VNIVERSITAT DE VALÈNCIA

Facultat de Farmàcia

Area de Nutrició i Bromatologia, Departament de Medicina Preventiva i Salut

Pública, Ciències de l´Alimentació, Toxicologia i Medicina Legal

EVALUATION OF BIOLOGICAL PROPERTIES OF EXTRACTS OBTAINED FROM MARINE BIOMASS ASSISTED BY PULSED ELECTRIC FIELDS (PEF) AND PRESSURIZED LIQUID EXTRACTION (PLE)

EVALUACIÓN DE LAS PROPIEDADES BIOLÓGICAS DE EXTRACTOS OBTENIDOS A PARTIR DE BIOMASA MARINA MEDIANTE PULSOS ELÉCTRICOS (PE) Y EXTRACCIÓN CON LÍQUIDOS PRESURIZADOS (PLE)

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La Graduada en "Food Science and Engineering" Dña. Min Wang ha realizado, bajo su dirección y en los laboratorios del área, el trabajo que lleva por título: **"Evaluation of biological properties of extracts obtained from marine biomass assisted by pulsed electric fields (PEF) and pressurized liquid extraction (PLE)** / "**Evaluación de las propiedades biológicas de extractos obtenidos a partir de biomasa marina mediante pulsos eléctricos (PE) y extracción con líquidos presurizados (PLE)**" y autorizan su presentación para optar al título de **Doctora Internacional** por la Universitat de València

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“Always do your best.

What you plant now,

you will harvest later.”

----Og Mandino

To all my family and

everyone who has helped me

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List of abbreviations

AAPH	2,2'-azobis-2-methyl-propanimidamide
ABTS	2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonic acid)
ACE	Angiotensin-converting enzyme
AGEs	Advanced glycation end products
APC	Allophycocyanin
ASE	Accelerated Solvent Extraction
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCFAs	Branched-chain fatty acids
BF	Bioaccessible fraction
BHI	Brain heart infusion medium
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
CS	Chondroitin sulfate
COX	Cyclooxygenase
DGM	Dynamic gastric model
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DRTA	Dynamic rheological thermal analysis
DSC	Differential scanning calorimetry
E. coli	*Escherichia coli*
EAA	Essential amino acids
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
ET	Electron transfer
FCS	Fucosylated chondroitin sulfate
FM	Fluorescence microscope

GC-MS	Gas chromatography mass spectrometry
GDS	Gastric digestion simulator
GM	Gut microbiota
HAT	Hydrogen atom transfer
HGS	Human gastric simulator
ICP-MS	Inductively coupled plasma mass spectrometer
IL-1β	Interleucina-1 beta
IL-6	Interleucina-6
ILs	Ionic liquids
ISP	Isopoint dissolution precipitation
L-cys	*L*-cysteine
LPS	Lipopolysaccharide
MOD	Maximal optical density
MRS	Man rogosa sharpe medium
MS	Microwave-assisted
MUFA	Monounsaturated fatty acids
NAs	Nitrosamines
NCDs	Non-communicable diseases
NMR	Nuclear magnetic resonance
NRV	Relative Nutrient Values
ORAC	Oxygen Radical Absorbance Capacity Assay
PBP	Phycobiliproteins
PCA	Principal component analysis
PC	Phycocyanin
PE	Phycoerythrin
PEC	Phycoerythrocyanin
PEF (PE)	Pulsed electric fields (Pulsos eléctricos)
PLE (PLE)	Pressurized liquid extraction (Extracción con líquidos presurizados)
PMSF	Phenylmethylsulfonyl fluorid

PUFA	Polyunsaturated fatty acids
RDAs	Recommended Dietary Allowances
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SCFAs (AGCC)	Short-chain fatty acids (Ácidos grasos de cadena corta)
SC-CO_2	Supercritical CO_2
SEAP	Secreted alkaline phosphatase
SEM	Scanning Electron Microscopy
SFE	Supercritical fluids extraction
SFA	Saturated fatty acids
SIP	Squid ink polysaccharides
S. aureus	Staphylococcus aureus
TAG	Triacylglycerols
TEAC	Trolox equivalent antioxidant capacity
TLR	Toll-like receptor
TNF-α	Tumor Necrosis Factor-α
UFAs	Unsaturated fatty acids

ABSTRACT

As an important part of biodiversity, marine resources are found to be an important source of variety of High-Added-Value Compounds (HAVCs), including protein, lipids, bioactive peptides, polyphenols, etc. Marine side streams such as fish head, viscera, skin, are discarded or not used effectively during the food processing, resulting in a waste of resources, however, they are a great source of HAVCs. Traditionally, conventional methods using high amounts of solvents, which in some cases are toxic, long extraction times and high temperatures, have been used. Therefore, at this stage of development there is a need for developing suitable, innovative, and sustainable extraction technologies to recover more efficiently the HAVCs from marine resources. In this doctoral dissertation, two innovative approaches, Pulsed Electric Fields (PEF) and Pressurized Liquid Extraction (PLE), also known as Accelerated Solvent Extraction (ASE), have been optimized and applied to recover HAVCs from fish (rainbow trout and sole) side streams and microalgae. The bioactivity of the extracts was also evaluated, including the antioxidant capacity, antibacterial properties, anti-inflammatory properties, and the impact on human gut microbiota by use of *in vitro* model.

The results showed that PEF and PLE significantly improved the protein extraction rate of fish side streams, and the molecular weight distribution of protein was also changed, which was mainly manifested as protein changes with molecular weighs between 5~250 kDa. Meanwhile, the antioxidant capacity of skin and head extracts from rainbow trout and sole was also significantly improved. In addition, the extracts can inhibit the growth of pathogenic bacteria. In this sense, PLE-assisted rainbow skin and

PEF-assisted sole viscera extracts had an inhibitory effect on the growth of *Staphylococcus aureus*. On the other hand, some extracts (such as fish head and skin extracts) can promote probiotics growth and anti-inflammatory activity. The impact of innovative extraction technologies on the mineral yield and bioaccessibility was also investigated, observing that PLE-assisted extraction enhanced the recovery of Mg and Fe from rainbow trout and sole viscera extracts. Furthermore, some extracts also showed a great potential to modulate the gut microbiota, for example sole viscera extract increased the levels of *Bifidobacterium* and *Lactobacillus* members and promoted the production of specific microbial metabolites (SCFAs).

Regarding microalgae samples, the extraction of bioactive compounds was affected by multiple factors, including the type of treatment (PEF, PLE or conventional extraction), solvent and extraction time. In this sense, PEF-assisted treatment increased the yields of most of the high-added-value compounds evaluated. For instance, the application of PEF+H_2O for 120 min, led to the highest protein (43.05±2.35 mg/g DW) and polyphenols (5.21±0.05 mg/g DW) extraction yields, respectively. On the other hand, the use of PEF+50% DMSO, allowed the higher chlorophyll *a*, chlorophyll *b* and carotenoids extraction yields (0.50, 0.60 and 0.15 mg/g, respectively). It was also found that the higher protein and polyphenols´content had a positive correlation with the Trolox equivalent antioxidant capacity (TEAC), while the chlorophyll and carotenoid contents had a high correlation with the oxygen radical absorption capacity (ORAC).

When PLE technique was used to recover the high-added-value compounds from microalgae, it was found that H_2O promoted a higher extraction rate of proteins, while

100% DMSO allowed a better recovery of polyphenols, chlorophyll and carotenoids. PLE+100% DMSO was a useful tool to recover antioxidants and minerals from different microalgae (*Spirulina*, *Chlorella* and *Phaeodactylum tricornutum*), being of particular interest the extraction of individual carotenoids such as β-carotene, lutein and zeaxanthin.

Using PEF and PLE to assist the recovery of HAVCs from aquaculture, can not only improve the extraction efficiency, but also protecting the bioactivity of the HAVCs from being destroyed. Moreover, selecting the appropriate methods to extract and recycle HAVCs is of great significance to reducing waste of resources and achieving sustainable development.

Keywords: Fish side streams, microalgae, pulsed electric fields, pressurized liquid extraction, bioactive compounds.

RESUMEN

Teniendo en cuenta el gran interés por la biodiversidad, los recursos marinos representan una fuente importante de compuestos de alto valor añadido como proteínas y péptidos bioactivos, lípidos y ácido grados vitaminas, minerales y compuestos bioactivos, etc. Los residuos y subproductos del pescado como cabeza, vísceras y piel, entre otros, se descartan o no se utilizan de manera efectiva durante el procesamiento de los alimentos, lo que resulta en un gran desperdicio de recursos, sin embargo, son una gran fuente de compuestos con alto valor añadido y que pueden tener un potencial uso considerable en las industrias alimentaria, farmacéutica, cosmética y otras, existiendo, por lo tanto, un interés creciente en la recuperación de estos compuestos. Tradicionalmente se han utilizado métodos convencionales que consumen altas cantidades de disolvente, siendo en algunos casos tóxicos, largos tiempos de extracción y altas temperaturas. Por lo tanto, en esta etapa de desarrollo existe la necesidad de desarrollar tecnologías de extracción adecuadas, innovadoras y sostenibles para recuperar de manera más eficiente el alto valor añadido de las fuentes marinas. En esta tesis doctoral, se han optimizado y aplicado dos enfoques innovadores, Pulsos Eléctricos (PE) y Extracción con Líquidos Presurizados (PLE) para recuperar compuestos de alto valor añadido a partir de subproductos de pescado (trucha arcoíris y lenguado) y microalgas. También se evaluó la bioactividad de los extractos, incluida la capacidad antioxidante, las propiedades antibacterianas, las propiedades antiinflamatorias y los efectos de éstos sobre la microbiota intestinal humana en modelo *in vitro*.

Los resultados mostraron que tanto la utilización de PE como PLE mejoraron significativamente la tasa de extracción de proteínas de los subproductos de pescado, y también cambió la distribución del peso molecular de las proteínas, lo cual se observó principalmente como cambios en las proteínas con pesos moleculares entre 5 y 250 kDa. Asimismo, la capacidad antioxidante de los extractos de piel y cabeza de trucha arcoíris y lenguado también mejoró significativamente. Además, se obtuvo que los extractos de algunos subproductos, como por ejemplo los obtenidos a partir de piel de trucha arco iris tras PLE y el extracto de vísceras de lenguado tras PE podían inhibir el crecimiento de bacterias patógenas, en particular *Staphylococcus aureus*. Algunos extractos también presentaron un alto potencial para promover el crecimiento de compuestos probióticos y antiinflamatorios. Por otro lado, se investigó el efecto de PE y PLE en la extracción de minerales y como afectaban a su bioaccesibilidad. En este sentido, se observó una mejor recuperación de Mg y Fe tras PLE, a partir de extractos de vísceras tanto de trucha arcoíris como de lenguado. También tuvo se obtuvo un efecto significativo en la bioaccesibilidad mineral. Además, algunos extractos también mostraron potencial para regular el microbiota intestinal, por ejemplo, la adición de extracto de vísceras de lenguado aumentó los niveles de los géneros *Bifidobacterium* y *Lactobacillus*, favoreciendo la producción de algunos metabolitos bacterianos como los ácidos grasos de cadena corta (AGCC).

Para las microalgas, la extracción de compuestos bioactivos se vió afectada por múltiples factores, incluidos el tiempo de extracción y el disolvente cuando se utilizaron los PE. El tratamiento asistido por PE aumentó los rendimientos de compuestos bioactivos antioxidantes. Así pues, la utilización de H_2O como disolvente combinado con

PE durante 120 min, permitió obtener los mayores rendimientos de extracción de proteínas (43,05±2,35 mg/g materia seca (MS)) y polifenoles (5,21±0,05 mg/g MS) y, respectivamente. Mientras que, cuando se utilizó DMSO al 50 % como disolvente, el rendimiento de extracción de clorofila *a* (0,50 mg/g), clorofila *b* (0,60 mg/g) y carotenoides (0.15 mg/g), respectivamente, fue mayor. Al mismo tiempo, el contenido de proteína y polifenoles tuvo una alta correlación con la capacidad antioxidante equivalente de Trolox (TEAC), y el contenido de clorofilas y carotenoides tuvo una alta correlación con los valores de ORAC (capacidad de absorción de radicales de oxígeno).

Tras utilizar la extracción con líquidos presurizados (PLE) para recuperar compuestos bioactivos a partir de microalgas, se observó que la tasa de extracción de proteína fue mejor cuando se usó H_2O como disolvente, mientras que la tasa de extracción de polifenoles, clorofila y carotenoides fue mayor cuando se usó el DMSO al 100% como disolvente. Además, la combinación de PLE+100% DMSO resultó una estrategia muy interesante para recuperar antioxidantes y minerales de diferentes microalgas (*Spirulina*, *Chlorella* y *Phaeodactylum tricornutum*), destacando en particular de los carotenoides β-caroteno, luteína y zeaxantina.

La utilización de PE y PLE para facilitar la recuperación de compuestos de alto valor añadido no solo mejora la eficiencia de extracción, sino que también protege la bioactividad de estos compuestos para que no se destruyan. También es importante tener en cuenta la selección de métodos apropiados para extraer y reciclar los compuestos de alto valor añadido para reducir así el desperdicio de recursos y lograr un desarrollo sostenible.

Palabras clave: Subproductos de pescado, microalgas, pulsos eléctricos, extracción con líquidos presurizados, compuestos bioactivos.

RESUMEN GLOBAL DE LA TEMÁTICA, PRINCIPALES RESULTADOS Y CONCLUSIONES

1. Extracción dirigida de compuestos potencialmente funcionales a partir de subproductos: Uso de Pulsos Eléctricos (PE) y Extracción con Líquidos Presurizados (PLE)

1.1 Extracción con líquidos presurizados y pulsos eléctricos para la valorización de subproductos de trucha arcoíris (Oncorhynchus mykiss) y lenguado (Dover sole): Evaluación del contenido proteico, distribución del peso molecular y potencial antioxidante de los extractos.

Se investigó el efecto de los métodos/tratamientos innovadores, PE y PLE, en la recuperación de proteínas a partir de subproductos (cabeza, piel y vísceras) de trucha arco iris y lenguado. Tras aplicar los tratamientos con PLE, se observó que la tasa de extracción de proteína de trucha arcoíris fue significativamente más elevada en vísceras (~80 %), seguida de la piel (~59 %) y la cabeza (~38 %), en comparación con el grupo control (sin PLE). Para los subproductos del lenguado, también se observó un aumento significativo de la tasa de extracción de proteínas a partir de las vísceras, sin embargo, este efecto no fue significativo ($p>0.05$) en la recuperación de proteínas a partir de piel y cabeza.

Se observaron cambios en la textura de la piel tras la liofilización en trucha arco iris y el lenguado, siendo la piel del lenguado más dura, dificultando así la mezcla con tierra de diatomeas, paso necesario para la extracción con PLE, y la tasa de extracción de proteínas de las vísceras de lenguado mejoró significativamente.

La aplicación del tratamiento por PE permitió aumentar de forma considerable la extracción de proteínas a partir de la piel de trucha arcoíris y lenguado obteniendo porcentajes de recuperación cercanos al 80%. También se observaron recuperaciones interesantes y significativas a partir de la cabeza y las vísceras tras aplicar el tratamiento por PE. Cabe destacar que para el tratamiento con PE se utilizaron muestras de piel fresca que no fueron liofilizadas. En este sentido, tras el tratamiento con PE y agitación durante 24 h, se observó una mejor destrucción de la estructura del tejido de la piel, permitiendo así una mejor extracción de las proteínas y mostrando un efecto más pronunciado en comparación con el tratamiento asistido por PLE. Sin embargo, cabe resaltar que, aunque se observa una mejor extracción de las proteínas a partir de la piel y cabeza del lenguado tras aplicar el tratamiento asistido por PE no se observó un efecto significativo cuando se utilizaron los subproductos de la trucha arcoíris.

En este sentido, los subproductos del pescado han sido ampliamente estudiados como una de las principales fuentes de proteína marina. En un estudio previo, se revisó la aplicación de diferentes métodos de extracción para recuperar colágeno a partir de subproductos de pescado (Ahmed et al., 2020), incluida la hidrólisis enzimática, la hidrólisis química asistida por ultrasonido y otros métodos que han resultado ser métodos efectivos para recuperar el colágeno. En otro estudio, Veeruraj et al. (2013) obtuvieron una recuperación de un 80% y 7.1 % de colágeno a partir de la piel de anguilas mediante extracción con ácido y pepsina, respectivamente, observando como el método de extracción con ácido fue el más eficiente, y obteniendo tasas de extracción similares al tratamiento asistido por PE, utilizado en la presente tesis. Sin embargo, la aplicación del

tratamiento de extracción con ácido presenta una duración larga (~3 días), lo que también aumenta de forma considerable el costo de producción. Álvarez et al. (2018) utilizaron la extracción ácido-base asistida por ultrasonidos para mejorar la tasa de extracción de proteína de los subproductos de caballa, y los resultados mostraron que las tasas de extracción del tratamiento por ultrasonidos asistido por ácido o base fueron de aproximadamente 100% y 95 %, respectivamente, concluyendo el impacto positivo del tratamiento por ultrasonidos para favorecer la extracción de proteínas.

En la presente tesis, se han empleado dos técnicas innovadoras de extracción de compuestos. Por un lado, los PE son un método de tratamiento basado en la electricidad, generando pulsos eléctricos de corta duración y alto voltaje que pueden mantener los efectos térmicos a un nivel bajo y alterar la estructura celular de la matriz alimentaria, protegiendo las propiedades sensoriales y nutricionales de los alimentos. Se ha demostrado que la aplicación de pulsos eléctricos cortos de 100~300 kV/cm a 20~80 kV/cm puede desintegrar la membrana celular y promover la formación de poros en la membrana. En este sentido, Zhou, He, y Zhou (2017) exploraron el efecto de los PE en la extracción de proteínas de mejillón, obteniendo las condiciones óptimas cuando aplicaron un campo eléctrico de 20 kV/cm, 8 pulsos, e hidrólisis enzimática durante 2 h, encontrando una tasa máxima de extracción de proteínas de alrededor del 77%.

A diferencia de los PE, el tratamiento con PLE reduce la fuerte interacción entre el soluto y la matriz de la muestra a altas presiones y también reduce la viscosidad del disolvente, lo que promueve la difusión del disolvente en la muestra. Las distribuciones de peso molecular de proteínas en los extractos obtenidos a partir de los subproductos de

pescado se obtuvieron mediante la utilización de una electroforesis en gel de poliacrilamida (SDS-PAGE). En este sentido, la distribución del peso molecular de la proteína de los extractos de cabeza, piel y vísceras de trucha arcoíris obtenidos por PLE fue de 150~10 kDa (grupo de control: 75~10 kDa), 100~10 kDa (grupo de control: 100~75 kDa) y 50~5 kDa (grupo control: 25~5 kDa). Se puede ver como el tratamiento por PLE enriqueció las especies de proteínas en el extracto de subproducto de trucha arcoíris. Sin embargo, el tratamiento asistido por PE no tuvo un efecto significativo en cuanto a las distribuciones de peso molecular de las proteínas respecto al tratamiento control.

En el caso de los extractos obtenidos a partir de subproductos de lenguado mediante PLE se observó un comportamiento similar al obtenido para la trucha arcoíris. Asimismo, también se observó un aumento en la proteína con un peso molecular de 150~100 kDa, 75 kDa y 50 kDa procedente del extracto de cabeza y una disminución de la proteína con un peso molecular de 50~37 kDa, 25 kDa, 20 ~15 kDa y 15 kDa tras aplicar el tratamiento por PE. Así pues, es posible concluir que el tratamiento asistido por PE permitió la extracción de proteínas de gran peso molecular e inhibió la disolución de proteínas de pequeño peso molecular, mostrando así el diferente comportamiento de las proteínas en función de los diferentes tratamientos.

En el caso de la extracción con PLE, la presión es un factor importante que afecta a los rendimientos de extracción, así como a las propiedades del extracto. En este sentido, Gómez-Guillén et al. (2005) aplicaron un tratamiento de alta presión de 250~400 MPa a la piel de pescado, que no solo aumentó la producción de colágeno de la piel, sino que

también cambió la distribución del peso molecular de las proteínas. La presurización también puede provocar cambios en la estructura molecular de la proteína en la matriz alimentaria. Estudios previos han demostrado que la presión puede provocar la interrupción de las interacciones no covalentes y cambios intermoleculares, alteraciones en las interacciones intermoleculares/intramoleculares y disolvente-proteína, que alteran la conformación de las proteínas (Jia, et al., 2021).

Por otro lado, a cierta temperatura, la presurización puede aumentar la permeabilidad del disolvente, facilitando el ingreso de éste en el interior de la matriz alimentaria y prolongando el tiempo de contacto, lo que tiene implicaciones importantes para la recuperación de proteínas (Jia et al., 2021).

Para los PE, las moléculas de las proteínas se polarizan bajo el tratamiento de campos eléctricos de baja potencia, a medida que aumenta la fuerza, exponiéndose más aminoácidos hidrofóbicos al disolvente. Cuando la fuerza del campo eléctrico excede un cierto límite, el efecto especial causado por el arco conducirá a la desnaturalización y agregación de proteínas termosensibles. Además, otros estudios han confirmado que los PE pueden provocar daños en la estructura secundaria de las proteínas (Zhao & Yang, 2009). El trabajo de esta Tesis demuestra que los tratamientos por PE tienen un efecto sobre la distribución del peso molecular de las proteínas. Una de las razones que pueden permitir explicar este comportamiento es que el tratamiento asistido por PE puede provocar la ruptura de las células y acelerar la disolución de las proteínas. Entonces, la exposición de los aminoácidos hidrófobos de las proteínas también conduce a la

agregación de proteínas, lo que puede provocar cambios en el peso molecular de las mismas.

Además de evaluar el impacto de los tratamientos por PLE y PE en la extracción de proteínas y distribución del peso molecular, también se evaluó la capacidad antioxidante de los extractos, mediante dos ensayos (TEAC y ORAC), los cuales se utilizaron como indicadores para evaluar la bioactividad de los extractos.

Para la trucha arcoíris, los extractos obtenidos a partir de la piel mostraron una mayor capacidad de absorción de radicales de oxígeno (ORAC) independientemente del tratamiento (PLE o PE), seguidos por los extractos de vísceras y cabeza. Mediante el tratamiento asistido por PLE, la capacidad de absorción de radicales de oxígeno de la piel y las vísceras de la trucha arcoíris mejoró significativamente, mientras que los PE no tuvieron un efecto significativo.

En los extractos obtenidos a partir de los subproductos del lenguado, el tratamiento por PLE podría mejorar la capacidad de absorción de radicales de oxígeno de los extractos de cabeza y vísceras, mientras los PE presentan un mayor interés para los extractos de cabeza y vísceras. Además, se evaluó la capacidad de eliminación del radical ABTS+· de los extractos, observando como el tratamiento con PLE mejoró significativamente la capacidad de eliminación de éste en los extractos de cabezas de lenguado y trucha arcoíris. Sin embargo, para los extractos viscerales se observó el efecto contrario. En cuanto al tratamiento con PE, cabe señalar que aumentaron los valores de TEAC de los extractos de piel y cabeza de lenguado, pero se encontró el efecto contrario para las vísceras.

1.2 Rol de los extractos de pescado en la modulación de las actividades bacterianas y antiinflamatorias

Aunque algunos estudios han demostrado que diferentes compuestos derivados del pescado tienen un gran potencial antibacteriano y antiinflamatorio, rara vez se han informado sobre las propiedades biológicas de éstos tras su obtención por tecnologías innovadoras como PE y PLE.

En la presente tesis, se evaluaron los efectos de los extractos sobre el crecimiento de cuatro microorganismos potencialmente patógenos o alterantes. Se monitorizó la cinética de crecimiento mediante el modelo de Gompertz (Zwietering et al., 1990). Los extractos obtenidos tras aplicar los tratamientos de PE/PLE en subproductos de trucha arcoíris promovieron el crecimiento de cepas de *Listeria (L. innocua)* y *Escherichia* (*E. coli*), obteniendo los efectos más evidentes en los extractos procedentes de vísceras. Por otro lado, no se observó un efecto significativo sobre el crecimiento de *Staphylococcus* spp. (*S. aureus*) tras utilizar los extractos de cabeza y piel obtenidos por PE. Sin embargo, la adición de extractos de vísceras redujo la tasa de crecimiento de *S. aureus*. Para los subproductos de trucha arco iris, se observaron diferentes comportamientos en función del tratamiento utilizado. En este sentido, los extractos de cabeza y piel obtenidos por PLE inhibieron el crecimiento de *S. aureus* pero no tuvieron efecto sobre el crecimiento de *Salmonella*.

Respecto a los subproductos de lenguado, cabe resaltar, de forma negativa, que la adición de extractos de vísceras de lenguado promovió el crecimiento de *Listeria*, mientras que los extractos de cabeza y piel no tuvieron un efecto significativo sobre ésta.

Tampoco tuvo un impacto significativo la utilización de PE o PLE en la obtención de los extractos. Además, el extracto de vísceras de lenguado obtenido por PE tuvo un efecto inhibitorio sobre el crecimiento de *S. aureus*. Asimismo, el extracto de cabeza obtenido mediante PLE también inhibió la tasa de crecimiento de *S. aureus*.

También se investigó el efecto de los extractos en el crecimiento de dos cepas potencialmente probióticas de los géneros *Bifidobacterium (B. lactis)* y *Lacticaseibacillus (L. casei)*. Cuando se exploró la utilización de la extracción asistida por PE se observó como el extracto de cabeza de trucha arcoíris promovió el crecimiento de *L. casei*, pero no hubo diferencias significativas entre los grupos control y PE. Además, los extractos no tuvieron efecto sobre el crecimiento de *B. lactis*. Mientras tanto, los extractos obtenidos a partir de trucha arcoíris mediante tratamiento PLE no mostraron un efecto significativo sobre el crecimiento de *L. casei* en comparación con el grupo control.

Asimismo, también se estudiaron los subproductos del lenguado, observando que los extractos obtenidos a partir de cabeza tras PE promovieron el crecimiento de *L. casei*, pero no hubo diferencias significativas entre el grupo asistido por PE y el control. Para el tratamiento asistido por PLE, los extractos de vísceras y cabeza de lenguado no tuvieron un efecto significativo sobre el crecimiento de *L. casei*, mientras que el extracto de vísceras del grupo de control inhibió el crecimiento de *L. casei*. Además, los extractos de cabeza y piel de lenguado obtenidos mediante PLE también aumentaron la densidad óptica de *B. lactis* en comparación con el grupo de control.

En los últimos años, los investigadores han explorado los efectos de los compuestos presentes en los subproductos de pescado sobre el crecimiento de bacterias patógenas y

probióticos, como *Yersinia ruckeri* de hidrolizados de subproductos de tilapia e hidrolizados de piel de caballa del Atlántico (Robert et al., 2015, Ennaas et al., 2015) obteniendo que los subproductos de la piel de caballa del Atlántico obtenidos por hidrólisis han presentado efectos inhibitorios sobre las bacterias. Además, el hidrolizado de cabeza de *Thunnus albacares* puede promover el crecimiento de bacterias ácido-lácticas, incluidas *L. acidophilus*, *L. delbrukii* and *L. casei* (Safari et al., 2012). En la presente tesis, se investigó el efecto de los extractos de subproductos de pescado obtenidos con tecnologías innovadoras sobre el crecimiento bacteriano. La utilización de PE/PLE no solo mejoró la tasa de extracción de los diferentes compuestos a partir de los subproductos del pescado, sino que también asegura sus propiedades antibacterianas y tiene un efecto positivo sobre las cepas potencialmente probióticas.

Además, también se evaluó el potencial antiinflamatorio de los extractos obtenidos a partir de los diferentes subproductos. En los extractos obtenidos a partir de los subproductos de trucha arcoíris, los extractos obtenidos por PE no mostraron potencial antiinflamatorio, mientras que los extractos obtenidos por PLE tuvieron efectos inhibitorios de la activación de la ruta de inflamación (NF-κB), especialmente el extracto de vísceras, que inhibe ≈40–45 % de actividad inflamatoria inducida por el factor de necrosis tumoral alfa (TNF-α). Por otro lado, para los extractos de subproductos de lenguado, el extracto de piel obtenido por PE exhibió un potencial antiinflamatorio más importante y significativo (con reducciones de ≈35%). Curiosamente, en el grupo de extractos de trucha arcoíris obtenidos por PE, los extractos de piel y vísceras (sin tratamientos con PE y PLE) aumentaron la actividad inflamatoria (150% y 126%,

respectivamente), lo que sugiere que el tratamiento con PE podría alterar algunos componentes de estos extractos y reducir su potencial proinflamatorio intrínseco.

1.3 Efectos de la digestión in vitro *sobre la capacidad antioxidante de los extractos de subproductos de pescado y bioaccesibilidad de minerales*

Se estableció un modelo de digestión estática *in vitro* para explorar el efecto de la digestión sobre la capacidad antioxidante de los extractos, y también se evaluó la bioaccesibilidad de los minerales. En primer lugar, se determinó la contaminación potencial de los subproductos del pescado, incluidos los niveles de metales pesados (As, Cd, Hg y Pb) y las micotoxinas. En todos los subproductos de pescado, el contenido de Cd, Hg y Pb estuvo por debajo del límite máximo permitido y no se detectó contaminación por micotoxinas en los extractos.

La digestión *in vitro* tuvo diferentes efectos sobre la capacidad antioxidante de los extractos. Para los extractos obtenidos a partir de subproductos de trucha arcoíris y lenguado, los PE no mostraron un efecto significativo en la capacidad antioxidante de los mismos, mientras que la utilización de PLE aumentó la capacidad antioxidante de los extractos de trucha arcoíris y cabeza de lenguado tras la digestión in vitro en comparación con el grupo control.

La digestión *in vitro* puede tener un efecto sobre la capacidad antioxidante del extracto. En el proceso de digestión *in vitro*, la digestión de proteínas comienza en el estómago y se degrada en pequeños péptidos moleculares bajo la acción de enzimas digestivas, luego se degrada aún más por las células epiteliales del intestino delgado para formar aminoácidos y entrar en la circulación de los fluidos corporales. Los péptidos bioactivos

en la proteína de pescado tienen capacidad antioxidante, y la digestión, así como las tecnologías innovadoras pueden cambiar la secuencia de péptidos contenidos en los extractos, afectando así la capacidad antioxidante.

Además, se estudió el impacto de los PE y PLE en la recuperación de minerales a partir de subproductos del pescado. Los subproductos de pescado seleccionados en este estudio tenían un contenido relativamente alto de Mg, Ca y P. Se observó como los PE promovieron la recuperación de minerales como Mg y P a partir de cabeza de trucha arcoíris, así como de Fe y Ca a partir de cabeza de lenguado.

También se evaluó el efecto del tratamiento PLE en la recuperación de minerales de subproductos de pescado, observando como éste aumentó los contenidos de Fe y Zn en los extractos de cabeza de trucha arcoíris, así como los contenidos de Fe y Se en los extractos de vísceras. También se observó un efecto positivo de los PLE en la recuperación de Zn a partir de extracto de cabeza de lenguado.

Asimismo, también se evaluó la bioaccesibilidad de los minerales contenidos en los subproductos del pescado. La utilización de PE y PLE mejoró la bioaccesibilidad de los minerales en algunos extractos obtenidos a partir de los subproductos evaluados, pero no en todos.

En este sentido, cabe destacar que el contenido de minerales y su bioaccesibilidad se ven afectados por varios factores, como el tipo de pez, el entorno de cultivo, los métodos de procesamiento, etc. Además, las interacciones con otros componentes también pueden tener un impacto en la bioaccesibilidad de los minerales. Sin embargo, se han realizado pocas investigaciones sobre el impacto de los PE y PLE sobre los minerales en

subproductos de pescado. Por lo tanto, es crucial comprender los cambios en la acción de diferentes técnicas de procesamiento de minerales que contienen otros constituyentes y su impacto en la digestión y absorción.

1.4 Efectos de los extractos de subproductos de pescado en la microbiota intestinal

Para evaluar de manera más completa la función saludable de los extractos de subproductos de pescado obtenidos, se exploró su impacto en la microbiota intestinal mediante un modelo de fermentación colónica *in vitro*. Se observo un aumentó en los niveles de bacterias totales tras 48h de incubación, fundamentalmente en los miembros de la familia *Enterobacteriaceae*, y los géneros *Bacteroides* y *Streptococcus*. En paralelo, se determinaron los ácidos grasos de cadena corta (AGCC), como principales metabolitos de la microbiota intestinal, y se observó un aumento en la producción de ácido acético y ácido propiónico, en paralelo a la incubación y el aumento en los niveles bacterianos. Específicamente, la adición de extracto de vísceras de lenguado incrementó los niveles de *Bifidobacterium* spp. y promovió una reducción en los niveles de bacterias pro-inflamatorias de la familia *Enterobacteriaceae*. Además, la adición de extractos de vísceras de trucha arcoíris y lenguado también redujo los niveles de *Streptococcus* en comparación con los controles (sin el extracto). Entre los extractos de subproductos de pescado, el extracto de vísceras de lenguado tuvo un efecto más positivo, probablemente debido a la presencia de proteínas de bajo peso molecular en los extractos de vísceras, pero aún es necesario explorar más mecanismos.

Para los AGCC, el contenido de ácido acético fue mayor en presencia de los extractos (vísceras de lenguado, cabezas de lenguado y trucha arco iris) que en el grupo control tras

24 h de incubación, mientras que tras 48h incubación, el contenido de ácido acético aumentó en presencia de los extractos de vísceras de lenguado y trucha arco iris. Tras 48 h de incubación, el ácido propiónico aumentó significativamente en el grupo que contenía el extracto de vísceras de trucha arcoíris, mientras que los otros grupos fueron más bajos que el grupo control (sin el extracto). Además, los extractos de vísceras de lenguado y de trucha arco iris mostraron efectos positivos en el aumento del contenido de ácido acético y ácido propiónico, respectivamente.

2. Recuperación de nutrientes y compuestos bioactivos de alto valor añadido a partir de microalgas utilizando tecnologías innovadoras

2.1 Recuperación de biomoléculas asistida por pulsos eléctricos a partir de Chlorella

En este estudio se investigó el efecto del tratamiento por PE para recuperar nutrientes y compuestos bioactivos a partir de *Chlorella*. En primer lugar, se exploró el impacto del disolvente (100% H_2O y 50% H_2O + 50% DMSO) y el tiempo de extracción en la recuperación de nutrientes y compuestos bioactivos a partir de *Chlorella*. En comparación con el grupo de control (sin PE), los PE aumentaron la extracción de biomoléculas de *Chlorella*, incluidas proteínas, polifenoles, clorofila *a*, clorofila *b* y carotenoides. Entre ellos, la utilización de DMSO (50%) se mostró el más beneficioso para la extracción de pigmentos liposolubles, mientras que la utilización de H_2O al 100% presentó la mayor extracción de proteínas. Esto puede deberse a que el grupo sulfonilo hidrofílico y el grupo metilo hidrofóbico del DMSO pueden disolver compuestos solubles en agua y liposolubles, lo que promueve la extracción de pigmentos (Mueller et al., 2019). Mientras tanto, la adición de DMSO dio como resultado la precipitación de proteínas, lo que afectó

de forma considerable a la tasa de extracción. En este sentido, Parniakov et al. (2015) utilizaron los PE en un estudio previo para mejorar la extracción de nutrientes y compuestos bioactivos a partir de la microalga *Nannochloropsis* spp., obteniendo una conclusión similar a la obtenida en la presente tesis. Además, se analizaron las proporciones de biomoléculas específicas de los extractos en diferentes momentos de la extracción, encontrando que las proporciones entre las diferentes biomoléculas también se vio modificada con el transcurso del tiempo.

Al evaluar la capacidad antioxidante de los extractos, se observó que el orden de las muestras en cuanto a los valores de ORAC en orden decreciente fue el siguiente: PE-+50% DMSO>control+50% DMSO>PE+ H_2O>control+H_2O, mientras que el orden de los valores de TEAC, también en orden decreciente fue: PEF+H_2O>PE+50% DMSO>control+H_2O/control+50% DMSO. Asimismo, los resultados del análisis de componentes principales mostraron que los contenidos de carotenoides y clorofila a/b mostraron una fuerte correlación con el método ORAC, mientras que los valores del método TEAC se relacionaron principalmente con las proteínas y los polifenoles, lo que podría explicar la diferente capacidad antioxidante de las muestras en función del ensayo analizado.

En este trabajo también se evaluó el contenido de minerales (Mg, P, Ca, Fe y Zn) en *Chlorella* y sus extractos. No se observó un efecto significativo de los PE sobre el contenido de Mg, mientras que sí que se observó un aumento en el contenido de P/Zn y una disminución de la concentración de Ca/Fe. El contenido de Ca y Fe en los extractos obtenidos por el tratamiento asistido por PE fue menor que en el grupo control. En este

sentido, se ha demostrado en algunos estudios que los PE alteran las células de microalgas y aumenta la tasa de extracción de algunas biomoléculas. Entre estos minerales, el Ca y el Fe pueden estar involucrados en la estructura celular mediante la quelación con proteínas, pudiendo los PE alterar esta unión y cambiar así las propiedades funcionales de las proteínas, provocando la sedimentación de proteínas que contienen Ca/Fe.

El efecto del tratamiento asistido por PE en la estructura celular de *Chlorella* se observó mediante microscopio de fluorescencia (FM) y microscopía electrónica de barrido (SEM), observando la aparición de algunas "grietas" en los agregados circulares de múltiples *Chlorella* tras el tratamiento con PE. A mayores aumentos, se vió de forma más evidente la ruptura de las células de *Chlorella*, lo que indica que se produce una "electroporación" durante el tratamiento con PE, lo que aumenta la tasa de extracción de biomoléculas.

2.2 Efectos de la combinación de la extracción con PLE y DMSO en la recuperación de compuestos valiosos a partir de microalgas

Se evaluó la combinación de PLE con diferentes concentraciones de DMSO para recuperar compuestos bioactivos de microalgas. En primer lugar, se utilizó la microalga *Chlorella* como matriz, obteniéndose que tanto el tratamiento con PLE como la concentración de DMSO podrían afectar a la recuperación de nutrientes y compuestos bioactivos de *Chlorella*, incluidos proteínas, polifenoles, clorofila a, clorofila b y carotenoides. Entre ellos, la combinación de PLE+H_2O como disolvente fue útil para la recuperación de proteínas, mientras que el DMSO tuvo un efecto más significativo sobre la recuperación de polifenoles, clorofila a, clorofila b y carotenoides.

También se evaluó la capacidad antioxidante de los extractos y su asociación con los diferentes nutrientes y compuestos bioactivos. Los resultados del método ORAC mostraron que la extracción con PLE aumentó la capacidad antioxidante de los extractos en comparación con el grupo de control. Asimismo, la concentración de DMSO también tuvo una influencia significativa en la recuperación de los diferentes compuestos. En este sentido, cuando la concentración de DMSO fue superior al 50%, el aumento de la capacidad antioxidante se relacionó con el aumento del contenido de polifenoles, clorofila y carotenoides. En cuanto a los valores de TEAC, cuando la concentración de DMSO superó el 50%, los extractos obtenidos por PLE y control mostraron una capacidad antioxidante similar, mientras que cuando la concentración de DMSO estuvo por debajo del 50%, los extractos obtenidos por PLE presentaron una mayor capacidad antioxidante. Esto puede deberse a que los métodos ORAC y TEAC presentan dos mecanismos de acción antioxidante diferentes, y el principio del primero está basado en que los antioxidantes y el sustrato compiten por los radicales peroxilo generados térmicamente a través de la descomposición de los compuestos azo, mientras que el TEAC mide la capacidad de los antioxidantes para reducir los oxidantes (Zulueta et al., 2009).

El análisis de componentes principales mostró que cuando se utilizó DMSO al 100% como disolvente de extracción, el contenido de polifenoles, clorofila y carotenoides en el extracto fue mayor y tenía una fuerte capacidad antioxidante. Por lo tanto, se exploró el uso de DMSO al 100% en combinación con el tratamiento PLE como disolvente para recuperar biomasa de alto valor de *Spirulina* y *Phaeodactylum tricornutum*. Se puede encontrar que, entre las tres microalgas seleccionadas en este estudio, los valores de

proteína, polifenoles, carotenoides y capacidad antioxidante de *Spirulina* y *Phaeodactylum tricornutum* son más altos que los de *Chlorella*, mientras que *Chlorella* presenta un mayor contenido de clorofila. Esto se puede atribuir a la diferente especie y morfología celular. Además, la distribución de carotenoides y minerales en los extractos también es diferente, teniendo la espirulina una mayor cantidad de zeaxantina y siendo una fuente importante de Zn y P, mientras que *Chlorella* presenta valores relativamente altos de luteína.

Por lo tanto, el tratamiento asistido por PLE en combinación con DMSO como disolvente puede considerarse un método valioso para recuperar compuestos de alto valor de las microalgas, lo que puede acortar significativamente el tiempo de procesamiento y mejorar el rendimiento de la extracción.

Las microalgas contienen una gran cantidad de biomasa de alto valor. Este estudio utilizó dos técnicas innovadoras para la recuperación de biomasa a partir de microalgas. Los estudios han demostrado que ambos son métodos eficientes para recuperar biomasa de microalgas y que la elección del disolvente de extracción también puede tener un impacto en el contenido de las diferentes biomoléculas. Sobre esta base, las microalgas tienen el potencial de convertirse en productos de alto valor, de modo que la biomasa se pueda utilizar muy bien. Sin embargo, vale la pena señalar que hay muchas especies de microalgas y tienen diferentes morfologías celulares, también es necesaria la exploración de métodos más eficientes para cada especie en particular.

Conclusiones

De los resultados obtenidos en la presente tesis doctoral se puede concluir que:

1) Las tecnologías innovadoras (pulsos eléctricos, PE y extracción con líquidos presurizados, PLE) se pueden utilizar para extraer nutrientes y compuestos bioactivos a partir de subproductos de pescado, pudiendo aumentar la extracción de proteínas y capacidad antioxidante.

2) La distribución del peso molecular de las proteínas en los extractos de subproductos de pescado puede verse afectada tanto por el tratamiento por PE como por PLE.

3) La influencia de los extractos de subproductos de pescado en el crecimiento de microorganismos específicos (potencialmente patógenos, alterantes y beneficiosos) y la actividad inflamatoria, dependen de la fuente marina (lenguado, trucha arcoíris), del origen (víscera, piel, etc..), y de la metodología empleada (PLE, PE).

4) La digestión *in vitro* puede afectar la capacidad antioxidante de los extractos de subproductos de pescado.

5) La recuperación de minerales en extractos de subproductos de pescado se vio afectada por los tratamientos de PE y PLE, estando íntimamente relacionada con el tipo de pez y mineral extraído. Los PE y el tratamiento por PLE permitieron mejorar la bioaccesibilidad de algunos minerales, pero no en todos los casos se mostró un efecto positivo.

6) El extracto de vísceras de lenguado modula la microbiota intestinal en el modelo *in vitro* de fermentación colónico mediante el aumento en el contenido de bacterias el género *Bifidobacterium*, además de favorecer la producción de ácidos grasos de cadena corta.

7) Los PE puede mejorar la tasa de recuperación de compuestos bioactivos antioxidantes en *Chlorella*, lo que se relaciona principalmente con la "electroporación" inducida por esta tecnología.

8) La extracción asistida con PLE+DMSO como disolvente también es un método eficiente para recuperar compuestos de alto valor (ej. pigmentos y carotenoides) a partir de microalgas.

Estas conclusiones demuestran que el uso de tecnologías innovadoras de extracción (PE y PLE) para recuperar compuestos de alto valor a partir de subproductos de pescado y microalgas son métodos eficaces, que no solo pueden mejorar el rendimiento de extracción de los compuestos, sino que también evitan que se degraden las propiedades biológicas. Sobre esta base, es necesario explorar el mecanismo de la actividad funcional de los componentes y más propiedades nutricionales. Mientras tanto, también es factible evaluar la aplicación de más tecnologías innovadoras como los fluidos supercríticos, microondas, etc. En conclusión, es de gran importancia estudiar la aplicación de compuestos de alto valor añadido a partir de fuentes marinas para su potencial uso en industrias alimentarias, farmacéuticas, cosméticas y otras, no solo para lograr una utilización sostenible de los recursos, sino también para reducir el desperdicio de recursos y la contaminación ambiental.

1. INTRODUCTION

1. Introduction and review literature

Oceans are an important part of Earth's biodiversity, with more than two million life forms present in oceans, including animal, algae, microorganisms, and more (Mora et al., 2011). A large number of high-added-value compounds from marine organisms have been identified, such as protein and bioactive peptides, polyunsaturated fatty acids (PUFAs), bioactive enzymes, carotenoids, etc. (Ali et al., 2021). Compared with terrestrial organisms, marine organisms can often adapt to more extreme environments and reduce the use of freshwater and arable land, making them more valuable for development and utilization (Poojary et al., 2022). These compounds are being considered to be used in food, pharmaceutical and other industries to exert their application potential (Ferraro et al., 2010; Zhang & Rehmmann, 2022).

The fishing and development of seafood plays a vital role in the economies of many countries. As a growing industry, total fish production has increased from 1.58 million tons in 2012 to 1.75 million tons in 2017 and is expected to reach 1.94 million tons in 2026. In addition, large amounts of algae, mollusks, and crustaceans are also farmed or fished (Food and Agriculture Organization, 2000; Lopes et al., 2015). Among them, around 40~50% of the weight is used for processing and human consumption, while the rest is often discarded as a waste, or to produce low-added-value animal feed and so on. For example, in the production and processing of fish, head, viscera, backbone, etc. are not commonly used as waste, and they may account ~50% of the whole fish weight (Korkmaz & Tokur, 2022). For crustaceans, only about 10% can be consumed. These side

streams are often a great source of high-added-value compounds (i.e. protein, lipids, etc.) that are not well utilized (Deng et al., 2020).

Therefore, effectively disposing of these side streams and recovering their high-added-value compounds, applying them as functional ingredients in different industries will bring more opportunities for the processing of marine organisms, and is also conducive to the sustainability of resources.

1.1 High-added-value compounds (HAVCs) from fish side streams and algae

1.1.1 Protein

The crude protein content can reach 8~35 % of the fish side streams. Recycling these high-protein side streams can largely alleviate potential environmental problems (Korkmaz & Tokur, 2022; Sila & Bougatef, 2016). Most fish protein exist in an inactive form and have complete protein sequences. At present, most of the research on protein in fish side streams is to obtain protein hydrolysates through different methods, that is peptides with bioactivity (Desai et al., 2022). These bioactive peptides are mostly composed of 3~20 amino acid residues. Different amino acid sequences make the hydrolysates show different physiological activities, including antioxidant, anti-inflammatory, antibacterial, neuroprotective, ability to prevent high blood pressure, etc. (Wang, 2021) (**Table 1**).

Likewise, algae are one of the most important sources of marine protein. The protein content of algae varies with species, growth environment and other factors. Generally, red algae have higher protein content, while brown algae have lower protein content.

Similar to fish protein, algae derived protein and its hydrolysates have biological functions, including antimicrobial, anti-hypertension, anti-coagulation, etc. (Pina-Pérez et al., 2017) (**Table 1**).

Table 1. Bioactivity of proteins in fish side stream and algae

	Sample	Bioactivity	Treatment method	Bioactive fraction	Ref.
Fish side streams	*Navodon septentrionalis* head	Antioxidant capacity	Hydrolysis (papain) +ultrafiltration	Trp-Glu-Gly-Pro-Lys (615.69 kDa), Gly-Pro-Pro (269.33 kDa), Gly-Val-Pro-Leu-Thr (485.59 kDa)	(Chi et al., 2015)
	Tuna dark muscle by-product	Antioxidant capacity	Hydrolysis (Alcalase), filtration	Peptide fraction <1 kDa	(Saidi et al., 2014)
	Salmon pectoral fin	Antioxidant, anti-inflammatory	Hydrolysis (proteases)	Peptide fraction 1~2 kDa	(Ahn et al., 2012)
	Rachycentron canadum skin	ACE inhibitory activity	Hydrolysis (Protamex and Protease N)	Peptide fraction 450~630 Da, Trp-Ala-Ala, Ala-Trp-Trp, Ile-Trp-Trp, Trp-Leu	(Lin et al., 2019)
	Salmon pectoral fin	Anti-inflammatory	Hydrolysis (proteases)	Pro-Ala-Tyr	(Ahn et al., 2015)
	Ctenopharyngodon idella	Antioxidant, neuroprotective	Hydrolysis (Protamex)	Glutathione	(Cai et al., 2015)
	Fish sauce	Antioxidant	Proteases and fermentation	Low molecular weight FSB fraction	(Choksawangkarn et al., 2018)
	Trout viscera	Antibacterial	Hydrolysis (pepsin)	The degree of hydrolysis is 30%	(Wald et al., 2016)
	Grass carp fish scale	Inhibit tyrosinase	*In vitro* digestion	Molecular weight<2023 Da	(Hu et al., 2022)
	Salmon backbones	ACE-inhibitory, antidiabetic	Hydrolysis	Molecular weight<1200 Da	(Slizyte et al., 2016)
	Carp fish skin	Inhibits ice crystal growth	Hydrolysis (Alcalase)	Peptides: 1000~2500 Da	(Damodaran & Wang, 2017)
	Tuna skin	Inhibits lipid accumulation	Subcritical water treatment		(Lee et al., 2017)

Table 1. (cont.)

	Sample	Bioactivity	Treatment method	Bioactive fraction	Ref.
Algae	*Ulva sp. Gracilaria sp.*	Antioxidant	Solvent extraction	Trp, Tyr, Met	(Kazir et al., 2019)
	Macrocysis pyrifera	Antioxidant, ACE inhibitory	Hydrolysis (cellulase)		(Vásquez et al., 2019)
	Navicula incerta	Antioxidant, hepatic fibrosis	Hydrolysis (pepsin)	Pro-Gly-Trp-Asn-Gln-Trp-Phe-Leu Val-Glu-Val-Leu-Pro-Ala-Glu-Leu	(Kang et al., 2011)
	Caulerpa lentillifera	ACE inhibitory activity	Hydrolysis (thermolysin)	Short-chain peptides	(Joel et al., 2018)
	Porphyra yezoensis	Anti-coagulant	Hydrolysis (pepsin)	NMEKGSSSVVSSR M (+15.99) KQ	(Indumathi & Mehta, 2016)

*Part of the research on the extraction and bioactivity evaluation of fish side stream and algae proteins in the past ten years. ACE: Angiotension converting enzyme.

