and glutathione peroxidase (GPx) activities have been reported to increase in fish exposed to temperatures outside their optimal range (Islam et al., 2020; Madeira et al., 2013; Vinagre et al., 2014) and in this study, CAT and GPx were affected by temperature and feed treatment. In fish fed the control diet (20Mix), GPx activity followed the previously reported pattern, and CAT activity showed no significant effect on rearing temperature. Interestingly, fish fed the fermented diet (20Mix-SSF) exhibited CAT and GPx activities significantly lower when exposed to suboptimal temperatures. Indeed, the combined effect of temperature and salinity stress has been reported to reduce CAT and GPx activity (Dawood et al., 2022) However, this effect was only observed in fish fed the fermented diet, which may indicate that 20Mix-SSF feed may have offered a lower protective effect on seabass. Nonetheless, since dietary treatment had no significant effect on hepatic lipid damage, it can be assumed that the observed reduction in antioxidant enzyme activities did not compromise overall oxidative status.

Environmental salinity has also been observed to affect the oxidative status of euryhaline fish (Chang et al., 2021). In the current study, when comparing the different groups

reared at 21ºC (21ºC O.S. and 21ºC F.S.), it was observed that weekly salinity variation resulted in lower TG, mainly due to a reduction in GSH, resulting in reduced GSH/GSSG. The decrease in GSH may be caused by glutathione-dependent GST, whose activity has been widely described in response to osmotic stress (Moniruzzaman et al., 2022; Sinha et al., 2015; Yin et al., 2011). Indeed, when comparing the different groups reared at 21 °C, GST activity was 59% higher in fish exposed to salinity fluctuation than in those reared in fixed salinity. This may have contributed significantly to the fact that osmotic stress had no effect on hepatic lipid damage in seabass, as the activities of CAT and GPx showed no response to salinity, as also observed by Chang et al. (2021).

When comparing the different groups reared at 21ºC (O.S. and F.S.) a distinct dietary effect was observed on the oxidative status of fish. Fish fed the fermented diet (20Mix-SSF) showed higher GPx activity, higher levels of GSH and lower levels of GSSG and consequently higher GSH/GSSG. Such improvement in cell redox status may also be explained by the higher activity of GR (Sánchez-Nuño et al., 2018).

Although higher antioxidant enzyme activity and the ability to improve apparent cellular redox status appear to be beneficial to fish welfare, no significant dietary effect on lipid peroxidation was observed, which may suggest that the higher antioxidant enzyme activity was compensatory for the significantly lower amount of bioactive antioxidant compounds in the fermented diet (20Mix-SSF).

Humoral Innate immunity

Rearing conditions such as water temperature and salinity, as well as the supplementation of immunostimulatory substances such as β-glucans, have been shown to influence innate immune responses in teleosts, often in response to infection (Alcorn et al., 2002; Bagni et al., 2005; Mozanzadeh et al., 2021).

Macrophages, eosinophils, and neutrophils are central cells of the innate immune system, important phagocytic cells, and producers of reactive oxygen species, such as hydrogen peroxide and myeloperoxidase, and peroxidase. Studies on the effects of suboptimal rearing conditions on macrophages and neutrophils have generally reported either no change or environmental modulation of their activity. In this study, peroxidase activity was negatively correlated with temperature, being significantly higher at lower rearing temperatures. This is not consistent with previously reported studies on the effects of water temperature on peroxidase activity in European seabass (Pereira et al., 2018; Valero et al., 2014), where rearing temperature had no significant effect. Despite this modulation not being reported in seabass, similar results were reported in Tench

(*Tinca tinca*) (Collazos et al., 1994) and Common carp (*Cyprinus carpio*) (Le Morvan et al., 1997).