In addition to fish and algae, there are many crustaceans in the ocean, such as shrimp, shells and crab, many by-products are also produced during the processing. Among them, it contains high-quality protein, minerals, polysaccharides, etc., showing great economic value (Hajji et al., 2021).

1.1.2 Lipids

Lipids are one of the most important nutrients in the human body, which can provide the body with the necessary energy to maintain metabolism. Marine organisms are rich in omega-3 PUFAs, such as eicosapentaenoic acid (EPA) and (docosahexaenoic acid) DHA, which are essential fatty acids. The lipid content of fish is up to about 30% and varies according to the species, growing environment, and diet, etc. Studies have shown that diet

containing fish lipids can help preventing and regulating a wide variety of noncommunicable diseases, such as cardiovascular diseases, inflammation, diabetes, and more (Chen et al., 2022) (**Table 2**).

Table 2. Effects of fish side and algae lipids on health

Source	Method/model	Bioactive fraction	Function	Ref.
Menhaden	Animal model (mice)	Fish oil	Regulate metabolic disorders and alleviate obesity	(Monk et al., 2019)
Sardines	Human (patients with type 2 diabetes)	Fish oil	Relieve diabetes	(Balfegò et al., 2016)
Fish	Animal model (mice)	Fish oil	Inhibited intestinal inflammation	(Yamamoto et al., 2018)
Fish/krill	Animal model (mice)	Fish and krill oil	Lower cholesterol levels	(Cui et al., 2017)
Tuna and algae	Animal model (mice)	Lipid	Improve cognitive ability	(Zhang et al., 2018)
Chlorella	*In vitro* study-Human peripheral blood mononuclear cells	Lipid extracts	Antioxidant and alleviate the acne vulgaris	(Sibi, 2015)
Chlorella pyrenoidosa, Spirulina platensis	Animal model (mice)	Ethanol extracts	Anti-diabetes	(Wan et al., 2019)
Spirulina	Animal model (rabbit platelets)	Lipid extracts	Anti-thrombotic	(Koukouraki et al., 2020)
Nannochloropsis, Schizochytrium	Animal model (mice)	Lipids	Protective neuron	(P. A. Lopes et al., 2017)
Nannochloropsis gaditana	*In vitro* model (Human acute monocytic leukemia cell line THP-1)	Lipid extracts	Anti-inflammatory	(Ávila-Román et al., 2018)

*Part of the research on the lipid bioactivity from fish side stream and algae in the past ten years.

The lipids content of algae is lower than that of fish, but it also can be used as a substitute for fish lipids due to its higher content of PUFAs. The type of algae, light

conditions, temperature, water quality and other factors all affect the lipids content in algae, these lipids rich in PUFAs have also been shown to have antioxidant, anti-inflammatory, antibacterial, immunomodulatory and other activities (Zhou et al., 2022) (**Table 2**).

1.1.3 Carbohydrates (polysaccharides)

Carbohydrates also make up large portion of the human diet and are used as high-quality active components due to their water-soluble and degradable properties. They are widely found in animals, plants and microorganisms in nature (Tan & Nie, 2022). There are various kinds of polysaccharides in the ocean, such as chitosan and chitin from animas; alginate and fucoidan from algae, which have been widely used in food, medicine, textile, and other industries. Among them, carbohydrate with a simple structure can be digested and absorbed in the digestive system, while some carbohydrates with a complex structure can partially or completely resist the degradation of enzymes in the upper digestive tract and reach the colon (Gibson et al., 2017). There is a complex microbial community in the colon, known as the gut microbiota (GM), participating in the host's metabolism, and having an impact on host health. These indigestible polysaccharides can be used as potential prebiotics by anaerobic bacteria in the gut to product short-chain fatty acids (SCFAs) and other metabolites, which have positive effects on relieving obesity, diabetes, improving gut health and others (Shang et al., 2018).

Animal-derived marine polysaccharides are mostly from fish, crustaceans and mollusks, such as chitosan, chitin, chondroitin sulfate, etc., and have been proven to have

various physiological functions such as anti-inflammatory, relieving diabetes, regulating GM and so on (Nie et al., 2019). For example, Liu et al. (2015) found that the intake of chitosan reduced the accumulation of lipids in the liver of rats, which could significantly reduce plasma glucose and improve impaired glucose tolerance and insulin tolerance. Ai et al. (2018) found that sulfated polysaccharides from abalone can inhibit obesity caused by high-diet diet by regulating the ratio of Firmicutes/Bacteroidetes in the mice gut microbiota and promoting the production of SCFAs.

Algae are an important source of marine polysaccharides (i.e., alginate, agarose, fucoidan, etc.), changing its content according to algae species and ranging about 20~75 %. Moreover, their bioactivities have also been studied. For instance, alginate can be used as a prebiotic by specific GM to improve metabolic syndrome, while it also can alleviate inflammation and obesity (Bai et al., 2017). Liu et al. (2017) investigated the effects of brown algae-derived alginates in a high-fat, high-sucrose diet mouse model, observing that alginate significantly reduced blood lipopolysaccharide levels in mice and suppressed colonic inflammation. In addition, it also influences the composition of gut microbiota, which can increase the abundance of the probiotic *Limosilactobacillus reuteri*. Fucoidan has also been shown to have the potential to alleviate inflammation in the host and combat Alzheimer's diseases. By exploring the effect of fucoidan on type-2 diabetic mice, Liu et al. (2022) found that the addition of fucoidan can improve glucose metabolism in mice by regulating TLR/NF-κB signal pathway, and also can enhance the barrier function of the intestinal tract by upregulating tight junction proteins. Carrageenan and agar, which are unique in red algae, have also been confirmed to have physiological

activities such as inhibitor of human papillomavirus (HPV), immune regulation, cholesterol level regulation and so on (Laurie et al., 2021; Valado et al., 2020). In addition to exerting physiological activities and being used as prebiotics, marine-derived polysaccharides are also used in food packaging materials, drug delivery and other applications (Beaumont et al., 2021).

An important challenge in making the most of high-added-value compounds in fish side streams and algae is to select efficient recovery and extraction methods for the target compounds. Over the last years, various techniques have been used to extract proteins and their hydrolysates from fish side streams and algae (Thirukumaran et al., 2022). Traditionally, methods such as acid/base extraction have been used for protein extraction and hydrolysis, while they are also considered to be simple and inexpensive methods. However, it is difficult to control the molecular weight of the products and may also affect the bioactivity of the target compounds. Furthermore, fermentation and enzymatic hydrolysis, as safe and environmentally friendly methods, have been also applied to recover and hydrolyze protein to obtain components with stronger antioxidant capacity (Choksawangkarn et al., 2018; Zamora-sillero et al., 2018).

To improve the recovery and added value of resources to a greater extent, some innovative technologies have also been explored. For water-soluble proteins, pulsed electric field (PEF), ultrasound-assisted extraction, isopoint dissolution precipitation (ISP), etc. are commonly used (Álvarez et al., 2018; Gehring et al., 2011). These methods have development potential due to their high efficiency and environmental protection.

Like protein, extraction is an important part of the development and utilization of fish side streams and algae lipids. Classical extraction methods such as Soxhlet, Folch and Bligh-Dyer, are characterized by simple operation and low cost, which promotes their widespread application in lipids´ extraction. To further improve the extraction rate and reduce the reagents pollution, some innovative technologies have also been used for the extraction of lipids, such as supercritical fluid extraction (SFE), subcritical fluid extraction, ionic liquids (ILs), etc., especially for algae, the use of innovative technology can overcome the phenomenon of low extraction rate caused by thick cell walls.

The extraction, separation and purification of polysaccharides are also the focus of several research. Generally, hot water extraction is the most common method for extraction, but it also has the disadvantages of long extraction time and low efficiency. The assisted extraction of innovative methods has also been used for the recovery of polysaccharides, such as ultrasound-assisted, microwave-assisted, enzymatic extraction, etc. (Qiu et al., 2022; Shi et al., 2018). At the same time, the combination of organic reagent precipitation, column chromatography, membrane separation and other technologies can effectively remove proteins, pigments and other impurities contained in the extract, while improve the purity of the extract (Tang et al., 2020). Polysaccharides from aquaculture have been widely used in food, medicine, cosmetics and other industries.

1.1.4 Other high-added-value compounds

There are also many compounds from fish side streams and algae have been also found to have a positive impact on human health (i.e. polyphenols, vitamins, minerals and

pigments) (Wang et al. 2021). The main source of polyphenol in the ocean is algae. Among them, the polyphenol content of brown algae is usually higher than that of red and green algae (Freile-pelegr & Robledo, 2014). Due to the different growth environment, polyphenols derived from algae exhibit stronger antioxidant properties, meanwhile, it has also been proven to have various health benefits such as inhibiting cancer cell proliferation, anti-diabetes, and lowering cholesterol. Moreover, fish side streams and algae are a good source of vitamins and minerals, which are micronutrients and are involved in different biological functions, including antioxidant, anti-inflammatory, anti-tumor, etc. (**Table 3**). These bioactive components from fish side stream and algae can be used as functional ingredients in food and medicine, reducing waste and obtaining more healthy products.

Table 3. Bioactivities of other compounds from algae

Object	Bioactive fraction	Function	Ref.
Palmaria, Ascophyllum* and *Alaria	Phenolic-rich extracts	Anti-proliferative, potential anti-diabetic	(Nwosu et al., 2011)
Padina tetrastramatica	Polyphenols	Antioxidant, antidiabetic	(Naveen et al., 2021)
Sargassum horneri	Polyphenols	Induced inflammatory response	(Herath et al., 2021)
Ecklonia stolonifera	Phlorotannins	Anti-adipogenic activity	(Jung et al., 2014)
Sargassum horneri	Fucoxanthin	Induced neurotoxicity	(Wei et al., 2021)
Chondrus crispus	Flavonoids, polyphenols and tannins	Antioxidant, anti-inflammatory, antitumor	(Alkhalaf, 2021)
Ascophyllum nodosum	Phlorotannin-rich extracts	Antioxidant, anti-genotoxic activity	(Corona et al., 2017)
Laminaria japonica	γ-Aminobutyric Acid	Anti-inflammatory	(J. Il Choi et al., 2012)

1.2 Extraction methods

One of the challenges in the valorization/exploitation of fish side streams and algae resources is related to the low recovery rates of high-added-value compounds. For instance, it is of a paramount importance selecting, developing, and optimizing the appropriate pretreatment and recovery technology to obtain the natural high-added-value compounds on the premise of ensuring good bioactivities. Below will be discussed some innovative approaches such as pulsed electric field (PEF), accelerated solvent extraction (ASE), ultrasonic-assisted treatment (US) and supercritical CO_2 (SC-CO_2).

1.2.1 Pulsed electric fields (PEF)

Pulsed electric fields (PEF) mainly relies on short-term transient high-voltage pulses to rupture the cell membrane, resulting in temporary or permanent pores, thereby increasing permeability, making it easier for intracellular and extracellular substances to diffuse, which will help the extraction of target components and the improvement of extraction efficiency. This technology has the advantages of high extraction efficiency, short treatment time, and better protection of product structure and flavor (Barba et al., 2015). PEF has been studied as an assisted extraction technology to high-added-value components recovery from a wide variety of marine organisms (**Table 4**).

Table 4. Application of various innovative extraction methods in the extraction of microalgae lipids.

Sources	Objective	Conditions	Results	Ref.
Fish bone	Recovery chondroitin sulfate	Electric field intensity: 16.88 kV/cm Pulse number: 9	The maximum yield is 6.92 g/L, reduced extraction time and increased efficiency.	(He et al., 2014)

Fish side stream	Recovery ptirotein	Electric field intensity: 1~3 kV/cm Specific energy: 123~300 kJ/kg Time: 15~24 h	Improved protein recovery and antioxidant capacity.	(Wang et al. 2021)
Hoki roe	Lipids extraction	Electric field intensity: 0.625, 1.25 and 1.875 kV/cm Voltages: 25, 50 and 100 Hz Pulses duration: 20 µs	The highest total lipid extraction rate and phospholipid were obtained when electric field intensity at 1.875 kV/cm.	(Ahmmed et al., 2022)
Sea bass	Improve the quality	Voltages: 50~500 Hz Time: 1~600 s Pulses duration: 5~20 µs	Shorten the marinating time and speed up the diffusion of the brine in the muscles	(Cropotova et al., 2021)
Salmon	Improve the quality	Electric field intensity: 1~2 kV/cm Voltages: 20 Hz Pulses duration: 100 µs Time: 15 s	Reduces protease and lipase activity, extending shelf life	(Pérez-Won et al., 2021)
Arthrospira platensis	Antioxidant compounds and pigments recoveries	Electric field intensity: 1 kV/cm Time: 5~180 min Solvent: DMSO, Ethanol, H_2O	Improved recovery rate of antioxidant components.	(Martí-Quijal et al., 2021)
Chlorella vulgaris	Protein recovery	Electric field intensity: 40 kV/cm Pulses duration: 1 µs	Promotes the release of protein	(Scherer et al., 2019)
Scenedesmus almeriensis	Enzymatic hydrolysis of protein	Energy: 75/150 kJ/kg	Preserves cell shape and promotes protein enzymatic hydrolysis.	(Akaberi et al., 2019)
***Sparus aurata* side stream**	Antioxidant protein recovery	Solvent: H_2O Pressure: 1500 psi Time: 5/15 min pH: 7	Improved protein recovery and antioxidant capacity.	(de la Fuente et al. 2021)
***Nannochloropsis* sp**	Polar lipids recovery	Pressure: 10 MPa Temperature: 125 °C Time: 5 min (3 cycles)	Polar lipids rich in EPA were obtained.	(Jiménez Callejón et al., 2022)
***Geitlerinema* sp.**	Bioactive ingredients	Solvent: H_2O/Ethanol Temperature: 20~200 °C	The extraction rate of phycocyanin was increased	(Ruiz-Domínguez et al., 2021)

1.2.2. Pressurized Liquid Extraction (PLE)

Pressurized liquid extraction (PLE), also known as pressurized fluid extraction (PFE), or accelerated solvent extraction (ASE), is a gentle and efficient extraction method. When water is used as solvent, it is called subcritical water extraction (Ruiz-Domínguez et al., 2021). In this process, the water is heated above the boiling point and below the critical point, meanwhile, the dielectric constant of the water also varies with temperature and pressure, much lower than dielectric constant at normal temperature and pressure, which can be used for the recovery of low polarity compounds (Essien et al., 2020). The advantage of PLE is that it can shorten the extraction time and protect the bioactivity of the target components, as well as it requires less amount of solvents. The application of PLE in the recovery of high-added-value components in aquaculture and fish side streams has been explored (see **Table 4**).

1.2.3. Other technologies

In addition to the above mentioned two techniques, there are other innovative technologies used to recover high-added-value bioactive components from aquaculture and fish side streams. For example, ultrasound technology (USN) which is simple to operate and has low energy consumption, is used in the processing of natural fish flavoring, which has been proved to significantly enhance the content and type of aroma compounds and reduce bitterness (Siewe et al., 2020). At the same time, when USN is used for lipid extraction of algae, it can also effectively destroy the cell wall and improving the extraction rate (Yao et al., 2018). Supercritical fluid extraction (SFE) is also one of the effective methods to recover fat-soluble components, ethanol and CO_2 are

often used as co-solvents to accelerate the recovery of lipids, which have the advantages of low cost and non-toxicity. When SFE is used to recover fish and algae lipids, it is found that this technique not only improves the extraction rate, but also affects the fatty acid composition (Kuvendziev et al., 2018). Moreover, a variety of innovative technologies are being used for the recovery of marine-derived bioactive components, such as microwave-assisted (MS), ionic fluids (ILs) and enzyme-assisted (Zhou et al., 2022). Compared with traditional technologies (including Soxhlet, chemical reagent extraction, etc.), these innovative technologies always have higher extraction rates, reduce the pollution of toxic reagents, showing higher application value.

1.3 Evaluation of the potential health benefits to humans

1.3.1 Bioaccessibility

In order for the high-added-value compounds to better perform physiological functions in the human body, they should be digested and absorbed by the human body and enter the fluid circulation to reach the target location (Fernández-García et al., 2009). Therefore, the evaluation of bioaccessibility, which refers to the fraction of nutrients released from the food matrix into the gastrointestinal tract and able to be absorbed normal routes (Picciano & Raiten, 2001) is helpful to understand the digestion and metabolic processes. Different methods have been used to determine the bioaccessibility of bioactive components, including *in vivo* and *in vitro* methods (Cardoso et al., 2015). However, *in vivo* experiments in humans and animals always face high cost or ethical limitations, so more and more *in vitro* digestion models are being developed, such as static digestion

models (i.e., INFOGEST model) and a variety of dynamic digestion models, including the dynamic gastric model (DGM), the human gastric simulator (HGS), the gastric digestion simulator (GDS) and so on (Sensoy, 2021). These *in vitro* digestion models can simulate the peristalsis, temperature, and pH of the human gastrointestinal tract by stirring, water bath, pH meter and other equipment. Meanwhile, salivary amylase, pepsin and trypsin have been added to the models as digestive enzymes to hydrolyse the food matrix. These models can simulate the digestion process from the oral to the intestinal phase. During the oral stage, food is broken down into smaller fragments by the action of tooth and salivary amylase. Because the whole process in oral stage takes less time, some models ignore the mouth stage, which directly simulates the digestion of stomach and intestinal stage (Hur et al., 2011). These models are used for bioaccessibility evaluation of bioactive components, including bioactive peptides, phenols, minerals, and others.

1.3.2 In vitro *Colonic fermentation: impact on gut microbiota*

Some *in vitro* digestion models include an intact digestive tract from the mouth to the colon, combining gastrointestinal digestion with gut microbial fermentation to better assess the interaction between food matrix and gut microbiota (Cardoso et al., 2015). Gut microbiota is an important part of the human microbial system, the amounts of microorganisms in the ileum and colon can reach 10^{12} CFU/mL, and most of them are anaerobic bacteria such as *Bacteroides* and *Bifidobacterium* (Wang et al. 2021). The gut microbiota plays an important role in the immune system, which exists in a balanced coexistence relationship. At the same time, the gut microbiota can participate in the host

nutrient metabolism, regulate the health of the central nervous system, and play an important role in human health (Pellegrini et al., 2018). Fermentation is one of the special functions of the colon. Some components in the food matrix that are not fully digested by the upper digestive tract undergo anaerobic fermentation under the help of gut microbiota to produce short-chain fatty acids (SCFAs) and other metabolites, which in turn effect on human health (Liu et al. 2021; Ferreira-Lazarte et al. 2019). Therefore, exploring the fermentation behavior of food components in the colon is of great significance to health. At present, *in vitro* colonic fermentation models can be established to explore the fermentation of food components in the colon, including static batch models and continuous dynamic models. These models use fecal samples as inoculum and simulate the colonic anaerobic fermentation by controlling temperature, pH and gas environment (Barry et al., 1995). Meanwhile, a variety of metabolism-related technologies are used to analyze microbial composition and their metabolites quantitatively and qualitatively, such as 16S rRNA gene amplification, real-time PCR, chromatography and mass spectrometry, which can obtain more information about gut microbiota and their potential relevance for the prevention of some diseases (Chen et al. 2019).

1.3.3 Regulation of the inflammation signalling

Inflammation is a complex organic reaction that occurs in response to cellular damage, infection, and repair. Inflammation generally consists of four parts, including pro-inflammatory factors, receptors, mediators, and affected target organs/tissues (Medzhitov, 2010). A variety of factors can induce inflammation (including bacteria, fungi, toxic

ingredients and so on), which triggers the response of inflammatory signalling pathways, and further induces the expression of pro-inflammatory factors, such as tumor necrosis factor (TNF-α), interleukins (ILs, such as IL-1, IL-8 and IL-6), etc (Gupta et al., 2021). In the inflammatory response, activated macrophages can produce reactive oxygen species (ROS) and reactive nitrogen species (RNS), which induce the oxidation of proteins, lipids, nucleic acids, etc. Meanwhile, inflammatory factors and cytokines also induce epithelial cells to grow ROS, thereby inducing tumor suppressor genes (such as p53 and BRCA1) (Kaur & Singh, 2022). Under normal circumstances, cells and inflammatory factors can maintain tissue homeostasis, but the overexpression of inflammatory mediators can lead to immune system disorders and cause several diseases, such as inflammatory bowel disease, arthritis, etc. (Esser et al., 2014). Some conventional treatment methods use cyclooxygenase (COX), TNF-α, interleukins, etc. are used as molecular targets, and the changes of ROS, IL-1, IL-6, TNF-α, etc. are measured to reflect the regulation effect of the research object on the inflammatory pathway (Ge et al., 2022). However, long-term use of medicines will bring a range of side effect, so finding natural products and exploring anti-inflammatory mechanisms is promising.

The development and research of bioactive compounds is always based on the improvement of human health. Therefore, in addition to exploring its bioactivities, it will be very important to evaluate the potential health benefits of bioactive compounds various methods to find more promising applications.

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2. OBJECTIVES

2. Rationale and Objectives

This thesis explores the application of innovative extraction technologies (pulsed electric fields, PEF, and pressurized liquid extraction, PLE) to recover high-added-value compounds (HAVCs) from fish side streams and microalgae. Moreover, it also investigates the bioactivity and potential health effects of the obtained extracts. The purpose is to maximize the recovery of HAVCs coming from fish side streams and microalgae, as well as to protect the bioactivities. Based on this, the marine side streams and aquaculture products can be fully utilized, which not only reduces environmental pollution and waste, but also contributes to the sustainable utilization of resources.

Then, the **general objective** of this PhD thesis is to develop and optimize innovative extraction methodologies (PEF and PLE) to extract and recover high-added-value compounds from marine resources including fish (rainbow trout and sole) side streams and microalgae and evaluating their biological activities. These objectives would promote the reduction of the waste of marine resources and promoting the circular economy and the sustainability of those bioactive compounds being used in food, pharmacy and medicine.

The specific objectives are:

a) To evaluate the effect of PEF and PLE treatments on the recovery of protein from fish side stream and evaluating the molecular size distribution, antioxidant capacity antibacterial and anti-inflammatory properties of the extracts obtained.

b) To identify and quantify minerals in fish side stream extracts and their bioaccessibility.

c) To evaluate the *in vitro* effects of fish side stream extracts on the gut microbiota.

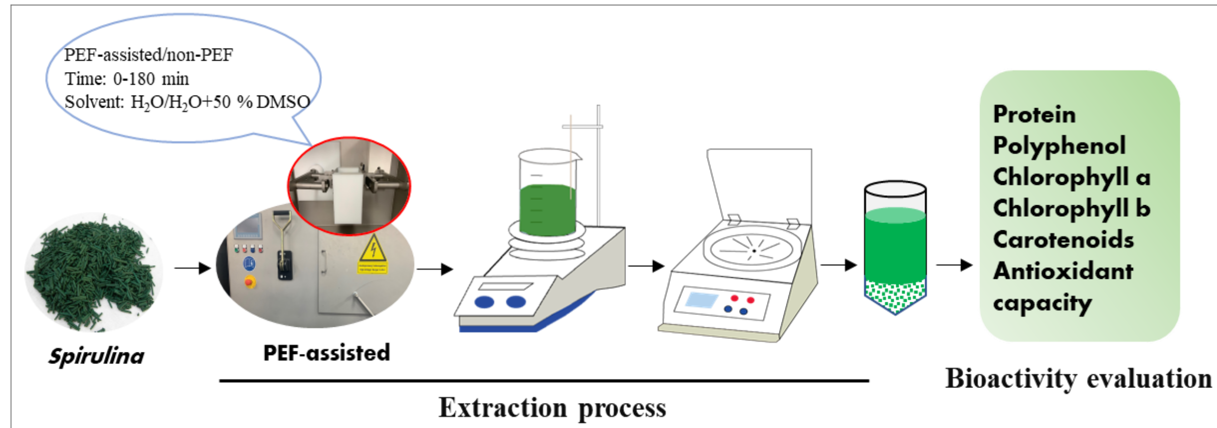
d) To understand the impact of PEF and PLE treatments, as well as the combination PLE +DMSO on the recovery of biomolecules from *Chlorella*

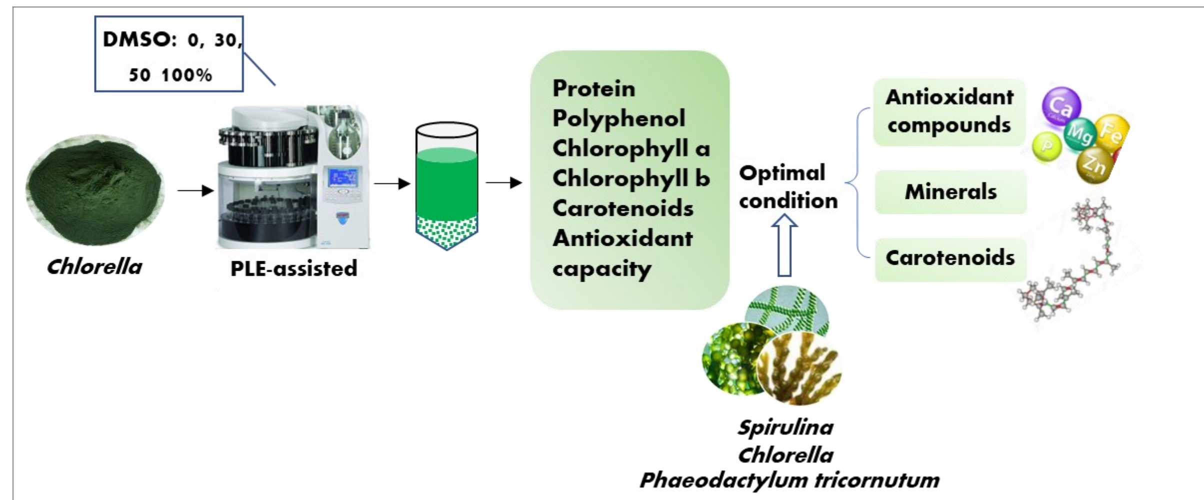
3. EXPERIMENTAL PROCESS

3. Experimental process

Fish side streams

Microalgae





4. RESULTS

4.1 Accelerated solvent extraction and pulsed electric fields for valorization of rainbow trout (*Oncorhynchus mykiss*) and sole (*Dover sole*) by-products: Protein content, molecular weight distribution and antioxidant potential of the extracts

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Accelerated Solvent Extraction and Pulsed Electric Fields for valorization of rainbow trout (*Oncorhynchus mykiss*) and sole (*Dover sole*) by-products: Protein content, molecular weight distribution and antioxidant potential of the extracts

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Abstract

Fishery by-products are rich in biologically active substances and the use of green and efficient extraction methods to recover these high-added-value compounds is of particular importance. In this study, head, skin and viscera of rainbow trout and sole were used as the target matrices and accelerated solvent extraction (ASE) (45–55 °C, 15 min, pH 5.2–6.8, 103.4 bars) as well as pulsed electric fields (PEF) (1–3 kV/cm, 123–300 kJ/kg, 15–24 h) were applied as extraction technologies. The results showed that ASE and PEF significantly increased the protein extract efficiency of the fish by-products ($p<0.05$) by up to 80%. SDS-PAGE results showed that ASE and PEF treatments changed the molecular size distribution of the protein in the extracts, which was specifically expressed as the change in the area or number of bands between 5 and 250 kDa. The antioxidant capacity of the extracts was evaluated by ORAC and ABTS assays. The results showed that both ASE and PEF treatments significantly increased the antioxidant capacity of rainbow trout and sole skin and head extracts ($p<0.05$). ASE and PEF extraction processes can be used as new technologies to extract high-added-value compounds from fish by-products.

Keywords: ASE; PEF; fish by-products; protein; SDS-PAGE; antioxidant

1. Introduction

According to the Food and Agriculture Organization of the United Nations report, global fish production has increased from 19 million tons in 1950 to 178.5 million tons in 2018, about a ninefold increase; per capita, consumption of fish products has increased from 9 kg in 1961 to 20.5 kg in 2018, an increase of about 2.2 times (Cordeiro, 2019; Food and Agriculture Organization of the United Nations, 2009).

At the same time, in the manufacturing process of fish products, a large number of by-products are also produced, including viscera, skin, bones, fins, heads, etc., and these by-products account for 30~70% of the total weight of the fish (Ideia et al., 2019; Marti-Quijal et al., 2020; Wang et al., 2019). Fish by-products have attracted a growing interest over recent years due to society's awareness regarding the sustainable use of resources and the search for alternative nutrient sources. Fish by-products have healthy nutritional and bioactive compounds (such as protein and bioactive peptides, polyunsaturated fatty acids, etc.) (Shen et al., 2019; Zhou et al., 2021).

In the past, due to the dual constraints of technology and knowledge, fish by-products were usually directly discarded. The spoilage of the by-products created a great burden on the environment, such as nourishing microorganisms, promoting the release of harmful gases, and polluting land and water (Chaklader et al., 2020; He et al., 2013).

In recent years, researchers have focus on the development of high nutritional value components in fish by-products, including collagen, phospholipids and fish oil (Ideia et al., 2019; Marti-Quijal et al., 2020; Wang et al., 2019). Studies have shown that these high-added-value compounds play an important role in human health. For example, some researchers have shown that collagen in fish skin is a natural ingredient, which can promote skin cell regeneration and metabolism, and improve skin elasticity, with long-term consumption of fish collagen leading to a delay in skin aging and reducing the formation of wrinkles and stains in human skin (Jia et al., 2010; Kato et al., 2011; Yunoki

et al., 2004). The phospholipids found in the fish head is an important source of neurotransmitter synthesis in the human brain, which can enhance human memory, thinking and analysis capabilities, and can control the degeneration of brain cells and delay aging (Mahmoud et al., 2008; Topuz et al., 2021). Moreover, fish oil is rich in docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids, which can help reducing the risk of cardiovascular and cerebrovascular diseases, such as atherosclerosis, heart disease, high blood pressure and other diseases (Asari et al., 2019; Grinevicius et al., 2019; Naeini et al., 2020).

During the process of the extraction of high-added-value compounds from fish by-products, the extraction efficiency, green conditions and the impact of labile nutrients and bioactive compounds must be considered. Accelerated solvent extraction (ASE) has been used in the food industry as an effective extraction method. For example, ASE has been used to extract natural compounds with antioxidants and anti-inflammatory properties from Passiflora species, seaweed and other plants (Gomes et al., 2017; Kraujalis et al., 2013; Toubane et al., 2017). In these studies, ASE not only shortened the extraction time, but also reserved the activity of natural products. In fishery-related research, ASE has been used to evaluate polychlorinated biphenyls, polybrominated diphenyl ethers and organochlorines in fish samples (Ottonello et al., 2014; Wang et al., 2010; Zhuang et al., 2004). However, the available literature regarding the application of ASE to obtain nutrients and bioactive compounds from fish by-products is scarce.

Meanwhile, pulsed electric fields (PEF), as a short-term electrical pulse effect, can keep the thermal effect at a low level and retain the flavor and quality of the food to a great extent. The use of PEF-assisted extraction will only destroy the biological cells in the food matrix without harmful effects on the food, and at the same time increase the extraction rate of juice and valuable compounds (Gómez et al., 2019; Zhang et al., 2020). PEF has also been used in aquatic products. For example, PEF has been used to extract

bioactive compounds from fish bones and has also been evaluated the potential of PEF for obtaining antioxidant compounds from fish residues (Franco et al., 2020).

Taking into account the large consumption demand for rainbow trout and sole and the high-value nutrients in their by-products, and PEF and ASE are less used in the recovery of these two fish by-products, this study uses ASE and PEF to extract its bioactive compounds. And further analyzed the protein content, protein molecular distribution and antioxidant capacity of the extract.

2. Materials and Methods

2.1 Samples

The experimental rainbow trout and sole samples were obtained from a local supermarket. Fish samples were dissected in the laboratory to obtain skin, head and viscera. The sample for the ASE extraction experiment needs to be freeze-dried under vacuum at -48 °C for 72 hours, then the sample is crushed into powder and stored at -20 °C for later use. The experimental samples of PEF were freshly dissected fish by-products.

2.2 Chemicals and reagents

2,2'-azobis-2-methyl-propanimidamide (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein sodium salt and potassium persulfate ($K2s2O_8$) were purchased from Sigma-Aldrich (Steinheim, Baden-Württemberg, Germany). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) -related reagents were purchased from Bio-Rad. Diatomaceous earth and other materials for the generation of ASE® extracts were purchased from Dionex (Dionex, Leeds, UK).

2.3. Extraction technologies

2.3.1 ASE extraction

The ASE extraction conditions were selected based on some experimental work carried out in the laboratory with sea bass samples (Fuente et al., 2021). According to those

experiments, the mix ratios of fish head, viscera, skin and diatomaceous earth were 1.0:2.0, 1.5:3.0 and 2.0:2.0 (g/g), respectively. Fish by-products and sample and diatomaceous earth were thoroughly mixed in a mortar and placed into the extraction tank and the ASE extraction conditions were settled using a pressure of 103.4 bars. For these experiments an ASE-200 Accelerated Solvent Extractor (Sunnyvale, CA, USA) was used. The standard operating conditions were as follows: preheating period (1 min), heating period (5 min), flush volume (60%), nitrogen purge (60 s), and extraction pressure (103.4 bars). More detailed extraction conditions are shown in **Table 2**. After the ASE extraction was completed, the extracts were collected in extraction bottles and kept at -20 °C until needed for analyzes. At the sample time, the control experiment was set up. Deionized water was used as the extraction reagent at normal pressure to ensure that the extraction time, pH and temperature were consistent with the ASE experimental group. After the extraction was completed, the extract was filtered using filter paper, and the obtained sample was stored at -20 °C for later use.

Table 1. Extraction conditions for recovering bioactive compounds from rainbow trout and sole by-products using accelerated solvent extraction (ASE) and pulsed electric fields (PEF).

Methodology	**ASE**				**PEF**		
Rainbow trout/ Sole	T (°C)	t (min)	pH	Pressure (bars)	Field strength (kV/cm)	Specific energy (kJ/kg)	t* (h)
Head-optimal	55	15	5.2	103.4	1.00	219.765	21.329
Head-control	55	15	5.2	No	No	No	21.329
Skin-optimal	45	15	6.5	103.4	3.00	300	24
Skin-control	45	15	6.5	No	No	No	24
Viscera-optimal	50	15	6.8	103.4	3.00	123.750	15.169
Viscera-control	50	15	6.8	No	No	No	15.169

t*: Time of supplementary extraction

Figures 1-2 show the ASE and PEF extraction processes followed for the recovery of high-added-value compounds from rainbow trout and sole by-products, respectively.

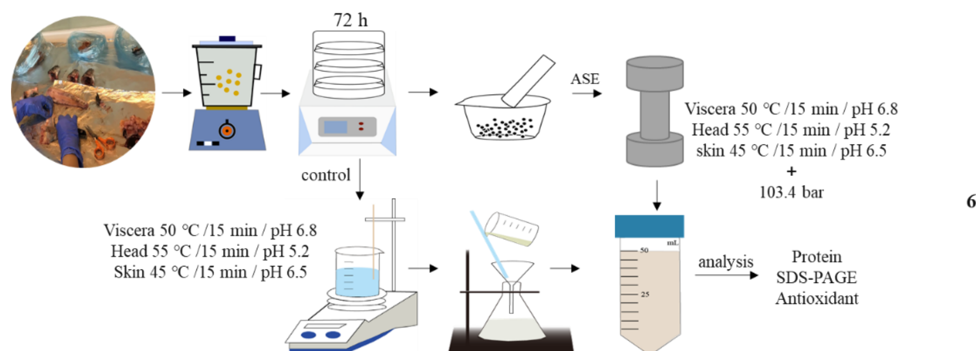


Figure 1. The accelerated solvent extraction (ASE) processes followed for the recovery of high-added-value compounds from rainbow trout and sole by-products.

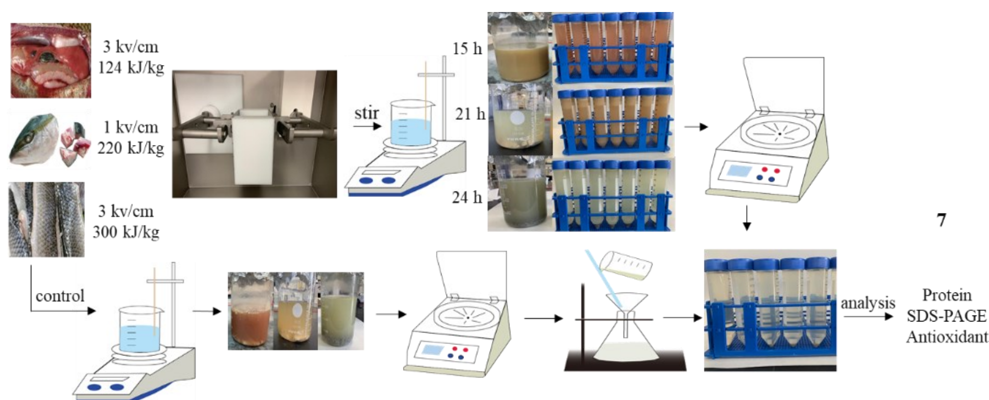


Figure 2. The pulsed electric fields (PEF) extraction processes followed for the recovery of high-added-value compounds from rainbow trout and sole by-products.

2.3.2 PEF extraction

Similarly, PEF optimal extraction conditions were previously selected at the laboratory (data not shown) using a PEF-Cellcrack III (German Institute of Food Technologies (DIL)) equipment (ELEA, Quakenbrück, Osnabrück, Germany). Specifically, the fresh fish by-products (head, skin and internal organs) were placed in the processing chamber, and a certain amount of tap water was added. The conductivity was maintained between 1000 and ~2000 µs/cm. Then, the samples were PEF-processed according to the experimental conditions (**Table 1**). The processed samples were introduced into a beaker and were continuously stirred using a magnetic stirrer at room temperature. Finally, the obtained extracts were centrifuged at 4000*x*rpm at 4 °C for 15 minutes and the supernatant was taken and filtered to obtain the sample.

2.4. Chemical analyzes

2.4.1. Proximate composition

Firstly, the moisture and protein content of the fish by-product samples were tested. Moisture was determined by oven-drying until constant weight at 103 ± 2 °C (Karásková et al., 2011). Protein was determined according to the Kjeldahl method (De Lourdes Mendes Finete et al., 2013), (see section 2.4.2.)

2.4.2 Protein content

The protein content was determined according to the Kjeldahl method (Barba et al., 2013). Five grams of zeolite, 3 g of potassium sulphate, 5 drops of copper sulphate solution and 2 ml sample were added into the nitrification tube, then 5 ml of 98% concentrated sulphuric acid were added and the mixture was heated at 120 ℃ until the solution was clear and translucent. The automatic Kjeldahl analyzer was used to convert the ammonia ions in the sample into ammonia gas, and boric acid was used as the absorption liquid. Finally, 0.1 M hydrochloric acid was used as the titration reagent, and methyl orange as the end point indicator of the titration. The calculation formula of nitrogen content was:

$$\mathbf{Nitrogen}\ \% = \frac{[(\mathbf{mL\ standard\ acid} - \mathbf{mL\ blank}) * \mathbf{N\ of\ acid} * \mathbf{1.400}]}{(\mathbf{Weight\ of\ sample\ in\ grams})} \qquad (\mathbf{1})$$

The calculation formula of protein content is:

$$\mathbf{Protein}\ \% = \mathbf{6.5} * \mathbf{Nitrogen}\ \% \qquad (2)$$

2.4.3 Molecular size distribution (SDS-PAGE)

SDS-PAGE method refers to the relevant literature and slightly modified (De la Fuente et al., 2021). Prior to the SDS-PAGE experiment, the sample buffer was prepared. Five hundred milligrams of SDS, 2.46 g of Tris-HCl, 6.25 mL of glycerol, 2.5 mL bromophenol blue reagent and 13 mL deionized water were introduced into a beaker and mixed on a magnetic stirrer. Then, the pH was adjusted to 6.8 with the diluting of a sodium hydroxide solution, and then deionized water was added up to 25 mL. Eight milligrams

of dithiothreitol (DTT) were added to 500 µL of the sample buffer, and then the mixture was stored under darkness and marked as 'A'.

In order to configure the electrophoresis strip fixing solution, 400 mL of methanol, 100 mL of acetic acid and 500 mL of water were mixed. Two hundred milliliters of methanol, 100 mL of acetone and 700 mL of deionized water as the decolorizing solution were mixed. Then, the electrophoresis solution was prepared, that is, 0.5 g SDS, 7.2 g glycine and 1.515 g Trizma base were weighed and then dissolved in 1000 mL of distilled water.

To prepare the sample, 100 µL of extracts and 400 µL of acetone were mixed, vortexed for 10 seconds, and centrifuged at 11,000 rpm at 4 °C for 10 minutes. The supernatant was removed and placed in a fume hood for 5 minutes, then the remaining acetone reagent was volatilized. The precipitate was then rinsed with 100 µL of deionized water and sonicated at 25 °C for 20 seconds to completely dissolve the precipitate, named as 'B'. Then A and B were mixed (*v/v*, 20 µL/20 µL) and heated at 95 °C for 5 minutes.

Bio-Rad equipment was used for gel electrophoresis. The label and sample loading volumes were 10 µL and 25 µL, respectively. The equipment was maintained under a constant voltage of 80 V, and when the marker band reached the bottom of the gel, the electrophoresis experiment was finished. Then, the gel was removed, and the fixative was added to soak for 30 minutes. Next, the fixative was removed and Coomassie brilliant blue dye was added for 30 minutes. Finally, the decolorizing solution was added and was kept shaking for 24 hours until the protein bars in the gel were clear.

2.4.3. Total antioxidant capacity

2.4.3.1. Oxygen radical absorbance capacity test

The oxygen radical absorption capacity (ORAC) test is used to evaluate the oxygen radical absorption capacity of the sample. The fluorimetric method described by Barba et al. was applied (Barba et al., 2012). We added 50 µL of phosphate buffer (pH 7.0~7.4), 1

mM Trolox and sample to 96-well plates, then added 50 μL of fluorescein sodium salt, incubated at 37 °C for 10 minutes, and added 25 μL of AAPH (120 mg/mL). The absorbance value of the sample was measured at 520 nm and a total of 45 cycles were tested. Each group of samples set five holes in parallel. The test was repeated three times, and the coefficient of variation of the data was less than 15%. The calculation formula was:

$$\mathbf{ORAC(trolox)} = \frac{\mathbf{Asample - Ablank}}{\mathbf{Atrolox - Ablank}} \qquad \mathbf{(3)}$$

2.4.3.2. Trolox equivalent antioxidant capacity assay (TEAC)

The spectrophotometric method proposed by Barba et al. was used (Barba et al., 2013). The 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) test method referred to the relevant literature and had slight modifications. We mixed 25 mL of 7 mM ABTS with 440 μL of 140 mM potassium thiosulphate solution to obtain a working solution and stored it at room temperature in the dark for 12~16 hours before use. Then 7 mM of the working solution were diluted with 96% ethanol to keep the absorbance value between 0.700±0.020. During the test, we mixed 0.1 mL of the sample or standard with 2 mL of working solution and read the absorbance value of the reaction solution at 734 nm after it had reacted in a dark room for 3 minutes. The standard curve was calculated with the absorbance and concentration values of the Trolox solution and the Trolox equivalent used as the antioxidant capacity of the sample. The standard curve equation is $y=0.0014x+0.6504$, $R^2=0.999$.

2.5. Software and statistics

IMAGE-J software was used for electrophoretic band analysis. GraphPad Prism (GraphPad Software Company, La Jolla, California, USA) was used for graph rendering, and SPSS (IBM Cop., Armonk, NY, USA) was used for data significance analysis. Analysis of variance (ANOVA) and Duncan's multiple range test were used to estimate

the significance of the difference in the mean. Each repeated analysis was performed three times, and the statistical significance was estimated at 5% level ($p<0.05$).

3. Results and Discussion

3.1 Protein and moisture content in rainbow trout and sole by-products

The total protein and moisture content of all rainbow trout and sole by-products are shown in Table 2. As can be seen in the table, the protein content of rainbow trout skin is higher than that of head and viscera, and the wet basis accounts for close to 21% (*w/w)*. The sole viscera had the highest protein content (≈21%). In terms of protein content, rainbow trout and sole by-products have high recycling value. The water content of the two fish by-products is relatively close, approximately distributed between 60 and ~70% (*w/w*).

Table 2. Protein and moisture content in rainbow trout and sole by-products

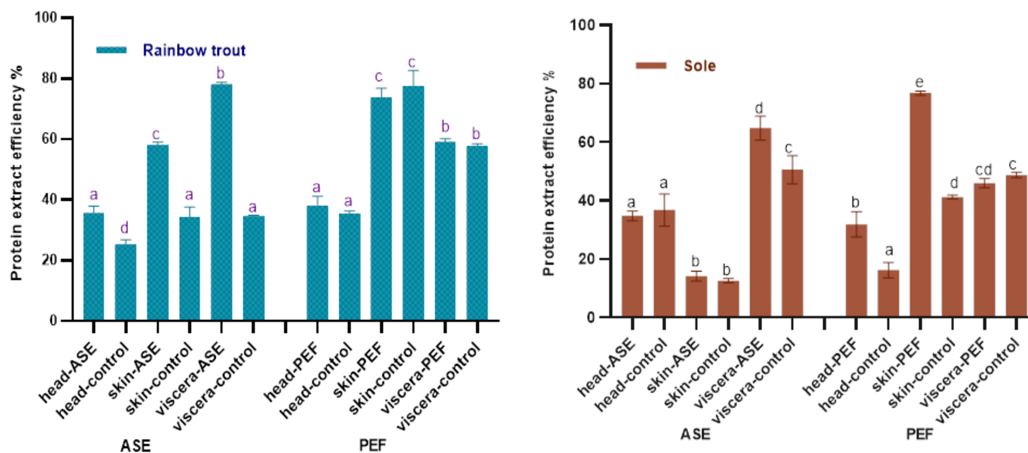
	protein（wet basis%）		**moisture（%）**	
	Rainbow trout	Sole	Rainbow trout	Sole
Head	$12.9±0.4^a$	$15.8±1.1^a$	$70.3±0.3^b$	$71.1±1.6^c$
Skin	$20.8±0.6^b$	$18.8±2.4^a$	$58.3±0.5^a$	$60.3±0.8^a$
Viscera	$13.4±0.6^a$	$21.7±1.9^b$	$69.2±0.9^b$	$65.6±0.7^b$

*Different letters in the table indicate statistically significant differences ($p<0.05$).

3.2 Protein extraction efficiency

Figure 3 shows the effects of ASE and PEF on the protein extraction rate of rainbow trout and sole, respectively. With regard to the ASE extraction groups, for rainbow trout, the highest protein extraction rate is the viscera group, which is close to 80%, followed by skin and head samples. The application of ASE significantly increased the protein extraction rate of each rainbow trout side stream ($p<0.05$). As for the sole, the highest protein extraction efficiency was obtained for viscera, close to 60%, followed by head and skin. This is related to the different skin tissue structures of the two fishes. Compared with rainbow trout skin samples, the texture of sole skin is harder after freeze-drying, and it is more difficult to grind and mix well with diatomaceous earth, which results in a lower protein extraction rate. It can be also seen that the application of ASE significantly increased the protein extraction rate from sole viscera ($p<0.05$), but the protein extraction rate from head and skin did not increase significantly ($p>0.05$).

Figure 3. Protein content in control and optimal ASE/PEF extracts from by-products of



Rainbow trout and Sole (head, skin, viscera). Results are expressed as mean ± standard deviation. Different letters in the bars indicate statistically significant differences ($p<0.05$).

The results also showed that after using PEF, the protein extraction efficiency of both two fish skins is higher-nearly 80%-followed by the viscera and head. From a sample

point of view, the protein content in fish skin is as high as 70%, which is higher than head and viscera. After PEF treatment, the sole skin samples were soaked in water for 24 hours and continuously whipped. Compared with the sole skin samples of the ASE group, the hard sole skin fragments were mixed with diatomaceous earth, this process being better in promoting the extraction of protein from fish skin and thus improving the efficiency of protein extraction from fish skin. Interestingly, compared with the control group, PEF only significantly increased the protein extraction rate of sole skin and head ($p<0.05$), but did not significantly increase the protein extraction rate of by-products in rainbow trout ($p>0.05$).

In recent years, there has been relatively abundant research on the extraction of protein from fish by-products. Among the extraction of fish by-products protein, there are relatively many studied on collagen. For instance, a review by Ahmed et al. summarized the application research of different extraction methods in the extraction of collagen from the by-products of different fish (Ahmed et al., 2020). For example, Veeruraj et al. used acid extraction and pepsin extraction to extract 80% and 7.1% of collagen (dry basis) from ocean eel skin, respectively. In their study, the acid extraction method obtained higher protein extract efficiency in eel skin, which was similar to the PEF and higher than the ASE extract efficiency in our study (Veeruraj et al., 2013). However, in terms of extraction time, the process of soaking fish skin in the acid extraction method takes 3 days, which consumes a lot of extraction time compared with our study and will increase the cost of actual industrial production. Similarly, Yu et al. used the response surface method to study the effect of extraction parameters on the extraction of collagen from the skin of large yellow croaker. When the pepsin concentration was 1389 U/g, the solid–liquid ratio was 1:57, and the hydrolysis time was 8.67 h, the extraction rate of collagen reached 84.85% (Yu et al., 2018). Similar to this study, the new extraction technology has been also applied in the extraction of fish by-product crude protein. Approximately

40% by weight in mackerel is regarded as a by-product, and studies have shown that as a new extraction technology like ASE and PEF, the ultrasound-assisted method has achieved good results in the protein extraction of mackerel by-products. Carlos et al. used the ultrasonic-assisted acid/alkali method to increase the yield of protein. Their research results showed that ultrasonic-assisted sequential acid and ultrasonic-assisted alkaline extraction can obtain almost 100% and 95% of the protein in mackerel by-products respectively (ultrasound-assisted extraction time approximately 75 mins). Compared with this study, although the protein extraction rate in this study was less than 95%, this study chose water as the extraction reagent, which reduced the cost of the reagent, and the ASE and PEF treatment time was less than 75 minutes, which greatly shortened the extraction time (Yu et al., 2018).

From the principle of ASE, the strong interaction force between the solute and the matrix caused by the van der Waals force, hydrogen bonds, the dipole attraction of the solute molecules and the active site of the sample matrix can be greatly reduced under high temperature and pressure. This accelerates the analytical kinetics process of the solute molecule, reduces the activation energy required for the analytical process, reduces the viscosity of the solvent, thus reducing the blocking of the solvent entering the sample matrix, and increases the diffusion of the solvent into the sample matrix (Zhuang et al., 2004).

Unlike ASE, PEF is one of the processing technologies based on electricity (F.J. Barba et al., 2015). The application of short electrical pulses at high voltages can keep the control of thermal effects at a low level, making it different from thermoelectric technologies such as ohmic heating (Gavahian & Farahnaky, 2018). These properties make PEF a promising technology, able to destroy the biological cells in the food matrix without any harmful effects on the properties of the food (Puértolas & Barba, 2016). Studies have shown that short pulse electric fields (μs to ms) in the range of 100–300

V/cm to 20–80 kV/cm applied by PEF can cause cell membranes to disintegrate and form membrane pores (temporary or permanent) (Barba et al., 2015; Puértolas & Barba, 2016). Zhou et al. (Zhou et al., 2017) studied the extraction effect of PEF technology to obtain protein from mussels. In the case of PEF (triangular pulse power waveform and pulse duration are 2 μs) and under the best estimation conditions (electric field strength is 20 kV/cm, pulse number 8, enzymatic hydrolysis 2 h), the maximum yield of protein extracted can reach 77.08%. In our study, the protein content of the sole head and skin samples increased significantly under the PEF treatment, which may be due to the phenomenon of 'electroporation', which promoted the dissolution of protein in the cells.

3.3 Protein molecular weight distribution in fish by-product extracts

The SDS-PAGE bands of rainbow trout and sole side stream extracts are shown in Figure 2. For rainbow trout, after ASE treatment, the protein molecular weights in the fish head, skin and viscera extract are respectively distributed in 150–10 kDa, 100–10 kDa and 50–5 kDa; the corresponding control group corresponds to the distribution in 75–10 kDa, 100–75 kDa and 25–5 kDa. This shows that ASE makes the protein species in the rainbow trout side stream extract more abundant. The difference is that the protein distribution of the PEF treatment group and PEF control group is the same. The molecular weights of proteins extracted from head, skin and viscera are distributed in 75–15 kDa, 75–15 kDa and 150–5 kDa, respectively. For sole, after ASE extraction, the protein molecular weights in head, skin and viscera extracts were distributed in 250–15 kDa, 250–15 kDa and 50–25 kDa, respectively; the corresponding control group results were 75–15 kDa, 100–15 kDa and 25 kDa. This also shows that ASE increased the protein abundance in

the sole side stream extract. The PEF treatment group only increased the protein of the fish head group at the molecular weight of 150–100 kDa.

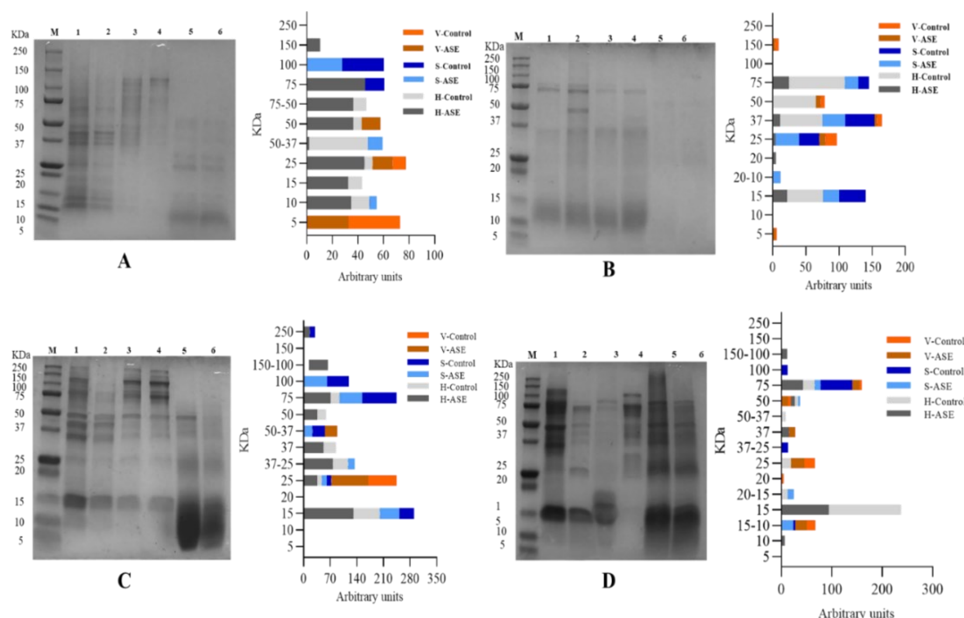


Figure 4. SDS-PAGE. A: Rainbow trout-Accelerated Solvent Extraction (ASE); B: Rainbow trout- Pulsed Electric Fields (PEF); C: Sole-Accelerated Solvent Extraction (ASE); D: Sole- Pulsed Electric Fields (PEF). 1–6 corresponds to Head-ASE/PEF, Head-Control, Skin-ASE/PEF, Skin-Control, Viscera-ASE/PEF and Viscera-Control, M means molecular weight standard.

In order to compare the amount of protein extracted at different molecular weights more directly, ImageJ was used to calculate the area of each band. The results of the by-products' protein distribution from rainbow trout-ASE and PEF, and sole-ASE and PEF are also shown in **Figure 4**. It can be seen from **Figure 4 (A)** that ASE promoted the extraction of proteins with molecular weights of 150 kDa and 75 kDa from rainbow trout heads, which does not occur in the control group. At the same time, from the results of the percentage of the strip area, ASE extraction increased the protein content of 75–50 kDa, 25 kDa, 15 kDa and 10 kDa in the rainbow trout head group. The distribution of protein bands in rainbow trout skin and viscera is relatively simple. ASE extraction increased the protein distribution at 50–37 kDa and 10 kDa in the skin group and the

protein with a molecular weight of 50 kDa in the viscera group. It is not difficult to see that the ASE extraction method increased the abundance of proteins extracted from rainbow trout by-products. However, the effect of PEF on the protein band distribution of rainbow trout by-products is not consistent. Specifically, it can be seen from **Figure 2 (B)** that the proportion of proteins at 75 kDa, 37 kDa and 15 kDa in the rainbow trout head samples treated with PEF decreased, and only the control group appeared with 50 kDa molecular weight proteins. In the fish skin group, although PEF extracted new proteins with a molecular weight of 20–10 kDa, the proportion of proteins at 37 kDa and 15 kDa was lower than that of the control group, and the proportion decreased by more than 10%. The band distribution of PEF in the viscera group is consistent with the control group. However, the protein ratio at 37 kDa and 25 kDa in the PEF group is nearly 1 time lower than that in the control group. This indicates that PEF failed to promote the extraction of different molecular weight proteins in rainbow trout by-products, which is consistent with the results of the protein extraction rate.

For sole, **Figure 4 (C)** shows that ASE promoted the extraction of proteins with molecular weights of 250 kDa and 150–100 kDa from sole heads, which did not occur in the control group. From the perspective of the relative area ratio of the bands, ASE extraction greatly increased the area ratio of the protein bands at 75 kDa, 50 kDa, 37 kDa, 37–25 kDa, 25 kDa and 15 kDa in the sole head sample. Compared with the skin control group, the band area ratio of the ASE extraction group at 100 kDa, 75 kDa, 50–37 kDa, 37 kDa decreased, and the protein ratio at 37–25 kDa and 15 kDa increased, which shows that the ASE can promote extraction of small molecular weight proteins from sole skin. Similarly, ASE increased the ratio of 50–37 kDa and 25 kDa molecular weight proteins in viscera. Consistent with rainbow trout, ASE promoted the extraction of proteins of different molecular weights in the sole by-products and obtained some band distributions that did not exist in the control group. The results showed that PEF (**Figure 4 (D)**) mainly

promoted the extraction of protein from sole viscera, manifested by increasing the protein content of 75 kDa, 50 kDa, 37 kDa, 20 kDa and 15–10 kDa. After sole fish head was treated with PEF, the proteins with molecular weights of 150–100 kDa, 75 kDa and 50 kDa increased, and the proteins at 50–37 kDa, 25 kDa, 20–15 kDa and 15 kDa decreased. This shows that PEF treatment increases the extraction of large molecular weight proteins in sole head samples and inhibits the dissolution of small molecular weight proteins. In contrast, after sole skin was treated with PEF, the protein of 20–15 kDa and 15–10 kDa increased, and the ratio of protein of 100 kDa, 75 kDa and 37–25 kDa decreased. This shows that PEF promotes the extraction of small molecular weight proteins in fish skin and is not conducive to the dissolution of large molecular weight proteins in sole skin. There have been research reports on analysing the peptide distribution of fish skin. Álvarez et al. (Álvarez et al., 2018) extracted 19.27 ± 0.05 mg/g collagen from sole skin under the conditions of acetic acid concentration of 0.54 M, sodium chloride concentration of 1.90 M, a liquid-to-solid ratio of 8.97 mL/g and 32.32 hours, and SDS-PAGE analysis revealed that the molecular weight of collagen peptides was between 118 kDa and 116 kDa. The results of this study showed that the ASE group extracted 100 kDa protein from sole skin, while the PEF group lacked the band distribution at 100 kDa. This shows that different extraction methods differ in the extraction of the same type of fish skin.

As for ASE, pressure extraction is an important factor in changing the protein content and properties of the extract. Gómez-Guillén et al. (Gómez-Guillén et al., 2005) used high pressures (250–400 MPa) to treat fish skin and found that high pressure not only increased the yield of fish skin collagen, but also changed the molecular weight distribution of fish skin protein, showing that a high-pressure extraction method is superior to traditional methods in fish skin collagen extraction. The pressure in the extraction of fish side streams protein in our ASE methodology was nearly 10 MPa, which is lower than 250–

400 MPa, but the result still shows that pressure changes the molecular weight of fish side stream protein, which is conducive to extracting more low-molecular-weight proteins from fish by-products. Related studies have confirmed that pressurization will have a certain impact on the structure of protein molecules in food. For example, a pressure lower than 150 MPa can affect the quaternary structure of a protein, 200 MPa can affect the tertiary structure, and 300–700 MPa can change the secondary structure (Briones-Labarca et al., 2012; Valérie Lullien-Pellerin, 2002). The pressure in this study was only close to 10 MPa, which may affect the spatial conception of protein molecules. Relevant studies have shown that during the pressurization process, the pressure may lead to the destruction of non-covalent interactions and changes in intermolecular and intramolecular and solvent–protein interactions, thereby changing the natural conformation of proteins (Jia et al., 2021). Normally, high pressure will not affect the covalent bonds of protein molecules and will not destroy the peptide bond structure in protein molecules (Escobedo-Avellaneda et al., 2020). From the perspective of the working principle of the ASE method, at a certain temperature, pressurization can increase the permeability of the solvent, making it easier to enter the sample matrix and increase the contact time between the sample and the solvent (Ahmad et al., 2020). The related effect is shown in Figure 6. Therefore, it can be inferred that the change in SDS-PAGE results is due to ASE changing the solubility of different molecular weight proteins in fish by-products in water. ASE extraction not only increased the total protein content in the extract, the SDS-PAGE results further showed that ASE also promoted the increase in the content of certain specific molecular weight proteins, which is meaningful for obtaining specific molecular weight proteins from fish by-products.

For PEF, the specific mechanism of protein distribution changes caused by PEF has not been accurately determined. However, related studies have proved that protein molecules will be polarized at low PEF intensity, and their hydrophobic amino acids will

gradually be exposed to the solvent as the electric field intensity increases. The final unfolded protein may become an aggregate of weak covalent and non-covalent bonds under relatively high field strength (Zhao & Yang, 2009). After a certain PEF intensity is exceeded, the thermal effect caused by the arc will cause the denaturation and aggregation of heat-sensitive proteins (Hermawan et al., 2004). Studies have also shown that PEF can destroy the secondary structure of proteins, such as increasing the ratio of β-sheets and reducing the content of α-helices (Li, 2012; Zhong et al., 2007). Since no relevant studies have been found to show that PEF can cause the breakage of the primary structure of the protein–peptide bond, the changes in the distribution of protein bands in this study are mainly related to two aspects. One is that PEF causes cell breakage in fish by-products to accelerate protein dissolution (**Figure 5**); the other is that the PEF electric field causes the exposure of protein hydrophobic amino acids and further protein aggregation occurs, which may cause some protein molecular weight changes during the extraction process.

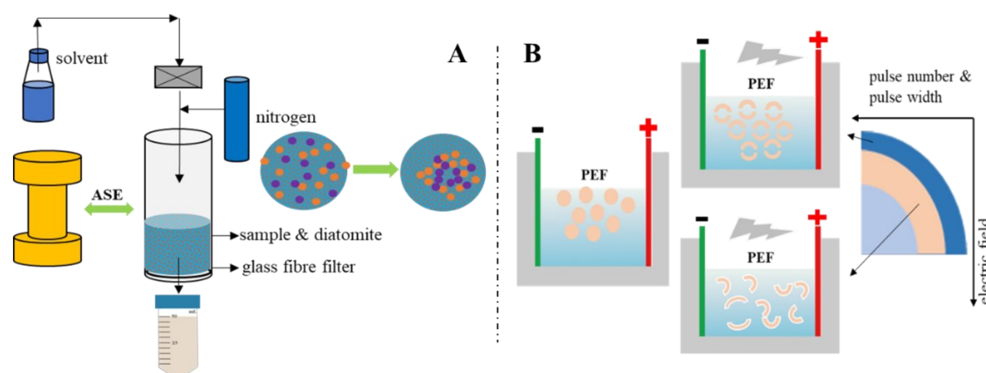


Figure 5. Schematic diagram of the accelerated solvent extraction (ASE) and pulsed electric fields (PEF) effect, respectively.

3.4 Antioxidant capacity

3.4.1 Oxygen radical absorbance capacity (ORAC)

The antioxidant properties in the extracts are worthy of attention. In this study, the oxygen radical absorbance capacity (ORAC) and $ABTS^+$ scavenging ability of the fish by-product extracts were used to judge the antioxidant capacity of the bioactive compounds in the extracts, and the results are shown in **Figure 6** and **Figure 7**. For rainbow trout by-product extracts, whether in the ASE or PEF group, skin extract has the strongest oxygen radical absorbance capacity, followed by viscera and head. From **Figure 6**, ASE significantly increased the oxygen radical absorbance capacity of rainbow trout skin and viscera extracts ($p<0.05$), while PEF had no significant effect on its oxygen radical absorbance capacity ($p>0.05$). For sole, the visceral anti-oxygen free radicals in ASE and PEF extract products are the strongest, followed by skin and head. ASE extraction significantly increased the oxygen radical absorbance capacity of sole head and viscera ($p<0.05$), and slightly increased the antioxidant capacity of sole skin extracts ($p>0.05$), and PEF significantly increased the oxygen radical absorbance capacity of head and viscera ($p<0.05$), and slightly increased the anti-oxygen free radical ability of skin ($p>0.05$).

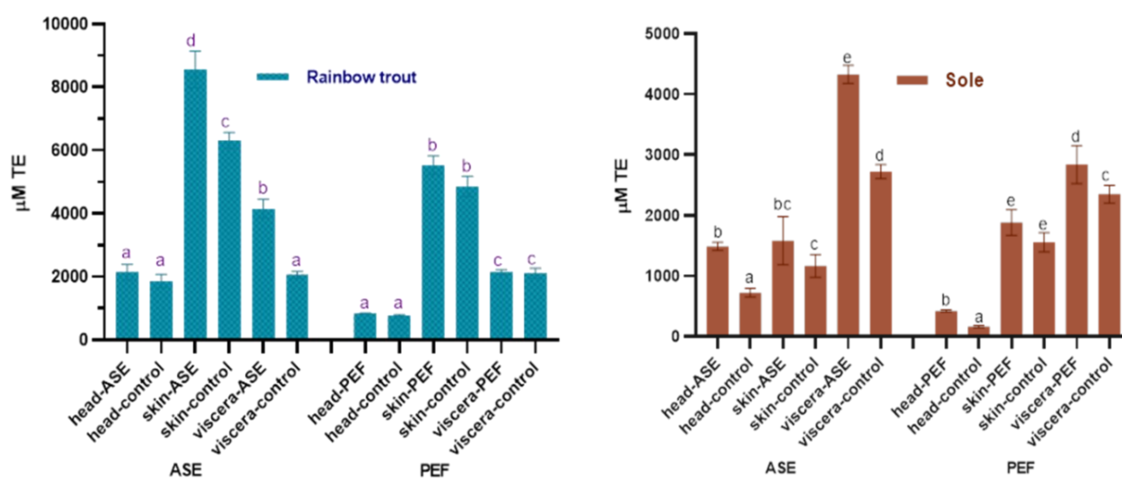


Figure 6. Total antioxidant capacity (ORAC) in control and optimal ASE/PEF extracts from by-products (head, skin and viscera) of Rainbow trout and Sole. ORAC: Oxygen radical absorbance capacity; ASE: accelerated solvent extraction; PEF: pulsed electric fields. Results are expressed as mean ± standard deviation. Different letters in the bars indicate statistically significant differences ($p<0.05$).

3.4.2 ABTS^{+} scavenging ability

Figure 7 shows the effects of ASE and PEF extraction on the ABTS+ scavenging ability of two fish by-products. The results showed that ASE significantly increased the anti-ABTS+ ability of rainbow trout and sole head and skin extracts ($p<0.05$), but meanwhile significantly reduced the anti-ABTS+ ability of the two fish viscera extracts ($p<0.05$).

The application of PEF technology significantly increased the ability of rainbow trout skin extract to resist ABTS^{+} ($p<0.05$) and did not affect the head and viscera significantly ($p>0.05$). Unlike rainbow trout, the use of PEF significantly increased the ABTS+ scavenging capacity of sole head and skin extract ($p<0.05$), but significantly reduced the antioxidant capacity of viscera extract ($p<0.05$). From the results of the ORAC and ABTS experiments, both ASE and PEF increased the Trolox equivalent value of rainbow trout and sole skin and head extracts, indicating that the antioxidant properties of the corresponding extracts were enhanced. However, the calculation results of ASE and PEF on the antioxidant capacity of visceral extracts are inconsistent. Both ORAC and ABTS can characterize the antioxidant activity of active substances, but the principles of the two are different. The principle of the ORAC experiment is to observe the ability of Trolox or the antioxidants in the extract to resist 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) to reduce the fluorescence intensity (Gregório et al., 2020), while the ABTS method is to measure the ability of the extracts or Trolox to eliminate ABTS^{+} (Leng et al., 2020). Although the sources of free radicals corresponding to the two methods are inconsistent, and the final Trolox quantification is not completely consistent, the results still show that both ASE and PEF treatments increase the antioxidant activity of the extract.

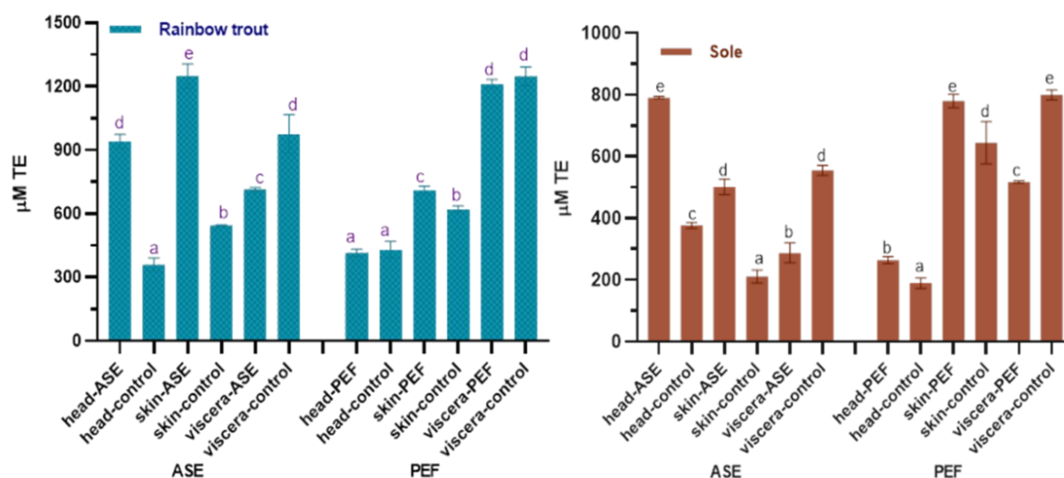


Figure 7. Total antioxidant capacity (ABTS) in control and optimal ASE/PEF extracts from by-products (head, skin and viscera) from Rainbow trout and Sole. ABTS: Trolox equivalent antioxidant capacity; ASE: accelerated solvent ex-traction; PEF: pulsed electric fields. Results are expressed as mean ± standard deviation. Different letters in the bars indicate statistically significant differences (*p<0.05*).

4. Conclusions

As a green and efficient extraction technology, this study shows that ASE and PEF have shown good results in the extraction of active substances from fish by-products. The treatment of ASE and PEF made the protein extraction rate of fish by-products reach 80 % and changed the distribution of molecular size. In addition, after evaluating the antioxidant capacity of the extracts, it can be shown that the treatment of ASE and PEF improves the antioxidant capacity of the skin and head from sole. Both the pressurization in the ASE extraction process and the electric field in the PEF extraction are beneficial to the extraction of soluble proteins in the by-products, which not only replaces the pollution of organic reagents in traditional extraction techniques, but also retains the antioxidant properties of active substances to the greatest extent. This is in line with the requirements of modern industry and environmental development and will play a huge role in the transformation and utilization of aquatic resources in the future.

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4.2 Role of extracts obtained from rainbow trout and sole side streams by accelerated solvent extraction and pulsed electric fields on modulating bacterial and anti-inflammatory activities

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Role of extracts obtained from rainbow trout and sole side streams by Accelerated Solvent Extraction and Pulsed Electric Fields on modulating bacterial and anti-inflammatory activities

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Abstract

In this study, accelerated solvent extraction (ASE) and pulsed electric field (PEF) were used as innovative approaches to recover extracts from rainbow trout and sole side streams rich in high-added-value compounds. Then, after aseptic filtration, the impact of the obtained extracts on bacterial growth and anti-inflammatory potential was evaluated. Moreover, the protein content and the total antioxidant capacity of the samples were determined. The results showed that some extracts could inhibit the growth of pathogenic bacteria, including the ASE rainbow trout skin and the PEF sole viscera extracts, which showed significant antibacterial activity on *Staphylococcus aureus*. The PEF sole viscera extract also showed an inhibitory effect on the growth of *Salmonella*. In addition, some extracts promoted probiotic bacteria growth. For example, the PEF rainbow trout head and skin extracts promoted *Lacticaseibacillus casei* growth, while the ASE rainbow trout head and skin extracts promoted *Bifidobacterium lactis* growth. In addition, some samples, such as the ASE rainbow trout viscera and the PEF sole skin extracts had interesting anti-inflammatory properties. Therefore, the use of ASE and PEF can be considered as useful strategies to recover antimicrobial, prebiotic and anti-inflammatory extracts from rainbow trout and sole side streams, although it is necessary to evaluate each specific side stream.

Keywords: fish side streams; rainbow trout; sole; accelerated solvent extraction; pulsed electric fields; antimicrobial; prebiotic; anti-inflammatory

1. Introduction

Over the last decades, growing attention has been paid to the development of natural and alternative antibiotics, especially due to the large use of the traditional ones, which has led to an increase in drug-resistant bacteria (Lewis, 2017). At the same time, food safety problems caused by food-borne pathogens are also of concern for consumers, which has led researchers to urgently seek new natural antibacterial compounds from food and side streams (Negi, 2012). There are thousands of naturally active compounds or foods that are thought to have antibacterial potential. Marine species in the oceans are an interesting potential source of these antimicrobial compounds (Enan, 2006; Tong et al., 2021).

For instance, around 70% of the earth's surface is covered by water. Marine species represent ≈ 50% of global biodiversity, among which fish resources occupy an important position. As the annual output of fish products increases, some by-products from processing side streams are produced. In the industrial processing of fish, each ton of fish processed produces ≈ 350–600 kg of waste, including head, viscera, bones and so on (Pérez-gálvez et al., 2018; Stevens et al., 2018). These side stream by-products may be used as fertilizers, livestock feeds or directly discarded. The high-value-added bioactive compounds in the side streams have not been utilized very well, causing a waste of resources (Ananey-Obiri et al., 2019).

These side streams contain several biologically active ingredients, such as protein, fish oil, gelatin, etc., which have high application value. For example, fish protein can be used as an important source of high-quality protein, in addition to containing a large amount of collagen. Moreover, it is an important source of bioactive peptides with antioxidant properties (Ananey-Obiri et al., 2019). In addition, previous studies have also shown that some components in fish and their side streams show interesting antibacterial and antiviral capacities. For example, Beaulieu et al. (Beaulieu et al., 2009) confirmed that the enzymatic hydrolysates of mackerel by-products show antibacterial effects on *Listeria*

and *Escherichia coli*; Fuochi et al. (Fuochi et al., 2017) also found that the skin mucus of the *Dasyatis pastinaca* (Linnaeus, 1758) showed antibacterial and anti-fungal effects. So, at this stage of development, there is a growing interest in the valorization of these side streams as potential sources of high-added-value compounds for the development of antioxidants, antimicrobials or antiviral compounds.

Traditionally, heat treatment and/or organic solvent extraction, etc., have been used as conventional extraction methodologies to recover valuable compounds from the food side streams. However, these techniques are not in full correspondence with the green extraction concept as they use large amounts of solvents, which in some cases are toxic, have long extraction times and can have negative effects on thermolabile valuable compounds due to the high temperatures used, among other drawbacks (Chemat et al., 2020; Fuochi et al., 2017).

In this study, two innovative non-thermal approaches, such as pulsed electric fields (PEF) and accelerated solvent extraction (ASE) were applied to improve the extraction rate according to the green extraction concept. As a short-time pulse effect, PEF has been widely studied in non-thermal food processing. The application of PEF disintegrates the biological cell membrane of the food matrix and forms temporary or permanent membrane pores, which can retain the nutritional and health characteristics of the food to a large extent, ensure the taste and improve the extraction rate (Barba et al., 2012). The use of PEF to pretreat fish and algae to extract bioactive compounds has been reported (Luo et al., 2019; Scherer et al., 2019). ASE is also a green and efficient extraction method which works in a high-pressure environment and can increase the extraction rate of the samples through the accumulation of heat and pressure. Due to its environmentally friendly and safe characteristics, ASE has been widely used in the extraction of a variety of high-added-value compounds (Ahmad et al., 2020, 2021) and has recently been shown

to be a useful technique to recover bioactive peptides with antioxidant and antimicrobial properties from salmon side streams (De la Fuente, Pallarés, et al., 2021).

Therefore, in the present work based on a previous study (Wang et al., 2021), the fish side streams of two fish species (rainbow trout and sole) with high nutritional values were selected as the target matrices to recover high-value-added compounds with potential antioxidant, antimicrobial and prebiotic activities. For this purpose, PEF and ASE were used to recover the bioactive compounds from fish side streams (head, skin and viscera), then the protein content and the total antioxidant capacity of the recovered compounds were evaluated. Afterwards, the effect of these recovered compounds on bacterial growth (pathogenic and probiotic) and anti-inflammatory activity was explored.

2. Materials and Methods

2.1. Chemicals and Reagents

Sodium carbonate (Na_2CO_3) was purchased from VWR (Saint-Prix, France). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), AAPH (2,2′-azobis-2-methyl-propanimidamide), ABTS (2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonic acid)), fluorescein sodium salt and potassium persulfate ($K_2S_2O_8$) were purchased from Sigma-Aldrich (Steinheim, Baden-Württemberg, Germany). Ethanol (99%) was acquired from Baker (Deventer, Overijssel, The Netherlands). Potassium dihydrogen phosphate and sodium phosphate dibasic were purchased from VWR International Eurolab S.L. (Barcelona, Spain). Deionized water was obtained by a Milli-Q SP Reagent Water System (Millipore Corporation, Bedford, MA, USA).

2.2. Sample Preparation

The rainbow trout (*Oncorhynchus mykiss*) and sole (*Dover sole*) samples used in the experiments were purchased from a local market in Valencia (Spain). The whole fish was separated in the laboratory to obtain the different fish side streams, including the fish head, skin and viscera for extraction. For PEF treatments, fresh samples were used, while for

the ASE experiments, samples were pretreated and stored at −20 °C for more than 12 h, then freeze-dried at −48 °C for 72 h. The freeze-dried samples were stored at −20 °C until needed.

2.3. Extraction Conditions

2.3.1. PEF-assisted extraction

A PEF-Cellcrack III (German Institute of Food Technologies (DIL)) equipment (ELEA, Quakenbrück, Osnabrück, Germany) was used for the extractions. Specifically, the fish side streams were placed in the treatment chamber and tap water was added. Then, the conductivity was measured to be between 1000 and 2000 µs/cm. The samples were pretreated according to the best conditions previously obtained in the laboratory (**Table 1a**). Samples were transferred to a beaker and were kept under agitation using a magnetic stirrer for a certain period at room temperature. Then, the supernatant was filtered through a 0.22 µm sterile filter membrane to obtain the samples. The control group was obtained under the same conditions but without the application of PEF pretreatment.

Table 1. (**a**) Pulsed electric fields (PEF)-assisted extraction experimental conditions, (**b**) Accelerated solvent extraction (ASE) experimental conditions.

(**a**)

Sample	Weight (g)	Field Strength (kV/cm)	H_2O (mL)	Specific Energy (kJ/kg)	Time (h) [1]
Head	100.25	1.00	1500	219.76	21.33
Skin	45.30	3.00	675	300.00	24.00
Viscera	45.30	3.00	675	123.75	15.17

(**b**)

Sample	T (°C)	Time (min)	pH	Pressure
Head	55	15	5.2	103.4
Skin	45	15	6.5	103.4
Viscera	50	15	6.8	103.4

[1] time of supplementary extraction.

2.3.2. ASE-assisted extraction

Similarly, the selection of ASE conditions was also based on the optimal conditions obtained previously in the laboratory (Fuente et al., 2021). An ASE-200 accelerated solvent extractor (ASE 200 Dionex, Sunnyvale, CA, USA) was used in this study. According to the different samples, it was modified as the ratio of diatomaceous earth: the sample utilized was comprised of 1.0:2.0 g/g, 1.5:3.0 g/g and 2.0:2.0 g/g for the head, skin and viscera, respectively. The samples and diatomaceous earth were mixed in a mortar and transferred to the extraction tank. The standard parameters used for ASE extraction are preheating time (1 min), heating time (5 min), flushing volume (60%), nitrogen scanning (60 s) and pumping pressure (103.4 bar). The other conditions are shown in **Table 1b**. The samples processed by ASE were filtered through a 0.22 μm sterile filter membrane and the control groups were also prepared.

2.4. Chemical Analyzes

2.4.1. Protein Content

The BCA (Bicinchoninic acid) assay was used to determine the protein content of the extracts (Parniakov et al., 2015). The working solution was prepared according to the BCA kit. Bovine serum albumin (0–2000 mg/L) was used as a standard to prepare the standard curve. Ten microliters of sample/standard and 200 μL of BCA working solution were added to the microplate, then the mixture was mixed well and incubated at 37 °C for 30 min. The absorbance of the samples was measured at 562 nm.

2.4.2. Total Antioxidant Capacity

Oxygen Radical Absorbance Capacity Assay (ORAC)

The determination of the ORAC values was carried out according to a previously described method (Cao et al., 1993; de la Fuente, Pallarés, et al., 2021). A phosphate buffer (pH 7.0–7.4) was used as the blank group and 1 mM Trolox solution was the standard. Fifty microliters of the sample and the 50 μL fluorescein sodium salt were added,

respectively, to a 96-well plate, then the 25 μL AAPH was added, and the plate was kept under 37 °C for 10 min. Wavelengths of emission at 520 nm and excitation at 480 nm were established to record the results within 60 min. Then, the antioxidant capacity of the sample was calculated according to the formula:

$$\textbf{ORAC (trolox unit)} = \frac{A_{sample} - A_{blank}}{A_{trolox} - A_{blank}} \quad (1)$$

A measured ORAC value of 1 unit indicates that the antioxidant capacity of the sample solution is equivalent to 100 μM Trolox solution.

Trolox Equivalent Antioxidant Capacity Assay (TEAC)

The TEAC assay was used to determine the ABTS free radical scavenging capacity of the extracts. According to De la Fuente's method and with some modifications, 25 mL ABTS (7 mM) and 440 μL $K_2S_2O_8$ (140 mM) were mixed to obtain an $ABTS^+$ working solution, which was stored at room temperature in the dark for about 12–16 h for use (de la Fuente, Pallarés, et al., 2021). The $ABTS^+$ working solution was diluted with 96% (*v/v*) ethanol to maintain the absorbance of 0.700 ± 0.020 at 734 nm. During the test, the samples were diluted to obtain a 50% free radical inhibition rate. The absorbance of 2 mL of the working solution was recorded as the initial value, then 100 μL of the correct dilution of the samples was added and the absorbance was recorded after 3 min of reaction. Different concentrations of Trolox (0–250 μM) were used as the standard to prepare the standard curve and to calculate the total antioxidant capacity of the samples.

2.4.3. Impact of the Extracts on Bacterial Growth

The impact of the different extracts obtained on several pathogenic and probiotic bacteria was investigated. The culture conditions of the different bacteria are shown in **Table 2**.

Table 2. The culture conditions of the bacteria used in this study.

Bacterial	Collection Number	Culture Medium	Culture Conditions
Listeria innocua	(CECT 910)	BHI [1]	37 °C, 24 h, aerobic
Escherichia coli	(CECT 99)		
Staphylococcus aureus	(CECT 86)		
Salmonella enterica	(CECT 4138)		
Lacticaseibacillus casei	(BB 12)	MRS	37 °C, 48 h, anaerobic
Bifidobacterium lactis	(NCC 2818)	MRS + 0.05% *L*-cys [2]	

[1] BHI: Brain heart infusion medium. [2] MRS: Man rogosa sharpe medium; *L*-cys: *L*-cysteine hydrochloride; CECT: Spanish National Culture Collection (www.cect.org).

In this study, four common pathogenic and two probiotic bacteria that modulate human health were selected to evaluate growth patterns in the presence or absence of the extracts. Bacterial cultures were collected by centrifugation and inoculated in the corresponding medium at a final optical density at 595 nm of 0.05. The culture medium (200 μL) and sample (20 μL) were added to 96-well microplates and incubated in POLARstar (BMG, Labtech, Offenburg, Germany) equipment at 37 °C for 20 h and the optical density was recorded. The Gompertz equation was used to describe the bacterial growth rate and the maximum optical density:

$$\mathbf{y} = \mathrm{K} + \mathrm{A}\exp\left[-\exp\left(-\frac{\mu_{max}e}{\mathrm{A}}\right) + 1\right) \quad (2)$$

where y is the extent of growth at time t (h), K is an initial cell number, A is the change in the number of cells between the inoculum and the stationary phase, μ_{max} is the maximum growth rate (the variation in the number of cells per unit of time), λ is the length of the lag phase (h) and e is a constant (2.7182).

2.4.4. Anti-Inflammatory Analysis

Cell Culture

To investigate the anti-inflammatory potential of the extracts, a reporter gene assay to analyze the activation of the pro-inflammatory transcription factor NF-κB was performed. Therefore, the human colon tumorigenic cell line HT-29 was previously stably transfected with the plasmid pNiFty2-SEAP (Invivogen, CA, USA) containing a secreted alkaline phosphatase (SEAP) reporter gene (Rocchetti et al., 2020). The cell line was routinely cultured in a DMEM high glucose medium, supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin and 150 μg/mL zeocin. Cells were cultured at 37 °C and 5% CO_2 under a humidified atmosphere.

Analysis of NF-κB Activation

In the experiment, 65.000 cells/well were seeded into 96-well plates and grown for 24 h. Then, to investigate the activation of NF-κB, 10 μL of pro-inflammatory cytokine TNF-α was added to achieve a final concentration of 10 ng/mL and 10 μL of the extracts was added to achieve a total volume of 100 μL in each well. After 24 h of stimulation, the supernatant was collected and the cells were lysed in PBS containing 1% Triton, 1 mM PMSF (phenylmethylsulfonyl fluorid) and 1 mM EDTA (ethylenediaminetetraacetic acid). The protein content of each well was determined using the Bradford Protein Assay (Biorad). SEAP activity in the supernatant was measured using *p*-nitrophenyl phosphate as the phosphatase substrate and was normalized to the protein content of each well. The absorbance at 414 nm was measured with a microplate analyzer and the activity of NF-κB induced by TNF-α was recognized to be 100%.

2.5. Statistical Analysis

Significant differences between the results were analyzed by analysis of variance (ANOVA). A Tukey's Multiple Comparison test was used to indicate the significant differences in the means. All statistical analyzes were performed using the software Statgraphics Centurion XVI.I software (Statpoint Technologies, Inc., The Plains, VA, USA).

3. Results and Discussion

3.1. Protein Content

Although the antimicrobial, prebiotic and anti-inflammatory activities were the main focus of this study, first of all, the protein content was evaluated. **Figure 1** shows the effect of the two selected alternative technologies (PEF and ASE) to assist the recovery of protein from the rainbow trout and sole side streams. As can be seen in the table, when ASE-assisted extraction was used, the protein content of the fish head extracts was significantly increased ($p < 0.05$) and the content reached values that were almost 2-fold higher compared to the control group. ASE also significantly ($p < 0.05$) increased the protein content of the fish skin and viscera extracts. Moreover, the impact of the ASE extraction conditions on the recovery of protein from the rainbow trout was even stronger than the pattern found for the sole samples. Conversely, the effect on the sole viscera was stronger than that of rainbow trout, which may be related to the different composition and structure of the fish side streams according to the species evaluated.

Compared with ASE, PEF-assisted extraction had less effect on the protein content of fish side streams extracts. For rainbow trout, PEF-assisted treatment had no significant ($p > 0.05$) effect on the increase in the protein content compared with the control group; the protein content of the fish head and skin extracts was slightly lower than that observed for the control group, while no significant effect was observed for the viscera extracts ($p > 0.05$). The PEF-assisted treatment significantly increased ($p < 0.05$) the protein content of the sole head extract by three times compared to the control group. However, it had a negative impact on the protein content of the sole skin extracts, while there was no

significant difference in the viscera; the results were similar to those found for the rainbow trout.

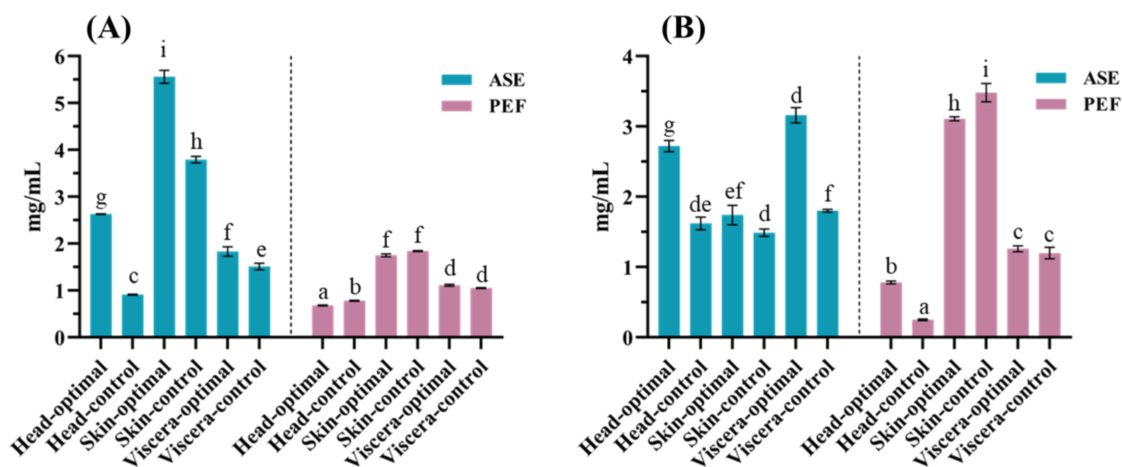

Figure 1. Protein content in the control and optimal PEF/ASE assisted extracts from fish by-products. (**A**) rainbow trout and (**B**) sole; PEF: pulsed electric fields; ASE: accelerated solvent extraction; Different lowercase letters (a–i) in the figure indicate statistically significant differences ($p < 0.05$).

Fish side streams contain a large amount of protein and other bioactive compounds with high-added value, which can be used as sources of high-quality amino acids. In recent years, in order to reduce the waste of fish side streams, a variety of technologies have been used for the recoanalyvery of protein from fish side streams. In this line, Álvarez et al. (Álvarez et al., 2018) used ultrasound-assisted acid/alkaline isoelectric solubilization precipitation to recover the protein in mackerel. The results showed that compared with traditional methods, ultrasound-assisted acid/alkaline isoelectric solubilization precipitation can significantly increase the protein recovery rate from 50–64% to 94%. Similarly, Khawli et al. (Khawli et al., 2021) also used ultrasound to assist the extraction of protein from the sea bass side stream. Under ultrasound-assisted treatment, the protein content of the fish side stream extracts was significantly increased, with the highest modifications observed for the viscera samples. ASE and PEF have also

recently been used as green processing technologies to assist the recovery of nutrients and bioactive compounds from the side streams of different species. For instance, De la Fuente et al. (De la Fuente, et al., 2021) used pressurized liquid-extraction (PLE)-assisted technology to obtain protein with antioxidant activity from the sea bream side stream and showed that the protein recovery rate could reach 1.2–4.5 times that of the control group. During PLE processing, high pressures and temperature increase the solubility and diffusion rate of high-added-value compounds, thereby improving their extraction efficiency.

3.2. Total Antioxidant Capacity

Figure 2 shows the antioxidant capacity of different extracts. Oxygen-free radical absorbance capacity (ORAC) and Trolox equivalent antioxidant capacity (TEAC) were used to evaluate the antioxidant capacity of the extracts. For the rainbow trout side stream extracts, both the ASE and PEF treatments improved the ORAC values of the extracts, showing significant ($p < 0.05$) differences, except for the PEF-assisted skin sample. Among the different side stream extracts, the skin extract obtained under ASE-assisted treatment showed the most obvious difference, which was about three times higher than that observed for the control group. The TEAC values also showed the same trend after the application of ASE, with a significant increase observed for all the extracts independently of the target side streams evaluated. For instance, compared with the control group, the TEAC values increased by about 1.4–3.3 times. On the contrary, after PEF-assisted treatment, the TEAC values of the PEF fish head extracts were slightly lower than those of the control samples. Moreover, no significant effect on the TEAC values was observed after applying PEF to the skin compared to the control samples ($p > 0.05$). However, interestingly, PEF enhanced the TEAC values of the viscera extracts to a certain extent.

Regarding the sole side streams, the behavior of the ORAC values of the extracts was similar to that found for rainbow trout and thus showed a considerable improvement in the ORAC values compared to control samples. However, no significant ($p > 0.05$) differences in the ORAC values were observed for ASE sole skin compared to the control group. The PEF treatment also contributed to an enhancement of the ORAC values of the sole side stream extracts; however, no significant differences ($p > 0.05$) in the ORAC values of the extracts were found for the PEF skin and viscera compared to the control group.

ASE also significantly increased the TEAC values of the extracts, which was about 1.7–2.0 times higher than that of the control group (without ASE). However, the impact of PEF on the TEAC values of the sole extracts was different from that found after applying ASE. For instance, PEF increased ($p > 0.05$) the TEAC values of the head extracts without any significant differences being observed. Two different methods have shown different effects on the antioxidant capacity of the extracts. Previously, the antioxidant capacity of the extracts without sterile membrane filtration was also measured (Wang et al., 2021). Comparing the results before and after filtration, it was found that filtration had relatively little effect on the antioxidant capacity of the extracts, meaning the nutrients and antioxidant compounds in the extracts were well retained, which provides a basis for further experiments.

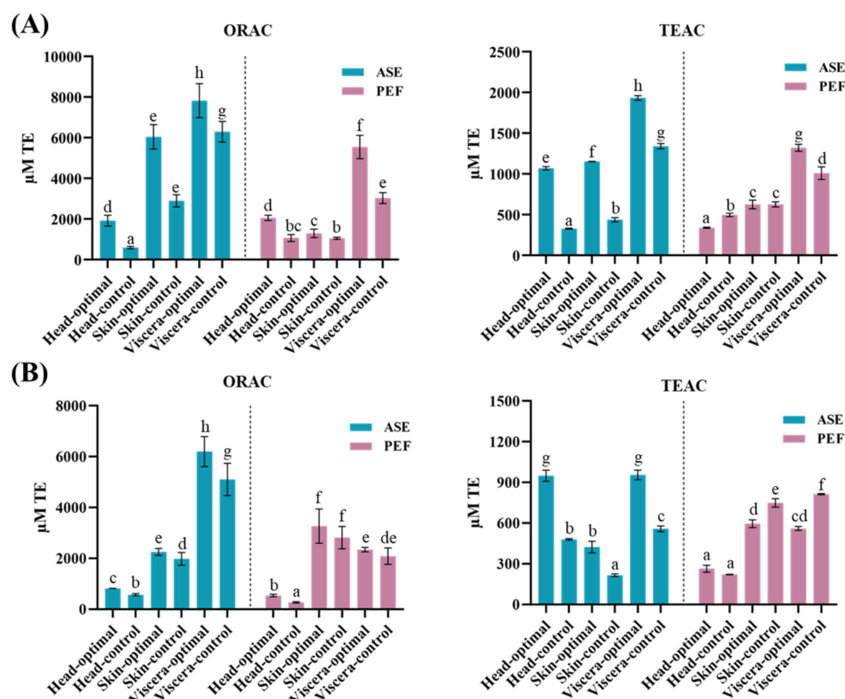

Figure 2. Total antioxidant capacity (ORAC and TEAC) in the control and optimal PEF/ASE-assisted extracts from fish by-products. (**A**) rainbow trout and (**B**) sole; PEF: pulsed electric fields; ASE: accelerated solvent extraction; Different lowercase letters in the same fish species indicate significant statistical differences between samples ($p < 0.05$). ORAC: oxygen radical absorbance capacity; ABTS: $ABTS^+$ scavenging ability.

3.3. Impact of Fish Side Stream Extracts on Bacterial Growth

3.3.1. Antibacterial Activity Against Pathogenic Bacteria

The effects of the different extracts on the growth of four common pathogens are presented in **Tables 3** and **4**. The growth rate of different bacteria in 20 h and the optical density of bacterial strains were obtained by fitting the Gompertz equation (Zwietering et al., 1990). As can be depicted from Table 3, the extracts obtained from the rainbow trout side streams after applying PEF and ASE induced the growth of *Listeria* and *E. coli*, with the viscera extracts showing the most obvious effect. In addition, the viscera extract also significantly increased the optical density of bacteria. When the effect of PEF was evaluated, it was observed that the extracts obtained from the head and skin had a

significant effect on the growth of *E. coli* ($p < 0.05$), but there was not a significant effect on the optical density ($p > 0.05$) independently of the PEF treatment. Compared to the control group, the ASE-assisted extracts reduced the optimal density of the viscera extract, with no significant differences observed with the blank group (without the ASE extract).

Moreover, for the PEF extracts, no significant effect was found on the growth of *S. aureus*. For instance, the extracts increased the optical density of bacterial growth, showing a significant difference for the viscera extracts ($p < 0.05$). Unlike PEF, the head and skin extracts obtained by ASE reduced the growth rate of *S. aureus* ($p < 0.05$), but the viscera extract showed an inducing effect on the growth of *S. aureus.* The addition of the extracts did not show any inhibitory effect on the growth of *Salmonella* and had no significant effect on the optical density of the bacteria. In summary, among the four pathogens, the rainbow trout head, and the skin extracts had an inhibitory impact on *S. aureus*, which was significant ($p < 0.05$) when ASE was used.

Table 3. Effect of rainbow trout extracts on the growth rate and maximal optical density of four pathogenic bacteria strains.