The current study is in agreement with Pereira et al. (2018), as rearing temperature had no significant effect on lysozyme activity, which has been shown to be seasonal in European seabass (Pascoli et al., 2011; Valero et al., 2014), but only when temperature decreases below 15 ºC. Therefore, rearing temperatures tested in this study (17 – 26 ºC) may not have been impactful enough to modulate lysozyme activity. Despite temperature not having a significant affect, rearing salinity oscillation led to an increased lysozyme activity. This effect of salinity alteration has been reported in other fish species such as Yellowfin seabream (*Acanthopagrus latus*), Asian seabass (*Lates calcarifer*) (Mozanzadeh et al., 2021) and rainbow trout (*Oncorhynchus mykiss*) (Fast et al., 2002). Mozanzadeh et al. (2021) and Cuesta et al. (2005) reported that Asian seabass (*Lates calcarifer*) and gilthead seabream (*Sparus aurata*) respectively, exposed to osmotic stress by hyposaline and hypersaline conditions showed a reduction in complement pathway activity (ACH50). This is consistent with the results observed in this study, in which European seabass juveniles exposed to salinity fluctuation exhibited reduced ACH50 activity. Such reduction may also be further explained by the fact that these groups of seabass juveniles were exposed to hypersaline conditions (45 ppt) during the last seven days of the environmental stress trial. Alternative complement pathway activity was not only influenced by environmental salinity but also by dietary treatment, where fish fed the 20Mix-SSF diet showed significantly higher ACH50 activity than those fed the control diet, which can be explained by the inclusion of the β-glucan-rich cell wall of *A. niger*. Previous studies have reported the immunostimulatory effect of including β-glucan in the diet of teleosts (Aramli et al., 2015; Kumari & Sahoo, 2006; Lauridsen & Buchmann, 2010; Yamamoto et al., 2018). Indeed, supplementing this polysaccharide has been shown to be an effective strategy for enhancing innate immunity in fish, even when included as an integral component of the fungal cell wall. El-Boshy et al. (2010), Pal et al. (2007) and Chang et al. (2013) have shown that ingestion of feeds containing yeast (*S. cerevisiae*) cell wall preparations and the processed mycelia of mushrooms (*Ganoderma lucidum* and *Coriolus versicolor*) can improve the activity of innate immune system parameters. Chang et al. (2013) observed that orange spotted grouper (*Epinephelus coioides*) fed 1 g and 2 g of fungal β-glucan per kg of diet had significantly higher ACH50 activity against bacterial infections The results of this study are also consistent with the observations of Bagni et al. (2005), where European seabass fed a diet containing β-glucan for 30 days showed increased ACH50 activity.

6. CONCLUSION

The *in vivo* digestibility trial showed that solid-state fermented plant-based ingredients could be well digested by juvenile European seabass, with increased apparent digestibility coefficients for protein, dry matter, and energy as long as the dietary supplementation is maintained at a 20% fermented plant-based ingredient level. The reduced feed consumption and, consequently, lower weight gain of seabass fed the fermented diet suggest that diet palatability may be a significant disadvantage.

In the environmental stress trial, thermal and osmotic stress modulated the physiological response of European seabass, with dietary treatment not influencing digestive enzymes activity nor the overall oxidative status but modulating the activities of antioxidant mechanisms. Together, these results may indicate that the reduced antioxidant bioactive compounds in the fermented diet resulted in a compensatory response by endogenous antioxidant mechanisms. In contrast, fish fed fermented diet showed enhanced alternative complement pathway activity.

7. FUTURE WORKS

Future projects could benefit from further optimisation of solid-state fermentation processes to neutralise or mitigate any negative factors that may have resulted from using *A. niger* for the fermentation of plant-based ingredients. The dietary inclusion levels of fermented ingredients may also be reproached to ensure maximum feed efficiency.

In future environmental stress trials, the "fixed salinity" group could be expanded to include three rearing temperatures, as was designed for the "oscillatory salinity" group. This experimental design would undoubtedly allow a clearer understanding of the physiological responses of fish reared outside their optimal temperature range, as it was difficult to determine whether specific physiological responses were solely due to thermal stress or whether they were due to the combined effect of thermal and osmotic stress.

Future trials may benefit from further tissue analysis, e.g. gut, kidney and gill oxidative stress, haematological parameters and plasma biochemical factors, which would lead to a better understanding of the physiological responses of teleosts to environmental stress and their systemic interactions. These analyses would also complement the existing bibliography on the subject.

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9. ATTACHMENTS

Aquaculture Europe 2022

September 27 - 30, 2022 - Rimini, Italy

International Conference & Exposition

Presentation Certificate

Diogo Amaral

Diogo Filipe, Thais Cavalheri, Lúcia Vieira, Rui Magalhães, Helena Peres, Rodrigo Ozorio

for Poster Presentation of

EFFECT OF NOVEL DIETARY INGREDIENTS ON THE PHYSIOLOGICAL RESPONSES OF EUROPEAN SEABASS *Dicentrarchus labrax* SUBJECTED TO DIFFERENT REARING TEMPERATURES AND SALINITY OSCILLATION

Herve Migaud
Chair

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EFFECT OF SOLID-STATE FERMENTED PLANT-BASED INGREDIENTS IN DIET DIGESTIBILITY AND DIGESTIVE ENZYMES ACTIVITY IN EUROPEAN SEABASS *Dicentrarchus labrax* JUVENILES

Herve Migaud
Chair


Aqualmprove

Certificate of Appreciation

AWARDED TO

Diogo Amaral

FOR DELIVERING A POSTER PRESENTATION IN THE

6th Aqualmprove - Aquaculture Research Workshop

Porto, 19th October 2022

Prof. Aires Oliva-Teles

Helena Peres

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