Sample	PEF [1]		ASE [2]	
	Growth Rate ($\mu max \cdot h^{-1}$)	***MOD**	**Growth Rate ($\mu max \cdot h^{-1}$)**	***MOD**
Listeria				
Bacteria-control	0.442 ± 0.027 [a]	1.558 ± 0.039 [a]	0.435 ± 0.017 [a]	1.524 ± 0.015 [a]
Head	0.472 ± 0.004 [a]	1.538 ± 0.046 [a]	0.479 ± 0.025 [a]	1.511 ± 0.019 [a]
Head-control	0.461 ± 0.001 [a]	1.543 ± 0.022 [a]	0.454 ± 0.016 [a]	1.525 ± 0.031 [a]
Skin	0.464 ± 0.001 [a]	1.503 ± 0.001 [a]	0.467 ± 0.014 [a]	1.588 ± 0.017 [b]
Skin-control	0.475 ± 0.006 [a]	1.498 ± 0.026 [a]	0.435 ± 0.011 [a]	1.486 ± 0.020 [a]
Viscera	0.599 ± 0.007 [c]	1.692 ± 0.023 [b]	0.576 ± 0.007 [b]	1.666 ± 0.026 [c]
Viscera-control	0.526 ± 0.031 [b]	1.686 ± 0.015 [b]	0.614 ± 0.037 [b]	1.635 ± 0.028 [b,c]
E. coli				

Bacteria-control	0.176 ± 0.009 ^{a}	2.346 ± 0.009 ^{a}	0.176 ± 0.009 ^{a,b}	2.346 ± 0.009 ^{a,b}
Head	0.208 ± 0.002 ^{b}	2.300 ± 0.044 ^{a}	0.180 ± 0.002 ^{b}	2.307 ± 0.061 ^{a,b}
Head-control	0.201 ± 0.001 ^{b}	2.284 ± 0.030 ^{a}	0.193 ± 0.003 ^{c}	2.203 ± 0.034 ^{a}
Skin	0.194 ± 0.009 ^{b}	2.318 ± 0.033 ^{a}	0.185 ± 0.002 ^{b}	2.472 ± 0.103 ^{b}
Skin-control	0.195 ± 0.011 ^{b}	2.292 ± 0.006 ^{a}	0.168 ± 0.003 ^{a}	2.338 ± 0.120 ^{a,b}
Viscera	0.172 ± 0.000 ^{a}	2.644 ± 0.046 ^{b}	0.275 ± 0.002 ^{d}	2.188 ± 0.038 ^{a}
Viscera-control	0.178 ± 0.001 ^{a}	2.730 ± 0.064 ^{b}	0.167 ± 0.008 ^{a}	2.757 ± 0.135 ^{c}
S. aureus				
Bacteria-control	0.591 ± 0.039 ^{b}	2.216 ± 0.215 ^{a}	0.524 ± 0.056 ^{c,d}	2.401 ± 0.047 ^{a}
Head	0.560 ± 0.054 ^{a,b}	2.309 ± 0.142 ^{a}	0.441 ± 0.041 ^{a,b}	2.559 ± 0.018 ^{a,b}
Head-control	0.559 ± 0.003 ^{a,b}	2.309 ± 0.124 ^{a}	0.448 ± 0.026 ^{b}	2.455 ± 0.053 ^{a}
Skin	0.505 ± 0.043 ^{a,b}	2.545 ± 0.126 ^{a,b}	0.404 ± 0.008 ^{a}	2.796 ± 0.034 ^{c}
Skin-control	0.496 ± 0.036 ^{a}	2.533 ± 0.135 ^{a,b}	0.482 ± 0.003 ^{b,c}	2.492 ± 0.048 ^{a}
Viscera	0.550 ± 0.026 ^{a,b}	2.751 ± 0.067 ^{b}	0.579 ± 0.037 ^{d}	2.724 ± 0.034 ^{b,c}
Viscera-control	0.596 ± 0.041 ^{b}	2.579 ± 0.077 ^{a,b}	0.578 ± 0.049 ^{d}	2.642 ± 0.053 ^{b,c}
Salmonella				
Bacteria-control	0.335 ± 0.026 ^{a}	1.838 ± 0.065	0.335 ± 0.026 ^{a}	1.838 ± 0.065
Head	0.353 ± 0.030 ^{a,b}	1.831 ± 0.164	0.308 ± 0.002 ^{a}	1.714 ± 0.151
Head-control	0.361 ± 0.025 ^{a,b}	1.756 ± 0.151	0.308 ± 0.005 ^{a}	1.655 ± 0.055
Skin	0.323 ± 0.007 ^{a}	1.859 ± 0.043	0.315 ± 0.024 ^{a}	1.766 ± 0.157
Skin-control	0.346 ± 0.022 ^{a,b}	1.810 ± 0.171	0.302 ± 0.025 ^{a}	1.798 ± 0.049
Viscera	0.308 ± 0.021 ^{a}	1.863 ± 0.214	0.418 ± 0.021 ^{b}	1.687 ± 0.049
Viscera-contro	0.390 ± 0.005 ^{b}	1.678 ± 0.132	0.447 ± 0.031 ^{b}	1.675 ± 0.004

[1] PEF: pulsed electric fields. [2] ASE: accelerated solvent extraction. Results are expressed as mean ± standard deviation. Different lowercase letters in the same column (in group with the same bacteria) indicate a statistical difference ($p < 0.05$) based on the sample type and treatment methods used ($p < 0.05$). *MOD: maximal optical density measured at 595 nm (difference between initial and final optical density); Bacteria-control: bacterial growth without fish by-products extracts; Head-control/Skin-control/Viscera-control: Head/Skin/Viscera extracts without PEF/ASE-assisted treatment.

As is depicted in **Table 4**, the viscera extracts promoted the growth rate of *Listeria* and increased the optical density, while the sole head and skin extracts did not have a significant ($p > 0.05$) effect on the growth of *Listeria*. Independently of the treatment applied (PEF or ASE), no significant effect was observed. The addition of the extracts induced the growth of *E. coli* and the extracts from the head and viscera had a significant effect on the growth of bacteria ($p < 0.05$). However, the addition of the head extracts reduced the optical density of *E. coli*, while no effect was observed for the PEF extracts on the growth of *E. coli*. When ASE was used, similar to PEF, the extracts showed a promoting effect on *E. coli* growth and the application of ASE did not show any significant ($p > 0.05$) effect on the results.

For the extracts obtained after the application of PEF, the head and skin extracts did not have any significant effect on the growth and optical density of *S. aureus*, but the addition of viscera extracts reduced the growth rate of *S. aureu*s. Meanwhile, compared with the control group, the PEF-assisted viscera extracts showed significant differences ($p < 0.05$). In contrast to the PEF samples, the ASE viscera extracts did not have a significant effect on the growth rate of *S. aureus* but increased the optical density. The head and skin extracts showed the opposite trend on the growth rate of *S. aureus*. For instance, compared to the control group, the ASE head extracts significantly reduced the growth rate of *S. aureus* ($p < 0.05$), while the ASE skin extract had a weaker effect on the *S. aureus* growth rate than that of the extracts without ASE treatment. Except for the ASE head and skin extracts, the other extracts did not show any significant effect on the optical density of *S. aureus* ($p > 0.05$).

Table 4. Effect of the sole extracts on the growth rate and maximal optical density of four pathogenic bacteria strains.

Sample	PEF [1]		ASE [2]	
	Growth Rate ($\mu max \cdot h^{-1}$)	***MOD**	**Growth Rate ($\mu max \cdot h^{-1}$)**	***MOD**
Listeria				
Bacteria-control	0.442 ± 0.027 [a,b]	1.558 ± 0.039 [a,b]	0.435 ± 0.017 [a,b]	1.524 ± 0.015 [a]
Head	0.427 ± 0.032 [a,b]	1.518 ± 0.022 [a]	0.454 ± 0.009 [a,b]	1.603 ± 0.008 [b,c]
Head-control	0.426 ± 0.034 [a,b]	1.563 ± 0.025 [a,b]	0.442 ± 0.019 [a,b]	1.554 ± 0.041 [a,b]
Skin	0.395 ± 0.014 [a]	1.498 ± 0.040 [a]	0.412 ± 0.036 [a]	1.503 ± 0.037 [a]
Skin-control	0.396 ± 0.041 [a]	1.551 ± 0.005 [a]	0.456 ± 0.016 [a,b]	1.512 ± 0.016 [a]
Viscera	0.468 ± 0.000 [b]	1.634 ± 0.021 [c]	0.502 ± 0.009 [c]	1.613 ± 0.015 [c]
Viscera-control	0.473 ± 0.017 [b]	1.622 ± 0.017 [bc]	0.479 ± 0.016 [b,c]	1.616 ± 0.005 [c]
E. coli				
Bacteria-control	0.176 ± 0.009 [a]	2.346 ± 0.009 [b,c]	0.176 ± 0.009 [a]	2.346 ± 0.009 [c]
Head	0.208 ± 0.005 [c]	2.182 ± 0.070 [a]	0.207 ± 0.008 [c,d]	2.274 ± 0.039 [b]
Head-control	0.213 ± 0.007 [c]	2.218 ± 0.041 [a]	0.191 ± 0.002 [a,b]	2.256 ± 0.029 [b]
Skin	0.182 ± 0.005 [a,b]	2.343 ± 0.004 [b,c]	0.200 ± 0.003 [b,c]	2.202 ± 0.106 [a,b]
Skin-control	0.200 ± 0.009 [b,c]	2.273 ± 0.044 [a,b]	0.204 ± 0.000 [b,c,d]	2.159 ± 0.059 [a]
Viscera	0.212 ± 0.013 [c]	2.391 ± 0.038 [c]	0.216 ± 0.006 [d]	2.299 ± 0.003 [b,c]
Viscera-control	0.211 ± 0.003 [c]	2.337 ± 0.022 [b]	0.215 ± 0.003 [d]	2.243 ± 0.022 [a,b]
S.aureus				
Bacteria-control	0.591 ± 0.039 [b,c]	2.401 ± 0.047 [b,c]	0.524 ± 0.056 [c]	2.401 ± 0.047 [a]
Head	0.533 ± 0.046 [b,c]	2.546 ± 0.178 [c,d]	0.452 ± 0.026 [b]	2.721 ± 0.009 [c]
Head-control	0.616 ± 0.025 [c]	2.216 ± 0.086 [a]	0.474 ± 0.004 [b,c]	2.483 ± 0.056 [a,b]
Skin	0.623 ± 0.008 [c]	2.300 ± 0.067 [a,b]	0.425 ± 0.024 [a,b]	2.434 ± 0.104 [a]

Skin-control	0.533 ± 0.060 [b,c]	2.530 ± 0.123 [b,c,d]	0.378 ± 0.032 [a]	2.385 ± 0.078 [a]
Viscera	0.458 ± 0.046 [a]	2.726 ± 0.007 [d]	0.523 ± 0.043 [c]	2.634 ± 0.076 [b,c]
Viscera-control	0.513 ± 0.013 [a,b]	2.650 ± 0.115 [d]	0.531 ± 0.018 [c]	2.542 ± 0.101 [a,b]
Salmonella				
Bacteria-control	0.335 ± 0.026 [b]	1.838 ± 0.065	0.335 ± 0.026	1.838 ± 0.065
Head	0.300 ± 0.007 [a,b]	1.676 ± 0.037	0.284 ± 0.020	1.656 ± 0.071
Head-control	0.280 ± 0.016 [a]	1.715 ± 0.018	0.303 ± 0.011	1.658 ± 0.035
Skin	0.335 ± 0.029 [b]	1.817 ± 0.191	0.323 ± 0.029	1.696 ± 0.108
Skin-control	0.338 ± 0.020 [b]	1.771 ± 0.146	0.339 ± 0.022	1.686 ± 0.145
Viscera	0.287 ± 0.018 [a]	1.779 ± 0.146	0.282 ± 0.003	1.799 ± 0.138
Viscera-control	0.276 ± 0.001 [a]	1.784 ± 0.151	0.308 ± 0.018	1.796 ± 0.145

[1] PEF: pulsed electric fields. [2] ASE: accelerated solvent extraction. Results are expressed as mean ± standard deviation. Different lowercase letters in the same column (in group with the same bacteria) indicate a statistical difference ($p < 0.05$) based on the sample type and treatment methods used ($p < 0.05$). *MOD: maximal optical density measured at 595 nm (difference between initial and final optical density). Bacteria-control: bacterial growth without fish by-products extracts; Head-control/Skin-control/Viscera-control: Head/Skin/Viscera extracts without PEF/ASE-assisted treatment.

Since fish side streams contain a large number of high-added-value compounds, many people have explored their antibacterial properties over the last years to further expand their applications in food and health. For instance, Robert et al. (Robert et al., 2015) evaluated the *in vitro* antibacterial activity of the tilapia by-product hydrolysate. The peptides produced by hydrolysis showed important antibacterial activity against *Yersinia ruckeri*. Moreover, they also observed the resistance of these hydrolysates against *Edwardsiella tarda* and *Bacillus megaterium*, thus indicating that tilapia by-products have important antimicrobial activity. In another study, Ennaas et al. (Ennaas et al., 2015) used different proteases to hydrolyse the by-products of Atlantic mackerel skin and the antibacterial properties of hydrolyzed collagen were evaluated. The results showed that

the crude hydrolysates of mackerel had an inhibitory effect on *Listeria* and *E. coli*, while the inhibition rate varied according to the different hydrolysates used. In addition to the properties against the different pathogens, the impact on the antibacterial properties varied with the type of fish. Previous studies have shown that fish by-products with a large number of low-molecular-weight peptides had a higher activity (He et al., 2013). In this study, two kinds of fish showed inhibitory effects on *S. aureus* and could be considered as potential sources of new antibacterial products.

3.3.2. Effect on the Growth of Probiotic Bacteria

Table 5 shows the effect of different extracts on the growth of two probiotics. By analysing the effect of rainbow trout on the growth of probiotics, it can be seen that the addition of the head and skin extracts in the PEF group promoted the growth of *Lacticaseibacillus casei* with significant differences ($p < 0.05$), while no significant differences were observed between PEF and the control group ($p > 0.05$). Moreover, these extracts also increased the optical density of *Lacticaseibacillus casei*. Compared to the control group, the optical density of the PEF extracts was lower than that of the control group, with no significant differences observed between the two skin extracts ($p > 0.05$). In addition, the viscera extracts did not have any significant effect on the growth rate and optical density of the *Lacticaseibacillus casei*. Studying the effect of the PEF extracts on *Bifidobacterium lactis*, it was observed that the addition of the extracts did not have any significant effect on the growth rate of *Bifidobacterium lactis*.

On the other hand, the addition of the ASE extracts reduced the growth rate of *Lacticaseibacillus casei*. Compared with the control group, the head and viscera extracts obtained with the ASE-assisted treatment did not show any significant difference on the *Lacticaseibacillus casei* growth rate. The ASE-assisted skin extract had a weaker effect on the growth rate of *Lacticaseibacillus casei* than the control group (skin-control) and this difference was significant ($p < 0.05$). At the same time, it was also seen that the head

and skin extracts increased the optical density of the *Lacticaseibacillus casei*, showing a significant difference compared to the control group, but there was not a significant effect of ASE-assisted extracts ($p > 0.05$). Compared to the control group, the ASE-assisted extracts increased the growth rate of *Bifidobacterium lactis*, but no significant differences were observed. For example, the addition of the ASE-assisted viscera extracts did not have any effect on the growth rate of *Bifidobacterium lactis*. On the other hand, the head and skin extracts increased the optical density of *Bifidobacterium lactis* and the optical density of the ASE-assisted extracts was significantly higher than that of the control group.

Table 5. Effect of the rainbow trout and sole extracts on the growth rate and maximal optical density of two probiotic bacteria strains.

Fish	Sample	PEF [1]		ASE [2]	
		Growth Rate (μmax·h $^{-1}$)	*MOD	Growth Rate (μmax·h $^{-1}$)	*MOD
Rainbow trout	*Lacticaseibacillus casei*				
	Bacteria-control	0.349 ± 0.008 [a,b]	3.597 ± 0.011 [a,b]	0.360 ± 0.012 [c]	1.524 ± 0.015 [a]
	Head	0.382 ± 0.011 [c,d]	3.681 ± 0.038 [b]	0.349 ± 0.007 [b,c]	1.603 ± 0.008 [b,c]
	Head-control	0.374 ± 0.004 [c,d]	3.836 ± 0.053 [c]	0.337 ± 0.009 [b]	1.554 ± 0.041 [ab]
	Skin	0.369 ± 0.002 b[c,d]	3.719 ± 0.062 [b,c]	0.334 ± 0.007 [b]	1.503 ± 0.037 [a]
	Skin-control	0.390 ± 0.017 [d]	3.683 ± 0.069 [b]	0.288 ± 0.011 [a]	1.512 ± 0.016 [a]
	Viscera	0.360 ± 0.000 [b,c]	3.533 ± 0.036 [a]	0.283 ± 0.006 [a]	1.613 ± 0.015 [c,d]
	Viscera-control	0.336 ± 0.012 [a]	3.595 ± 0.071 [a,b]	0.271 ± 0.005 [a]	1.616 ± 0.005 [d]
	Bifidobacterium lactis				
	Bacteria-control	0.536 ± 0.027	3.597 ± 0.011 [b]	0.536 ± 0.027 [a,b]	2.346 ± 0.009 [a,b,c]
	Head	0.542 ± 0.035	3.681 ± 0.006 [c]	0.557 ± 0.019 [b]	2.274 ± 0.039 [a,b]
	Head-control	0.544 ± 0.027	3.836 ± 0.006 [d]	0.536 ± 0.024 [a,b]	2.256 ± 0.029 [a,b]
	Skin	0.508 ± 0.028	3.719 ± 0.000 [d]	0.547 ± 0.014 [b]	2.202 ± 0.106 [ab]

	Skin-control	0.561 ± 0.014	3.683 ± 0.010 [c]	0.498 ± 0.008 [a]	2.159 ± 0.059 [a]
	Viscera	0.550 ± 0.021	3.533 ± 0.007 [a]	0.529 ± 0.010 [a,b]	2.299 ± 0.003 [a,b,c]
	Viscera-control	0.532 ± 0.023	3.595 ± 0.009 [a]	0.530 ± 0.007 [a,b]	2.243 ± 0.022 [a,b]
	Lacticaseibacillus casei				
	Bacteria-control	0.349 ± 0.008 [b,c]	3.597 ± 0.020 [a,b]	0.360 ± 0.012 [c]	2.401 ± 0.047 [a]
	Head	0.405 ± 0.003 [d]	3.565 ± 0.053 [a]	0.355 ± 0.010 [c]	2.721 ± 0.009 [c]
	Head-control	0.365 ± 0.007 [c]	3.572 ± 0.041 [a]	0.357 ± 0.002 [c]	2.483 ± 0.056 [a,b]
	Skin	0.338 ± 0.000 [b]	3.637 ± 0.076 [a,b]	0.273 ± 0.001 [a]	2.434 ± 0.104 [a]
	Skin-control	0.345 ± 0.025 [b,c]	3.662 ± 0.011 [a,b]	0.324 ± 0.016 [b]	2.385 ± 0.078 [a]
	Viscera	0.308 ± 0.004 [a]	3.663 ± 0.014 [ab]	0.363 ± 0.001 [c]	2.634 ± 0.076 [b,c]
Sole	Viscera-control	0.305 ± 0.002 [a]	3.695 ± 0.027 [b]	0.328 ± 0.007 [b]	2.542 ± 0.101 [a,b,c]
	Bifidobacterium lactis				
	Bacteria-control	0.536 ± 0.027	3.597 ± 0.011 [a]	0.536 ± 0.027 [b,c]	1.838 ± 0.065
	Head	0.526 ± 0.045	3.565 ± 0.003 [a]	0.499 ± 0.010 [a]	1.656 ± 0.071
	Head-control	0.517 ± 0.016	3.572 ± 0.008 [a]	0.483 ± 0.003 [a]	1.658 ± 0.035
	Skin	0.537 ± 0.001	3.637 ± 0.001 [b]	0.504 ± 0.009 [a,b]	1.696 ± 0.108
	Skin-control	0.545 ± 0.015	3.662 ± 0.004 [b]	0.553 ± 0.033 [d]	1.686 ± 0.145
	Viscera	0.541 ± 0.034	3.663 ± 0.029 [b]	0.544 ± 0.027 [c,d]	1.799 ± 0.138
	Viscera-control	0.527 ± 0.008	3.695 ± 0.036 [c]	0.530 ± 0.018 [b,c]	1.796 ± 0.145

[1] PEF: pulsed electric fields. [2] ASE: accelerated solvent extraction. Results are expressed as mean ± standard deviation. Different lowercase letters in the same column (in group with the same bacteria) indicate a statistical difference ($p < 0.05$) based on the sample type and treatment methods used ($p < 0.05$). *MOD: maximal optical density measured at 595 nm (difference between initial and final optical density). Bacteria-control: bacterial growth without fish by-products extracts; Head-control/Skin-control/Viscera-control: Head/Skin/Viscera extracts without PEF/ASE-assisted treatment.

The effect of the sole side stream extracts on probiotic growth was also studied in this work. As can be seen from **Table 5**, the PEF-assisted head extract increased the growth rate of *Lacticaseibacillus casei*, which was significantly different from the control group

(without PEF) and blank group. The viscera extract inhibited the growth of *Lacticaseibacillus casei* and the addition of the other side streams did not have any significant effect on the growth of *Lacticaseibacillus casei*. The addition of the PEF-assisted extracts did not have a significant effect on the optical density of the *Lacticaseibacillus casei*. At the same time, under the PEF-assisted treatment, the addition of different extracts did not have a significant effect on the growth rate of *Bifidobacterium lactis*, but the skin and viscera extracts increased the optical density of *Bifidobacterium lactis* and the optical density of the control group was higher than that of the PEF-assisted extraction.

Exploring the effect of ASE, it was observed that the skin extracts significantly ($p < 0.05$) reduced the growth rate of *Lacticaseibacillus casei*, thus ASE had an obvious impact on the results. The ASE-assisted viscera and head extracts did not have a significant effect on the growth rate of *Lacticaseibacillus casei*, while the viscera extract without ASE-assisted treatment reduced the growth rate of *Lacticaseibacillus casei*. In addition, all the extracts increased the optical density, but there was not a significant difference between them ($p > 0.05$). At the same time, the addition of the head extract decreased the growth rate of *Bifidobacterium lactis*, but ASE did not have any significant effect on the results. Moreover, the skin and viscera extracts did not show any significant effect on the growth rate of *Bifidobacterium lactis*. Compared to the control group, the ASE-assisted head and skin extracts increased the optical density of *Bifidobacterium lactis* and the viscera extract also increased the optical density, but no impact of ASE was observed ($p > 0.05$).

In recent years, many studies have shown that bioactive compounds and hydrolysates derived from fish side streams have antibacterial activity, which can inhibit the growth of pathogenic bacteria to a certain extent, although there are only a few studies on the growth of probiotics. Probiotics are used in the fermentation and preservation of food to help

maintain food quality and improve nutrition. In the study of Safari et al. (Safari et al., 2012), two different peptones were obtained from the yellowfin tuna (*Thunnus albacares*) head by enzymatic hydrolysis and their effects on the growth of a variety of bacteria including pathogens and probiotics were explored. The results obtained by these authors showed that the proteins obtained by hydrolysis promoted the growth of *Lactobacillus plantarum* and *Lactobacillus bulgaricus*, making their growth rate higher than that of *Lactobacillus sakei* and others. It has been speculated that the main reason for this may be that the growth of *Lactobacillus* from different sources has different requirements for the types of amino acids, while the matching degree of the peptides produced by enzymatic hydrolysis to different types of *Lactobacillus* is also different. Combined with the effect of the extract on the pathogenic bacteria, it can be seen that the type of peptide in the extract will affect the growth of bacteria, while peptides of appropriate molecular weight can make probiotics show greater growth activity. In addition, the difference in the form and concentration of the samples during PEF and ASE-assisted extraction is also one of the reasons for the different results.

3.4. Anti-Inflammatory Activity

The anti-inflammatory potential of the rainbow trout and sole fish side streams are shown in **Figure 3**. For rainbow trout, the PEF extracts did not show any significant anti-inflammatory potential, while the ASE extracts had a significant inhibitory effect on NF-κB activity, which could inhibit ≈ 40–45% of TNF-α-induced NF-κB activity in the viscera extracts. On the other hand, for sole side streams, the PEF skin extracts showed a significant anti-inflammatory potential, inhibiting the TNF-α activity by ≈ 35%. The sole head extract without ASE treatment showed significant anti-inflammatory potential, while the other extracts did not show any significant difference. Interestingly, in the PEF group of rainbow trout extracts, the extracts from the skin and viscera (without PEF and ASE treatments) enhanced the TNF-induced NF-κB activity to levels of 150% and 126%,

suggesting that the PEF treatment could alter some components of these extracts and reduce their intrinsic pro-inflammatory potential. A similar response in SEAP activity was observed for sole extracts in PEF treated skin *vs*. skin-control extracts.

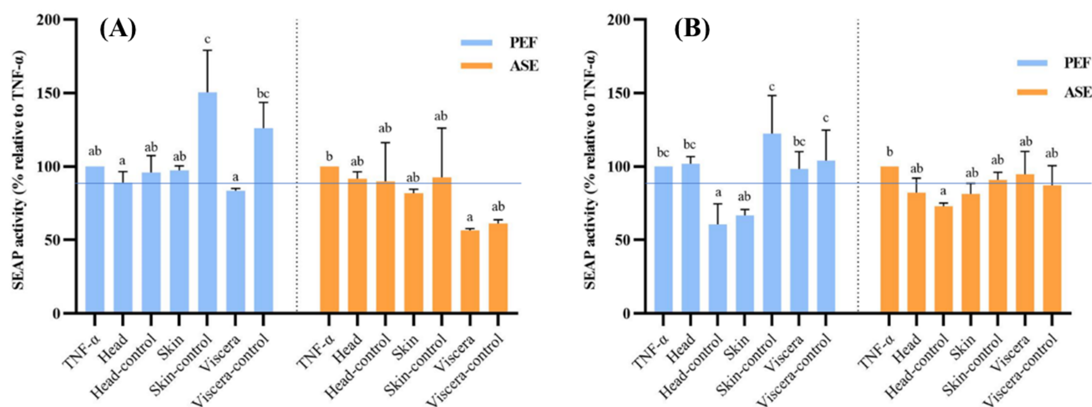

Figure 3. The NF-κB activation induced by TNF-α and the effect of fish by-product extracts using different treatment technology (PEF/ASE) were determined. (**A**) rainbow trout and (**B**) sole; the SEAP activity induced by TNF-α was considered to be 100%. PEF: pulsed electric fields; ASE: accelerated solvent extraction; Different lowercase letters in the same fish species indicate significant statistical differences between samples ($p < 0.05$).

Inflammation is the immune system's response that can effectively protect our body from injury and infection; however, the excessive release of inflammatory mediators can become chronic and lead to many inflammatory diseases. In the intestinal tract, pro-inflammatory stimulants activate in intestinal epithelial cells NF-κB, a master regulator of inflammatory processes among several others, which upregulates cytokines and chemokines (Ávila-Román et al., 2018; Peng et al., 2021). Studies have shown that protein polypeptides are anti-inflammatory, anti-hypertensive, etc. For instance, Gao et al. (Gao et al., 2020) obtained synthetic peptides from sturgeon muscle and found that they can effectively reduce the release of inflammatory mediators and cytokines. It can

be speculated that the anti-inflammatory potential of some extracts may be related to the bioactive peptide in extracts.

4. Conclusions

From the results obtained in this study, it can be concluded that PEF and ASE may be used as useful alternative approaches in recovering extracts with antimicrobial, prebiotic and anti-inflammatory properties. Some extracts showed antibacterial and anti-inflammatory effects, including those obtained by ASE rainbow trout and sole head and skin extracts that promoted inhibitory effects on the growth of *S. aureus* and *Salmonella*. When PEF was studied, the PEF rainbow trout head and skin extracts also showed an inhibitory effect on the growth of *S. aureus*. In addition, they also enhanced the growth of *Lacticaseibacillus casei*. It was also found that some extracts showed anti-inflammatory potential, including those obtained from the ASE and non-ASE rainbow trout viscera, the PEF sole skin and the non-PEF sole head. This may be because the bioactive peptides in them play a vital role. In general, these extracts can be considered as potentially valuable functional substances to further study their beneficial effects on humans.

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4.3 Evaluation of heavy metals, mycotoxins and mineral bioaccessibility through *in vitro* static digestion models of rainbow trout (*Oncorhynchus mykiss*) and sole (*Dover sole*) side stream extracts obtained by accelerated solvent extraction (ASE) treatment

(under review)

Evaluation of heavy metals, mycotoxins and mineral bioaccessibility through *in vitro* static digestion models of rainbow trout (*Oncorhynchus mykiss*) and sole (*Dover sole*) side stream extracts obtained by Accelerated Solvent Extraction (ASE) treatment

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Abstract

Fishery processing is often accompanied by the production of by-products containing high-value bioactive compounds. In this study, rainbow trout (*Oncorhynchus mykiss*) and sole (*Dover sole*) by-products (including head, skin and viscera) were used as the research object and accelerated solvent extraction (ASE) technologies as recovery methods to obtain high-value extracts. Potential contaminants (including mycotoxins and heavy metals) were evaluated in the extracts, the results showed that no mycotoxins were detected in the extracts and the content of heavy metals (Hg, Cd and Pb) was within the allowable range. Then, the effect of digestion on the antioxidant capacity and minerals contents of the extracts obtained under different treatment conditions (ASE-assisted and no-ASE) were explored by establishing an *in vitro* static digestion model, and the bioaccessibility of minerals in the extracts was assessed. The more significant effects included digestion reduced the antioxidant capacity of rainbow trout skin extract under ASE-assisted treatment. In addition, mineral recovery rates and their bioaccessibility in extracts with ASE-assisted treatment were also evaluated. ASE-assisted treatment can obviously improve the recovery rate of Mg and Fe in the rainbow trout viscera extract, and also promote the recovery of Mg, Fe and Zn in sole viscera extract. Moreover, the bioaccessibility of minerals in the extracts was also assessed, showing different effects. Understanding the digestion and absorption of high-value components can helps to maximize their utilization and achieve higher application value.

Keywords: Fish side stream, bioactive compounds, antioxidants, mineral, bioaccessibility

1. Introduction

In recent years, global seafood production has shown a continuous increase. According to the forecast of FAO (Food and Agriculture Organization of the United Nations), fish production will reach 195 million ton around 2025 (Food and Agriculture Organization of the United Nations, 2018). In many countries, fishery are the mainstay of the economy and an important source of animal protein, unsaturated fatty acids (UFAs) as well as many bioactive components (Rubio-Rodríguez et al., 2012; Sarker, 2020). A large number of fish by-products are produced in the fish processing process (including head, skin, backbone, etc.), which are mainly used to produce some low value-added products, but their quality may account for 50 % of the whole fish weight (Zamora-sillero et al., 2018). These by-products often contain high-value compounds that can be used by humans, such as proteins, bioactive peptides, minerals and so on. These high-value added components exert potential bioactive functions such as antioxidant, anti-inflammatory, microbiota modulation, etc. (Marti-Quijal et al., 2020). The discarding of fish by-products not only causes a waste of resources, but also has a negative impact on the environment and is not conducive to the sustainable development of ecology (Marti-Quijal et al., 2020; Zamorano-Apodaca et al., 2020). In order to reduce the waste of by-products and environmental pollution, researchers try to recover high-value bioactive components from fish by-products and serve them as functional ingredients in the food, medicine, cosmetics and other industries. Bioaccessibility are often used to assess the health risks of diet and the ease of utilization, providing reliable information for the utilization of bioactive compounds (Leufroy et al., 2012; Li & Wang, 2019).

Previously, we have applied non-thermal technologies (accelerated solvent extraction-ASE) to the recovery of high-value added compounds from fish by-products (Wang et al., 2021). These non-thermal technologies are green and efficient. During the treatment process, they can not only increase the extraction rate of high value-added compounds, but also ensure the bioactivity of the extracts. They can be used as innovative technologies for recovering high-value components in fish by-products, which can improve the extraction yields of protein and the antioxidant capacity of the extracts (Wang et al., 2021). These extracts also contain a variety of macro/trace elements required in human physiology. Mineral elements play an important role in the human metabolism. For example, iron (Fe) participates in the transport of oxygen and energy supply in the body; calcium (Ca) is important for maintaining bone-health; magnesium (Mg) and phosphorus (P) also have the potential to regulate protein activity and prevent some diseases (Jha et al., 2021; Mir-Marqués et al., 2016). However, the bioactivity effectiveness of these bioactive compounds obtained with different assisted treatment has not been evaluated. Based on previous experiments, this study used ASE-assisted treatment to obtain fish by-products extracts. First, the heavy metal content and mycotoxins of the extracts were evaluated, then an *in vitro* static digestion model was used to assess the effect of digestion on the antioxidant capacity of fish by-products extracts and the bioaccessibility of minerals, aiming to explore reasonable ways to improve the utilization rate of high-value compounds.

2. Materials and methods

2.1 Reagents

Potassium chloride (KCl), sodium chloride (NaCl), magnesium chloride hexahydrate ($MgCl_2(H_2O)_6$),potassium dihydrogen phosphate (KH_2PO_4), acetonitrile and methanol HPLC grade, absolute ethanol,chloroform,titrisol concentrated standards of macro/trace elements (Mg, Ca, Fe, Zn, K and P,andnitric acid (HNO_3) were purchased from Merck (Darmstadt, Germany); hydrochloric acid (37 %) was obtained from Scharlau (Barcelona, Spain); ammonium formate (99 %), formic acid (≥ 95 %) and mycotoxins standards including (AFB1, AFB2, AFG1, AFG2, OTA, ZEA, ENNA, ENNA1, ENNB, ENNB1etc.) were purchased from Sigma-Aldrich (St. Louis, MO, USA); ammonium carbonate ($(NH_4)_2CO_3$), sodium bicarbonate ($NaHCO_3$) and calcium chloride dihydrate ($CaCl_2(H_2O)_2$) were purchased from Sigma-Aldrich (St. Louis, MO, USA); the enzymes pepsin (975 units per protein, porcine), pancreatin (8 × USP specifications, porcine) and porcine bile extract were also supplied by Sigma-Aldrich (St. Louis, MO, USA); Trizma® base, ABTS (2,2’ -azinobis (3-ethylbenzothiazoline 6-sulfonic acid)), Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and fluorescein sodium salt were obtained from Sigma-Aldrich (St. Louis, MO, USA); AAPH (2,2’-azobis (2-amidinopropane)) (Acros Organics), sodium phosphate dibasic, potassium dihydrogen phosphate and potassium sulphate were obtained from VWR International Eurolab S. L. (Barcelona, Spain);

Ethyl acetate was obtained from Alfa Aesar (Karlsruhe, Germany); disodium phosphate and sodium dihydrogen phosphate were obtained from Panreac. Deionized water was obtained in the Milli-Q SP® reagent water system (Millipore Corporation, Bedford, MA, USA); syringe nylon filters (13 mm diameter and 0.22 μm pore size) were purchased

from Membrane Solutions (Plano, TX, USA); nylon filters (0.45 μm) were obtained from Scharlau.

Simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to the method from Minekus (Minekus et al., 2014).

2.2 Sample collection and processing

The rainbow trout and sole used in the experiments were purchased from local markets. Fish were dissected in the laboratory to obtain three by-products: head, skin and viscera, then fish by-products were stored at -20 ℃ for further experiments.

2.3 Extraction technologies

ASE processes used the same methodology as a previous study (Wang et al., 2021). During this procedure, ASE-200 Accelerated Solvent Extractor (Sunnyvale, CA, USA) was used. The fresh fish by-products were freeze-dried and mixed with diatomaceous earth in a certain proportion (head, skin and viscera, respectively: 1.0:2.0, 2.0:2.0 and 1.5:3.0 g/g). The mixture was added to the ASE extraction tank, and the operating conditions were set at: preheating time 1 min, heating time 5 min, flushing volume 60 %, and nitrogen purge for 60 s. Finally, the extracts were stored at -20 °C for subsequent analysis.

For different fish by-products, a control group without ASE-assisted was set up. The fish by-product samples after freeze-dying were extracted by stirring with distilled water for 15 min under certain conditions (head: 55 °C+pH 5.2, skin: 45 °C+pH 6.5, viscera: 50 °C+pH 6.8) to obtain an extract. The extracts were centrifuged and collected the supernatant, which was stored at -20 °C for analysis.

In order to evaluate the extract efficiency of ASE, the efficiency coefficient K_{ASE} is introduced:

$$K_{ASE} = \frac{M}{Mc} \quad (1)$$

M and Mc refer to the mineral content in ASE-assisted treatment and no-ASE-assisted group, respectively.

2.4 Mineral profile and Heavy metals

The content of heavy metals (As, Cd, Hg and Pb) and mineral profile (Mg, Ca, P, Fe, Zn and Se) in the fish by-products was determined according to the methodology proposed by De la Fuente et al. (De la Fuente et al., 2021). For this, different by-products were placed in a microwave oven (MARS, CEM, Vertex, Spain) for mineralization. 0.3 g samples were weighted into a Teflon digester, then HNO_3 (14 M, 4 mL) and H_2O_2 (30 % *v/v*, 1 mL) were added and digest at 800 W, 180 °C for 15 min. The digested samples were taken out and cooled to room temperature, after removing nitrogen, it was filtered, and the volume was made up with distilled water.

The inductively coupled plasma mass spectrometer (ICP-MS) was used to detect heavy metals and conditions were set as follows: carrier gas flow (1.07 L/min), helium (He) as reactant gas, high-frequency emission power (1550 W), Ar gas flow (15.0 L/min), nebulizer pump speed (0.10 rps), and radio frequency matching (1.80 V). For heavy metals, ^{72}Ge, ^{103}Rh and ^{193}Ir were used as internal standard solutions to correct the fluctuation and drift of the instrument signal. The 0~1000 μg/L standard calibration curve was used for quantitative analysis of As, Cd and Pb, and the 0~100 μg/L standard curve was used for the quantitative analysis of Hg. For mineral profile, ^{45}Sc and ^{72}Ge were used

as internal standard solution, the standards of 0~10000 μg/L were used for quantitative analysis of minerals.

2.5 Detection of mycotoxins in fish side streams

2.5.1 Dispersive liquid-liquid microextraction procedure (DLLME)

The DLLME process was employed to extract mycotoxins from the samples according to previous works (Khawli et al., 2021). 2 mL samples were added to a tube containing 0.2 g NaCl and vortex for 1 min. Then, the mixture of dispersant and extractant solvents (950 μL AcN/620 μL EtOAc) was added and vortexed for 1 min. Subsequently, was centrifuged at 4000 rpm for 5 min to achieve phase separation and the top organic phase was transferred to another tube. Then, the mixture of dispersant and extractant solvents (950 μL MeOH/620 μL $CHCl_3$) was added to the remaining residue. After vortexing and centrifugation, the organic phase at the bottom of the tube was separated and placed with the previously transferred organic phase. The mixture of the two organic phases was dried under a nitrogen stream using a Turvovoap LV evaporator (Zymark, Hoptikinton, MA, US). Finally, the residue was reconstituted with1 mL of 20 mM ammonium formate (MeOH/ACN: 50/50 *v/v*) and filtered through a 13 mm/0.22 μm nylon filter before injection into the LC-MS/MS-IT system.

2.5.2 LC-MS/MS-IT determination

Mycotoxins were determined using an Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a 3200 QTRAP system (Applied Biosystems, AB Sciex, Foster City, CA) with Turbo Ion Spray (ESI) electrospray ionization, which combines triple quadrupole and linear ion trap mass spectrometer with fully functions. The Gemini-NX column C18 (Phenomenex, 150 mm x 4.6 mm, 5 particle size) was used

for chromatographic separation. The mobile phase was composed of 5 mM ammonium formate, 0.1 % formic acid water and 5 mM ammonium formate, 0.1 % formic acid methanol. At the beginning of the gradient elution, the ratio of mobile phase B is 10 %, then increases to 100 % within 10 min, then decreases to 80 % within 5 min, and decreases to 70 % again within 2 min. Finally, the column was cleaned for 6 min and adjusted to the initial conditions until equilibrium is reached. The flow rate is set at 0.25 mL/min. During the analysis, the following parameters were used: Turbo Ion Spray in positive ionization mode (ESI+), nitrogen served as nebulizer and collision gas, the nebulizer (GS1) and TIS (GS2) gases were set to 50 psi, flow rate was 12.0 L/min, capillary voltage 5500 V, the air curtain was set to 20 (arbitrary units), crusher voltage 160 V, scanning range m/z 50~1500, and dry gas temperature was 370 °C. Mass Hunter Workstation was used for data analysis.

2.6 Evaluation of Bioaccessibility of Fish By-product Extracts

2.6.1 In vitro simulated gastrointestinal digestion

In vitro digestion was performed according to the method proposed by Minekus et al. (Minekus et al., 2014). In order to mimic the digestive processes in the oral cavity, stomach and intestine, simulated saliva fluid (SSF, pH=7), simulated gastric fluid (SGF, pH=3) and simulated intestinal fluid (SIF, pH=7) were prepared. The *in vitro* static digestion simulation includes three stages: chewing, gastric phase, and intestinal phase.

Oral stage (chewing): the oral simulated digestion stage was carried out without α-amylase, 2.5 mL of fish by-products extracts were mixed with 2 mL of SSF and shaken quickly for 1 min. Then 12.5 μL $CaCl_2$ was added, and the volume was made up to 5 mL with distilled water, the mixture was shaken in a water bath at 37 °C for 2 min.

Gastric stage: 4.55 mL of SGF were added to the above mixture and vortexed for 1 min. Then 8 mg pepsin (2000 U/mL) from porcine gastric mucosa and 2.5 μL $CaCl_2$ were added to the solution and vortexed for 1 min. The pH was adjusted to 3 with NaOH (1 M)/HCl (6 M), and the volume up to 10 mL with distilled water, before to incubate at 37 ℃ for 2 h.

Intestinal stage: 5.5 mL of SIF was added to the above solution and vortex 1 min. Then 2.5 mL pancreatin (800 U/mL) form porcine pancreas, 1.25 mL porcine bile extract (0.16 M) and 20 μL $CaCl_2$ were added andthe mixture was vortexed during 1 min. The pH was adjusted to 7 and the volume completed up to 20 mL. Then, the solution was incubated at 37 ℃ for 2 h and centrifuged at 4000 rpm for 40 min at 4 ℃, and supernatant (bioaccessible fraction (BF)) was collected for analysis of antioxidant capacity and mineral profile. A blank group was set, containing all the digestive juices, and replacing the fish by-product extracts with deionized water.

For bioaccessibility estimation, the digestion blank value needs to be subtracted from the sample value to eliminate the interference caused by reagents such as digestive enzymes. The results can be calculated as the ratio of the concentration of bioactive compounds in the digested sample to original samples, as follow equation (2):

$$\boldsymbol{Bioaccessibility}\ (\%) = \left(\frac{\boldsymbol{content\ in\ BF}}{\boldsymbol{original\ content}}\right) \times \mathbf{100} \qquad (2)$$

2.6.2 Antioxidant capacity

Total antioxidant capacity was evaluated by oxygen radical absorption capacity test (ORAC) and Trolox equivalent antioxidant capacity assay (TEAC) according to previous study (Wang et al., 2021). For the evaluation of oxygen radical absorption capacity,

Trolox was used as the antioxidant standard. Add 50 μL samples/Trolox standard and 50 μL fluorescein sodium salt to 96-well microplate and set phosphate (pH=7.4) as blank group. After 10 min incubation at 37 °C in the dark, 25 μL of peroxide AAPH was added to initiate the oxidation reaction. Use a microplate reader to measure the absorbance at 520 nm, and 45 cycles was set, and each cycle is 60 seconds. Five parallels were set for each group of samples and repeated three times, and the antioxidant capacity of the samples was calculated according to the formula:

$$\boldsymbol{ORAC(trolox)} = \frac{A_{sample} - A_{blank}}{A_{trolox} - A_{blank}} \quad (3)$$

For the Trolox equivalent antioxidant capacity assay, 25 mL ABTS and 440 μL sodium thiosulfate were mixed to obtain a working solution, which was kept at room temperature for 12~16 hours in the dark. Dilute the working solution with 96 % ethanol to maintain the absorbance between 0.700 ± 0.020. In the experiment, different concentrations of Trolox were used as the standard, 2 mL working solution wasmixed with 0.1 mL of sample/Trolox, the absorbance at 734 wea measured after 3 min reaction in the dark at room temperature.

For the digested samples, a group containing all the digested solution and no extracts was set as the background to eliminate the interference of the digested solution, the analysis was performed in triplicate, while the data were reported as the mean ± standard deviation.

2.7 Statistical analysis

All experiments were performed in triplicate. GraphPad Prism (GraphPad Software Company, La Jolla, CA, USA) and Statgraphics (version 5.1, Statpoint Technologies Inc., Warrenton, VA) were used for graph plotting and analysis of results, respectively. One-

way analysis of variance (ANOVA) and Duncan's multiple-level difference test were used for determining the significant differences among samples. For each analysis, a significance level of 5 % was assumed. The error bars presented on the figures correspond to the standard deviations, letters were used to label the significance of the difference.

3. Results

3.1 Heavy metal content and mycotoxins in the fish by-products extracts

Table 1. Heavy metals content in different fish by-products.

		As (mg/kg)	Cd (mg/kg)	Hg (mg/kg)	Pb (mg/kg)
Rainbow trout	Head	2.121±0.031	0.008±0.001	0.159±0.005	0.012±0.001
	Skin	1.949±0.030	0.010±0.001	0.110±0.003	0.032±0.001
	Viscera	2.040±0.020	0.046±0.001	0.099±0.003	0.065±0.001
Sole	Head	2.431±0.041	0.018±0.007	0.079±0.002	0.073±0.002
	Skin	2.852±0.039	0.022±0.002	0.050±0.002	0.025±0.001
	Viscera	2.070±0.014	0.054±0.003	0.126±0.006	0.072±0.001

*None of the extract pass the EU regulation maximum levels of the heavy metals for human consumption.

Heavy metals have been explored as one of the main pollutants in the water environment. **Table 1** shows the contents of As, Cd, Hg, and Pb in the fish by-products selected in this study. In the six by-products involved in this study, the As content was in the range of about 2.0~2.9 mg/kg, of which rainbow trout skin presented the lowest concentration (1.949±0.030 mg/kg). In this study, the contents of Cd in the viscera of both fish were higher than in the other by-products, 0.046±0.001 mg/kg in rainbow trout and 0.054±0.003 mg/kg in sole, respectively, while the head had lower content of Cd, 0.008±0.001 mg/kg in rainbow trout head and 0.018±0.007 mg/kg in sole head. All results

were in general below the maximum allowed limit (0.050 mg/kg) (Reglamento (CE) 1881/2006, 2008). In rainbow trout by-products, the order of Hg content was head>skin>viscera, and in sole it was viscera>head>skin. Among them, the highest content was 0.159±0.005 mg/kg for rainbow trout, which was lower than the maximum allowable limit (0.5 mg/kg). Furthermore, Pb levels were also measured. For all by-products, the content of Pb ranged from about 0.012~0.073 mg/kg, with the highest levels in sole head and viscera about 0.073 mg/kg, however the levels were also below the maximum allowable limit (0.3 mg/kg).

3.2 Evaluation of antioxidant capacity in fish by-products extracts

There are changes in the oxygen free radical absorption capacity (ORAC) and ABTS+ scavenging capacity (TEAC) of fish by-products extracts (ASE-assisted and no-ASE) before and after *in vitro* digestion (**Table 2**).

Table 2. Total antioxidant capacity (ORAC and TEAC) in control (no ASE-assisted) and ASE-assisted treatment extracts from fish by-products (head, skin and viscera)

	ORAC (μM TE)		TEAC (μM TE)	
Rainbow trout				
	Before digestion	After digestion	Before digestion	After digestion
Head-ASE	2147.56±24.88[c]	3779.36±20.80[c]	940.71±12.14[d]	995.92±4.39[f]
Head-control	1862.30±20.56[a]	1913.90±9.17[b]	358.71±7.14[a]	346.15±3.07[b]
Skin-ASE	8559.76±18.57[f]	4607.27±8.43[f]	1247.86±5.42[f]	761.46±6.85[d]
Skin-control	6313.88±24.40[e]	4217.93±13.48[e]	543.01±2.11[b]	269.23±3.88[a]
Viscera-ASE	4152.41±29.74[d]	1517.03±12.74[a]	713.71±8.57[c]	899.99±5.84[e]
Viscera-control	2060.48±11.22[b]	4091.94±12.11[d]	976.02±9.87[e]	684.62±8.46[c]
Sole				
Head-ASE	1489.20±18.31[c]	4599.27±16.57[f]	790.71±3.57[f]	149.23±3.07[a]
Head-control	722.90±17.10[a]	1286.93±16.31[a]	376.93±9.64[c]	307.69±18.46[b]
Skin-ASE	1583.20±19.30[d]	2192.54±18.22[b]	501.43±25.00[d]	430.77±7.69[c]
Skin-control	1168.71±18.88[b]	4462.82±12.37[e]	210.86±12.43[a]	ND
Viscera-ASE	4330.90±15.41[f]	2999.20±14.64[d]	287.14±13.14[b]	553.84±7.93[d]
Viscera-control	2724.41±11.43[e]	2651.24±15.75[c]	554.79±16.07[e]	607.69±15.38[e]

*ORAC: oxygen radical absorbance capacity; TEAC: Trolox equivalent antioxidant capacity; Results are expressed as mean±standard deviation. One-way ANOVA was performed using Duncan's multiple comparison post-hoc test to assess statistical significance between groups. For one type of fish, different letter in the same column indicates statistically significant differences ($p < 0.05$). Among them, antioxidant capacity before digestion has been published (Wang et al., 2021) and was used here to compare with the digested groups. ND: not detected.

Table 2 shows the changes in the oxygen free radical absorption capacity (ORAC) and $ABTS^+$ scavenging capacity (TEAC) of fish by-products extracts (ASE-assisted and no-

ASE) before and after *in vitro* digestion. In previous studies (Wang et al., 2021), we have confirmed that the extracts from ASE-assisted treatment have higher protein content and antioxidant capacity compared to samples without ASE-assisted treatment, and *in vitro* digestion showed different effect on the extract's antioxidant capacity. The antioxidant capacity of the fish by-products extracts with ASE-assisted before and after digestion was also determined (**Table 2**). Firstly, for rainbow trout, digestion improved the oxygen radical absorption capacity of head extract, and the ASE-assisted effect was more obvious, while $ABTS^+$ scavenging capacity did not change significant before and after digestion process. After *in vitro* digestion, the oxygen radical capacity and $ABTS^+$ scavenging capacity of skin extracts showed a decreasing trend. For the extracts obtained from sole by-products, the oxygen radical capacity of head and skin extracts was significantly enhanced after digestion. The difference is that in head extract, ASE-assisted showed a more positive effect. Moreover, digestion also enhance the $ABTS^+$ scavenging capacity of viscera extract, with more obvious increase assisted by ASE comparing with control group. As it can be seen, ASE-assisted treatment shows a certain effect in some extracts.

3.3 Mineral content in fish by-products extracts

Minerals, as micronutrients play an important role in human health, they can participate in variety of enzymatic reactions and the anabolism of nutrients (Lall, 2022). In this study, the mineral content of fish by-products extracts was shown in **Figure 1~2**.

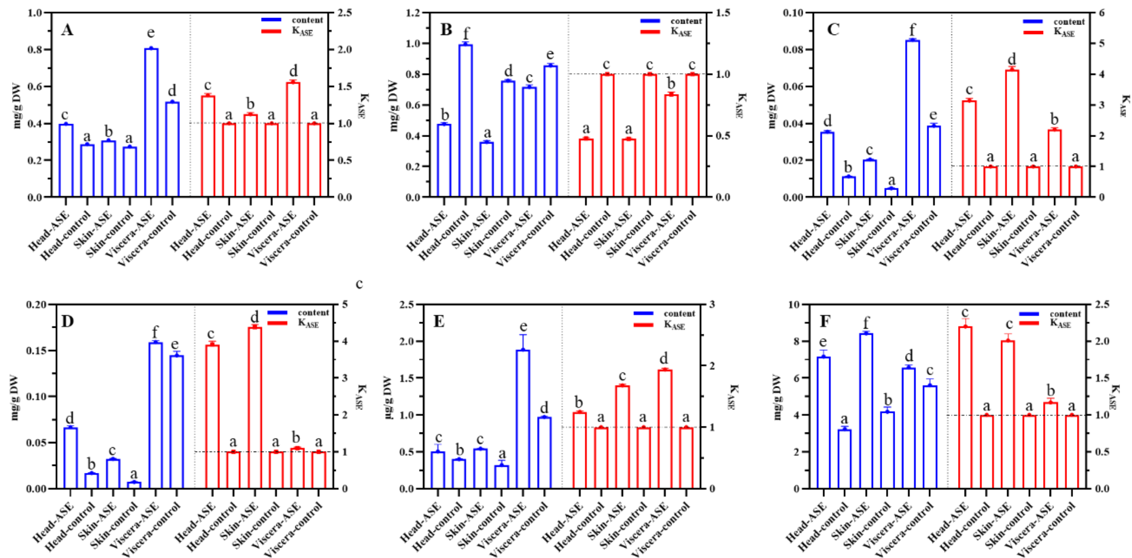

Figure 1: Mineral content in rainbow trout by-products extracts (ASE-assisted treatment and control group) and the efficiency coefficient K_{ASE}: A): Mg, B): Ca, C): Fe, D): Zn, E): Se, F): P. One-way ANOVA was performed using Duncan's multiple comparison post-hoc test to assess statistical significance between groups. Different letters in the bars indicate statistically significant differences ($p < 0.05$) for each mineral.

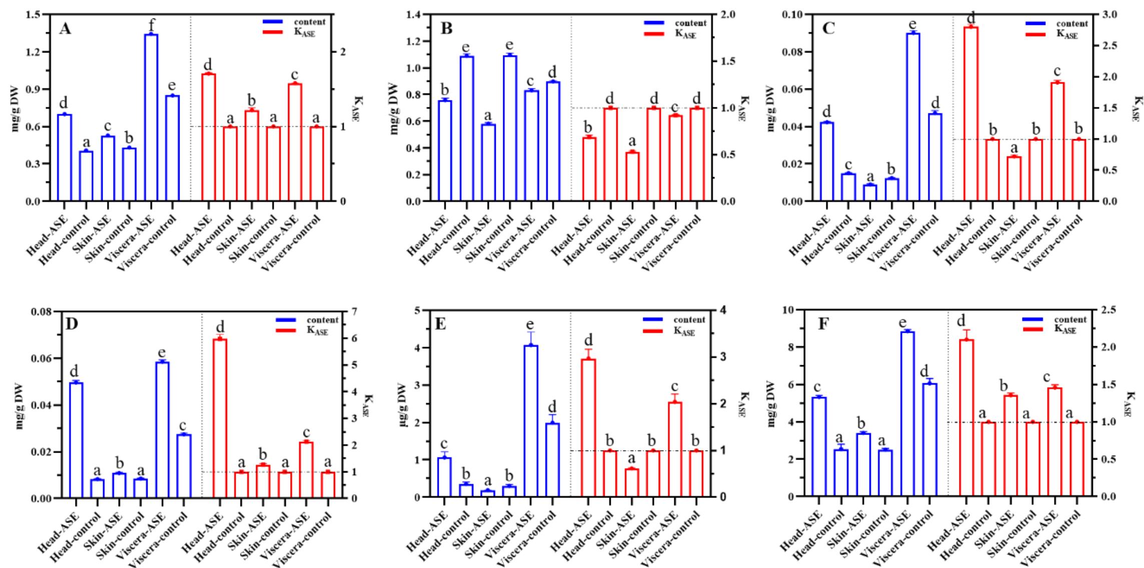

Figure 2: Mineral content in sole by-products extracts (ASE-assisted treatment and control group) and the efficiency coefficient K_{ASE}: A): Mg, B): Ca, C): Fe, D): Zn, E): Se,

F): P. One-way ANOVA was performed using Duncan's multiple comparison post-hoc test to assess statistical significance between groups. Different letters in the bars indicate statistically significant differences (p<0.05) for each mineral.

Figure 1 and **Figure 2** show the minerals content and ASE efficiency coefficients (K_{ASE}) in the two fish (rainbow trout and sole) by-products extract with and without ASE-assisted treatment, respectively. As can be seen from **Figure 1**, ASE-assisted treatment can significantly increase the content of various minerals in the rainbow trout by-products extracts, including Mg, Fe, Zn, Se and P (K_{ASE}>1). Concerning head extract, the ASE-assisted treatment had the most significant effect on the Fe and Zn contents, with K_{ASE} of 3.14 and 3.91, followed by P (K_{ASE}2.21)>Mg (K_{ASE}1.38)>Se (K_{ASE}1.25). Contrary, the content of Ca in the ASE-assisted treatment was half of that in control group (K_{ASE} 0.47). Similarly, in the skin extract, ASE-assisted treatment increased the content of Fe and Zn (Fe: K_{ASE} 4.15, Zn: K_{ASE} 4.38), moreover the effect was higher than in head extracts. ASE-assisted treatment also increased the content of Mg, Se and P in comparison with control group (Mg: K_{ASE} 1.12, Se: K_{SE} 1.68, P: K_{ASE} 2.01), however Ca content was about 50 % of the control group. Differently to those observed in head and skin extracts, ASE-assisted treatment showed a greater effect on Fe and Se contents in viscera extracts with K_{ASE} of 2.20 and 1.94, respectively. In addition, the ASE-assisted treatment also increased the Mg, Zn and P contents in the viscera extracts (Mg: K_{ASE} 1.56, Zn: K_{ASE} 1.10, P: K_{ASE} 1.17), while the content of Ca was slightly lower than in the control group (K_{ASE}=0.83), as was observed in head and skin by-products.

Figure 2 shows the mineral content in sole by-product extracts obtained by ASE and control group. The results show that ASE-assisted treatment can significantly increase the content of Zn in the head extract, which is about 6 times that the control group (K_{ASE} 5.97), and it also has a significant effect on the content of Fe (K_{ASE} 2.80), Se (K_{ASE} 2.97), Mg (K_{ASE} 1.71) and P (K_{ASE} 2.10). However, the content of Ca in the ASE-assisted treatment extract was lower than in the control group ((K_{ASE} 0.69). ASE-assisted treatment increased the contents of Mg, Zn and P in skin extracts (Mg: K_{ASE} 1.22, Zn: K_{ASE} 1.27, P: K_{ASE} 1.36), but the contents of Ca, Fe and Se were lower than in the control group (Ca: K_{ASE} 0.53. Fe: K_{ASE} 0.72, Se: K_{ASE} 0.61). In the viscera extracts, the content of Ca in the ASE-assisted treatment group was lower than in the control group (K_{ASE} 0.92), while the contents of other minerals were higher than the control group. Thus ASE-assisted treatment had a significant positive effect on minerals recovery from fish by-products.

3.4 Bioaccessibility of mineral in fish by-products extracts

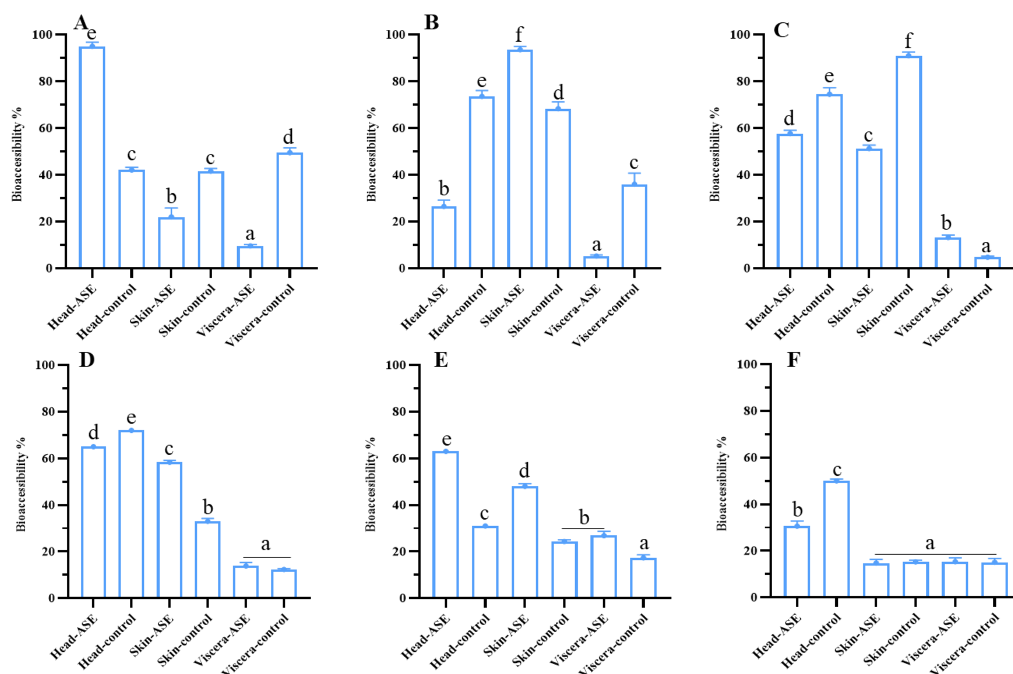

Figure 3. Bioaccessibility evaluation of minerals in rainbow trout by-products extracts with ASE-assisted treatment. A): Mg, B): Ca, C): Fe, D): Zn, E): Se, F): P. One-way ANOVA was performed using Duncan's multiple comparison post-hoc test to assess statistical significance between groups. Different letters in the bars indicate statistically significant differences (*p < 0.05*) for each mineral.

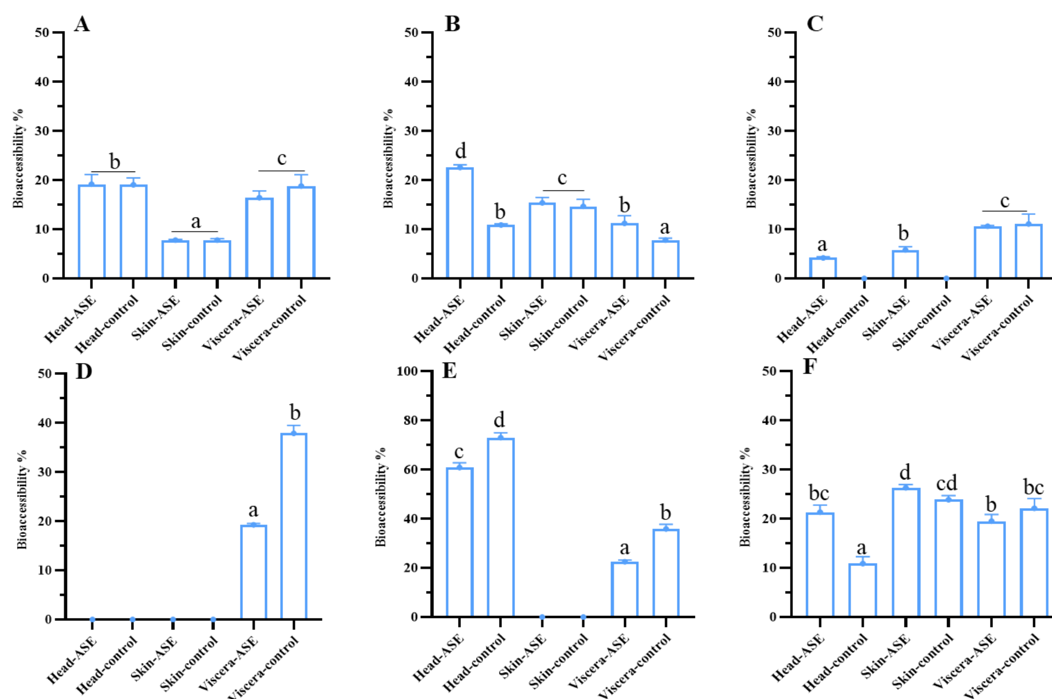

Figure 4. Bioaccessibility evaluation of minerals in sole by-products extracts with ASE-assisted treatment. A): Mg, B): Ca, C): Fe, D): Zn, E): Se, F): P. One-way ANOVA was performed using Duncan's multiple comparison post-hoc test to assess statistical significance between groups. Different letters in the bars indicate statistically significant differences ($p < 0.05$) for each mineral.

The bioaccessibility of minerals was assessed by establishing an in vitro digestion model. **Figure 3** and **Figure 4** show the bioaccessibility of minerals from fish by-products recoveries in the ASE-assisted treatment and control group based on *in vitro* static digestion model. For rainbow trout, the bioaccessibility of different minerals showed large differences, in head extract, ASE-assisted treatment significantly improved the bioaccessibility of Mg and Se, while the bioaccessibility of the other minerals was lower than in the control group. Inskin extracts, Ca, Zn and Se showed higher bioaccessibility under ASE-assisted treatment. Moreover, ASE-assisted treatment showed a positive

effect on Fe and Se bioaccessibility in viscera extract, , however the bioaccessibility of Mg and Ca in ASE-assisted extracts was significantly lower than in the control.

Figure 4 shows the bioaccessibility of minerals in sole by-products extracts under ASE-assisted treatment. Overall, the bioaccessibility of Mg, Ca and Fe was low in all extracts. Among the skin extract, only Fe presented a slightly higher bioaccessibility in the ASE-assisted treatment group than in the control group, while the other minerals were not significantly affected. In the viscera extract, the bioaccessibility of Zn and Se in the ASE-assisted treatment group was lower than in the control group, while the effect of ASE-assisted treatment on Mg, Fe and P was not significant.

4. Discussion

Heavy metals are non-degradable pollutants in nature, which in the aquatic environment mainly come from the crust, sediments and wastes produced by human activities, etc. (Habib et al., 2022). These compounds will enter the fish body through the gills and other organs and accumulate in different parts, which has become a concern of people. The concentrations and types of heavy metals contained in different type of fish are different, the main that can be detected are arsenic (As), cadmium (Cd), nickel (Ni), lead (Pb) and mercury (Hg) (Soltani et al., 2019).

Concerning the information available in literature, in farmed rainbow trout liver, As, Cd and Hg were reported at similar levels, ranging from 0.74 to 5.23 mg/kg. 0 to 0.61 mg/kg and 0.097-2.23 mg/kg respectively, however higher levels were reported for Pb (0.713-6.18 mg/kg) by these authors, concentrations that exceeded the legislation limits (Fallah et al., 2011). In other study performed in sole from the North-Eastern of Mediterranean Sea, Pb and Cd levels were detected in liver at 0.05-0.37 mg/kg and 0.02-

0.06 mg/kg respectively, in line with those observed in the present study (Kılıç et al., 2021).

Regarding mycotoxins, none of the studied mycotoxin was detected in the fish extracts obtained from rainbow trout and sole by-products under ASE-assisted treatments. The exposure of farm animals to mycotoxins can result in undesirable residues in animal derived food products. The mycotoxins carry-over from feed to edible fish tissues depends on several factors such as the mycotoxin structure and the animal species. In this regard, some studies are available in bibliography with different results (J. Tolosa et al., 2021).

However, in this work due the low solubility of mycotoxins in water, there is not to be expected an important pass of mycotoxins from the fish by-products to their resulted extracts. Employing water as extractant solvent, we pretended to extract high-added-value compounds from fish by products avowing the extraction of some contaminants like mycotoxins. Similarity to this study, in a previous one AFs, OTA, FUS-X, STG, FBs, ENNs, and BEA were not found in trout samples (Josefa Tolosa et al., 2019). Contrary to these results ENNs were reported in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) species (Josefa Tolosa et al., 2014).

According to the change of the oxidative capacity of the fish by-product extracts before and after digestion, it can be speculated that the changes in the antioxidant capacity of the extracts before and after digestion were mainly attributed to the bioactive components. In previous studies, the protein content of the extracts has been determined. During the *in vitro* digestion, the proteins digestion starts from the stomach and degraded into low molecular weight peptides in the stomach and intestine, which are further degraded into

amino acids by the small intestinal epithelial cells and enter the body fluid circulation. These fish protein-derived peptides often have antioxidant capacity, so the changes in antioxidant capacity before and after digestion may be attributed to the different sequences of peptides contained in the different extracts. Likewise, the effect of digestion on the antioxidant properties of fish-derived protein has also been reported in bibliography. In this regard, Ahn et al. (Ahn et al., 2014) evaluated salmon by-products proteolytic peptides and their antioxidant capacity by *in vitro* digestion. The digested octapeptides showed high oxygen radical scavenging capacity and reduced oxidative stress-induced DNA damage. Vásquez et al. (Vásquez et al., 2022) also explored the antioxidant properties of *Oncorhynchus mykiss* viscera hydrolysate. The oxygen radical absorption capacity of the viscera hydrolysate obtained by Alcalase® did not change significantly, but the hydrolysis increased the $ABTS^+$ scavenging capacity and reduced the antioxidant capacity of hydroxyl radicals, which is also related to the bioactive peptides. In other study, Mirzapour-Kouhdasht et al. observed that peptide concentration of all fractions obtained from barred mackerel by-products increased after *in vitro* gastrointestinal digestion while antioxidant activities were significantly decreased. These authors attributed this fact to the digestion of 3–10 kDa peptides, which present the high activity, to lower molecular weight peptides (< 3 kDa) (Mirzapour-Kouhdasht et al., 2021).

There is scarce information available in literature about the impact of non-thermal technologies on essential minerals. Emerging technologies do not affect minerals directly but induce changes in the physical properties and structure of the associated macromolecules (Galanakis, 2021). The mineral content in fish is related to various

factors such as species, growing environment, food, etc. At the same time, there are interactions between some mineral elements, resulting in antagonistic or synergistic effects, thus affecting the recovery of minerals. In addition, minerals such as Fe and Zn can exist in combination with proteins. In this regard, in a previous work, Abdollahi et al., explored the effect of mechanical separation and pH-shift processes on the mineral content of protein recovered from fish backbones. These authors observed that mineral content seemed to be dependent on whether the minerals are located, as well as their binding affinity to proteins vs. their water solubility. For instance, minerals enriched in the bone (Ca and Mg), resulted more effectively removed by pH-shift process, while the opposite was observed for minerals with high binding affinity to protein (Zn). Thus, factors such as fish species and composition of input materials define the content of minerals in the final protein-enriched extracts (Galanakis, 2021). In the present work the results obtained have also been influenced by the fish spice (rainbow trout or sole), the extraction technique (such as ASE), as well as the minerals solubility and binding affinity.In a previous study, we have confirmed that ASE-assisted treatments can change the molecular size distribution of protein in the recovered, which may also be one of the reasons affecting the minerals recovery (Wang et al., 2021).

ASE as non-thermal technology has been shown to affect the bioaccessibility of various bioactive compounds, such as phenols and carotenoids, which may be due to the fact that non-thermally assisted treatment could alter the interactions between food matrices or affect the release of the compounds (Cilla et al., 2018; Ribas-Agustí et al., 2019). However, to the best of our knowledge the effect of ASE-assisted treatment on mineral bioaccessibility in fish by-products has not been reported.

In other study performed in beef muscle, no significant decrease was observed in the release of Fe, K, P, Ca, Na and Mg minerals from the muscle after gastrointestinal digestion. In this sense, PEF treatment may induce higher membrane permeability resulting in higher minerals release. In the present study, PEF extraction also improved significatively the bioaccesibility of Fe in rainbow trout and sole by-products (head and skin) (Bhat et al., 2019). Minerals bioaccessibility is related to some factors such as food matrix, cooking method, among others (Jiang et al., 2021). In addition, complex structures formed between minerals and other components, can affect their absorption.

5. Conclusion

This study shows that the antioxidant capacity of high-value extracts from rainbow trout and sole by-products using ASE-assisted treatment can be affected by *in vitro* digestion, which can improve the oxygen radical absorption capacity and $ABTS^+$ scavenging capacity in some samples. Then, ASE-assisted treatment also has a certain positive effect on the recovery of minerals. The bioaccessibility of minerals in by-products extracts was evaluated after an *in vitro* digestion model, resulting in bioaccessibility increase in some extracts, however not in all cases was observed a positive effect. Understanding the absorption efficiency and properties of high-value components from fish by-products in the digestion process could help to find a more reasonable methods to improve the utilization rate of high-value compounds and make them more valuable.

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4.4 Bioaccessibility evaluation of antioxidants and minerals (Mg, Ca, P, Fe, Zn, Se) extracted from rainbow trout and sole side streams by pulsed electric field using the INFOGEST *in vitro* static gastrointestinal digestion model

(Under review)

Bioaccessibility evaluation of antioxidants and minerals (Mg, Ca, P, Fe, Zn, Se) extracted from rainbow trout and sole side streams by pulsed electric field using the INFOGEST *in vitro* static gastrointestinal digestion model

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Abstract

Rainbow trout and sole side streams were used as a target matrix to recover antioxidants compounds and minerals (Mg, Ca, P, Fe, Zn, Se) assisted by pulsed electric fields (PEF) (1-3 kV/cm, 124-300 kJ/kg, 15-24 h). Then, a static *in vitro* simulated gastrointestinal digestion model was used to evaluate the bioaccessibility of these compounds. A positive effect on $ABTS^+$ scavenging capacity of rainbow trout and sole skin extracts was found while the same behavior was obtained for the oxygen radical absorption capacity of sole head extracts. Furthermore, PEF increased Mg (156.06±1.52 mg/100g) and P (256.57±3.54 mg/100g) of rainbow trout head extract, but its bioaccessibility (12.00±0.13 % and 29.49±0.59 %) was lower than control. Ca and P contents of PEF sole viscera extracts (432.86±17.14 mg/100g and 441.43±17.14 mg/100g, respectively) were lower than control (1238.57±38.57 mg/100g and 1290.01±18.57 mg/100g, respectively), but PEF-assisted extraction extraction improved the bioaccessibility of Ca and P of viscera extracts (11.27±1.00 % vs 38.78±3.83%).

Keywords: fish by-product, *in vitro* digestion, antioxidant, mineral, bioaccessibility

1. Introduction

According to the Food and Agriculture Organization of the United Nations, the global production of fisheries and aquaculture production has grown from 19 million tons in 1950 to 178.5 million tons in 2018 (FAO, 2020). Fisheries and aquaculture play a vital role in reducing food stress and improving food nutrition. Meanwhile, the increased fish consumption promotes a higher amount of side streams, such as fish viscera, head, bones, etc., which account for around 30-70% of the total fish weight (Sarker, 2020). Fish side streams are often directly discarded or used to produce low-value products, such as animal feed, fertilizers, biofuels, etc., in which high-added-value compounds including nutrients (protein, and bioactive peptides, fat and fatty acids, minerals) and antioxidant bioactive compounds are not exploited (Shabani et al., 2018; Surasani et al., 2020; Wang et al., 2021). Therefore, it is of great importance to develop innovative tools to reuse fish side streams to obtain high-added-value compounds, which cannot only reduce the pollution caused by waste, but also contributes to the sustainable utilization of resources.

Minerals, as micronutrients play an important role in human health. They have an important role in several enzymatic reactions as well as in the anabolism of nutrients (Lall, 2022). For example, iron (Fe) is an important component of hemoglobin, which is involved in the transport and storage of oxygen in the body and promotes the growth and development of the body. Calcium (Ca) can participate in muscle contraction and reduces cholesterol concentration. In addition, magnesium (Mg) can also be involved in the body’s energy metabolism, protein synthesis, skeletal development, etc. (Jayakumar et al., 2022; Sujka et al., 2021). However, the human body cannot synthesize minerals by itself and must obtain them from the diet. Due to the low solubility and low absorption

rate of minerals, researchers are exploring more minerals sources as dietary supplements to maintain human health (Gharibzahedi & Jafari, 2017). Regarding, fish products, most of the studies available in the literature have been focused on protein, lipids, peptides and some bioactive compounds, but they are also an important source of minerals for human intake.

The data of elements concentration in food by itself does not provide a comprehensive assessment of their acceptance and health risks in the human body. Therefore, bioaccessibility refers to the part of the substance released into the gastrointestinal tract and absorbed by the intestinal tract (de Souza et al., 2019). The bioaccessibility of minerals in fish is affected by several factors, such as fish species, food processing and protein chelation, among others.

Previously, our research group explored the impact of pulsed electric fields (PEF), a technology able to damage the cell membrane, thus promoting electroporation phenomena and affecting the structure and recovery of some high-added-value compounds (Bhat et al., 2021). We found that PEF-assisted treatment contributes to an increased rate of nutrients and antioxidant bioactive compounds (Wang, Zhou, Collado, et al., 2021). However, at this stage of development, there is a lack of information regarding the impact of PEF technology on the bioaccessibility of minerals and antioxidants from fish side streams.

Therefore, in this study, a static *in vitro* digestion model was established to evaluate the bioaccessibility of minerals (Mg, Ca, P, Fe, Zn, Se) and antioxidant compounds from fish side streams assisted by PEF and conventional extraction.

2. Materials and methods

2.1 Reagents

Inorganic reagents, ammonium carbonate ($(NH_4)_2CO_3$), sodium bicarbonate ($NaHCO_3$) and calcium chloride dihydrate ($CaCl_2(H_2O)_2$) were purchased from Sigma-Aldrich (St. Louis, MO, USA); pepsin (975 units per protein, porcine), pancreatin (8 × USP specifications, porcine) and porcine bile were also acquired from Sigma-Aldrich (St. Louis, MO, USA). Potassium chloride (KCl), sodium chloride (NaCl), magnesium chloride hexahydrate ($MgCl_2(H_2O)_6$) and potassium dihydrogen phosphate (KH_2PO_4) were obtained from Merck (Darmstadt, Germany). Simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to the method proposed by Minekus et al., (2014). AAPH (2,2'-azobis (2-amidinopropane)) (Acros Organics), sodium phosphate dibasic, potassium dihydrogen phosphate and potassium sulphate were obtained from VWR International Eurolab S. L. (Barcelona, Spain). Trizma® base, ABTS (2,2' -azinobis (3-ethylbenzothiazoline 6-sulfonic acid)), Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and fluorescein sodium salt were obtained from Sigma-Aldrich (St. Louis, MO, USA). Absolute ethanol was purchased from J.T. Baker (Deventer, the Netherlands). Titrisol concentrated standards of macro/trace elements (Mg, Ca, Fe, Zn, K and P) and nitric acid (HNO_3) were purchased from Merck (Darmstadt, Germany); hydrochloric acid (37 %) was obtained from Scharlau (Barcelona, Spain).

2.2 Sample collection and preparation

The two types of fish selected for the experiment, rainbow trout and sole, were obtained from a local market in February-March 2021 (Valencia, Spain), and were dissected in the laboratory to obtain the head, skin, and viscera as research objects. Pulsed electric fields

(PEF) treatment used the same methodology described in a previous study (Wang, Zhou, Collado, et al., 2021). Fresh fish by-product samples were placed in the PEF processing chamber (PEF-Cellcrack III (German Institute of Food Technologies (DIL)) equipment (ELEA, Quakenbrück, Osnabrück, Germany) and a volume of tap water was added. Then, the samples were treated under PEF-assisted according to the optimized conditions obtained in the laboratory before (Table 1), after which the samples were transferred to a magnetic stirrer for continuous stirring. The extract was obtained after centrifugation at 4000 rpm for 10 min. Meanwhile, a control group without PEF-assisted treatment was prepared in parallel.

Table 1. PEF-assisted treatment conditions

Side streams	Time (h)*	Field strength (kV/cm)	Specific energy (kJ/kg)	Volume (mL)
Head	21.33	1.00	219.76	1500
Skin	24.00	3.00	300.00	675
Viscera	15.17	3.00	123.75	675

*Time of supplementary extraction.

2.3 In vitro digestion protocol

A static *in vitro* digestion adult model was established according to the method proposed by the COST INFOGEST network (Minekus et al., 2014). For this purpose, three solutions (SSF, SGF and SIF) were prepared to simulate the digestive juices in the saliva, gastric and intestinal stages. First, 2.5 mL of the sample was mixed with 2 mL of SSF and vortexed for 1 minute. 12.5 µL $CaCl_2$ was added, and the volume was made up to 5 mL with distilled water. Then, the mixture was placed in a water bath at 37 °C for 2 min to simulate the oral chewing stage of digestion.

Subsequently, 4.55 mL of SGF were added to the mixture and vortexed. After that, 8 mg of pepsin (2000 U/mL) from porcine gastric mucosa and 2.5 µL of $CaCl_2$ were added. The pH was adjusted to 3 employing NaOH/HCl, and the volume up to 10 mL with distilled water. Then, the mixture was incubated at 37 °C for 2 hours to simulate gastric digestion.

Finally, 5.5 mL of SIF, 2.5 µL of porcine pancreatin, 12.5 µL of porcine bile extract and 20 µL of $CaCl_2$ were added to the above mixture and mixed well. The pH was adjusted to 7 and the volume make up to 20 mL with distilled water. Then the mixture, was incubated at 37 °C for 2 hours to simulate intestinal digestion.

Finally, after digestion, the mixture was centrifuged at 4 °C, 4000 rpm for 40 min, and the supernatant was collected to obtain the bioaccessible fraction. At the same time, a blank group for digestion was prepared in parallel, in this case the sample was replaced by distilled water to eliminate the interference caused by the reagents and digestion enzymes. The bioaccessibility of a substance can be obtained according to the formula:

$$\boldsymbol{Bioaccessibility}\ (\%) = \left(\frac{\boldsymbol{content\ in\ bioaccessible\ fraction}}{\boldsymbol{total\ content}}\right) \times \mathbf{100} \qquad (1)$$

2.4 Determination of antioxidant capacity

The antioxidant capacity of the extracts was evaluated by measuring the oxygen radical absorption capacity (ORAC) and Trolox equivalent antioxidant capacity (TEAC), according to the protocols established in previous studies (De la Fuente et al., 2021).

ORAC was measured using Trolox as the standard solution and phosphate buffer (pH7.0~7.4) as the blank group. 50 µL of samples and 50 µL of fluorescein sodium salt were added to the 96-microplate and incubated at 37 °C for 10 min in the dark, then 25 µL AAPH was added. The emission wavelength was set at 520 nm and the excitation

wavelength at 480 nm, and the data were recorded within 60 min. The antioxidant capacity can be calculated according to the formula:

$$\boldsymbol{ORAC\ (trolox\ unit)} = \frac{A_{sample} - A_{blank}}{A_{trolox} - A_{blank}} \quad (2)$$

The determination of TEAC also used Trolox as a standard. To obtain $ABTS^+$ working solution, 25 mL of ABTS (7 mM) were mixed with 440 μL of potassium persulphate ($K_2S_2O_8$) (140 mM) and kept at room temperature for 12-16 hours. During the test, the working solution was diluted with 96 % ethanol until the absorbance was 0.700±0.020 at 734 nm, then 2 mL of working solution were mixed with 100 μL of the sample, and the absorbance was recorded after 3 min of reaction. At the same time, Trolox® at different concentrations was used to prepare a standard curve, which was used to calculate the antioxidant capacity of the samples.

2.5 Mineral profile

The main macro elements (Mg, Ca, P) and micro elements (Fe, Zn, Se) in the extracts before and after digestion were evaluated according to the methodology proposed by de la Fuente et al. (2019). In brief, 1 mL of sample was mixed with 1 mL of concentrated nitric acid (HNO_3, 69 %) and 250 μL H_2O_2, then the mixture was placed in a microwave digestion furnace for digestion, at maximum temperature of 180 °C. After that, the volume was adjusted to 5 mL with ultrapure water, subsequently a 100 μL aliquot was taken and the volume was adjusted to 10 mL with ultrapure water. ^{45}Sc and ^{72}Ge were used as internal standards, while standards ranging from 0~10000 μg/L were used for quantitative analysis. The mineral content was determined through an Agilent model 7990 ICP-MS (Agilent Technologies, CA, USA), under the following conditions: reactive gas (He),

carrier gas flow (1.07 L/min), transmit power (1550 W), RF matched (1.80 V) and nebulized pump speed (0.10 rps).

2.6 Statistical analysis

All experiments were performed in triplicate. GraphPad Prism (GraphPad Software Company, La Jolla, CA, USA) and Statgraphics (version 5.1, Statpoint Technologies Inc., Warrenton, VA) were used for graph plotting and analysis of results, respectively. One-way analysis of variance (ANOVA) and Duncan's multiple-level difference test were used for determining the significant differences among samples. For each analysis, a significance level of 5 % was assumed. The error bars presented on the figures correspond to the standard deviations and letters were used to label the significance of the difference.

3. Results

3.1 Evaluation of the antioxidant capacity of fish side stream extracts

In previous studies, our research team confirmed the potential of PEF as a useful tool to enhance the antioxidant capacity of fish side stream extracts (Wang, Zhou, Collado, et al., 2021). In this study, it was found that the oxygen free radical absorption capacity (ORAC) and $ABTS^+$ scavenging capacity (TEAC) of fish side stream extracts (PEF-assisted and no-PEF) changed before and after *in vitro* digestion (**Figure 1**).

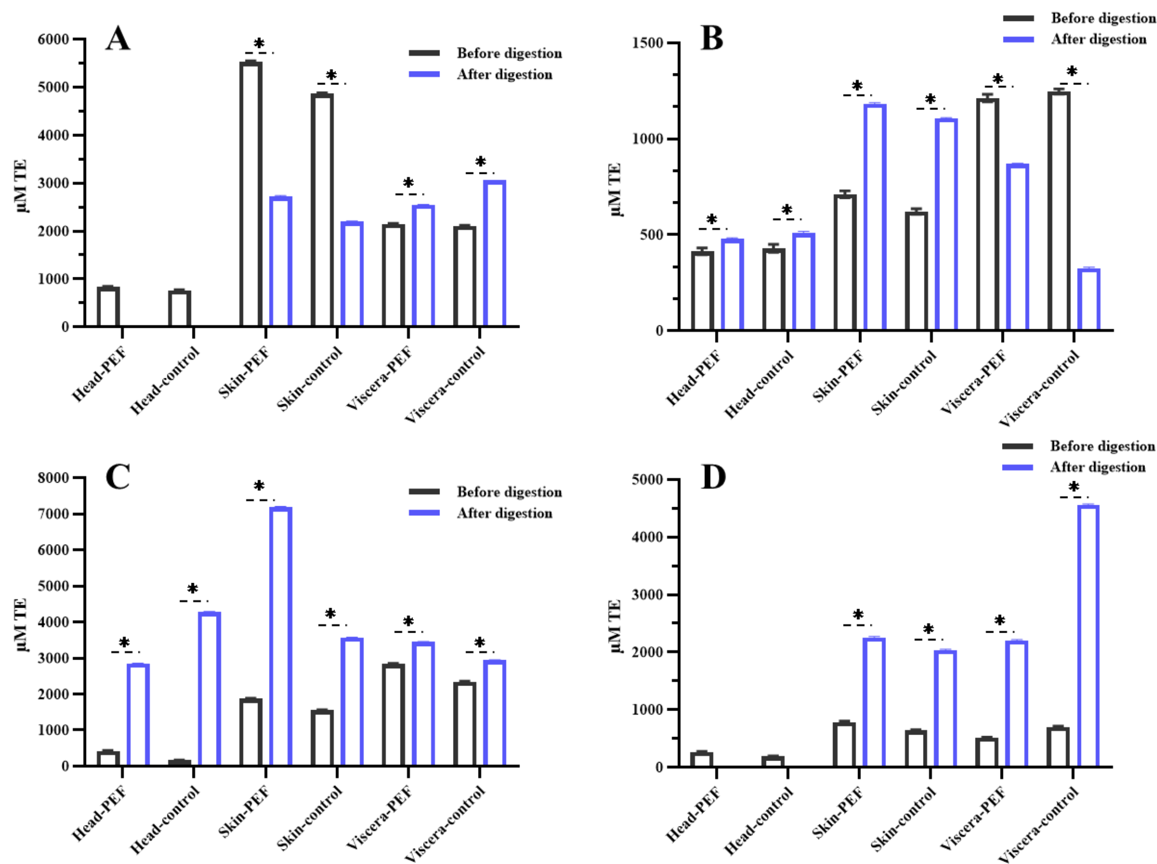

Figure 1. Total antioxidant capacity (ORAC and TEAC) in control and PEF-assisted treatment extracts from fish side streams (head, skin and viscera). ORAC: oxygen radical absorbance capacity; TEAC: Trolox equivalent antioxidant capacity. *The antioxidant capacity of the same sample changed significantly before and after digestion ($p<0.05$). Among them, antioxidant capacity before digestion has already been published (Wang et al., 2021) and was used here to compare with the digested samples. (A) and (B): the antioxidant capacity (ORAC and TEAC, respectively) of rainbow trout side streams; (C) and (D): the antioxidant capacity (ORAC and TEAC, respectively) of sole side streams.

As can be observed in **Figure 1**, no antioxidant capacity, measured as ORAC values, was found for rainbow trout (RT) heads after digestion, while the digestion process

increased the $ABTS^{+}$ scavenging capacity of the same matrix extracts, regardless of whether PEF was used to assist the extraction process or not.

It should be noted, that the increased (1.6~1.8 times higher) $ABTS^{+}$ scavenging capacity values were observed for RT skin extracts after the *in vitro* digestion, while the ORAC values for the same samples were reduced by about 45~50 % after digestion.

On the other hand, the RT viscera extract showed the opposite results compared to the skin samples. For instance, after digestion, the $ABTS^{+}$ scavenging capacity of the viscera extract was significantly reduced, showing the control group greater differences than the PEF-treated samples.

For sole, the head digested extracts did not show $ABTS^{+}$ scavenging capacity, but the ORAC values of the control and PEF extracts were increased after digestion. Among them, the ORAC values after digestion in the control group were more significantly increased, being up to 26-fold higher compared to the values before digestion. For sole skin and viscera, the *in vitro* digestion increased the ORAC and ABTS values of the extracts. After the *in vitro* digestion, the ORAC values of the skin extracts were increased by about 2.2~3.8 times, while the $ABTS^{+}$ scavenging capacity values augmented ~3 times. After digestion, the ORAC values of the viscera extract increased ~1.2 times, independently of PEF pretreatment, while the $ABTS^{+}$ scavenging capacity was enhanced by ~4 times (PEF-assisted) and 6 times (control group), respectively. In conclusion, *in vitro* digestion can change the antioxidant capacity of the extracts, but there is not a clear relationship with the use of PEF treatment. In the case of rainbow viscera and sole head extracts after digestion was observed lower $ABTS^{+}$ scavenging capacity but stronger ORAC values.

3.2 Content and bioaccessibility of minerals of fish side streams

The mineral contents of both rainbow trout and sole side stream extracts after PEF and control treatments are shown in **Tables 2-3.**

Table 2. Mineral content before and after simulated gastrointestinal digestion in rainbow trout by-product extracts (PEF-assisted treatment and control group)

Sample	Total content (mg/100 g)	BF (mg/100 g)	Bioaccessibility (%)
		Mg	
Head-PEF	156.06±1.52^{Ac}	18.72±1.41^{Bc}	12.00±0.13^{b}
Head-control	135.35±1.01^{Ab}	21.26±1.41^{Bc}	15.71±1.10^{c}
Skin-PEF	48.20±1.11^{Aa}	10.07±0.56^{Bb}	20.90±1.70^{d}
Skin-control	47.37±0.58^{Aa}	0.50±0.02^{Ba}	1.05±0.01^{a}
Viscera-PEF	151.95±2.44^{Ac}	27.85±2.28^{Bd}	18.33±1.73^{cd}
Viscera-control	239.61±4.87^{Ad}	28.03±2.11^{Bd}	11.70±1.11^{b}
		Ca	
Head-PEF	209.09±3.03^{Ac}	32.51±0.71^{Bd}	15.55±2.01^{cd}
Head-control	227.78±3.03^{Ad}	37.38±1.44^{Be}	16.41±1.42^{e}
Skin-PEF	137.77±2.88^{Aa}	8.96±0.02^{Ba}	6.50±0.22^{a}
Skin-control	131.65±1.80^{a}	ND	ND
Viscera-PEF	210.88±4.87^{Ac}	23.42±2.24^{Bb}	11.11±0.30^{b}
Viscera-control	244.97±3.89^{Ae}	29.64±2.83^{Bc}	12.10±0.92^{bc}
		Fe	
Head-PEF	0.87±0.02^{Ab}	0.60±0.01^{Bc}	69.20±1.40^{d}
Head-control	1.28±0.02^{Ac}	0.43±0.02^{Bb}	33.21±0.56^{a}
Skin-PEF	0.52±0.01^{Aa}	0.24±0.01^{Ba}	46.01±2.02^{b}
Skin-control	0.51±0.01^{a}	ND	ND

Viscera-PEF	1.51±0.02^{Ae}	0.78±0.04^{Bd}	51.92±1.74^{c}
Viscera-control	1.39±0.04^{Ad}	0.66±0.04^{Bc}	47.22±3.11^{bc}
		Zn	
Head-PEF	2.44±0.03^{Ab}	2.22±0.03^{Bc}	91.01±0.34^{d}
Head-control	3.66±0.05^{Ac}	2.12±0.00^{Bb}	58.00±0.81^{b}
Skin-PEF	0.52±0.01^{Aa}	0.43±0.08^{Aa}	82.82±1.17^{c}
Skin-control	0.41±0.01^{a}	ND	ND
Viscera-PEF	15.93±0.34^{Ad}	3.95±0.00^{Be}	24.79±1.68^{a}
Viscera-control	15.83±0.44^{Ad}	3.50±0.01^{Bd}	22.09±1.17^{a}
		Se (µg/100 g)	
Head-PEF	ND	ND	ND
Head-control	ND	ND	ND
Skin-PEF	21.58±3.96^{Aa}	11.43±0.85^{Ba}	52.98±1.22^{c}
Skin-control	19.42±3.60^{Aa}	11.07±0.31^{Ba}	57.01±2.81^{d}
Viscera-PEF	92.53±1.46^{Ac}	36.57±0.95^{Bb}	39.52±3.03^{a}
Viscera-control	77.92±1.46^{Ab}	38.21±1.69^{Bb}	49.04±0.94^{b}
		P	
Head-PEF	256.57±3.54^{Ab}	75.66±1.42^{Bb}	29.49±0.32^{b}
Head-control	223.74±7.58^{Aa}	106.05±2.93^{Bc}	47.40±0.59^{c}
Skin-PEF	388.49±7.99^{Ad}	120.86±5.65^{Bd}	31.11±0.76^{b}
Skin-control	346.40±3.24^{Ac}	27.64±1.75^{Ba}	7.98±0.12^{a}
Viscera-PEF	506.49±9.48^{Ae}	145.67±5.67^{Be}	28.76±0.72^{b}
Viscera-control	491.88±7.31^{Ae}	146.09±2.83^{Be}	29.70±0.03^{b}

BF: bioaccessible fraction. Data presented in dry weight. Results are expressed as mean±standard deviation. Different lowercase letters in the same column for each mineral compound indicate significant differences ($p < 0.05$). Different uppercase letters in the

same row for each mineral indicate significant differences before and after digestion for one sample ($p < 0.05$). ND: not detected.

Overall, the side stream extracts obtained from both fishes showed a relatively low content in Fe, selenium (Se) and zinc (Zn), while they had a high content in Mg, Ca and phosphorus (P). Meanwhile, it can be found that the bioaccesible fraction is lower than the total mineral content. From **Table 2**, it can be seen that for rainbow trout head extracts, the contents of Mg and P in the PEF-assisted treatment were higher (~1.15 times) in the than control group. However, the contents of Ca and Fe in the head extract after PEF were slightly lower than the control group. For skin extract, PEF-assisted treatment did not increase the recovery rate of Mg, Ca, Fe, Zn and Se, while the recovery rate of P was increased slightly (~1.12 times). Meanwhile, the PEF-assisted treatment enhanced the recovery of Fe and Se in the viscera extract ~1.1 and 1.2 times, respectively.

Considering the bioaccessibility of minerals in rainbow trout side stream extracts, the bioaccessibilities of Fe and Zn in head extracts were improved after PEF-assisted treatment (Fe: PEF 69.20% *vs* no-PEF 33.21%, Zn: PEF 91.01% *vs* no-PEF 58.00%). However, the bioaccessibility of Mg and P was lower in PEF-assisted extracts compared to control (Mg: PEF 12.00% *vs* no-PEF 15.71%, P: PEF 29.49% *vs* no-PEF 47.40%). For rainbow skin extracts, the bioaccessibilities of Mg, Ca, Fe, Zn and P in the control group were significantly lower than in the PEF group. In viscera extracts, PEF-assisted treatment increased the bioaccessibility of Mg, while the bioaccessibility of the other five minerals was not significantly increased with PEF-assisted extraction.

Table 3. Mineral content before and after simulated gastrointestinal digestion in sole by-product extracts (PEF-assisted treatment and control group)

Sample	Total content (mg/100 g)	BF (mg/100 g)	Bioaccessibility (%)
		Mg	
Head-PEF	201.90±3.63^{Ad}	31.90±2.12^{Bbc}	15.80±1.01
Head-control	191.01±1.56^{Ac}	30.56±0.56^{Bb}	16.00±0.71
Skin-PEF	156.05±2.27^{Ab}	25.73±0.56^{Ba}	16.49±1.21
Skin-control	146.22±2.64^{Aa}	24.13±1.25^{Ba}	16.50±1.50
Viscera-PEF	211.97±1.71^{Af}	33.91±0.71^{Bc}	16.00±0.88
Viscera-control	207.73±3.01^{Ae}	31.01±1.44^{Bbc}	14.93±1.47
		Ca	
Head-PEF	461.94±15.57^{Ad}	58.25±4.24^{Bb}	12.61±0.40^{b}
Head-control	420.42±20.76^{Ac}	66.72±4.43^{Bc}	15.87±1.01^{e}
Skin-PEF	377.83±15.11^{Ab}	51.38±1.89^{Ba}	13.60±0.22^{c}
Skin-control	333.25±6.81^{Aa}	48.02±0.86^{Ba}	14.43±0.20^{d}
Viscera-PEF	432.86±17.14^{Ac}	50.73±1.01^{Ba}	11.72±1.00^{b}
Viscera-control	1238.57±38.57^{Ae}	47.07±0.98^{Ba}	3.80±0.32^{a}
		Fe	
Head-PEF	0.77±0.02^{Aa}	0.69±0.01^{Bc}	89.99±1.01^{f}
Head-control	1.30±0.03^{Ab}	0.89±0.01^{Bd}	68.40±2.61^{e}
Skin-PEF	0.67±0.01^{Aa}	0.35±0.03^{Ba}	52.55±1.78^{d}
Skin-control	1.81±0.02^{Ac}	0.52±0.04^{Bb}	28.91±1.60^{c}

Viscera-PEF	6.68±0.12^{Ae}	1.19±0.04^{Be}	17.82±1.04^{a}
Viscera-control	5.57±0.04^{Ad}	1.13±0.06^{Be}	20.31±1.33^{b}
Zn			
Head-PEF	2.32±0.01^{Ac}	1.92±0.28^{Bc}	82.50±2.51^{d}
Head-control	1.93±0.01^{Ab}	1.62±0.01^{Bb}	83.83±2.77^{d}
Skin-PEF	1.24±0.01^{Aa}	0.89±0.14^{Ba}	72.00±2.71^{c}
Skin-control	2.27±0.00^{Ac}	1.55±0.01^{Bb}	68.11±0.72^{b}
Viscera-PEF	4.62±0.12^{Ad}	2.27±0.04^{Bd}	49.10±1.88^{a}
Viscera-control	4.67±0.08^{Ad}	2.48±0.00^{Bd}	53.20±2.90^{a}
Se (µg/100 g)			
Head-PEF	ND	ND	ND
Head-control	ND	ND	ND
Skin-PEF	ND	ND	ND
Skin-control	23.20±0.45^{Aa}	13.68±0.71^{Ba}	58.97±0.31^{b}
Viscera-PEF	188.57±11.14^{Ac}	66.41±2.01^{Bb}	35.22±1.91^{a}
Viscera-control	89.14±5.57^{Ab}	63.46±3.32^{Bb}	71.20±5.20^{c}
P			
Head-PEF	163.33±0.73^{Ab}	130.66±7.01^{Bc}	80.00±1.10^{d}
Head-control	143.77±5.19^{Aa}	120.78±1.69^{Bb}	84.01±1.42^{e}
Skin-PEF	203.65±4.53^{Ac}	78.63±4.55^{Ba}	38.61±1.58^{b}
Skin-control	160.58±5.29^{Ab}	75.63±1.86^{Ba}	47.10±1.61^{c}
Viscera-PEF	441.43±17.14^{Ad}	171.19±3.13^{Bd}	38.78±3.83^{b}

Viscera-control	1290.01±18.57^{Ae}	214.27±5.67^{Be}	16.61±1.04^{a}

BF: bioaccessible fraction. Date presented in dry weight. Results are expressed as mean ± standard deviation. Different lowercase letters in the same column for each mineral compound indicate significant differences ($p < 0.05$). Different uppercase letters in the same row for each mineralindicate significant differences before and after digestion for one sample ($p < 0.05$). ND: not detected.

Table 3 shows the minerals content from sole side stream extracts comparing PEF treated samples with controls and the digested samples with not digested controls. It can be seen that the PEF-assisted treatment can increase the content of Mg, Ca, Zn and P in the head extract. For skin extract, PEF treatments can increase the contents of Mg, Ca and P, but compared with the control group, the contents of Fe and Zn were lower. In the viscera extract, the Mg and Zn contents were not significantly affected by PEF-assisted treatment, while the content of Fe and Se increased about 1.2 and 2.1 times, respectively. In contrast, the PEF-assisted treatment reduced the Ca and P contents of viscera extracts.

Likewise, the bioaccessibility of minerals is also shown in **Table 3.** Among them, the bioaccessibility of Mg was not significantly different in all extracts. In the head extract, PEF-assisted treatment increased the bioaccessibility of Fe by 30 % compared to the control group, while the bioaccessibility of Ca and P was slightly lower after PEF compared to control group (Ca: PEF 12.6 % *vs* no-PEF 15.9 %, P: PEF 80.0 % *vs* no-PEF 84.1 %). For skin extract, PEF-assisted treatment increased the bioaccessibility of Fe (52.55 % *vs* 28.91 %), as well as Zn (72.00 % *vs* 68.11 %). At the same time, as can be observed in **Table 3**, the bioaccessibility of Ca and P in the control group was slightly higher than in the PEF treatment (Ca: PEF 13.60 % *vs* no-PEF 14.43 %, P: PEF 38.61 % *vs* no-PEF 47.10 %). In viscera extracts, PEF-assisted treatment enhanced the bioaccessibility of Ca and P (Ca: PEF 11.72 % vs no-PEF 3.80 %, P: PEF 38.78 % vs no-

PEF 16.61 %), showing a different effect compared with skin and head extracts. It can also be seen that PEF-assisted treatment resulted in a bioaccessibility decrease of Fe, Zn and Se in the viscera extract.

4. Discussion

Fish side stream extracts contain high amounts of antioxidants and minerals that may potentially contribute to health. Considering the content of these substances alone is not enough to assess their effects on health, due to the nutritional value of antioxidants and minerals is meaningful when they can be digested and absorbed (Manditsera et al., 2019). In this study, the antioxidant capacity of the extracts changed before and after simulated gastrointestinal digestion, but it was also found that the change was not directly related to the use of PEF treatment.

Fish side streams extracts contain high-added-value proteins, and their nutritional value is mostly attributed to the essential amino acids. In addition, protein structure, processing, modification, etc., can also affect protein digestion. For different fish side streams, the extracts may be enzymatically decomposed into short-chain peptides or amino acids during the digestion process, thereby changing the antioxidant capacity of the extracts after digestion (Kaur et al., 2022; Luo et al., 2020).

Processing also has an impact on protein digestion. When PEF is used, it can affect the function, sensory, and nutritional value of food matrices. In this study, low-strength PEF was used to treat the samples, which may increase the permeability of cell membranes, further affecting digestion (Bhat et al., 2021). The antioxidant properties of extracts may have different performances under the combined action of many factors. There are few studies on the use of PEF-assisted treatment to change the protein digestion properties of

fish. For animal protein, Chian et al. (Chian et al., 2021), observed that the combination of PEF and sous-vide could affect the digestion of beef brisket muscle protein, and the reason was attributed to the fact that PEF treatment could destroy the microstructure and ultrastructure of cooked muscle, enhancing digestive proteolysis.

The mineral content and the bioaccessibility of fish side stream extracts under PEF-assisted and control (without-PEF) treatments were also evaluated. Scarce information is available in the literature about the effect of PEF on the mineral content of food matrices. In this study, it was observed that PEF can increase the mineral content of some extracts, but it is worth noting that the PEF-assisted extraction did not show a positive effect in all samples. In general, several factors such as thermal effects during food processing, processing methods, and the combination with other substances have an impact on mineral content and digestion (Jiang et al., 2021). During the PEF-assisted extraction, the temperature was lower than 60 °C, thus ensuring that the bioaccessibility of minerals was not reduced due to the high temperature. It can be speculated that the digestion process may result in protein changes and interaction with other components. Studies have also shown that peptides derived from fish side streams can chelate mineral elements to form soluble chelates, thus affecting the recovery and absorption of minerals (Zamora-sillero et al., 2018). Taking into account the data on the mineral content and the amount that can be absorbed a more comprehensive assessment of the possible health benefits to the human body can be established.

5. Conclusions

From the results obtained in this study, it can be concluded that the *in vitro* simulated digestion caused an effect on the antioxidant capacity of the extracts, but the positive

effects of PEF extraction were not shown in all the extracts. Digestion and absorption of minerals were affected by several factors, having PEF a positive role to increase the content of some minerals in the extracts obtained. Moreover, it also has an important impact on the bioaccessibility of minerals, having a negative effect on some minerals. Selecting the appropriate treatment method according to the research purpose can help improving the efficient utilization of side streams, thus promoting the sustainable use of natural resources.

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4.5 Investigating the effects of fish side stream extracts on gut microbiota through an *in vitro* static colonic fermentation model

(Under review)

Investigating the effects of fish side stream extracts on gut microbiota through an *in vitro* static colonic fermentation model

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Abstract

The composition of gut microbiota and the content of its metabolites can be used as evaluation indicators of human health and some diseases. Based on previous studies, this study by established an *in vitro* static colonic fermentation model to investigate the effects of four fish by-products (sole-head, sole-viscera, rainbow trout-head and rainbow trout-viscera) extracts on the content of specific gut microbes and the main metabolite short-chain fatty acid (SCFAs) at different fermentation times. The results showed that the addition of the extract had an effect on the gut microbiota and the production of SCFAs. Among them, the more obvious ones are that the sole-viscera extract can increase the content of probiotics *Bifidobacterium* and *Lactobacillus*, while reducing the content of *Enterobacteriaceae* and *Bacteroides* group. At the same time, the addition of sole-viscera and rainbow trout-viscera extracts also promoted the accumulation of SCFAs. Therefore, the fish by-products extract effects the composition and metabolism of the gut microbiota, with potential implications for host health.

Key words: fish by-products, colonic fermentation, gut microbiota, SCFAs

1. Introduction

The human microbial community contains bacteria, fungi, archaea, viruses, etc., which interact with the immune system and affect human health. The intestine can host trillions of microbes and is considered a unique organ, these microbial communities present in the intestine are collectively referred to as the gut microbiota (D'Amelio and Sassi 2018). In the intestinal tract of healthy people, Firmicutes and Bacteroidetes are dominant, while the abundance of Proteobacteria and Actinobacteria are also high (Kumar et al. 2021). At the same time, the composition of gut microbiota is also affected by age, diet, genetics and other factors (Rothschild et al. 2018). In recent years, the importance of gut microbiota to human health has become more and more well known. It can participate in immune regulation as part of the immune system, and also can plays a regulatory role in human metabolism and central nervous system regulation. Normally, there is a balance between gut microbiota and human health. Once the balance is broken, it will cause a series disease such as obesity, diabetes, metabolic diseases and so on (Pellegrini et al. 2018; Song et al. 2022).

The colon is an anaerobic environment, and one of its most important functions is fermentation. Certain anaerobic bacteria are able to ferment undigested components form the diet and bio-transform the body's endogenous compounds. In the process of colonic fermentation, short-chain fatty acids (SCFAs), bile acids, carboxylic acids and their derivatives are produced (Wang et al. 2019). Among them, SCFAs have been studied the most as the most important metabolites. SCFAs is a class of saturated aliphatic organic acids containing 1~6 carbon backbones produced by fermentation of undigested

carbohydrates or certain protein in the diet, while it has been found that SCFAs can be used as an energy source to promote cell growth, it also can induce the production of anti-inflammatory factors to reduce inflammation and inhibit the proliferation of tumor cells. In addition, SCFAs can function as signaling molecules in blood pressure regulation and lipid metabolism (Macfarlane and Macfarlane 2003; Feng, Ao, and Peng 2018). Nutrients contained in the diet, including protein, lipids, carbohydrates, etc., have been proven to interfere with the metabolism of the gut microbiota, thereby affecting the composition of the gut microbiota. therefore, evaluating the regulatory effects of different nutrients on the gut microbiota can effectively prevent diseases related to gut microbiota imbalance (Zhang et al. 2018). At present, a series of *in vitro* colonic fermentation models have been developed, which can simulate specific pH, temperature, substrate concentration, etc., to evaluate the impact of dietary nutrients on gut microbiota. *In vitro* colonic fermentation models can be divided into two categories: static batch fermentation and dynamic continuous dynamic models (Barry et al. 1995; Ferreira-Lazarte et al. 2019). Most of these fermentation models use single or mixed fecal samples as inoculum and ferment in an anaerobic environment, while investigating the metabolic process and product accumulation of gut microbes. The advent of next-generation sequencing technologies has made it possible to clearly label the gut microbiota, in which 16S rRNA gene amplification combined with real-time PCR can classify and quantify the gut microbiota, while some chromatography-based mass spectrometry, NMR, capillary electrophoresis can analyze the metabolites of gut microbiota including SCFAs (Veintimilla-Gozalbo et al. 2021; García-Rivera et al. 2022). Using a variety of research methods to analyze the

composition of gut microbiota and its metabolites, more effective information can be obtained for the prevention and treatment of some diseases.

The oceans are a huge treasure trove of resources, with millions of life forms, including fish, crustaceans, algae and microorganisms. The rational development and utilization of marine resources can help alleviate the environment and food pressures brought about the population growth (Mora et al. 2011). Among them, fish, as the most important marine resources can provide high-value added compounds including protein, amino acids, lipids, etc. However, in the process of fish processing, a large number of by-products will be produced that cannot be used well, such as head, viscera, skins, which not only wastes resources, but also pollutes the environment (Min Wang et al. 2021). In this research, we established an in vitro static colonic fermentation model to explore the effects of fish by-product extracts on gut microbiota, aiming to improve the utilization of these high-value compounds and explore more potential application values.

2. Materials and methods

2.1 Sample preparation

The samples were prepared in the same way as in Chapter 4.3. In this section, ASE-assisted fish head and viscera extracts were selected.

2.2 In vitro colonic fermentation

An in vitro colonic fermentation model was established to evaluate the effect of fish by-products extracts on human gut microbiota. Based on Yousi's (Yousi et al. 2019) method with minor modifications, in brief, fresh fecal samples were collected from volunteers who had been free of digestive disorders and had not received antibiotics in the past three

months, and sterile saline was used to dilute the fecal samples to obtain 10 % fecal slurry. After 30 min of homogenization and 15 min for rest, 10.5 mL supernatant was transferred to a new tube filled with 5 mL Nycodenz and centrifuged at 4 °C/4000 rpm for 60 min. Discarded the supernatant, transfer the intermediate white layer (bacterial phases) to a centrifuge tube and wash twice with 0.9 % (*w/v*) NaCl. Before colonic fermentation, basal medium was added to the fermenter and boiled for about 5 min to remove oxygen. After cooling to room temperature, fecal microorganisms were added and incubated overnight at 37 °C under anaerobic conditions. The next day, the extracts from fish by-products were added to the fermentation tube and anaerobic fermentation continued at 37 °C, with samples collected at 24 and 48 h. After centrifugation, the supernatant and pellet were collected separately for SCFAs and DNA extraction.

2.3 Targeted microbial quantification by qPCR analysis

According to the study by Selma-Royo et al. (Selma-Royo et al. 2021), total DNA was extracted from fecal microorganisms after fermentation using Maxwell® RSC PureFood GMO and Authentication kits (Catalo No-AS1600; Promega Corporation, Madison, WI, USA). Specifically, the pellet obtained by centrifugation was homogenized with 900 µL CTAB buffer and mix with glass beads in a 2 mL centrifuge tube, while the FastPrep-24 5G (Lab Innovations, UK) device was used for two cycles at 6 m/s, each cycle lasting 60 s. Then, the samples were heated at 95 °C for 2.5 min, vortexed for 10 s and then heated 2.5 min again. Add protease (40 µL) and lysozyme (20 µL) to centrifuge tubes, vortex and heat at 70 °C for 10 min. DNA extraction was performed according to the manufacturer's instruction from Maxwell® RSC PureFood GMO and Authentication kits.

The DNA concentration in the samples was determined using a Qubit 2.0 Fluorometer (Life Technology, Carsbad, CA, USA) and stored at -80 °C for until use. Bacteria were quantified by 16S rRNA amplification and analysis. Add 3.5 µL H_20, 1 µL DNA, 5 µL SYBR Green Master Mix, 0.25 µL forward primer, 0.25 µL reverse primer, standard/water in 96-well microplate, record the number of 16S rRNA gene copies by real-time qPCR using LightCycle 480 device (Roche), set 40 cycles and calculate based on standard and dissociation curve. In this study, we measured 16s rRNA gene copies of total bacterial, *Bifidobacterium* genus, *Lactobacillus* spp., *Enterobacteriaceae* family, *Streptococcus*, and *Enterococcus* spp.

2.4 Determination of SCFAs by GC-MS

To analyze the main metabolite SCFAs during fermentation, take 200 µL supernatant mixed with 800 µL standard solution, 1 mL diethyl ether and 1 spoon Na_2SO_4, and vortex 10 s to prepare for GC-MS analysis. Agilent GC 7890B-5977 GC-MS with a multi-injector was used in the process, specifically: the column (Agilent DB-FATWAX, 30 cm ×0.25 mm×0.25µm) was operated in split mode, the oven temperature was ramped up to 100 °C at a rate of 5 $°C·min^{-1}$, to 150 °C within 1 min, and at a rate of 20 $°C·min^{-1}$ raised to 200 min^{-1} and held at 200 °C for 5 min, carrier gas was N_2, flow rate 1 $mL·min^{-1}$.

2.5 Statistical analysis

Statistical analysis was performed, and graphs created using GraphPad Prism 8.1.0. One-way ANOVA with Dunnett's multiple comparisons test was performed using Statgraphics® Centurion XV (Statpoint Technologies, Inc., USA). Statistical significance was accepted at $p < 0.05$.

3. Results

Table 1. Total and specific bacterial levels measured with qPCR in cultures with fecal adult's microbiota supplemented with fish by-products extracts

Total bacterial (initial content: 6.352±0.551 Log (16S rRNA gene copies/µg DNA))					
	control	S-head	S-viscera	R-head	R-viscera
24 h	6.548±0.076	6.900±0.245	6.755±0.774	6.361±0.049	6.721±0.059
48 h	7.143±0.144	7.066±0.002	7.384±0.622	7.152±0.155	6.818±0.068
Bifidobacterium (initial content: 2.338±0.163 Log (16S rRNA gene copies/µg DNA))					
	control	S-head	S-viscera	R-head	R-viscera
24 h	2.328±0.039[a]	2.177±0.131[a]	2.604±0.026[b]	2.310±0.035[a]	2.285±0.067[a]
48 h	2.517±0.042[b]	2.709±0.038[b]	2.980±0.006[c]	2.228±0.159[a]	2.515±0.436[b]
Lactobacillus (initial content: 2.657±0.063 Log (16S rRNA gene copies/µg DNA))					
	control	S-head	S-viscera	R-head	R-viscera
24 h	2.338±0.011[a]	2.532±0.228[ab]	2.666±0.017[b]	2.571±0.060[b]	2.564±0.095[ab]
48 h	2.572±0.004[a]	2.572±0.043[a]	3.138±0.213[b]	2.820±0.180[ab]	2.532±0.070[a]
Enterobacteriaceae (initial content: 4.571±0.847 Log (16S rRNA gene copies/µg DNA))					
	control	S-head	S-viscera	R-head	R-viscera
24 h	6.750±0.035[d]	6.892±0.139[d]	2.754±0.172[a]	4.599±0.136[b]	6.253±0.053[c]
48 h	7.156±0.031[b]	7.067±0.021[b]	2.953±0.027[a]	7.216±0.089[b]	7.149±0.044[b]
Bacteroides group (initial content: 4.093±0.190 Log (16S rRNA gene copies/µg DNA))					
	control	S-head	S-viscera	R-head	R-viscera
24 h	6.840±0.072[c]	4.091±0.096[b]	3.462±0.081[a]	3.236±0.273[a]	3.897±0.160[b]
48 h	6.906±0.008	6.453±0.192	6.372±0.468	5.960±0.079	5.892±0.085
Streptococcus (initial content: 1.837±0.133 Log (16S rRNA gene copies/µg DNA))					

	control	S-head	S-viscera	R-head	R-viscera
24 h	3.278±0.039[c]	1.687±0.071[a]	3.876±0.196[e]	3.529±0.111[d]	2.264±0.074[b]
48 h	3.659±0.003[b]	1.896±0.136[a]	3.736±0.091[b]	3.600±0.085[b]	1.950±0.015[a]

*S: sole, R: rainbow trout. Control groups are without extracts added and instead of the same volume of distilled water. Different letters mean significant differences between samples at the same fermentation time ($p<0.05$).

To evaluate the effect of fish by-product extracts on gut microbiota, the 16s rRNA gene copies/µg DNA of microbes after colonic fermentation was determined for 24 h and 48 h, including Total bacterial, *Bifidobacterium*, *Lactobacillus*, *Enterobacteriaceae*, *Bacteroides* group and *Streptococcus*. It can be seen that from **Table 1**, *Enterobacteriaceae* and *Bacteroides* group are dominant, followed by *Bifidobacterium* and *Lactobacillus*, while the proportion of *Streptococcus* is lower. With the extension of fermentation time to 48 h, the amounts of bacteria in most samples increased, while the more obvious ones were total bacterial, *Enterobacteriaceae*, *Bacteroides* group and *Streptococcus*.

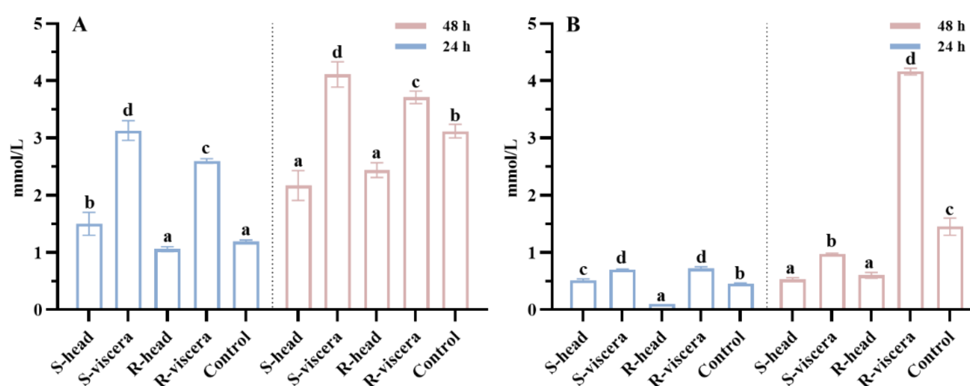

Figure 1. Short chain fatty acids (SCFAs) production in fecal adult's microbiota supplemented with fish by-products extracts after 24 and 48 h: A) Acetic acid, B) Propanoic acid. S: sole, R: rainbow trout. Control group is without extracts added and

instead of the same volume of distilled water. Different lowercase letter means significant differences between different samples at the same fermentation time ($p < 0.05$).

Short chain fatty acids (SCFAs) were determined as major metabolites of gut microbiota. **Figure 1** shows that the fermentation process is accompanied by the production of acetic acid and propionic acid, in which the content of acetic acid is about 1.1~4.2 mmol/L, and the content of propionic acid is 0.1~4.1 mmol/L, acetic acid was higher for most samples. At the same time, the prolongation of fermentation time also contributed to the accumulation of SCFAs, and the content of SCFAs at 48 h was significantly higher than that at 24 h.

4. Discussion

Gut microbiota plays a key role in human health and is also affected by a variety of factors. Among them, diet is one of the main factors affecting the composition and metabolism of gut microbiota. Moreover, the gut microbiota produces beneficial metabolites, such as SCFAs, that not only serve as a source of energy, but also have a positive impact on the health of host (Brial et al. 2021). In the gut microbiota, *Bifidobacterium* (Actinobacteria) and *Lactobacillus* (Firmicutes) are considered to be probiotics with positive effects, which have the potential to control cholesterol, regulate the immune system and promote digestion. Bacteroides is one of the bacteria involved in the degradation of polysaccharides. In addition, at the phylum level, the Firmicutes/Bacteroidetes ratio was found to be closely related to the balance in the host and change in this ration could lead to some diseases, such as obesity (Li et al. 2019).

It can be seen from **Table 1** that there was no significant difference in the content of total bacteria in the groups added with extracts compared with the control group when the fermentation time was 24 and 48 h. The amount of *Bifidobacterium* in the group containing the sole-viscera extract increased at both 24 and 48 h. The addition of all extracts increased the amount of *Lactobacillus* at 24 h compared to control group, while the sole-viscera and rainbow trout-head extracts showed significant differences when the fermentation time was extended to 48 h. For *Enterobacteriaceae*, the group with the sole-viscera extracts showed significantly lower than the control group at both fermentation 24 and 48 h. The addition of fish by-products extracts made the content of *Bacteroides* group in the samples lower than the control group at 24 h, while the content of *Bacteroides* group was not significantly different after 48 h. In addition, for *Streptococcus*, the group containing sole-head and rainbow trout-viscera extracts were significantly lower than the control group at 24 and 48 h. Overall, in the four extracts selected in the experiment, the sole-viscera extract showed a more positive effect, increasing the content of *Bifidobacterium* and *Lactobacillus*, while the contents of *Enterobacteriaceae* and *Streptococcus* were also lower than the control group. previously, we have explored the protein content and molecular weight in the extracts, while the small molecular weight protein (<15 kDa) in the sole-viscera extract is higher than others, which may be a potential factor for its positive effects (Wang et al. 2021). Wang et al. (Wang et al. 2020) also found that low-molecular weight bioactive peptides from fish can promote the increased in the abundance of probiotics such as *Lactobacillus* and *Akkermansia*, and also have the potential to inhibit inflammation.

The production of two SCFAs was detected in this study, including acetic acid and propionic acid. In the gut microbiota, *Bacteroidetes*, *Bifidobacteria* and *Lactobacillus* can produce acetic acid, which can be used as an energy source for the human after entering the body fluid circulation. *Bacteroidetes* also can produce propionic acid, which is involved in the metabolic synthesis of cholesterol (García-Rivera et al. 2022). Figure 1 shows the content of SCFAs in this study. For acetic acid, the addition of sole-viscera, sole-head and rainbow trout-viscera extracts significantly increased the content of acetic acid compared with the control group when the fermentation time was 24 h. At 48 h, the content of acetic acid was significantly higher in the groups containing the sole-viscera and rainbow trout-viscera extracts than in the control group. For propionic acid, the rainbow trout head extracts-added group was significantly lower than the control group, while the other three extracts were slightly higher than the control group. After 48 h of fermentation, the propionic acid content increased, most obviously in the group containing rainbow trout-viscera extract, while the content of propionic acid in the added sole-head, sole-viscera and rainbow trout-head extracts was lower than that in the control group. Sole-viscera and rainbow trout-viscera extracts showed positive effects in promoting acetic acid and propionic acid content, respectively.

This study investigated the effects of four by-product extracts from sole and rainbow trout on gut microbiota and SCFAs, with the aim of exploring the potential use of the extracts as functional ingredients. The addition of the extract had an effect on the gut microbiota and also affected the accumulation of SCFAs. However, the fish by-product

extracts in this study are a high-value mixture containing protein, and the protein in extracts has not been further hydrolyzed and fractionated, which has certain limitations.

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4.6 Pulsed electric field (PEF) recovery of biomolecules from *Chlorella*: Extract efficiency, nutrient relative value, and algae microstructure analysis

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Pulsed electric field (PEF) recovery of biomolecules from *Chlorella*: Extract efficiency, nutrient relative value, and algae morphology analysis

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Abstract

This study investigated the effects of pulsed electric field (PEF) (3 kV/cm, 44 pulses, 99 kJ/kg), solvent (H_2O or 50% DMSO) and time (0, 10, 20, 30, 60, 90, 120 and 180 min) on the extraction of Chlorella antioxidant biomolecules and minerals. The results showed that PEF treatment increased the biomolecules recovery. For the extraction time of 120 minutes, more proteins and polyphenols were obtained using water, while more chlorophyll a and b, and carotenoids were obtained using 50% DMSO as the extraction solvent. The extracts mineral concentration (PEF vs. control) were analyzed including Mg, P, Ca, Fe and Zn, and the Relative Nutrient Values results indicated that Chlorella H_2O-extracts could be used as a mineral source for different populations. Finally, the fluorescence and scanning electron microscopy revealed the electroporation effect of PEF.

Keywords: Pulsed electric fields (PEF), microalgae, antioxidants, minerals, microstructure

1. Introduction

One of the main challenges we face in the 21st century is feeding a growing population with increasingly limited natural resources (Torres-Tiji, Fields, & Mayfield, 2020). FAO and the Green Deal recommend that humanity should move towards a more sustainable and environmentally friendly global food system (FAO and WHO, 2019; Couto et al., 2022). In addition, the global food industry contraction and increased healthy food demand caused by Covid-19 make it necessary for the food industry to think about how to make good use of the existing natural edible resources to better overcome the potential crisis. Microalgae is an important part of marine and freshwater resources. Microalgae belongs to autotrophic microorganisms, which utilize light energy and inorganic nutrients (carbon dioxide, nitrogen, phosphorus, etc.) to synthesize valuable biomass (such as proteins, polysaccharides, polyphenols, minerals, etc.) (Markou & Nerantzis, 2013). Microalgae can be produced on a large scale without competing with conventional agriculture land (Liu et al., 2022). The chemical composition of microalgal biomass, including proteins, lipids, pigments, polysaccharides, etc., basically supports the development of microalgae products, which has attracted global interest (Song et al., 2018).

Among thousands of species of microalgae, *Chlorella* is the most industrially cultivated microalgae since the early 1960s, and it has been consumed as novel foods and studied worldwide (Couto et al., 2022). *Chlorella* is a unicellular green algae classified as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration, which is rich in protein (47.82 ± 0.05 % dw), lipids (13.32 ± 0.07 % dw), carbohydrates (8.08 ± 0.09 % dw), and mineral elements such as magnesium (Mg, 344.3±0.12 mg/100

g dw), phosphorus (P, 1761.5±0.02 mg/100 g dw), calcium (Ca, 593.7±0.07 mg/100 g dw), iron (Fe, 259.1±0.04 mg/100 g dw), zinc (Zn, 1.19±0.07 mg/100 g dw), and selenium (Se, 0.07±0.03 mg/100 g dw), (Song et al., 2018; Tokuşoglu & Ünal, 2003). Consuming *Chlorella* rich in the above nutrients has many benefits to human health, such as antioxidant, anti-inflammatory, anti-cardiac, anti-diabetic and regulating the balance of gut microbiota (Helene Gateau et al., 2017). Considerable research and efforts have been devoted to the use of *Chlorella* for food production, however, the high cost of production, cultivation and downstream processing is the biggest hurdle (Loke Show, 2022). In recent years, with the rise of the microalgae culture industry, the culture technology has gradually matured, and downstream processing, such as the efficient recovery of *Chlorella* nutrients, has become the main focus.

Chlorella has a multi-layered cell wall structure with a thickness of 100~200 nm, which is composed of polysaccharides, proteins, and inorganic salts, as a consequence nutrients in the cytoplasm are not easily available (Ahmed & Kumar, 2022). Traditional extraction techniques such as Soxhlet, Folch, hot water extraction, are used to obtain soluble bioactive compounds from *Chlorella*. Although these techniques can obtain most molecules from *Chlorella*, they are gradually replaced by new extraction techniques due to the disadvantages of long extraction time, high temperature, and the use of toxic reagents (Soleimani Khorramdashti, Samipoor Giri, & Majidian 2021). Compared with the traditional extraction technology, pulsed electric field (PEF) extraction is a novel and non-thermal technology that is being widely used for the recovery of microalgae biomolecules due to its advantages of cleanliness, safety, and high efficiency (Hélène Gateau et al., 2021). PEF device (laboratory configuration) typically includes an electrical

pulse generator, a treatment chamber, and electrodes, with the electrical pulse placed between or through two electrodes (Naliyadhara et al., 2022). The PEF principle is based on the application of short electrical pulses (from a few nanoseconds to a few milliseconds) of high voltage (from 100~300 V/cm to 80 kV/cm) to the product between two electrodes (Barba et al., 2015). PEF treatment can alter cell membrane properties due to the high-intensity electric field pulse discharges (electroporation phenomenon), resulting in increased cell membrane permeability and promotion of cytoplasmic dissolution (Zhou et al., 2022).

At present, the studies of PEF assisted extraction of biomolecules from *Chlorella* are mainly focused on biological macromolecules like proteins, polysaccharides ('t Lam et al., 2017; Carullo et al., 2018; Postma et al., 2016; Scherer et al., 2019) and lipid compounds (Canelli et al., 2022). In addition, Chlorella is also rich in minerals and antioxidants, however, there are not many related studies. Based on this, the recovery of a variety of antioxidant biomolecules (proteins, polyphenols, chlorophyll a, chlorophyll b, carotenoids) and minerals (Mg, P, Ca, Fe, Zn, Se) from Chlorella assisted by PEF was carried out in this study. Moreover, the effect of PEF treatment on the permeability of Chlorella cells was observed under a scanning electron microscope (SEM) and fluorescence microscope (FM), to comprehensively evaluate the effect of PEF on the extraction of nutrients from Chlorella.

2. Materials and methods

2.1. Chemicals and reagents

ABTS (2,2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid), AAPH (2,2'-Azobis(2-methylpropionamidine) dihydrochloride), Folin-Ciocalteu, gallic acid, Trolox (6-

hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), D-glucose, phenol, fluorescein sodium salt, potassium persulfate and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Steinheim, Baden-Württemberg, Germany). The methanol (>99%) was purchased from Merck (Whitehouse Station, NJ, USA). Sodium carbonate was acquired from VWR (Saint-Prix, France). Bicinchoninic acid (BCA) kit, sodium hydroxide, glacial acetic acid, and sulfuric acid were supplied by Fisher Scientific (Madrid, Spain). Deionized water (resistivity >18 MΩ cm^{-1}) was produced by a Milli-Q SP® Reagent Water System (Millipore Corporation, Bedford, MA, USA).

2.2. Samples

Chlorella vulgaris from Hainan Island (China, 3°30′~20°17'N, 108°15′~120°15′E) was produced in open raceway ponds and average temperatures varying from 21 °C to 33 °C and precipitations of 1600 mm per year. At the time of harvesting, biomass was washed and then spray-dried at 160 to 180 °C for 15 minutes. The final product was green powder with characteristic smell and taste, which was stored at -20 °C for experimental analysis.

2.3. Pulsed electric fields (PEF) extraction process

For the PEF pre-treatment of the *Chlorella* 2% (*w/v*) solution was used with the PEF-Cellcrack III equipment (German Institute for Food Technology (DIL)) (ELEA, Germany). According to the previous studies in our laboratory (Martí-Quijal et al., 2021), PEF treatment conditions of 3 kV/cm, 44 pulses, 99 kJ/kg were selected to extract antioxidants and minerals from *Chlorella* at room temperature (23±2 °C). The temperature and conductivity of each sample were measured with a portable conductivity meter ProfiLine Cond 3310 (WTW, Xylem Analytics, Weilheim in Oberbayern, Germany). In this study, the aqueous suspension of *Chlorella* (2 g/200 mL) was first

treated with PEF. The temperature was changed from 23±2 °C to 31±3 °C and the conductivity was changed from 1400±20 µs/cm to 1620±33 µs/cm after PEF treatment. Then, the same volume of water or DMSO (200 mL) was added after PEF treatment to make the final sample as 2g/400 mL water extracts or 2g/400 mL water: DMSO 1:1 extract (50% DMSO). Then, a magnetic stirrer was used to continuously stir the samples at room temperature (25 ºC) and the samples were collected at 0, 10, 20, 30, 60, 90, 120 and 180 minutes, respectively. The control experiment was carried out with 2 g Chlorella powder/400 mL water or 2 g *Chlorella* powder/400 mL 50% DMSO stirred at room temperature and the samples were collected at the same time as PEF extraction process. Finally, the samples were centrifuged (2504 × g, 4 °C, 15 minutes) using a 5810R centrifuge (Eppendorf Ibérica, Madrid, Spain), and the supernatants were collected and stored at -20 °C until analyzes. Each biomolecule amount was calculated based on the dry weight (DW) of *Chlorella* powder.

2.4. Bioactive molecules analysis

2.4.1. Protein

The BCA working solution was prepared according to the instructions of the BCA kit (Pierce Biotechnology, Inc., Waltham, MA, USA), that was, mixed reagents A and B at a ratio of 50:1 (*v/v*). 10 µL of samples or bovine serum albumin (BSA) and 200 µL of BCA working solution were added to a 96-well plate, mixed well, and incubated in a 37 °C oven for 30 minutes and measured the absorbance at 562 nm. The protein content (mg/g dw) was calculated by means of a calibration curve prepared with BSA from 0~2000 mg/L.

2.4.2. Polyphenol

The Folin-Ciocalteu method was used to analyze the total polyphenol content in the extract (Korzeniowska et al., 2020). Briefly, 0.2 mL samples, 1 mL Folin-Ciocalteu (diluted with water at a ratio of 1:1, v/v) and 0.8 mL sodium carbonate solution (75 g/L) were mixed and incubated in a water bath at 50 °C for 10 minutes. Then, the absorbance value was measured at 750 nm using a Perkin-Elmer UV/Vis Lambda 2 spectrophotometer (Perkin-Elmer, Jügesheim, Germany). Gallic acid was used as a standard to calculate the polyphenol content (mg/g dw) in the extracts.

2.4.3. Pigments (chlorophyll a, chlorophyll b, carotenoids)

The chlorophyll *a*, chlorophyll *b* and carotenoids concentration of *Chlorella* extracts were analyzed by spectrophotometry. The absorbance values and formulas used to analyze extracts varied with solvent, for 50% DMSO extracts, the equations were as follows (Wellburn, 1994):

$$C_{ch}^{a} = 12.47 * A_{665.1} - 3.62 * A_{649.1} \quad (1)$$

$$C_{ch}^{a} = 25.06 * A_{649.1} - 6.5 * A_{665.1} \quad (2)$$

$$C_{carotenoids} = \left(100 * A_{480} - 1.29 * C_{ch}^{a} - 53.78 * C_{ch}^{b}\right) \quad (3)$$

For H_2O-extracts, the equations were as follows (Kokkali et al., 2020):

$$C_{ch}^{a} = 16.82 * A_{665} - 9.28 * A_{653} \quad (4)$$

$$C_{ch}^{a} = 36.92 * A_{653} - 16.54 * A_{665} \quad (5)$$

$$C_{carotenoids} = \left(1000 * A_{470} - 1.91 * C_{ch}^{a} - 95.15 * C_{ch}^{b}\right)/225 \quad (6)$$

WhereC_{ch}^{a}, C_{ch}^{b}, and $C_{carotenoids}$ were the concentrations (mg/L dw) of chlorophyll *a*, chlorophyll *b* and total carotenoids, respectively.

2.4.4. Dynamic proportion

Based on the protein, polyphenols, chlorophyll a, chlorophyll b and carotenoids content, the dynamic proportion of these compounds in extracts collected at different times (0, 10, 20, 30, 60, 90, 120, 150, 180 min) was further calculated according to equation 7 (Zhang et al., 2020):

$$\boldsymbol{Dynamic\ proportion}\ (\%) = (\boldsymbol{X}/\boldsymbol{T}) * \mathbf{100} \qquad (7)$$

Where X was the content of specific antioxidant compounds (protein, polyphenol, chlorophyll a, chlorophyll b, carotenoids), T was the total amount of the compounds in the extracts (the sum of protein, polyphenol, chlorophyll a, chlorophyll b, carotenoids content).

2.4.5. Antiradical properties

The oxygen radical antioxidant capacity (ORAC) (Cao et al., 2019) and the Trolox equivalent antioxidant capacity (TEAC) (Miller et al., 2019) assays were used to evaluate the antioxidant capacity of the *Chlorella* extract. Briefly, 50 µL of extract and fluorescein sodium salt solution were added to a 96-well plate and incubated in a microplate reader at 37 °C for 10 minutes, then 25 µL AAPH solution was added, and the absorbance was recorded at 520 nm. Each group of samples was tested in 3 wells in parallel, and the experiment was repeated at least three times to make the coefficient of variation value within 10%. For TEAC experiments, the working solution was prepared as follows. 25 mL of 7 mM ABTS solution were mixed with 440 µL of 140 mM potassium thiosulfate solution (dissolved in distilled water) and incubated under darkness at room temperature for 12~16 hours. During the TEAC test, the working solution was diluted with 96% ethanol to obtain an absorbance value of 0.700 ± 0.020 at 734 nm. Then, 0.1 mL of the samples or Trolox standard solution were mixed with the above working solution

(absorbance value of 0.700 ± 0.020 at 734 nm), and after reacting for 3 minutes in a dark room, the absorbance at 734 nm was measured. Trolox was used as the standard solution to calculate the antioxidant capacity of the sample, and the unit for ORAC and TEAC was μm Trolox equivalent

2.5.Mineral analysis

According to the methods of relevant studies (De la Fuente B, López-García G, Máñez V, Alegría A, Barberá R, 2019), the macro elements (Mg, Ca, P) and micro elements (Fe, Zn, Se) in the samples were evaluated. In brief, 1 mL sample was mixed with 1 mL of concentrated nitric acid (HNO_3, 69 %) and 250 μL H_2O_2, then placed in a microwave digestion furnace for digestion, the maximum temperature was 180 °C. After that, the volume was adjusted to 5 mL with ultrapure water, a 100 μL aliquot was taken and the volume was adjusted to 10 mL with ultrapure water. The content of mineral elements was obtained through Agilent model 7990 ICP-MS (Agilent Technologies, CA, USA).

2.6.Nutrient Relative Value (NRV) Analysis (Mg, P, Ca, Fe, Zn, Se)

Table 1. Dietary Reference Intakes (DRIs): Elements

Life stage	Mg (mg/d)	P (mg/d)	Ca (mg/d)	Fe (mg/d)	Zn (mg/d)	Se (μg/d)
baby (6~12 months)	75	275	260	11	3	20
children (1~3 years)	80	460	700	7	3	20
children (4~8 years)	130	500	1000	10	5	30
males (9~13 years)	240	1250	1300	8	8	40
males (14~18 years)	410	1250	1300	11	11	55
males (19~30 years)	400	700	1000	8	11	55
males (31~50 years)	420	700	1000	8	11	55

males (51~70 years)	420	700	1000	8	11	55
males (>70 years)	420	700	1200	8	11	55
females (9~13 years)	240	1250	1300	8	8	40
females (14~18 years)	360	1250	1300	15	9	55
females (19~30 years)	310	700	1000	18	8	55
females (31~50 years)	320	700	1000	18	8	55
females (51~70 years)	320	700	1200	8	8	55
females (>70 years)	320	700	1200	8	8	55
pregnant (19~30 years)	350	700	1000	27	11	60
breastfeed (19~30 years)	310	700	1000	310	12	70

NOTES: This table (taken from the DRI reports, see *www.nap.edu*) presents Recommended Dietary Allowances (RDAs) in bold type. An RDAs is the average daily dietary intake level sufficient to meet the nutrient requirements of nearly all (97~98 %) healthy individuals in a group.

The contribution of minerals in extracts extract from 100 g dry *Chlorella* powder (Nutrient Relative Value) towards Dietary Reference Intake (DRI) was calculated as equation (Jalali & Fakhri, 2021) (8) :

$$\boldsymbol{NRV = (X/R) * 100} \qquad (8)$$

Where X and R corresponded to the mineral content in extracts (water as a solvent) from 100 g *Chlorella* dry powder and Recommended Dietary Allowances (RDAs) respectively (Ramu Ganesan et al., 2020).

2.7.Microalgae morphology

2.7.1. Fluorescence Microscope

The samples obtained after PEF treatment (3 kV/cm, 44 pulses, 99 kJ/kg) were

centrifuged at 157 × g for 10 min. After centrifugation, the supernatant was removed to collect the pellet, and it was washed with 90 % methanol and centrifuged to collect the precipitate again (157 × g/10 min). The sample was repeatedly washed until the supernatant was colorless, and the precipitate was collected and diluted with water. The control group was set as a mixed extract of microalgae and water without PEF treatment. To characterize Chlorella morphology, these samples were observed by means of an Eclipse 90i Nikon widefield microscope (Nikon corporation, Japan) equipped with 5-megapixels cooled digital color camera Nikon Digital Sight DS-5Mc (Nikon corporation, Japan). All microscopy images were acquired and processed by using Nis-Elements Br 3.2 Software (Nikon corporation, Japan). Nikon objective used for all images was CFI Plan Fluor DIC M/N2 40X (MRH00401). An optical zoom factor of 0.8x or 2x was combined with this objective. Brightfield images were acquired by illuminating with a halogen lamp for transmitted visible light, while fluorescent images were acquired by illuminating with a mercury lamp. Filter blocks used in fluorescent images were for red (Nikon reference G-2E/C) [EX 540/25, DM 565, BA 605/55], for ultraviolet excitation (Nikon reference UV2-A) [EX 330-380, DM 400, LP 420]. Main image properties were: RGB 24 bits; Frame size of 2560*1920 pixels; image dimensions of field of view were 270*200 microns for 32x images (0.11 microns/pixel) and 105*80 microns for 80x images (0.04 microns/pixel). Image with annotations has a frame size 1075*806 pixels and 0.25 microns/pixel, but same field of view dimensions of 32x original image.

2.7.2. Scanning Electron Microscopy

A scanning electron microscope (S-4800) was used to analyze the microstructure of Chlorella samples (aqueous extraction) after freeze drying (FreeZone 2.5 L, Labconco,

MI, USA) for 72 hours at a cold trap temperature of -65 °C. Freeze-dried Chlorella samples were then mounted on specimen stubs with colloidal silver, sputter-coated with gold-palladium and imaged with an SEM microscope (S-4800) at magnifications of 110×, 450× and 1500× (Fang et al., 2021).

2.8. Statistical analysis

All experiments and measurements of characteristics were repeated at least three times. One-way analysis of variance (ANOVA) was used for determining the significant differences among samples using the software Statgraphics plus (version 5.1, Statpoint Technologies Inc., Warrenton, VA). For each analysis, a significance level of 5% was assumed, a p-value < 0.05 was considered statistically significant. The error bars presented on the figures correspond to the standard deviations, letters were used to label the significance of the difference. The unsupervised principal component analysis (PCA) was performed by exporting the dataset in the software GraphPad 9 (GraphPad Software, San Diego, California, USA).

3. Results and discussion

3.1. Biomolecules' content and composition ratio analysis

The effects of PEF (3 kV/cm, 44 pulses, 99 kJ/kg), solvent (water or 50% DMSO) and extraction time (0, 10, 20, 30, 60, 90, 120 and 180 min) on the extraction of *Chlorella* components were shown in **Figure 1**. The results showed that the extraction of *Chlorella* protein was 12~42 mg/g dw, polyphenols 3.5~5.5 mg/g dw, chlorophyll *a* 0~0.5 mg/g dw, chlorophyll *b* 0~0.6 mg/g dw, carotenoids 0~0.15 mg/g dw. Compared with the biomolecules content of *Chlorella* powder: protein 480~600 mg/g dw (Liu, Gifuni, Mear, Frappart, & Couallier, 2021), polyphenols 7.06~19.16 mg/g dw (Jelínek, Procházková,

Quintelas, Beldíková, & Brányik, 2015), chlorophyll *a* 3.04~7.69 mg/g dw, chlorophyll *b* 0.39~10.34 mg/g dw, carotenoids 0.29~11.83 mg/g dw (Hynstova et al., 2018), the extraction yield of this study could theoretically be further improved. **Figure 1** showed that PEF treatment increased the extraction of biomolecules compared to the control group. For example, the protein content of PEF-H_2O was higher than that of control-H_2O for extraction times from 80 to 120 minutes, and the protein yield of PEF-50% DMSO was significantly higher ($p < 0.05$) than that of control-50% DMSO (from 0 to 180 minutes). Similarly, the polyphenol extraction results also showed that the yield of PEF-H_2O and PEF-50% DMSO extracts were significantly higher ($p < 0.05$) than those of control-H_2O and control-50% DMSO extracts, respectively, which could be attributed to the electroporation phenomenon of PEF.

Compared with the effect of PEF on protein and polyphenol yield, the extraction of chlorophyll *a*, chlorophyll *b* and carotenoids were more affected by solvent. The results showed that the extraction of pigments was higher in 50% DMSO extracts than in water extracts, regardless of whether a PEF treatment was applied or not. As we know, chlorophyll a, chlorophyll b and carotenoids are fat-soluble pigments, while DMSO ($(CH_3)_2SO$) has one hydrophilic sulfinyl group and two hydrophobic methyl groups, which can dissolve water-soluble and fat-soluble compounds and increase pigment extraction(García-Vaquero et al., 2021; Mueller et al., 2019). However, the opposite result was shown in protein yield, i.e., 50% DMSO decreased protein yield compared with water. This may be attributed to the precipitation of protein caused by increasing organic reagent concentration, thereby reducing the protein content in the extract (Arakawa, Kita, & Timasheff, 2007). Similar to the present study, Parniakov et al (2015)

investigated the potential of PEF-assisted extraction of nutrients from microalgae *Nannochloropsis spp*. using a mixture of organic solvents (DMSO) and water, and the results showed that PEF increased the yield of microalgae proteins, polyphenols, and pigments, and 50% DMSO was beneficial to increase the extraction yield of pigment, which was consistent with the results of our study (Parniakov et al., 2015b).

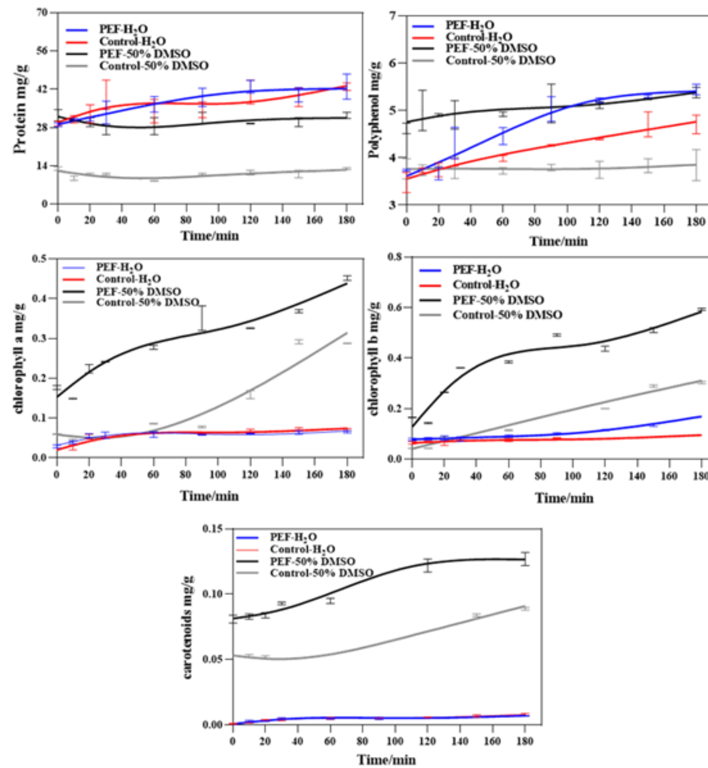

Figure 1. Biomolecules extraction yield (mg/g dw) from *Chlorella* treated with PEF/without PEF (control) under different extraction times (0~180 min) and solvents (H_2O, 50% DMSO)

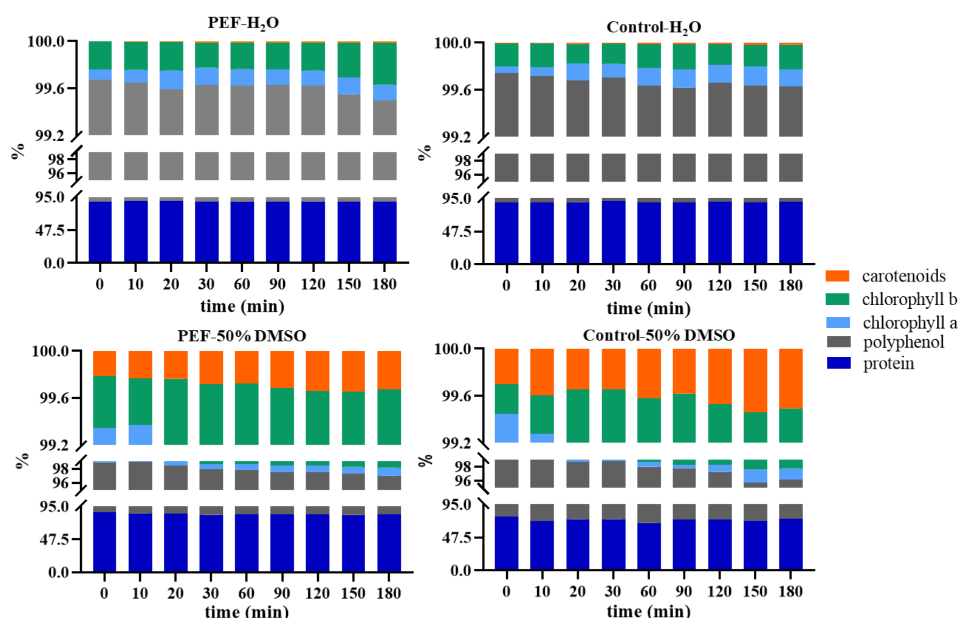

Figure 2. Dynamic proportion of biomolecules different time point (0~180 min). Dynamic proportion (%)= X / T×100%, Where X was the content of specific antioxidant compounds (protein, polyphenol, chlorophyll a, chlorophyll b, carotenoids), T was the total amount of the antioxidant compounds in the extracts (the sum of protein, polyphenol, chlorophyll a, chlorophyll b, carotenoids content).

The extraction time and biomolecules content determined the final extraction efficiency. The results in Figure 1 showed that the protein and polyphenol content reached a stable level at 120 minutes, and there was no significant difference between the extraction yields at 180 minutes. The contents of chlorophyll *a*, chlorophyll *b* and carotenoids in the 50% DMSO extract continued to increase from 0 to 180 minutes, whereas the pigment content in the water extract was almost unchanged (0~180 minutes). In order to better describe the dynamic proportion of these components in the extract, we further analyzed the proportion of specific biomolecules at each time point according to equation 7, and the results were shown in **Figure 2**. The results in **Figure 2** showed that

the proportions of molecules in different extraction techniques and solvents were different at specific times. Specifically, as the extraction time of PEF-H_2O and control-H_2O was extended to 180 min, the proportions of polyphenols and chlorophyll (chlorophyll *a* and chlorophyll *b*) slightly decreased and increased respectively, while the proportions of protein and carotenoids remained stable. However, different dynamic ratio changes occurred when 50% DMSO was used as the extraction solvent. From the PEF-50% DMSO and control-50% DMSO extracts, it was observed that the prolonged extraction time was accompanied by a significant increase in the proportions of chlorophyll *a*, chlorophyll *b* and carotenoids and a significant decrease in the proportion of polyphenols (Schoefs, 2003). Chlorophylls and carotenoids were sensitive to heat, light, acid, and alkali, and in studies of other extraction techniques, such as ultrasound-assisted extraction, the extraction process could result in increased solution temperature and reduced pigments content in the extract (Parniakov et al., 2015a). **Figure 2** showed that extending the extraction time to 180 min did not reduce the chlorophyll content in the extract, which was because the PEF treatment conditions (3 kV/cm, 44 pulses, 99 kJ/kg) of this study did not cause thermal effects to the extraction process, thus avoiding the thermal decomposition of heat-sensitive components. The dynamic components proportion results provided a reference for selecting recovery conditions for different antioxidant components (single or composite component), which facilitated the development of further processes such as separation and purification of antioxidant biomolecules from the *Chlorella* extracts after the extraction stage.

3.2.Antioxidant capacity and multi-factor correlation analysis

The measurement of the antioxidant capacity of the samples depended on the technique and free radical generator or oxidant used, so it is necessary to use different methods to evaluate the antioxidant properties of the extract (Siddeeg et al., 2021). In this study, oxygen radical antioxidant capacity (ORAC) and Trolox equivalent antioxidant capacity (TEAC) was used to analyze the effects of PEF, solvent, and extraction time on the antioxidant properties of *Chlorella* extracts. The results presented in **Figure 3** showed that the antioxidant capacity of PEF extracts was higher than that of the control group in both ORAC and TEAC analyzes, these results could be attributed to the higher biomolecules content (*section 3.1*) in the *Chlorella* PEF extracts.

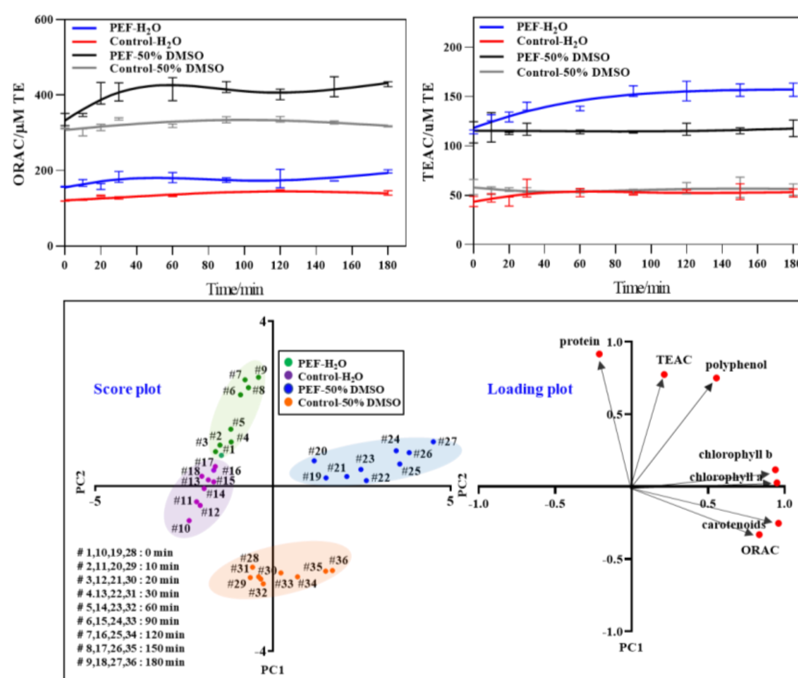

Figure 3. Antioxidant results and principal component analysis (PCA). ORAC (oxygen radical antioxidant capacity) and TEAC (Trolox equivalent antioxidant capacity) results of *Chlorella* extracts treated with PEF/without PEF (control) under different extraction times (0~180 min) and solvents (H_2O, 50% DMSO).

However, the results of ORAC and TEAC were inconsistent in the evaluation of the antioxidant capacity of *Chlorella* extracts. In terms of antioxidant capacity, ORAC results showed that PEF-50% DMSO > control-50% DMSO > PEF-H_2O > control-H_2O, while TEAC results showed that PEF-H_2O > PEF-50% DMSO > control-H_2O/control-50% DMSO. Combined with the PCA loading plot in **Figure 3**, ORAC was strongly correlated with carotenoids, chlorophyll *a*, and chlorophyll *b*, while TEAC was strongly correlated with proteins and polyphenols, which could explain the antioxidant capacity test results, that was, PEF-50% DMSO recovered more chlorophyll a, chlorophyll b and carotenoids, while PEF-H_2O recovered more protein and polyphenols, which caused the different antioxidant capacity results in the ORAC and TEAC test. In addition to this study, inconsistencies in the results of ORAC and TEAC have also been reported in other studies. For example, studies have shown that the antioxidant capacity of carotenoids measured by TEAC and ORAC corresponded to β-carotene > lutein > zeaxanthin and lutein > zeaxanthin > β-carotene respectively, and these differences could be attributed to the different reaction mechanisms of TEAC and ORAC (Zulueta, Esteve, & Frígola, 2009). Methods for measuring antioxidant capacity were divided into two categories: methods based on hydrogen atom transfer (HAT) and methods based on electron transfer (ET) (Huang, Ou, & Prior, 2005). The assay principle of HAT was followed as antioxidants and substrates competed for thermally generated peroxyl radicals through the decomposition of azo compounds, such as ORAC, while the assay of ET measured the ability of antioxidants to reduce oxidants, such as TEAC (scavenging ABTS cationic radicals) (Zulueta et al., 2009). Therefore, when the samples are complex or contain

different kinds of antioxidants (proteins, polyphenols, pigments, etc.), the ORAC and TEAC methods could be less relevant due to different kinetics and reaction mechanisms.

The antioxidant capacity of PEF-50% DMSO extract in ORAC results and PEF-H_2O extract in TEAC results showed an increasing trend with time, while other curves remained stable. On the one hand, the ORAC results in this study were correlated with pigments content in PCA analysis, so the increased antioxidant activity of PEF-50% DMSO extract could be related to the increase in chlorophyll and carotenoid content over time. The TEAC results were related to polyphenol content in the PCA analysis, so the increased antioxidant activity of the PEF-H_2O extract was related to the increased polyphenol content over time, which corresponded to the results in **Figure 3**. Furthermore, the score plot in **Figure 3** showed that all extraction conditions were divided into 4 groups, based on the presence or absence of PEF treatment and solvent type (water, 50% DMSO), indicating that the extraction efficiency was more affected by extraction technique and solvent than extraction time, which provided a reference for selecting the recovery conditions of *Chlorella* biomolecules. Finally, considering the extraction yield of all biomolecules and the cleanliness of the solvent, water was selected as the solvent in this study, and the extraction time of 120 minutes was used as the experimental condition to analyze the effect of PEF treatment on the recovery of trace minerals and morphology of *Chlorella*.

3.3.Minerals yield and analysis of NRV (Nutrient relative value)

Minerals are necessary for physical growth due to their diverse functionalities in body metabolism and homeostasis and are important for the normal structural and physiological growth, maintenance of hormonal and regulatory functions of the body as

well as build of muscles and bones. Moreover, minerals are essential nutrients because they cannot be synthesized in the body and must be obtained through food or as supplements to meet daily requirements (Chongtham, Bisht, Santosh, Bajwa, & Indira, 2021).

On this line, this study analyzed the content of minerals in *Chlorella* dry powder and *Chlorella* extract, including magnesium, phosphorus, calcium, iron, and zinc, and further calculated the NRV of minerals (extracted from 100 g *Chlorella* dry powder) with reference to RDAs, the results were shown in **Table 2**. The results showed that *Chlorella* powder was rich in Mg, P, Ca, Fe and Zn, and compared with the average values in other studies, i.e., Mg (3443 mg/kg dw), P (17615 mg/kg dw), Ca (5927 mg/kg dw), Fe (2591 mg/kg dw), Zn (11.9 mg/kg dw) (Tokuşoglu & Ünal, 2003), the P, Ca and Fe contents of the *Chlorella* powder in this study were relatively low, while the contents of Mg and Zn were relatively high, which could be attributed to the differences of cultivation conditions. The mineral content results showed that PEF treatment had no significant effect on Mg ($p > 0.05$), significantly increased P and Zn content ($p < 0.05$) and decreased Ca and Fe concentration respectively ($p < 0.05$). The PEF induced phenomenon that reduced Ca and Fe yield was worth considering, as most reports showed that PEF treatment disrupted microalgae cells and increased biomolecule yields, and the following explanations could be given according to the related studies (Oleksii Parniakov et al., 2014). Ca and Fe can be present in microalgal cells by chelating with proteins (Yang et al., 2022), and electrostatic interactions induced by PEF treatment may alter the spatial structure of proteins, such as unfolding and aggregation, which altered the functional properties of

proteins (solubility, etc.) (Dong et al., 2020), resulting in Ca/Fe-containing proteins sedimentation during extraction.

In this study, water was used as the mineral extraction solvent, which was safe and edible. On these basics, the contribution of macro (Ca, Mg, P) and trace minerals (Fe, Zn, Se) in *Chlorella* extracts (NRV (Nutrient relative value), extracted from 100 g dry *Chlorella* powder) towards Recommended Dietary Allowances (RDAs) was calculated using equation 8, the results were shown in **Table 2**.

Mg is an indispensable mineral required in the human diet for processing ATP (adenosine triphosphate) and bones. Mg deficiency could cause various diseases, such as type-2 diabetes, metabolic syndrome, hypertension, atherosclerotic vascular disease, etc (Eggleston et al., 2022). *Chlorella* extract (control extracts) met more than 45% of the Mg RDAs for infants (6~12 months) and children (1~3 years old), and about 30% Mg RDAs for children (4~8 years old), as well as more than 8% Mg RDAs for male/female (> 9 years old, 8.7~15.2%) and pregnant/breastfeed female (19~30 years old, 10.4~11.8%). P is a multifunctional component, which is an important component of bones and cells, and also plays an important role in the body's energy processing due to its presence in ATP and DNA (Eggleston, Triplett, Bett-Garber, Boue, & Bechtel, 2022). The NRV results showed that *Chlorella extracts* (PEF extracts) met 41.2% of the P RDAs for infants (6~12 months), and more than 20% of the P RDAs for children (1~8 years), as well as more than 9% P RDAs for male/female (> 9 years old, 9.1~16.2 %) and pregnant/breastfeed female (19~30 years old, 16.2%). Ca is the most abundant mineral in the body, which is essential for muscle, bones, teeth, heart, and digestive system health, as well as the synthesis and function of blood cells (Michos, Cainzos-Achirica, Heravi,

& Appel, 2021). The NRV results showed that the Ca NRV in the *Chlorella* extracts (control extracts) was relatively low, specifically, it met 7.7% of the Ca RDAs for infants (6~12 months), and 2~3% calcium RDAs for children (1~8 years old), as well as 1.5~2.0% Ca RDAs for male/female (> 9 years old) and pregnant/breastfeed female (19~30 years old). Fe is an essential metal for biological processes, which is a fundamental inorganic nutrient in the human body, playing an important role in DNA synthesis and repair, ATP production and oxygen transport (Salnikow, 2021). The NRV results showed that *Chlorella* extracts (PEF extracts) could meet 20~30 % of Fe RDAs for infants (6~12 months), children (1~8 years old), males (> 9 years old) and females (9~13, > 51 years old), as well as 8.5 % and 0.7% Fe RDAs for pregnant and breastfeed female respectively. Zn is an essential micronutrient in our diet, which is a key component for the function of numerous proteins, including Zn-containing metalloenzymes and zinc-associated transcription factors, and is an essential micronutrient required for numerous cellular processes and immune system development (Ho, Wong, & King, 2022). NRV results showed that chlorella extracts (PEF extracts) met 17.7% of the Zn RDAs for infants (6~12 months) and children (1~3 years old), and 10.6% of the Zn RDAs for children (4~8 years old), as well as 4.8~6.6% of the Zn RDAs for male/female (> 9 years old) and pregnant/breastfeed female (19~30 years old). Among them, the NRV of Mg, P and Fe were relatively high, and the NRV of Ca and Zn were relatively low, which depended on the mineral content of *Chlorella* and the extraction process. From these results, *Chlorella* extract could be used as a source of minerals. Moreover, the bioavailability of minerals in *Chlorella* extracts should be considered, and the corresponding research has been gradually carried out in our laboratory. At present, there are few reports on the use of PEF

to extract minerals from *Chlorella*. This study showed that PEF increased the yield of some minerals, which provided a possibility for the application of PEF in the recovery of microalgae minerals in the future.

Table 2. Mg, P, Ca, Fe, Zn, Se (Nd) yield and Nutrient relative value (NRV) analysis

mineral	Mg		P		Ca		Fe		Zn	
powder (mg/kg dw)	3660±30		10894±180		1593±30		679±6		21.8±0.3	
Reference data (mg/kg dw)	3443±0.1		1762±0.2		5937±0.7		2591±0.4		11.9±0.7	
extracts	PEF-	control-	PEF-	control-	PEF-	control-	PEF-	control-	PEF-	control-
yield (mg/kg dw)	352±2 4a	364.8±4.4[a]	1132±20[a]	940±40[b]	70±5[a]	200±8[b]	3.98±0.03[a]	23.0±0.1[b]	5.31±0.07[a]	3.07±0.07[b]
	NRV: contribution of minerals in extracts (extract from 100 g dry *Chlorella* powder) towards DRI									
mineral	Mg		P		Ca		Fe		Zn	
life stages	PEF-	control-	PEF-	control-	PEF-	control-	PEF-	control-	PEF-	control-
baby (6~12 months)	46.9	48.7	41.2	34.2	2.7	7.7	3.6	20.9	17.7	10.3
children (1~3 years)	44.0	45.6	24.6	20.4	1.0	2.9	5.7	32.9	17.7	10.3
children (4~8 years)	27.1	28.1	22.6	18.8	0.7	2.0	4.0	23.0	10.6	6.2
males (9~13 years)	14.7	15.2	9.1	7.5	0.5	1.5	5.0	28.8	6.6	3.9
males (14~18 years)	8.6	8.9	9.1	7.5	0.5	1.5	3.6	20.9	4.8	2.8

males (19~30 years)	8.8	9.1	16.2	13.4	0.7	2.0	5.0	28.8	4.8	2.8
males (31~50 years)	8.4	8.7	16.2	13.4	0.7	2.0	5.0	28.8	4.8	2.8
males (51~70 years)	8.4	8.7	16.2	13.4	0.7	2.0	5.0	28.8	4.8	2.8
males (>70 years)	8.4	8.7	16.2	13.4	0.6	1.7	5.0	28.8	4.8	2.8
females (9~13 years)	14.7	15.2	9.1	7.5	0.5	1.5	5.0	28.8	6.6	3.9
females (14~18 years)	9.8	10.1	9.1	7.5	0.5	1.5	2.7	15.3	5.9	3.4
females (19~30 years)	11.4	11.8	16.2	13.4	0.7	2.0	2.2	12.8	6.6	3.9
females (31~50 years)	11.0	11.4	16.2	13.4	0.7	2.0	2.2	12.8	6.6	3.9
females (51~70 years)	11.0	11.4	16.2	13.4	0.6	1.7	5.0	28.8	6.6	3.9
females (>70 years)	11.0	11.4	16.2	13.4	0.6	1.7	5.0	28.8	6.6	3.9
pregnant (19~30 years)	10.1	10.4	16.2	13.4	0.7	2.0	1.5	8.5	4.8	2.8
breastfeed (19~30 years)	11.4	11.8	16.2	13.4	0.7	2.0	0.1	0.7	4.4	2.6

3.4.Fluorescence microscope (FM)

Previous reports have shown that high electric field strength altered cell membrane properties during PEF treatment, resulting in increased membrane permeability and enhanced cytoplasmic extraction (Saulis, 2010). However, the effect of PEF treatment on the microalgae morphology depended on the cell structure (cell wall thickness) and treatment conditions (pulse, electric field strength, time, etc.), which should be specifically explored. In this study, fluorescence microscopy was used to analyze the effect of PEF (3 kV/cm, 44 pulses, 99 kJ/kg) on the morphology of *Chlorella*, and the results were shown in Figure 4.

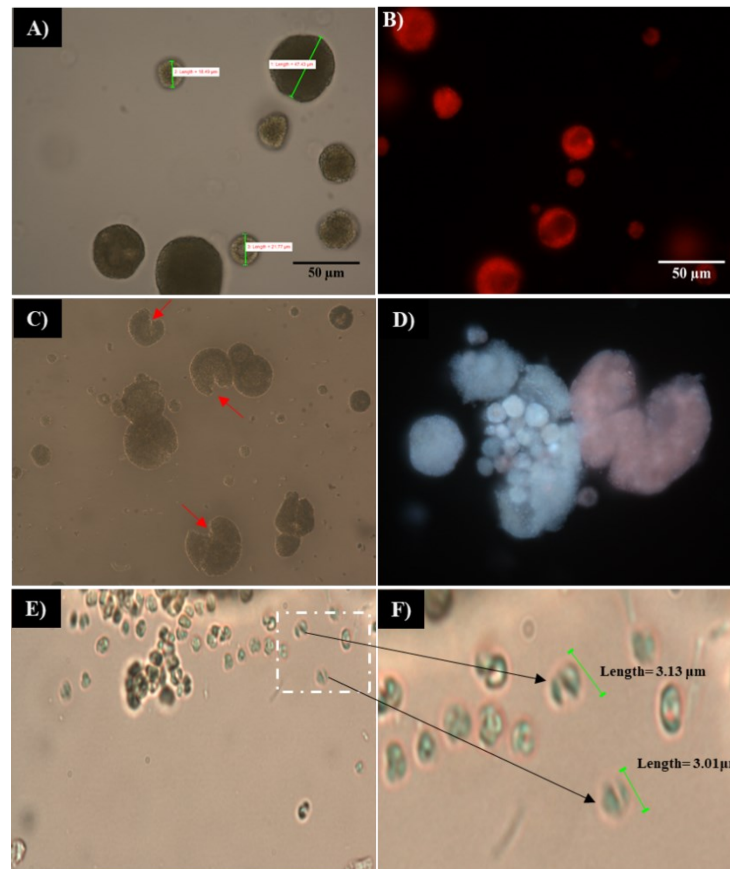

Figure 4. Microstructure of *Chlorella* under fluorescence microscope. Figure 4A (16×)/4B (64×)-control extraction, Figure 4C (16×)/4D (64×)-PEF extraction, Figure 4E (32×)/4F (160×)-PEF extraction.

Figures 4A (16×) and **4B** (64×) were the morphology of *Chlorella* in no-PEF treated (control) suspension, showing no single cells but circular aggregates of multiple *Chlorella* in this field of view. **Figures 4C** (16×) and **4D** (64×) were *Chlorella* in a PEF-treated suspension, and some 'cracks' in the circular *Chlorella* aggregates could be observed. To confirm that the increased biomass yield of Chlorella is related to the change of cell morphology by the electric field effect of PEF, this study further focused on observing the single *Chlorella* cells, the results were shown in **Figures 4E** and **4F**. **Figures 4E** (32×) and **4F** (160×) were single *Chlorella* cell morphology in PEF-treated suspension. Figure 4F shows that *Chlorella* cells were approximately 3 microns in diameter, which was consistent with previous reports on *Chlorella* (3~10 microns) (Hyunkuk, L., Dawoon, J., SungJu, I., & Am, J, 2020). **Figures 4E** and **4F** showed that *Chlorella* cells were ruptured or perforated after PEF treatment, indicating that the 'electroporation phenomenon' occurred during the PEF extraction process, increased the recovery of *Chlorella* biomolecules. Similarly, Scherer et al. (2019) analyzed the effect of PEF on the permeability of *Chlorella* cells through a microscope (63×). The results showed that PEF-treated *Chlorella* cells could be stained with Evans blue, a dye that could accumulate in permeabilized cells, indicating that PEF treatment caused the rupture of *Chlorella* cells, which was consistent with the results of this study (Scherer et al., 2019).

3.5.Scanning Electron Microscopy (SEM)

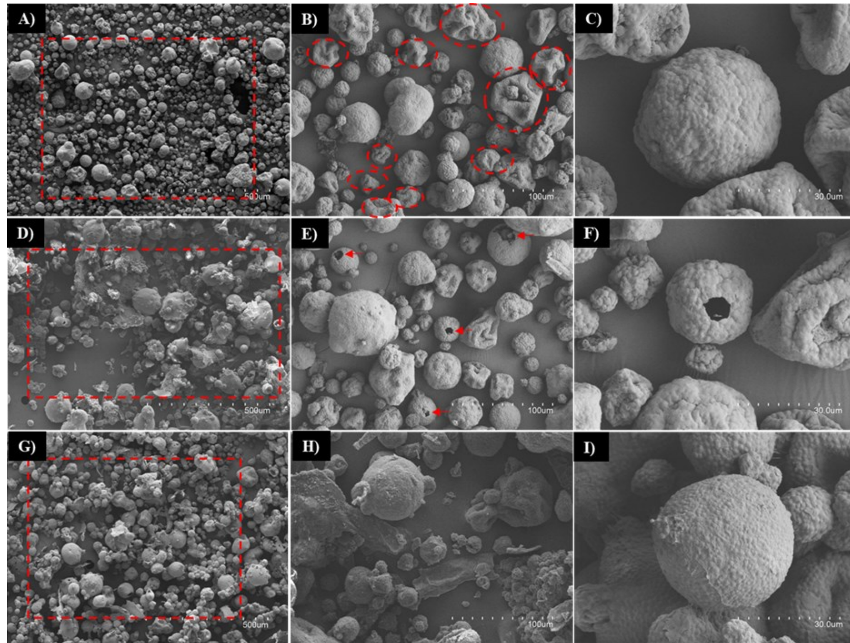

Figure 5. Microstructure of *Chlorella* under SEM (scanning electron microscope). Figure 5A (110×)/5B (450×)/5C (1500×)-*Chlorella* powder (before extraction), Figure 5D (110×)/5E (450×)/5F (1500×)-PEF-H_2O extraction, Figure 5G (110×)/5H (450×)/5I (1500×)-control-H_2O extraction.

In this study, SEM was used to observe the effect of PEF on the cell surface structure of *Chlorella*, the results were shown in **Figure 5**. **Figure 5A** (110×), **5B** (450×), **5C** (1500×) were the SEM results of the *Chlorella* samples before extraction, showing that most of the *Chlorella* were spherical, and some of them were shrunken (Figure 5B, marked with red circles), which may be related to the drying and dehydration process of microalgae harvesting. **Figure 5D** (110×), **5E** (450×), **5F** (1500×) were the results of PEF-extracted *Chlorella* samples. Non-spherical debris was shown in Figure **5D** (110×), which were the cell debris caused by PEF or biomolecules leached during extraction. Pores on the surface of *Chlorella* cells could be seen in **Figure 5E** (450×) and **5F** (1500×), which could be caused by the electroporation effect of PEF, thus promoting the dissolution of biomolecules. For the control group, non-spherical

debris was also shown in **Figure 5G** (110×), **5H** (450×) and **5I** (1500×), which were generated during the extraction process, and compared to PEF, no obvious holes appeared on the cell surface. The SEM results indicated that PEF-induced pores in *Chlorella* cells could be the key factor to change the extraction efficiency of *Chlorella* biomolecules in this study. A recent study also analyzed the effect of PEF on the surface structure of *Chlorella*, however, the SEM results showed that PEF treatment (5 μs at 20 kV cm^{-1}, 31.8 kJ kg^{-1}) had no visible effect on the cell structure of *Chlorella* (Canelli et al., 2022). While in another study, Carullo et al. (2018) found that PEF-treated *Chlorella* cells were deformed (shrunken), which was attributed to the increased permeability of cells membrane by PEF treatment and the release of biomass (Carullo et al., 2018). These studies showed that the effect of PEF on the surface structure of *Chlorella* was different, which was related to the processing parameters of PEF, such as the number of pulses, electric field strength, processing time, etc. For example, during PEF treatment, high-intensity voltage and more pulses may aggravate microalgal cell rupture, while low-intensity voltage and relatively few pulses may not cause significant damage to microalgae, so different PEF conditions correspond to different results. Moreover, the properties of microalgae, such as cell size, cell wall thickness, microalgae harvesting process, etc., will also affect the effect of PEF extraction, which should be analyzed comprehensively.

4. Conclusions

The recovery of *Chlorella* biomolecules was affected by different factors (PEF, extraction solution and time), and the proportion of biomolecules changed dynamically with the extraction time during the extraction process. The PEF treatment increased the yield of antioxidant biomolecules in *Chlorella*, and microscopic analysis indicated that this was mainly related to

the PEF electroporation mechanism. *Chlorella* contained various minerals, and the NRV values calculated based on RDAs suggest that *Chlorella*-water PEF-extract could be used as a mineral source for different populations, and similar studies have not been reported yet. In addition, the content of antioxidant biomolecules and minerals in PEF extract relative to *Chlorella* total nutrient content could be further improved, which could be achieved by changing the processing conditions of PEF or combining with other extraction technologies.

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4.7 Effects of Pressurized Liquid Extraction with dimethyl sulfoxide on the recovery of dietary valuable compounds from the microalgae *Spirulina*, *Chlorella* and *Phaeodactylum tricornutum*

(Under review)

Effects of Pressurized Liquid Extraction with dimethyl sulfoxide on the recovery of dietary valuable compounds from the microalgae *Spirulina*, *Chlorella* and *Phaeodactylum tricornutum*

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Abstract

The impact of pressurized liquid extraction (PLE) and different concentrations of DMSO (0, 30, 50, 100%) on the yield of antioxidant bioactive compounds and minerals from *Chlorella* were investigated. The results showed that PLE extraction increased the antioxidants yield. Regarding the solvent, water extracted more proteins, while with 100% DMSO more polyphenols, chlorophylls, and carotenoids were obtained. The efficiency coefficient (K_{PLE}) results showed that PLE+100% DMSO was more suitable for the recovery of antioxidants from *Chlorella* (polyphenols 10.465 mg/g, chlorophyll a 6.206 mg/g, chlorophyll b 3.003 mg/g, carotenoids 0.971 mg/g). On this basis, PLE+100% DMSO was used for the recovery of antioxidants and minerals from *Spirulina*, *Chlorella*, and *Phaeodactylum tricornutum*. The carotenoid profile was clearly different, and the total carotenoid content was considerably higher in the Spirulina extract (59.06 mg/L). Finally, the mineral yields of the extracts were analyzed, and Relative Nutrient Values results were calculated based on Recommended Dietary Allowances. The results indicated that the extracts could be used as a mineral source for different populations.

Keywords: carotenoids, chlorophylls, minerals, polyphenols, proteins, sustainability

1. Introduction

The global shortage of food resources, the increase in demand for healthy food brought about by Covid-19, and the war between Russia and Ukraine, make it necessary for the food industry to make full use of the existing natural food resources as a solution. From the perspective of environmentally friendly strategies and nutritional value, marine resources show a great potential as edible resources, especially marine microalgae. On the one hand, marine microalgae can use light and sequestrate CO_2 to synthesize high-value nutrients, such as proteins, polysaccharides, pigments, etc. (Markou & Nerantzis, 2013). On the other hand, the cultivation of marine microalgae does not compete with traditional agriculture for land space, which allows marine microalgae to play a huge potential advantage in helping humans cope with food resource crises (Vaz et al., 2016). Moreover, microalgae are rich in bioactive compounds such as carotenoids, polyphenols, or polysaccharides, that are thought to be involved in health-promoting biological actions such as antioxidant, anti-inflammatory, anti-cardiac, or anti-diabetic actions or even in the regulation of gut microbiota (Laamanen et al., 2021).

Extraction with solvents is a key process to recover microalgae valuable compounds. For instance, most of marine microalgae contain hard cell walls, with a great range of thicknesses depending on the microalgae species. Overcoming the cell wall barrier of microalgae to dissolve cytoplasmic nutrients into the extraction solution is currently an important issue (Zhang et al., 2022). Traditional techniques such as Soxhlet, Folch, and hot water extractions have been used to extract valuable compounds from marine microalgae. Although these techniques can yield important quantities of such compounds, they have been gradually

replaced by new efficient extraction techniques due to their disadvantages such as long extraction time and the use of large amounts of organic solvents (Zhou, Wang, Saraiva, Martins, Pinto, Prieto, Simal-Gandara, et al., 2022). Pressurized liquid extraction (PLE) is a highly efficient extraction technique compared to traditional extraction techniques. The PLE process is proceeding in a tightly closed stainless-steel cell, in order to be submitted to high temperature and pressure (Hoff & Pizzolato, 2018). During the extraction process, the solvent is kept below the critical point of the liquid phase. The pressure and temperature conditions are selected to increase the mass transfer rate by reducing the surface tension and viscosity of the solvent and increasing the solubility of the components, thus facilitating the penetration of the solvent into the matrix of the microalgae (Hoff & Pizzolato, 2018). Briefly, the PLE facilitates the process of matrix-solvent interaction during the extraction process and greatly shortens the extraction time. Moreover, PLE has additional advantages such as small sample and solvent usage or automatic extraction of multiple samples. Currently, it is widely used in the extraction of bioactive components (Zhou, Wang, Berrada, Zhu, Grimi, & Barba, 2022).

Although the duration of the high-temperature phase has been greatly shortened in the PLE process compared to traditional extraction techniques, this process is still not conducive to the protection of the properties of heat-sensitive bioactive components which may lead to the degradation of substances such as carotenoids, chlorophylls, or proteins, etc. Therefore, PLE combined with a suitable extraction solvent to reduce the dependence on high-temperature extraction conditions is an issue worth exploring. Compared with conventional extraction solvents (i.e., ethanol, methanol, or acetone), DMSO has the following advantages: (A) extraction is easy and fast because multiple grinding and centrifuging are not required. (B) high

stability of microalgae bioactive components (chlorophyll, carotenoids, etc.) in DMSO solvents (Nikolopoulos et al., 2008). (C) low toxicity, high fluidity, and good selectivity at room temperature and pressure (Liu, Zhao, et al., 2021). The above advantages indicate that with DMSO as the solvent, PLE may perform the extraction of microalgae bioactive compounds at mild temperature conditions, although this is rarely reported. In this study, PLE combined with different concentrations of DMSO was used to recover dietary valuable compounds (proteins, polyphenols, chlorophylls, carotenoids, Mg, Ca, P, Fe, Zn, Se) at room temperature from *Chlorella*, *Spirulina,* and *Phaeodactylum tricornutum*. Principal component analysis was conducted to analyze the intrinsic correlation of bioactive components and antioxidant properties in the extracts, and the relative nutrient values of minerals in the extracts were assessed to provide a reference for the edible value of marine microalgae.

2. Materials and methods

2.1 Chemicals and reagents

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ABTS (2,2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid), AAPH (2,2'-Azobis(2-methylpropionamidine) dihydrochloride), fluorescein sodium salt, Folin-Ciocalteu, gallic acid, D-glucose, phenol, potassium persulfate and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Steinheim, Baden-Württemberg, Germany). Sodium carbonate was acquired from VWR (Saint-Prix, France). Sodium hydroxide, bicinchoninic acid (BCA) kit, glacial acetic acid, and sulfuric acid were supplied by Fisher Scientific (Madrid, Spain). Deionized water (resistivity >18 MΩ cm-1) was produced by a Milli-Q SP® Reagent Water System (Millipore Corporation, Bedford, MA, USA). β-carotene (≥95.0% purity), lutein (≥96.0% purity) and

zeaxanthin (≥95.0% purity), and fucoxanthin ((≥95.0% purity) were from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany)

2.2 Samples

Chlorella from Hainan Island (China) was produced in open raceway ponds, where the average temperatures varying from 21 °C to 33 °C and precipitations of 1600 mm per year. At the time of harvesting, biomass was washed and spray-dried at 160 to 180 °C. The final product was green powder with characteristic smell and taste, which was stored at -20 °C for experimental analysis. *Spirulina* biomass came from *Arthrospira platensis* species, strain paracas 15016, being Paracas the lake where it was originally isolated (Lima, Peru). Cultivation took place at EcoSpirulina company (Serra, Valencia, Spain) in raceway ponds using a greenhouse under natural sunlight. The day-time temperature was 32 °C on average, while temperature decreased to 24 °C at night. The biomass was used for further experiments after freeze-drying. *Phaeodactylum tricornutum* was produced in four 800 L GemTube (LGEM, Rotterdam, The Netherlands) photobioreactors at the National Algae pilot plant in Mongstad (NAM), Norway. The photobioreactors were in a greenhouse exposed to natural light and additionally equipped with artificial illumination (EAX 170W LED lights, Evolys AS, Oslo, Norway) with an average incident artificial light of 200 μmol $m^{-2}\cdot s^{-1}$. The culture temperature of *Phaeodactylum tricornutum* were maintained between 15~35 °C by heating the greenhouse or spraying the reactors with water to cool them down, and the biomass after freezing dried was used for further experiments.

2.3 PLE extraction process

The PLE extraction was carried out based on a previous study (Moret et al., 2014) with slight modifications. Microalgae and diatomaceous earth were thoroughly mixed (0.5:1.5, *w:w*) in a mortar and then placed into the PLE extraction tank. An ASE-200 Accelerated Solvent Extractor (Sunnyvale, CA, USA) was used to perform the extraction, and the operating conditions were referred to our previous study: preheating time of 1 min, heating time of 5 min, flush volume 60%, nitrogen purge 60 s, extraction pressure of 103.4 bars, extraction temperature of 40 °C, extraction time of 15 minutes. Different proportions of DMSO (0, 30%, 50%,100%) were used for PLE extraction to evaluate the effect of DMSO concentration on the extraction yield. For 0.5 g microalgae (dw), the final extracts volume was near 20 mL. According to the PLE extracts volume, the control experiment was carried out as 0.5 g algae powder/20 mL solvent (0, 30%, 50%,100%) stirred at 40 °C for 15 minutes. The samples were centrifuged (2504*x*g, 4 °C, 15 minutes), and the supernatants were stored at -20 °C for further analyzes.

2.4 Analysis of dietary valuable compounds

2.4.1 Protein yield

The bicinchoninic acid (BCA) method was used to analyze the protein content of the microalgae extracts (Al Khawli et al., 2021). Specifically, 10 µL of samples or BSA and 200 µL of BCA working solution were added to a 96-well plate, mixed well, and incubated at 37 °C for 30 minutes. Finally, the absorbances were measured at 562 nm. The protein content was determined using a calibration curve (0~2000 mg/L) with bovine serum albumin (BSA) as a standard.

2.4.2 Pigments (chlorophylls and carotenoids) and polyphenols yield

The concentration of pigments and polyphenols in the microalgae extracts was analyzed following the procedures previously reported by Zhou et al. (2021). The absorbance values and formulas used to analyze the DMSO and 50% DMSO extracts were as follows (Zhou et al., 2021):

$$C_a = 12.47 \times Abs_{665.1} - 3.62 \times Abs_{649.1} \quad (1)$$

$$C_b = 25.06 \times Abs_{649.1} - 6.5 \times Abs_{665.1} \quad (2)$$

$$C_{carotenoids} = (100 \times Abs_{480} - 1.29 \times C_a - 53.78 \times C_b)/220 \quad (3)$$

$$C_{total\ chlorophylls} = C_a + C_b \quad (4)$$

The formulas used to analyze the H_2O extracts was as follows (Kokkali et al., 2020) :

$$C_a = 16.82 \times Abs_{665.1} - 9.28 \times Abs_{653} \quad (1)$$

$$C_b = 36.92 \times Abs_{653} - 16.54 \times Abs_{665} \quad (2)$$

$$C_{carotenoids} = (100 \times Abs_{470} - 1.91 \times C_a - 95.15 \times C_b)/225 \quad (3)$$

$$C_{total\ chlorophylls} = C_a + C_b \quad (4)$$

Where C_a, C_b, $C_{carotenoids}$ and $C_{total\ chlorophylls}$ corresponded to the concentrations (mg/L) of chlorophyll *a*, chlorophyll *b*, carotenoids, and total chlorophylls respectively. The final yield of pigments was calculated based on the dry weight of microalgae (mg/g dw).

Total phenolic content was determined by the Folin-Ciocalteu assay. For instance, 0.2 mL of extracts, 1 mL of Folin-Ciocalteu (diluted with water at a ratio of 1:10, v/v), and 0.8 mL of Na_2CO_3 (75 g/L) were mixed and incubated in a water bath at 50 °C for 10 minutes. Then, the absorbances were measured at 750 nm using a spectrophotometer. Gallic acid was used as a standard to prepare the calibration curve to quantify the content of total polyphenols in the microalgae extracts.

2.4.3 Extraction of carotenoids and HPLC profile

For the extraction of carotenoids, 3 mL of trichloromethane were added to 1 mL of *Chlorella*, *Spirulina,* and *Phaeodactylum tricornutum* (PLE+100% DMSO extracts). The samples were vortexed for 2 min and then centrifuged at 20 °C (to avoid DMSO freezing) for 3 min. The supernatant was removed. After washing the solution with 4 mL of 10% NaCl, the samples were evaporated to dryness in a rotary evaporator (< 30 °C). Finally, the carotenoid extracts were dissolved in 200 µl of ethyl acetate and injected to identify the carotenoid profile by HPLC.

The HPLC analysis was performed on an Agilent 1260 system (Agilent, Palo Alto, CA). The carotenoids were separated on a YMC C_{30} column (5 µm, 250 × 4.6 mm) (YMC, Wilmington, NC), which was kept at 20 °C. The mobile phase, which comprised methanol, methyl *tert*-butyl ether, and water, was pumped at 1 mL/min. The linear gradient elution used is described elsewhere (Stinco et al., 2012).

The chromatograms were monitored for absorbance at 450 nm. The identification of carotenoids was carried out by comparison of their chromatographic and UV/vis spectroscopic characteristics with those of standards. The carotenoid content was measured using the calibration curves described by Stinco et al. (2019) and a calibration curve performed with a fucoxanthin standard. The quantification of the carotenoids for which standards were not available was carried out using the calibration curves of carotenoids with a similar spectrum. Thus, myxoxanthophyll and diatoxanthin were quantified using the calibration curves of lycopene and zeaxanthin, respectively. The total carotenoid content was calculated as the sum of the content of the individual compounds.

2.4.4 *Dynamic proportion and PLE efficiency coefficient (K_{PLE})*

In order to characterize the impact of PLE on the extraction efficiency, the PLE efficiency coefficient (Parniakov et al., 2015) was evaluated, being K_{PLE} defined as the yields of the extracts obtained for PLE and control extraction procedures according to equation 9 :

$$\mathbf{K_{PLE} = Y_{compounds}(PLE)/Y_{compounds}(control)} \quad (9)$$

Where $Y_{compounds}$ was the yield of specific bioactive compounds (protein, polyphenol, chlorophyll a, chlorophyll b, carotenoids) in PLE or control extracts.

2.4.5 *Antioxidant properties*

The antioxidant capacity of the microalgae extracts was evaluated through the oxygen radical antioxidant capacity (ORAC) and the Trolox equivalent antioxidant capacity (TEAC) assays (Zhou et al., 2021). For the ORAC assay, Trolox, and 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were used as antioxidants and oxygen free radicals, respectively, and phosphate buffer was used as a blank control. Specifically, 50 µL of extract and 50 µL of the fluorescein sodium salt solution were added to a 96-well plate and incubated in a microplate reader at 37 °C for 10 minutes, then 25 µL AAPH solution was added, and the absorbance was recorded at 520 nm. For the TEAC assay, 25 mL of 7 mM ABTS were mixed with 440 µL of 140 mM potassium thiosulfate solution and incubated under darkness at room temperature for 12-16 hours to obtain the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) working solution. During the measurement, the ABTS working solution was diluted with 96% ethanol to obtain an absorbance value of 0.700 ± 0.020 at 734 nm. Then, 0.1 mL of the extracts or Trolox solution and 2 mL of the working solution were mixed, and after reacting for 3 minutes in a dark room, the absorbance at 734 nm was recorded.

2.4.6 ICP-MS mineral determination and Nutrient Relative Value (NRV) analysis

The contents of Mg, Ca, P, Fe, Zn, and Se in the extracts were evaluated considering methodologies described elsewhere (De la Fuente et al., 2019). Specifically, 1 mL extracts were mixed with 1 mL of concentrated nitric acid (HNO_3, 69 %) and 250 µL H_2O_2, then placed in a microwave furnace for digestion with a maximum temperature of 180 °C. After that, the volume was adjusted to 5 mL with ultrapure water, a 100 µL-aliquot was taken, and the volume was adjusted to 10 mL with ultrapure water. Finally, the mineral content was analyzed using a ICP-MS (7990 ICP-MS, Agilent Technologies, CA, USA). Furthermore, the contribution of minerals in extracts from 100 g microalgae dry basics (Nutrient Relative Value, NRV) towards DRI was calculated as equation (10) (Jalali & Fakhri, 2021):

$$\mathbf{NRV = (X/R) \times 100} \qquad (10)$$

Where X and R corresponded to the mineral content in PLE extracts (DMSO as a solvent) from 100 g microalgae dry powder and Recommended Dietary Allowances (RDAs, as shown in Supplementary Table 1), respectively.

Table 1. Dietary Reference Intakes (DRIs): Minerals

Life stage	Mg (mg/d)	P (mg/d)	Ca (mg/d)	Fe (mg/d)	Zn (mg/d)	Se (µg/d)
baby (6~12 months)	75	275	260	11	3	20
children (1~3 years)	80	460	700	7	3	20
children (4~8 years)	130	500	1000	10	5	30
males (9~13 years)	240	1250	1300	8	8	40
males (14~18 years)	410	1250	1300	11	11	55
males (19~30 years)	400	700	1000	8	11	55
males (31~50 years)	420	700	1000	8	11	55

males (51~70 years)	420	700	1000	8	11	55
males (>70 years)	420	700	1200	8	11	55
females (9~13 years)	240	1250	1300	8	8	40
females (14~18 years)	360	1250	1300	15	9	55
females (19~30 years)	310	700	1000	18	8	55
females (31~50 years)	320	700	1000	18	8	55
females (51~70 years)	320	700	1200	8	8	55
females (>70 years)	320	700	1200	8	8	55
pregnant (19~30 years)	350	700	1000	27	11	60
breastfeed (19~30 years)	310	700	1000	310	12	70

NOTES: This table (taken from the DRI reports, see *www.nap.edu*) presents Recommended Dietary Allowances (RDAs) in bold type. An RDAs is the average daily dietary intake level sufficient to meet the nutrient requirements of nearly all (97~98 %) healthy individuals in a group.

2.5 Statistical analysis

The statistical analysis was performed by analysis of variance (ANOVA), using SPSS19.0 analysis software. The means were compared by Duncan's multiple range test ($p < 0.05$).

3 Results and discussion

3.1 Evaluation of Chlorella extraction process: yields, antioxidant properties and efficiency coefficient

3.1.1 Extraction yields

The extraction effect of DMSO was evaluated with *Chlorella* as the raw material and the extraction conditions were determined, which were subsequently used for the recovery of the bioactive components of *Spirulina* and *Phaeodactylum tricornutum.* The effects of PLE and DMSO concentrations on the yield of *Chlorella* valuable compounds are shown in **Figure 1**.

PLE extraction increased the protein yield of *Chlorella* samples compared to the control group. The highest yield was obtained when water was used as the extraction solvent and decreased as the DMSO concentration increased, thus indicating that high concentrations of DMSO are not suitable for protein recovery from *Chlorella* samples. In this line, a previous study showed the negative impact of DMSO on protein recovery from *Nannochloropsis*, which was attributed to protein precipitation promoted when high concentrations of DMSO are used (Parniakov, Apicella, et al., 2015).

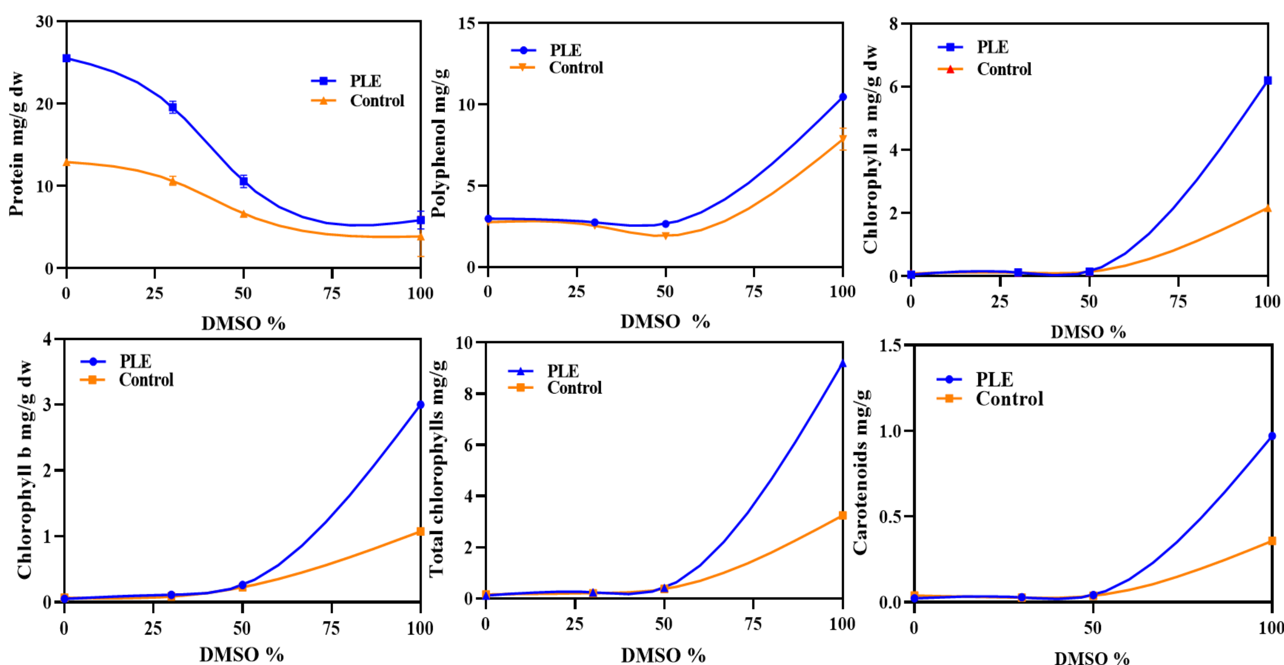

Figure 1. Effects of pressurized liquid extraction (PLE) and dimethyl sulfoxide (DMSO) concentration on *Chlorella* valuable compounds yield (mg/g dw)

On the other hand, the effect of PLE on the extraction yield of polyphenols was affected by the concentration of DMSO. The polyphenol contents of the PLE extracts were higher than the control group when the concentration of DMSO was higher than 30%. The polyphenol yield augmented with the increase of DMSO concentration until reaching about 10 mg/g dw at 100%

DMSO, thus indicating that DMSO concentration over 30% could increase the polyphenol yield.

Up to a DMSO concentration of 50% no significant differences were found between the PLE and the control group in the values of the dynamic curves of pigments, including chlorophyll *a*, chlorophyll *b* and carotenoids. Concentrations of DMSO higher than 50%, resulted in a significant increase in the pigment yield of the PLE extracts compared to control group. It should be also noted that when DMSO at 100% was used as the extraction solvent, the contents of chlorophyll *a*, chlorophyll *b*, total chlorophylls, and carotenoids of the PLE extracts reached values up to 6.3±0.1 mg/g dw, 3.0±0.1 mg/g dw, 9.3±0.2 mg/g dw and 1.08±0.03 mg/g dw respectively. Chlorophyll *a*, chlorophyll *b* and carotenoids are fat-soluble pigments whereas DMSO has amphiphilic properties and can be used to dissolve polar and non-polar compounds in various solvent systems, thus promoting the increase of the extraction yield of those pigments when DMSO concentrations higher than 50% are used (Selvakumar et al., 2021). Similar to this study, Parniakov et al. used the combination of pulsed electric field-DMSO to recover about 20 mg/g dw and 10 mg/g dw of total chlorophylls and carotenoids, respectively, from *Nannochloropsis*. The results were attributed to the good solubility of pigments in DMSO (Parniakov et al., 2015).

3.1.2 Efficiency coefficient

To evaluate the effect of PLE on the yields, the PLE efficiency coefficient, K_{PLE}, was evaluated. The K_{PLE} value was calculated according to equation 9, and the results are shown in **Figure 2**. The K_{PLE} values of proteins and polyphenols both exceeded 1, corresponding their maximum K_{PLE} values to 2.0 (water as the extraction solution) and 1.25 (50% or 100% DMSO as the

extraction solution), respectively. When the DMSO concentration was 0%, 30% and 50%, the K_{PLE} values of the pigments were all close to 1, while when the DMSO concentration was 100%, the K_{PLE} values of chlorophyll *a*, chlorophyll *b* and total chlorophylls were close to 3.0, while the K_{PLE} values of carotenoids were close to 2.7. From the overall K_{PLE} results observed in **Figure 2**, it can be depicted that PLE combined with 100% DMSO appeared to be more suitable for the recovery of chlorophylls, carotenoids and proteins from *Chlorella*. The microalgae mechanical disruption treatment process was not involved in this study, so the extraction of bioactive compounds is mainly attributed to the behavior of the solvent diffusion into the interior of microalgal cells (Nikolopoulos et al., 2008). Moreover, the pressurization process of PLE can reduce the strong interaction of solute with the matrix caused by van der Waals forces or hydrogen bonds in the system (Cao et al., 2021), thus facilitating the extraction process, resulting in K_{PLE} values of 2 or even 3.

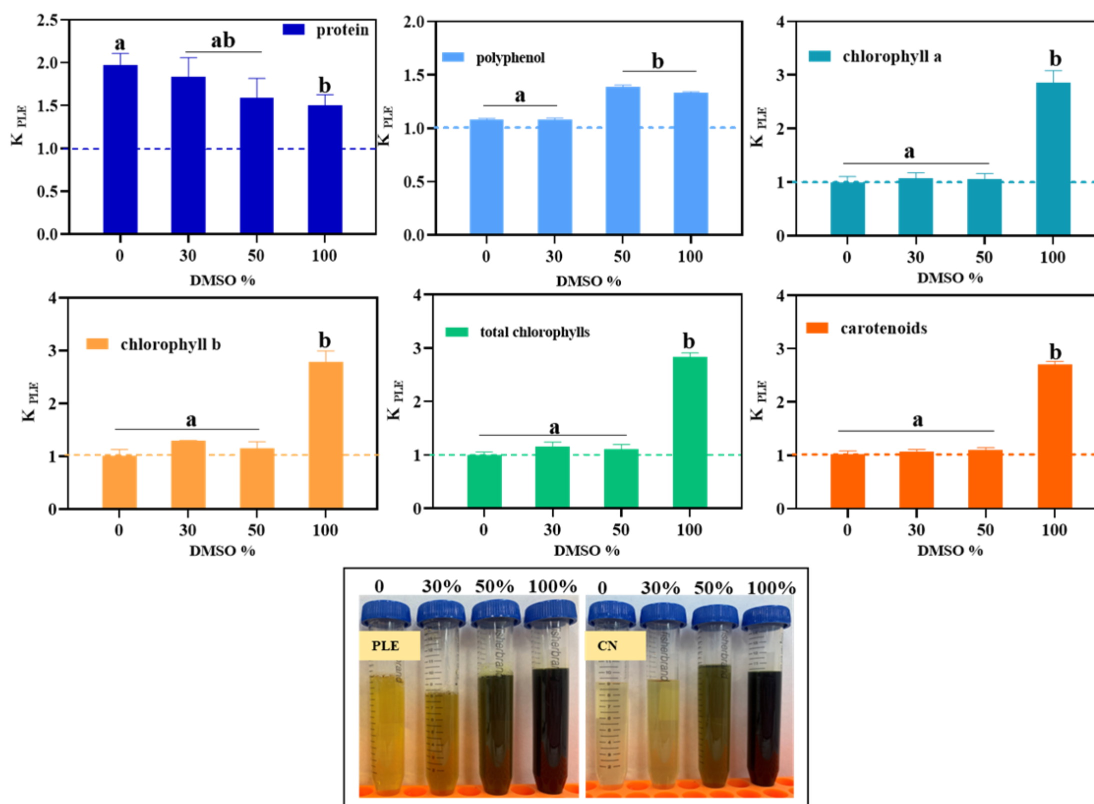

Figure 2. Effects of dimethyl sulfoxide (DMSO) concentration on efficiency coefficient of pressurized liquid extraction (PLE) (K_{PLE}). CN, control extraction.

3.1.3 Antioxidant properties and principal component analysis (PCA)

Oxygen radical antioxidant capacity (ORAC) and Trolox equivalent antioxidant capacity (TEAC) were used to analyze the antioxidant properties of *Chlorella* extracts. The results are shown in **Figures 3a-b**. The ORAC results (**Fig. 3a**) showed that the *in vitro* antioxidant capacity of the PLE extracts was higher than that of the control group. At DMSO concentrations lower than 30% the main difference between PLE and control extracts was a higher content of proteins in the former (Figure 1). Whether this higher antioxidant capacity was due to the original proteins or derivatives that may have formed requires further investigation. When the concentration of DMSO was higher than 50%, the increase in the antioxidant capacity of the PLE extracts was correlated with the increased content of polyphenols, chlorophylls, and

carotenoids. Contrastingly, the TEAC results (**Fig. 3b**) showed that the antioxidant capacity of the PLE and control extracts were very similar at concentrations of DMOS over 50%, but higher in the PLE extracts below such concentration. The inconsistency between the ORAC and TEAC results could be attributed to the different oxidizing agents and mechanisms of both methodologies. Overall, methods for measuring antioxidant capacity could be divided into two categories: methods based on hydrogen atom transfer (HAT) or electron transfer (ET) (Huang, Ou, & Prior, 2005). The assay principle of HAT followed that antioxidants and substrate compete for thermally generated peroxy radicals through the decomposition of azo compounds (such as ORAC), while the assay of ET measures the ability of antioxidants to reduce oxidants, such as TEAC (scavenging ABTS cationic radicals) (Zulueta, Esteve, & Frígola, 2009). Therefore, in complex extracts containing different kinds of antioxidants (polyphenols, pigments, etc.), the relevance of the ORAC and TEAC methods could be reduced due to different kinetics and reaction mechanisms. To further explain the association of bioactive components with antioxidant activity, a principal component analysis (PCA) was carried out (**Figure 3C)**. The results showed that 100%-PLE, 100%-Control were on the same side of the first principal component (PC1), while the other extraction conditions were on the other side (PC1). Moreover, 100%-PLE, 100%-Control, polyphenols, carotenoids, chlorophyll a, chlorophyll b, ORAC, and TEAC were distributed on the same side of PC1, thus indicating that DMSO had a more signicant effect on the yield and antioxidant activity of bioactive compounds than the technology used (PLE). ORAC and TEAC were distributed in the same quadrant (the first quadrant), thus indicating a positive correlation between these assays. More specifically, ORAC was closely distributed with carotenoids and chlorophyll *a*, while TEAC

was more closely distributed with polyphenols, which may be the reason for the difference in the results observed for the ORAC and TEAC assays. Overall, the extracts with higher content of polyphenols, chlorophyll and carotenoids as well as higher antioxidant properties were recovered when 100% DMSO was used as the extraction solvent. Therefore, 100% DMSO can be selected as the PLE extraction solvent for the recovery of these compounds from *Spirulina* and *Phaeodactylum tricornutum.*

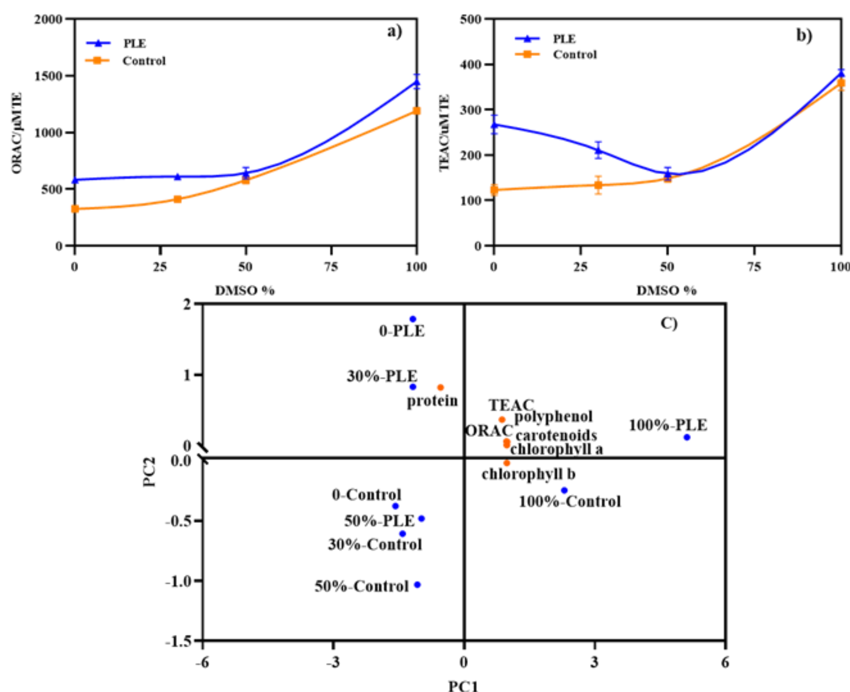

Figure 3. Effects of pressurized liquid extraction (PLE) and dimethyl sulfoxide (DMSO) concentration on the *in vitro* antioxidant capacity of *Chlorella* extracts. Figure 3a). Oxygen radical antioxidant capacity (ORAC), Figure 3b). Trolox equivalent antioxidant capacity (TEAC), Figure 3c). Principle component analysis (PCA).

3.2 Comparison of extraction effect on Spirulina, Chlorella and Phaeodactylum tricornutum

3.2.1 Yields, antioxidant properties and efficiency coefficient

The results of the extractions with 100% DMSO are shown in **Figure 4**. Under these conditions, the highest yields for the recovery of protein (45 mg/g dw), polyphenols (12.5 mg/g dw), chlorophyll a (8.0 mg/g dw), chlorophyll b (3.0 mg/g dw), and carotenoids (1.9 mg/g dw) from *Spirulina* were found. In the case of *Phaeodactylum tricornutum*, the highest yields of protein (33 mg/g dw), polyphenols (11.5 mg/g dw), chlorophyll a (5.7 mg/g dw), chlorophyll b (1.5 mg/g dw) and carotenoids (1 .6 mg/g dw) were lower. When the yields of protein and other valuable compounds of the three microalgae extracts were compared it was found that protein, polyphenols, carotenoids and antioxidant capacity yield of the extracts obtained from *Spirulina* and *Phaeodactylum tricornutum* were significantly higher than those of *Chlorella* ($p < 0.05$), while the chlorophylls *of Chlorella* extracts were significantly higher than that of *Phaeodactylum tricornutum* ($p < 0.05$), which maight be attributed not only to differences in the biosynthesis of the compounds across species but also to distinct characteristics, such as cell wall thickness or cell particle size, etc. (Zhou et al., 2022).

Figure 4 shows that all K_{PLE} values exceeded the value of 1. Specifically, for *Spirulina* (S-P) the K_{PLE} values of protein, polyphenols, chlorophyll a, chlorophyll b, and carotenoids were 8.2, 3.5, 4.0, 8.0, and 5.5, respectively. In the case of *P. tricornutum* (P-P), the K_{PLE} values of protein, polyphenols, chlorophyll a, chlorophyll b, and carotenoids were 4.8, 2.0, 3.0, 1.5 and 3.7, respectively. The results indicate that PLE enables the efficient recovery of the compounds studied when 100 % DMSO was used as the solvent. A recent study compared the extraction efficiency of pigments from mixed microalgae consortium using DMSO, methanol, chloroform and acetone. The maximum chlorophyll a (4.62 µg/ml), chlorophyll b (4.78 µg/ml) and total carotenoid (1.76 µg/ml) contents where obtained when DMSO was used as the extraction

solvent for 16 h (Jain, Behera, & Paramasivan, 2021). Compared with the extraction time used in the study of (Jain et al., 2021), the whole PLE extraction process of our study took 15 minutes, so it could be concluded that the combination of PLE+100% DMSO greatly improved the extraction efficiency of microalgae pigments. Overall, the pressurized process of PLE extraction and the superior diffusivity of DMSO synergistically improved the extraction yield of microalgae biomass, while similar studies were rarely reported and deserve further exploration.

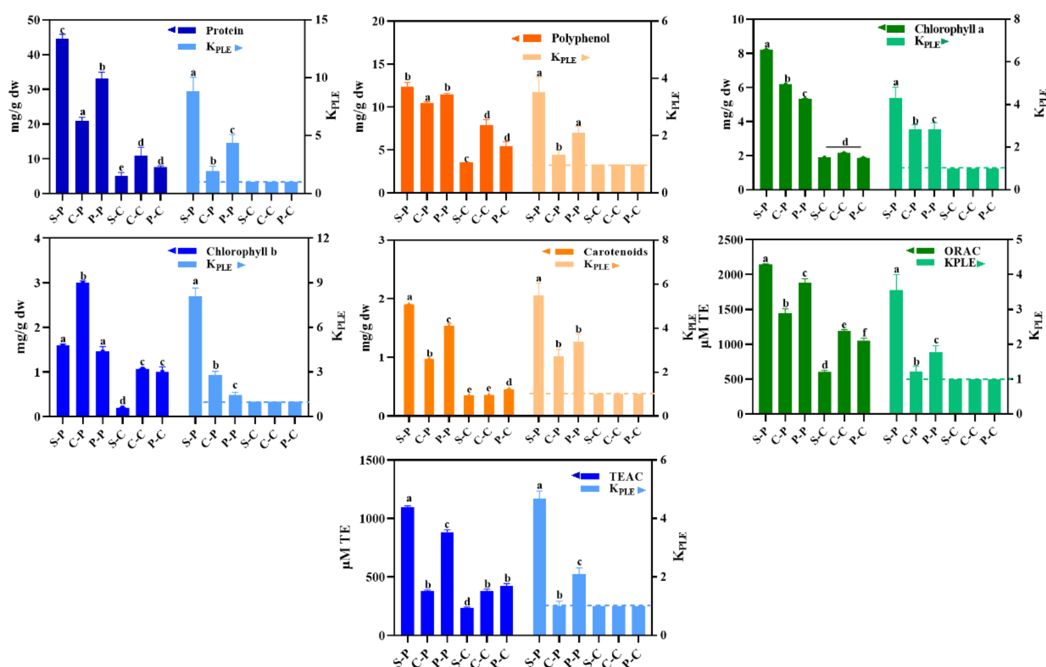

Figure 4. Effects of pressurized liquid extraction (PLE) and dimethyl sulfoxide (DMSO) concentration on the yield, antioxidant properties, and efficient coefficient (KPLE) of Spirulina, Chlorella, and Phaeodactylum tricornutum extracts. Different lowercase letters indicate significant differences ($p < 0.05$), same lowercase letters indicate no significant differences ($p > 0.05$). S-P, *Spirulina*-PLE; C-P, *Chlorella*-PLE; P-P, *Phaeodactylum tricornutum*-PLE; S-C, *Spirulina*-Control; C-C, *Chlorella*-Control; P-C, *Phaeodactylum tricornutum*-Control.

3.2.2 Carotenoid content

Carotenoids are isoprenoids biosynthesized by all photosynthetic organisms (plants, algae, cyanobacteria) as well as in some fungi, bacteria and arthropods. They are important as natural colorants, precursors of vitamin A, antioxidants and for health promotion as evidence has accumulated that they can contribute to reduce the risk of developing cancer, cardiovascular diseases, eye conditions, metabolic diseases and other conditions (Meléndez-Martínez, 2019; Rodriguez-Concepcion et al., 2018). Being present in high amounts in microalgae, these matrices are important in the context of healthy diets and the development of innovative products, including functional foods or nutricosmetics, among others (Meléndez-Martínez et al., 2021). The major carotenoids found in the samples analyzed were β-carotene, zeaxanthin and lutein. The total carotenoid content of *Spirulina* was considerably higher relative to *Chlorella* and *P. tricornutum* (~ 16-fold) (**Figure 5)**. The major carotenoids in the *Spirulina* extracts were zeaxanthin, β-carotene and (9*Z*)-β-carotene, in this order. Therefore, predominan tones in *Chlorella* and *P. tricornutum* extract were mainly lutein, β-carotene and (9*Z*)-β-carotene, in this order. Previous studies have shown that *Spirulina* is an industrial-scale source of carotenoids (0.5 mg to 2 g β-carotene/kg dry matter), with β-carotene being the main type (Santos Assunção et al., 2021). Similarly, *Chlorella* is also considered as a good source of carotenoids, including β-carotene, α-carotene, lutein, and zeaxanthin, being a genus commonly used for large-scale production of carotenoids (Chang Liu et al., 2021). Other studies have shown that *Phaeodactylum tricornutum* is a good source of fucoxanthin (Gille et al., 2019), however, only lutein, β-carotene and 9Z β-carotene were detected from *Phaeodactylum tricornutum* extracts in this study, which could be related to the differences in the extraction processes followed in the different studies.

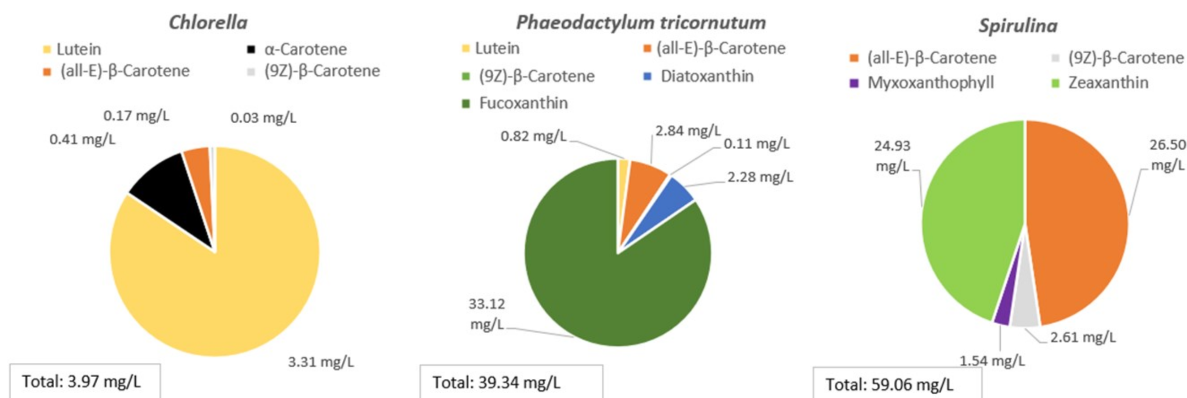

Figure 5. Carotenoids profile determination in PLE-100% DMSO extracts of *Spirulina*, *Chlorella* and *Phaeodactylum tricornutum*

Ultrasound, microwave and supercritical extraction have been the most studied techniques for extracting carotenoids from plant tissues and microalgae (Elik et al., 2020). In this study, different types of carotenoids were obtained from microalgae using PLE extraction with DMSO as a solvent, which provided a new idea for the recovery of carotenoids from microalgae. Overall, the present results showed that microalgae extracts were rich in carotenoids, and studies have shown that the carotenoid release could be limited by the cell wall when the complete microalgae was used as a diet, resulting in low carotenoid bioavailability (Fernandes et al., 2021).

3.2.3 Minerals and nutrient reference value (NRV)

The application of innovative and efficient extraction technologies to obtain macromolecular substances such as lipids, polysaccharides and proteins from microalgae has been widely studied (Vernès, Abert-Vian, El Maâtaoui, Tao, Bornard, 2019; Parniakov et al., 2015; Scherer et al., 2019). However, the information regarding the efficient recovery of mineral elements from microalgae is more limited. Some previous studies have shown that the mineral content of *Spirulina* mainly consists of Mg (383.5 mg/100g dw), P (752.5 mg/100g dw), Ca (798

mg/100g dw), Fe (96.8 mg/100g dw), Zn (2.73 mg/100g dw), Se (0.11mg/100g dw), while the main minerals found in*Chlorella* are Mg (344.3 mg/100 g dw), P (1761.5 mg/100 g dw), Ca (593.7 mg/100 g dw), Fe (259.1 mg/100 g), Zn (1.19 mg/100 g), Se (0.07 mg/100 g) (Tokuşoglu & Ünal, 2003), and the mineral content of *Phaeodactylum tricornutum* is mainly Mg (555 mg/100g dw), P (269 mg/100g dw), Ca (1910 mg/100g dw), and Zn (373 mg/100g dw) (Rebolloso-Fuentes, Navarro-Perez, 2000). At this stage of development there is a need for efficient tools to to recover these minerals, therefore, in this study the potential of PLE extraction process to recover Mg, Ca, P, Fe, Zn, Se was evaluated (**Table 2)**. When the total mineral content of the different microalgae was compared it was observed that the Zn content was relatively higher in *Spirulina* (44±1 mg/kg dw), being the extraction rate close to 100%. On the other hand, the P content was relatively higher in *Chlorella* (10647±248 mg/kg dw), beingthe extraction rate ~14%. Moreover, the contents of Mg, Ca and Fe were relatively higher in *Phaeodactylum tricornutum*, with values of 6206±99 mg/kg dw, 27333±2401 mg/kg dw and 2194±6 mg/kg dw, respectively, which corresponded to extraction rates of to 12.4% and 0.6 % and 4.8% respectively.

Table 2. Mg, P, Ca, Fe, Zn, Se (Nd) yield and Nutrient Relative Value (NRV) analysis

mineral	Mg			P			Ca			Fe			Zn		
algae	S-	C-	P-	S-	C-	P-	S-	C-	P-	S-	C-	P-	S-	C-	P-
total (mg/kg dw)	3239±89	3605±55	6206±99	9312±868	10647±248	6753±158	790±6	1574±20	27333±2401	290±13	674±5	2194±6	44±1	22±1	27±2
extracts (mg/kg dw)	595±2	324±6	772±8	1120±72	1504±28	309±8	109±4	36±2	169±5	63±1	20±1	106±1	44±1	8±1	18±1
	NRV: contribution of minerals in extracts (extract from 100 g microalgae dry matter) towards DRI														
	Mg			P			Ca			Fe			Zn		
life stages	S-	C-	P-	S-	C-	P-	S-	C-	P-	S-	C-	P-	S-	C-	P-
baby (6~12 months)	74.4	40.5	96.5	24.3	32.7	6.7	1.6	0.5	2.4	90.0	28.6	151.4	146.7	26.7	60.0
children (1~3 years)	45.8	24.9	59.4	22.4	30.1	6.2	1.1	0.4	1.7	63.0	20.0	106.0	88.0	16.0	36.0
children (4~8 years)	24.8	13.5	32.2	9.0	12.0	2.5	0.8	0.3	1.3	78.8	25.0	132.5	55.0	10.0	22.5
males (9~13 years)	14.5	7.9	18.8	9.0	12.0	2.5	0.8	0.3	1.3	57.3	18.2	96.4	40.0	7.3	16.4
males (14~18 years)	14.9	8.1	19.3	16.0	21.5	4.4	1.1	0.4	1.7	78.8	25.0	132.5	40.0	7.3	16.4

males (19~30 years)	14.2	7.7	18.4	16.0	21.5	4.4	1.1	0.4	1.7	78.8	25.0	132.5	40.0	7.3	16.4
males (31~50 years)	14.2	7.7	18.4	16.0	21.5	4.4	1.1	0.4	1.7	78.8	25.0	132.5	40.0	7.3	16.4
males (51~70 years)	14.2	7.7	18.4	16.0	21.5	4.4	0.9	0.3	1.4	78.8	25.0	132.5	40.0	7.3	16.4
males (>70 years)	24.8	13.5	32.2	9.0	12.0	2.5	0.8	0.3	1.3	78.8	25.0	132.5	55.0	10.0	22.5
females (9~13 years)	16.5	9.0	21.4	9.0	12.0	2.5	0.8	0.3	1.3	42.0	13.3	70.7	48.9	8.9	20.0
females (14~18 years)	19.2	10.5	24.9	16.0	21.5	4.4	1.1	0.4	1.7	35.0	11.1	58.9	55.0	10.0	22.5
females (19~30 years)	18.6	10.1	24.1	16.0	21.5	4.4	1.1	0.4	1.7	35.0	11.1	58.9	55.0	10.0	22.5
females (31~50 years)	18.6	10.1	24.1	16.0	21.5	4.4	0.9	0.3	1.4	78.8	25.0	132.5	55.0	10.0	22.5
females (51~70 years)	18.6	10.1	24.1	16.0	21.5	4.4	0.9	0.3	1.4	78.8	25.0	132.5	55.0	10.0	22.5
females (>70 years)	17.0	9.3	22.1	16.0	21.5	4.4	1.1	0.4	1.7	23.3	7.4	39.3	40.0	7.3	16.4
pregnant (19~30 years)	19.2	10.5	24.9	16.0	21.5	4.4	1.1	0.4	1.7	2.0	0.6	3.4	36.7	6.7	15.0
breastfeed (19~30 years)	79.3	43.2	102.9	40.7	54.7	11.2	4.2	1.4	6.5	57.3	18.2	96.4	146.7	26.7	60.0

Note. The Nutrient Relative Value (NRV)– contribution of minerals in extracts (extract from 100 g microalgae dry matter) towards DRI was calculated as: NRV = X/R 100%, Where X and R corresponded to the mineral content in microalgae extracts (form 100 g microalgae dry matter) and Recommended Dietary Allowances (RDAs) respectively. S-, C-, P- corresponds to *Spirulina*, *Chlorella* and *Phaeodactylum tricornutum* respectively, Nd-not detected.

The minerals evaluated in this study (Mg, Ca, P, Fe, Zn) are of great significance to human health. Specifically, Mg is an indispensable mineral in the human diet for the processing of ATP (adenosine triphosphate) and bones in the human diet, P is an important component of bones and cells, and Ca is the most abundant mineral in the body and is essential for muscles, bones, teeth, the health of the heart and digestive system, and the synthesis and function of blood. Fe plays an important role in DNA synthesis and repair, ATP production and oxygen transport and Zn is a key component of many protein functions and also an essential micronutrient required for many cellular processes and the development of the immune system, and the lack of these minerals in the human body can trigger related diseases (Eggleston, Triplett, Bett-Garber, Boue, & Bechtel, 2022; Erin D. Michos, Miguel Cainzos-Achirica, Amir S. Heravi, 2021; Ho, Wong, & King, 2022; Salnikow, 2021). Therefore, it is meaningful to discuss the mineral contents found in microalgae extracts to meet the needs of human health. Based on this, we further calculated the Nutrient Relative Values (NRV, %) of minerals in the extract (from 100 g of microalgae dry matter) with reference to Recommended Dietary Allowances (RDAs), being the results shown in **Table 2**. From the NRV results in **Table 2**, *Spirulina* and *Chlorella* extracts can be considered as suitable sources of Zn and P, respectively, while *Phaeodactylum tricornutum* is an interesting source of Mg and Fe. More specifically, for Mg, *Phaeodactylum tricornutum* extract could satisfy breastfeed (19-30 years), baby (6-12 months), children (1-3 years) 100%, 96.5% and 59.4% of Mg requirements respectively, and could cover the requirements for other populations (>4 years) 18.4-32.2% of Mg requirement. For P, *Chlorella* extract can satisfy breastfeed (19-30 years), baby (6-12 months), children (1-3 years) 54.7%, 32.7% and 30.1% of Mg

requirements respectively, and can meet the requirements established for other populations (>4 years old) 12.0-21.5% Mg requirement. Compared with other minerals, the microalgae extracts can only provide a limited amount of calcium for human body, i.e., less than 7%.

Phaeodactylum tricornutum and *Spirulina* can meet the Fe requirements of the human body to a large extent, among which *Phaeodactylum tricornutum* extract could meet the total Fe requirements of babies (6-12 months), children (1-8 years), males (>14 years), and females (31-70 years), whereas it could cover above 58.9% of that of other populations (except for pregnant (19-30 years), meeting 3.4% of its Fe requirement). For Zn, *Spirulina* extract could satisfy 146.7, 146.7, 88.0, and 36.7-55 % of this mineral requirement for breastfeeding (19-30 years), babies (6-12 months), children (1-3 years), and other populations (>4 years), respectively.

From the NRV results, in addition to Ca, microalgae extract can meet the body's mineral requirements to a large extent. It should be noted that in this study, we selected DMSO as the extraction solvent based on the yield, so the extract cannot be directly ingested. Nowadays, DMSO can be completely removed by methods such as oil pump vacuum distillation (60 °C) in the laboratory, which provided the possibility for the preparation of safe microalgae mineral supplements.

4 Conclusions

The total carotenoid content of *Spirulina* biomass was considerably higher relative to *P. tricornutum* and *C. vulgaris* (~1.5- and 15-fold, respectively). Fucoxanthin, all-*trans*-β-carotene, diatoxanthin, lutein, and (9*Z*)-β-carotene were the major carotenoids in *P. tricornutum.* β-Carotene, zeaxanthin, (9*Z*)-β-carotene, and myxoxanthophyll in *S. maxima*

and lutein, α-carotene, β-carotene, and (9*Z*)-β-carotene in *C. vulgaris*. PLE+H_2O can be considered as a useful strategy to recover proteins from microalgae, while PLE+DMSO is an interesting tool to recover lipophilic pigments, observing an increase when the DMSO concentration was augmented, especially over 30%. The NRV value based on the mineral content and DRIs indicated that the PLE microalgae extracts could be used after drying as Mg, P, Ca, Fe, and Zn supplements for different populations. Similar studies have rarely been reported so far.

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4.8 Potential benefits of high-added-value compounds from aquaculture and fish side streams on human gut microbiota

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Potential benefits of high-added-value compounds from aquaculture and fish side streams on human gut microbiota

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Abstract:

Background: Human gut microbiota dysbiosis has been linked to a higher risk of non-communicable diseases (NCDs) such us inflammatory disorders, allergy and obesity. Specific dietary strategies, including the use of specific food supplements targeted to microbiota modulation, have been suggested to be especially relevant in reducing the risk of NCDs. In this regard, marine environment is considered as a pivotal source of nutrients and bioactive compounds such as polyunsaturated fatty acids, polysaccharides and active peptides. These compounds, including algae- (alginate, fucoidan) and animal-derived polysaccharides (chitin, chitosan), among others, have been widely studied. The use of these active substances from marine organisms as a food supplement has been reported to affect human health.

Scope and approach: This review provides the evidence-base information on the potential effects of various active substances from marine organisms, including fatty acids, proteins and polysaccharides, on the structure of gut microbiota and their effects on host health.

Key findings and conclusions: These compounds could regulate the gut microbiota structure and thus, intestinal and systemic level with potential human health benefits. The exploration and evaluation of the relationship between these substances and gut microbiota may provide a new direction for further exploration of the influence of high-added-value components on gut microbiota with potential health effects. These high-added-value compounds have been explored to not only improve the utilization rate of aquatic products, but also reduce waste and contribute to the environment and economy sustainability. Meanwhile, it is possible to expand the commercial applications of these products by the industry.

Keywords: Aquatic life; side streams; fats; protein; polysaccharides; gut microbiota.

1. Introduction

Human Microbiota is considered as an "organ" that plays an important role in human health by interacting with the immune system. The vast majority of human microbes reside in the gastro-intestinal tract and these complex microbial community is known as "gut microbiota (GM)" (D'Amelio & Sassi, 2018; Zhang et al., 2012) and the predominant phyla are Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria (Matijašić et al., 2014).

The number of bacteria in the stomach (10^2~10^4 CFU/mL) with high gastrointestinal flow is lower than that in the large intestine and they mainly consist of Gram-positive aerobic organisms (*Streptococcus* and *Lactobacillus* genus), while in the ileum and colon, the number is significantly higher (up to 10^{12} CFU/mL), and the concentration of anaerobic bacteria exceeds that of aerobic bacteria, including the genus *Bacteroides, Bifidobacterium,* and also *Clostridium,* among other bacterial groups.

In addition, GM composition and structure changes along human life being early life microbiota less diverse and more sensitive to exogenous factors as compared with adults where microbiota is complex and resilient to changes, and again, during elderly, the microbiota is characterized by the loss of diversity and richness as well as shifts in GM composition (Li, Wang, Wang, Hu, & Chen, 2016). GM and immune system coexist in a balanced relationship to maintain a homeostatic state that can induce the body's protective response to pathogens and antigens (Malys et al., 2015). The disruption of this balance due a microbial dysbiosis causes a series of physiological processes which has been related to some diseases such as obesity and diabetes (Pellegrini et al., 2018).

Although many factors have been described to shape the GM, including genetics, age, health status drugs, antibiotics, smoking, stress, etc., diet is one of the most important exogenous factor affecting the GM composition, diversity and activity (Emge et al., 2016; Rothschild et al., 2018). The relationship between nutrients and GM is essential for understanding the crucial role of microbiota for human health. GM is involved in important host functions, at physiological and metabolic level, it can participate in the metabolism of carbohydrates and proteins, producing metabolism products including vitamins and short chain fatty acids (SCFAs), which plays an important role in human health. As an example, Specific gut Anaerobic bacteria have the ability to ferment undigested foods, such as polysaccharides, dietary fiber and proteins, and then, produce SCFAs (Kasubuchi et al., 2015). SCFAs can be used as an energy source by colonic mucosa cells and peripheral tissues, promoting cell growth, and also affect the water absorption and pH of the colon. In addition, SCFAs can induce the production of anti-inflammatory molecules and the reduction of inflammatory responses, inhibit the proliferation of tumor cells, thus acting as an anti-tumor (Felizardo, Mizuno Watanabe, Dardi, Venturini Rossoni, & Olsen Saraiva Câmara, 2019; Wang et al., 2019).

On the other side, Food nutrients such as polysaccharides, proteins, fats, and dietary fiber, have been shown to affect the structure and composition of GM. The interventions of these compounds highlight the relationship between GM changes and health outcomes (Tidjani Alou, Lagier, & Raoult, 2016; Zhang, Ju, & Zuo, 2018). Thus, the identification of new nutrient sources and products targeted to GM modulation, would be especially

relevant in the prevention and treatment of microbial dysbiosis linked to higher risk of NCDs.

In this regard, there are about 2,210,000 life forms in the marine environment, including animals, algae and microbes, which are an important source of life-active compounds (Mora et al., 2011). In addition to its rich nutritional value, they can also play an active physiological role in the body (Grienke et al., 2014). Many bioactive compounds derived from aquatic products are used in the food field, such as proteins, polyunsaturated fatty acid (PUFA), polysaccharides and minerals. These compounds can be obtained from fish, algae, crustaceans, etc., and have a great application potential. In recent years, the global fish consumption per capita increased from 9 kg/person in 1961 to 20.2 kg/person in 2015 (Sarker, 2020). At the same time, the annual production of fish is also increasing. According to statistics from the Food and Organization of the United States (FAO), aquaculture "is understood to mean the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants” (FAO, 1988). The global aquaculture fish caught reached 171 million tons in 2016 (FAO, 2018). The increase in fish consumption also brought about the waste of fish resources. In the industrial processing of fish, each ton of fish processed produce ~350-600 kg of waste, including fish head, viscera, bones and so on (Stevens et al., 2018). In the processing of shrimps and crabs, edible accounts for only about 9% of the total weight, resulting in a large amount of crustacean waste (Deng et al., 2020). These wastes are often used as fertilizers, livestock feed or directly discarded, so that the protein, fish oil, chitosan, etc. contained in them are not properly used, and they also can cause an important damage to the environment (Ananey-Obiri et

al., 2019). In addition, there are about 25000 to 30000 kinds of algae in the ocean, which contains a lot of high-quality protein, polysaccharides, etc. They are considered a good source of biologically active substances due to their fast growth and due they do not use arable land (Charoensiddhi et al., 2017). Therefore, there is a growing interest of researcher on how to make a better use of the biologically active substances obtained from these aquatic products and more and more studies are being carried out (Atef & Ojagh, 2017; Suleria et al., 2016). In this line, this paper reviews and summarizes the effects of specific bioactive compounds isolated from aquatic products and fish side streams on GM and their potential value as food ingredients and food supplements (**Figure 1**), aiming to improve the aquatic resources utilization rate and pollution reduction, while exploring the potential application value of these bioactive components in the field of food and medicine

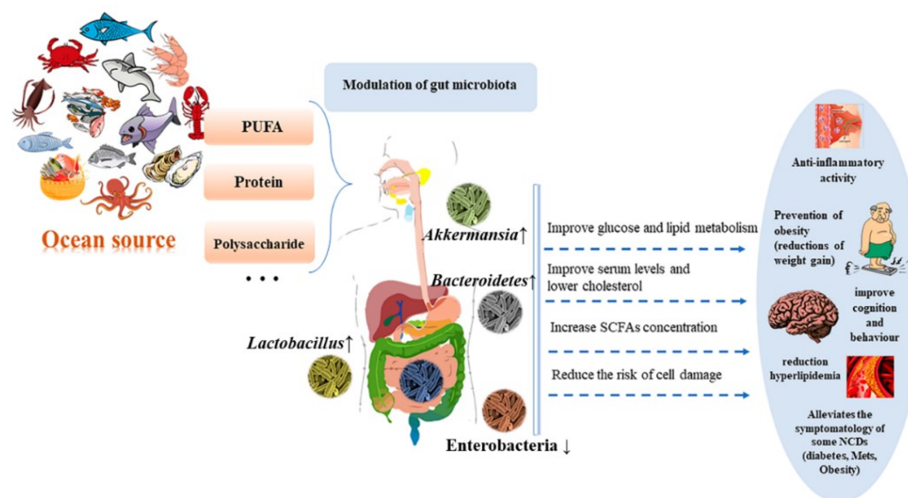

Figure 1. Role of ocean-source derived nutrients such as fat, protein and polysaccharides and their potential effect on the gut microbiota composition and diversity. Images for "Anti-inflammatory activity" and "reduction hyperlipidemia" are from Wikimedia (commons.wikimedia.org).

2. Effect of fat from fish-sources on gut microbiota

Dietary fat is one of the most important nutrients for the human body as it provides energy and is basic for human nutrition and the development of some metabolic functions (Pateiro et al., 2019). It is mainly obtained by the consumption of animal-derived foods, nuts and edible vegetable oil (Suwapat et al., 2018). Most of the fat in the diet exists in the form of triacylglycerol, which contains three fatty acids combined with the main chain of glycerol. According to chain lengths and the number of saturations, fatty acids can be divided into saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA).

Fat in diet has a significant role in GM that could vary depending on the type of fatty acid (Wolters et al., 2019). For instance, through dietary intervention, it was found that PUFA rich in ω-3 have been associated with increased levels of lactic acid bacteria and *Bifidobacterium* spp. in the intestine while SFA were suggested to disrupt the balance between the GM microbial components, increasing some genera such as *Bilophila* or *Bacteroides*, thus promoting inflammation (Noriega et al., 2016; Shin et al., 2015). On the other hand, by analyzing the microbial composition of women's diet and feces during pregnancy, the authors observed that high MUFA diet over a long period affected GM richness and diversity, increasing the relative abundance of *Salmonella spp.*, with possible negative effects (Roÿtiö et al., 2017). Dietary fats from other different sources have been shown to have an effect on GM. For instance, the role of olive oil on anti-inflammatory activity and regulatory effect of arterial pressure by affecting GM was recently reviewed (Gavahian et al., 2019). On the other hand, the impact of sweet orange essential oil and

kiwi seed oil as dietary additives to reduce the proportion of *Bacteroides* to suppress obesity was shown (Li, Wu, Dou, Guo, & Huang, 2018; Qu et al., 2019). In addition, corn oil, linseed oil, etc. have been also reported (Awoyemi, Trøseid, Arnesen, Solheim, & Seljeflot, 2019; Zhu et al., 2020).

Marine organisms are rich in ω-3 PUFA such as eicosapentanoic (EPA) and docosahexanoic acid (DHA) (Rai et al., 2013; Shen et al., 2020). Both ω-3 PUFA have been proven to have positive effects on the human host such as anti-inflammatory activity, obesity alleviation and reduction of the incidence of cardiovascular disease (Monk et al., 2019) (**Table 1**). Besides, they could also affect the structure and composition of GM (Imamura et al., 2016; Zhang et al., 2018). In a study conducted by Monk et al., the effects of fish oil on the intestinal environment, including GM composition and epithelial barrier, were explored using male mice fed by high-fat diet (Monk et al., 2019). The results showed that, compared with a simple high-fat diet meal, a high-fat diet supplemented with fish oil feeding reduced the proportion of Firmicutes and increased proportions of Bacteroidetes phylum. Also, the barrier function of the intestinal epithelium and oral glucose tolerance were improved after using fish oil rich in ω-3 PUFA, thus the authors concluded that the addition of fish oil could improve intestinal health and metabolic dysfunction associated with obesity.

Similarly, other authors have investigated the effects of fish and krill oils as dietary supplements to reduce weight gain in mice on a high-fat diet (Cui et al., 2017; Han et al., 2018). It has been reported that mice receiving dietary supplements of fish oil and krill

oil gained less weight, and gradually reduced their liver index and total cholesterol, being the effect greater on male mice than female mice.

On the other hand, the effect of fish or algal oils on the structure of GM has also been studied by different authors. For example, Balfegò et al. (2016) explored the effect of a sardine-rich diet (6 weeks) on type 2 diabetes and compared it with a normal diet. They observed that the addition of sardines reduced the ratio of *Phytoplankton/*Bacteroidetes. In another work, the addition of fish oil rich in ω-3 PUFA significantly changed the GM composition in mice, which may partially explain the health effects of the fish oil addition (Yu et al., 2014).

Table 1. Effects of marine fat on gut microbiota (GM)

Source	Methods	Study	Microbiota analysis	Daily dosage	Effects on GM	Ref.
Menhaden fish oil	high-fat diets were supplemented with fish oil	mice	Fecal samples 16S rRNA sequencing	60% /5.3 % kcal fat	fish oil ↑ Bacteroidetes phyla, improved the barrier function of intestinal epithelial cells, and alleviated the metabolic dysfunction associated with obesity.	(Monk et al., 2019)
Fish oil	fish oil, soybean and lard oil were compared	mice	Fecal samples 16S rRNA sequencing	15 % fat diet 30 days	fish oil diet ↑bile acids in feces ↑relative abundance of Firmicutes.	(Hosomi et al., 2019)
Sardines fish oil	Patients with type 2 diabetes were given a sardine diet	human	Fecal samples Quantitative PCR	100 g, 5 day/week	↓ ratio *Phytoplankton*/Bacteroidetes	(Balfegò et al., 2016)
Fish oil	high levels of fish oil (40 and 27 %) were supplemented	mice	Fecal samples 16S rRNA sequencing	5 mg/kg 10 mg/kg/day	fish oil ↓*Helicobacter*, *Uncultured bacterium Clone WD2_aaf07d12 (GenBank: EU511712.1)*, *Clostridiales bacterium*	(Yu et al., 2014)

Fish oil	fish oil and soybean oil as contrast	mice	Fecal samples 16S rRNA sequencing	1.6 fish oil/100 g of diet	↓ ratio of *E. coli* and *Escherichia*, inhibiting inflammatory bowel diseases.	(Yamamoto et al., 2018)
Fish oil	fish and lard oil were added into the diet make a comparison	mice	Cecal samples 16S rRNA sequencing	45 % kcal fat 11 weeks	↑the relative abundance of *Akkermansia* and *Lactobacillus* genus in the cecal of mice	(Caesar et al., 2015)
Fish/Krill oil	high-fat diets were supplemented with fish/krill oil	mice	Fecal samples 16S rRNA sequencing	10/40 % kcal fat 12 weeks	↓weight gain, liver index and total cholesterol in high-fat mice, ↓the ratio of *Firmicutes* to *Bacteroidetes*	(Cui et al., 2017)
Krill oil	different doses of krill oil	mice	Ileum and colon samples 16S rRNA sequencing	100, 200, 600 mg/kg/day	↑the relative abundance of *Bacteroidales* and *Lactobacillales*	(Lu et al., 2018)
Tuna/ Algae oil	fish oil and algae oil were supplemented	mice	Fecal samples 16S rRNA sequencing	600 mg/kg/day 12 weeks	↑ the learning and cognitive abilities of mice.	(Zhang et al., 2018)

Microalgae oil	Pregnant female mice and male offspring were fed microalgae oil	mice	Fecal samples 16S rRNA sequencing		↑ the relative abundance of *Bifidobacterium* and *Lactobacillus* genus in feces of mice and showed stronger cognitive ability.	(Robertson et al., 2017)
PUFA from *Spirulina platensis*	The lipid metabolism of mice was evaluated.	mice	Liver tissues real time-qPCR	150 mg/kg/day 4/8 weeks	↓ *Turicibacter*, *Clostridium_XIVa*, and *Romboutsia*, which were positively associated with lipid metabolism.	(Li et al., 2019)

*GM: gut microbiota

In order to investigate the effects of ω-3 PUFA intake on mothers and their offspring, Robertson et al. fed pregnant female mice and their male offspring with ω-3 PUFA from microalgae and assessed the cognitive and social skills of the male offspring. The analysis showed that the addition of ω-3 PUFA triggered slight changes in the behavior of the offspring and showed strong cognitive modulation ability (Robertson et al., 2017). In addition, Firmicutes to Bacteroidetes phylum ratio increased as well as the abundance of the genus *Bifidobacterium* (Actinobacteria phyla) and *Lactobacillus (*Firmicutes phyla). The use of PUFA of algae (*Spirulina*) as a dietary supplement to high-fat diet, triggered the inhibition of hepatic lipid accumulation and steatosis, as well as the reduction of relative abundance of *Turicibacter*, *Clostridium_XIVa*, and *Romboutsia* genus, which were positively associated with lipid metabolism (Li et al., 2019). Therefore, the authors suggested spirulina could be used as a functional food to improve lipid metabolism disorders.

Fish oils have also been suggested to have an effect on intestinal disorders. For example, an elderly population was fed with a diet supplemented with fish oil (12 weeks) (with and without HIV) and the gut barrier function and inflammatory factors related to tract were determined (Zhang, Xia, Lu, & Sun, 2018). The results showed that the addition of fish oil could reduce inflammation and intestinal permeability. In summary, fish and algae oils have been suggested to have a positive role in regulating the structure and composition of GM as well as alleviating some symptoms of obesity and other diseases.

3. Effect of protein on gut microbiota

Dietary proteins, being an important source of nitrogen, can provide energy for the human body apart from being involved in several physiological functions. Indeed, proteins can be also used as fermentation substrate for GM (Esteghlal et al., 2019). The amount of protein that enters the intestine depends on protein intake and digestibility. Regarding this, about 6~18 g of nitrogen-containing compounds are obtained daily from the diet, in addition to a small amount of endogenous nitrogen-containing compounds (Yao et al., 2016). Undigested and endogenous proteins can be fermented by GM in the intestine to produce a wide range of metabolites, including SCFAs, branched-chain fatty acids (BCFAs), ammonia, indole, N-nitroso compounds, sulfur metabolites, etc.

The amount of these metabolites is also related to the ratio of carbohydrate to nitrogen (Portune et al., 2016). The bacteria can degrade the proteins into smaller peptides, which will be further metabolized. In the colon, carbohydrate fermentation produces SCFAs, which are quickly absorbed by the colon and have been described to have beneficial effects on the host. At the end of the colon, where the pH increases, the protein fermentation becomes more active. The SCFAs, which are fatty acids produced by the fermentation of branched-amino groups, are metabolic products of colonic microorganisms with phenol and indole, and their excretion can be used to evaluate the fermentation of protein in the intestine (Yang et al., 2020). These metabolites are related to the mucosal function of the intestinal tract and can interact with mucosal cells (Le Leu & Young, 2007). In addition, since protein fermentation is more active at the end of the large intestine, it may be associated with diseases such as colon cancer (Windey et al., 2012).

Table 2. Effects of marine proteins on gut microbiota (GM)

Marine proteins	Methods	Study	Microbiota analysis	Daily dosage	Effects on GM	Ref.
Phycocianin	Phycocyanin was supplemented	mice	Fecal samples 16S rRNA sequencing	50 mg/kg/day	↑the relative abundance of *Bacteroidetes* and *Actinobacteria* ↓lipopolysaccharide level.	(W. Li et al., 2020)
Glycosylated fish protein	glycosylated fish protein was fed	mice	Cecal and fecal samples 16S rRNA sequencing		↓protein digestibility ↑the abundance of *Allobaculum*, *Akkermansia, Lactobacillus animalis*	(K. Han et al., 2018)
Collagen peptides	Collagen peptides were supplemented to the diet	mice	Fecal samples 16S rRNA sequencing	40 mg/kg Orlista 800 mg/kg collagen peptides	↑relative abundance of *Lactobacillus* and *Akkermansia* ↓the abundance of bacteria associated with intestinal inflammation	(Wang et al., 2020)
Antioxidant peptides	antioxidant peptides were supplemented by gavage	mice	Fecal samples	100 mg/kg/day	↑the abundance of 3-indolepropionic acid and SCFAs	(J. Han et al., 2020)

			16S rRNA sequencing		↑the abundance of *Lactobacillus* ↓the abundance of *Helicobacter*	
Herring milt hydrolysate (protein: 47~94 %)	high-fat diets were supplemented with herring milt hydrolysate	mice	Fecal samples 16S rRNA sequencing	208.8 mg/kg	↑the abundance of *Lactobacillus* ↓ metabolic associated with obesity and inflammatory disease	(Durand et al., 2020)
Alaska pollock protein (APP)	alaska pollock protein was fed	mice	Fecal samples 16S rRNA sequencing	15 % fat 30 days	↑the relative abundance of *Lactobacillus*, *Bacteroides* and *Akkermansia*	(Hosomi et al., 2020)
Fish muscle protein	lard, soybean protein and fish protein were compared	mice	Cecal sample 16S rRNA sequencing		fish protein ↓the abundance of *Bacteroidete*	(Y. Zhu et al., 2015)

*SCFAs: short-chain fatty acids, GM: gut microbiota

Marine products are rich in protein (**Table 2**), such as fish and algae, which are less restricted by cultivated land, which makes them especially relevant for commercialization (Aiello et al., 2019; de Boer et al., 2020; Ganesan et al., 2020). Limited evidence has been reported on the effect of aquatic product- protein derived on human GM. Fish or algae protein have been used as feed for fish breeding to explore its influence on the growth of artificially farmed juvenile barramundi (Siddik et al., 2020). Xie et al. (2019) used a bleomycin-induced pulmonary fibrosis model to determine the role of phycocyanin from *Spirulina* in vivo studies. The results showed that phycocyanin reduced lung fibrosis, and reduced the relative abundance of pro-inflammatory in parallel to an increased on GM diversity and richness and also, an increase on the total concentration of SCFAs (Xie et al., 2019).

In related studies, the effect of glycosylated fish protein on rats GM has also been explored (Han et al., 2018). The Maillard reaction between protein and reducing sugar causes the glycosylation of the protein, triggering the loss of essential amino acids and the decreasing of protein digestibility. Thus, there may be an increase in protein entering the colon and being fermented by microbes. At the same time, glycosylation of proteins is also a chemical modification method, which can change the function of proteins to a certain extent. The intermediate product of the Maillard reaction may be used by GM and could affect it (Delgado-Andrade & Fogliano, 2018).

In the study of Han et al., mice were fed with different fish proteins (heated fish protein, and glycosylated fish protein) and mice fed with glycosylated fish protein showed a reduction in the weight gain, in the colonic protein fermentation and in *Escherichia-*

Shigella and *Fusobacterium abundances.* However, is the same group an increased on the butyrate levels and also, on the relative abundance of *Allobaculum*, *Akkermansia*, *Turicibacter* genera were observed (Han et al., 2018). It has been studied the impact of heating times (24 h and 48 h, 50 °C) on the fermentation characteristics of glycosylated fish protein. Higher abundance of *Lactococcus* spp. in the mice intestine were observed at 48 h (71%) compared to those observed at 24 h (47%) (Han et al., 2018). In addition, the glycosylated fish protein heated for 48 h triggered a higher increase in the relative abundance of the *Streptococcus* and *Enterococci* spp. than that heated for 24 h. The glycosylation of fish protein also reduces the production of ammonia and indole.

Bioactive peptides are also studied as the constituent fragments of protein. In Wang's research, Walleye pollock skin was used as the object to enzymatically hydrolyzed produce collagen peptides with a molecular weight between 500 and 5000 (Wang, Lv, Zhao, Wang, & He, 2020). Studies have found that collagen peptides not only have anti-obesity effects, but also can regulate the overall composition of the GM in mice and increase the relative abundance of specific genus as *Lactobacillus* and *Akkermansia* and decrease the abundance of pro-inflammatory bacteria (Wang et al., 2020). In addition, Han et al. (Han et al., 2020) also screened out antioxidant peptides in tuna roe and found that they could inhibit the release of pro-inflammatory cytokines in mice models and increase the abundance of 3-indolepropionic acid and SCFAs has and also, influence the GM. In addition to protein from aquatic products, mung bean protein and tartary buckwheat protein have been also found to relieve obesity and improve cholesterol metabolism by regulating GM (Nakatani et al., 2018; Zhou et al., 2018).

4. Effect of Polysaccharides on Gut Microbiota

Carbohydrates and polysaccharides comprise a large part of people's daily diet and dietary intakes affecting the structure and function of GM (**Table 3**). Simple carbohydrates (such as starch and monosaccharides) in the diet can be directly digested and absorbed by the body. More complex carbohydrates (non-starch and resistant starch), that are resistant to human digestive enzymes, are not absorbed in the upper digestive tract and reach the distal gastrointestinal tract (Leong et al., 2019). These indigestible carbohydrates can be used as potential prebiotics compounds as they could be used by GM (Gibson et al., 2017). favoring specific bacteria and resulting in the production of SCFAs, which have a positive effect on improving glycometabolism, obesity, diabetes, among others (Nie et al., 2019). As a kind of abundant macromolecular compounds found in nature, polysaccharides have a wide range of sources. *Apocynum venetum* leaves, *Aralia echinocaulis*, mushrooms, fungus and other polysaccharides from multiple sources have been proven to be an important source of energy for GM, improving the structure of GM and alleviating diseases (Li, Dai, Wang, & Wang, 2021; Yin et al., 2020; Zhang, Zhao, Xie, & Liu, 2020). The polysaccharides in aquatic products are distributed among algae, animals and microorganisms. Algae- (fucoidans, alginate) and animal-derived (chitin and chitosan) polysaccharides have been shown to improve intestinal health, regulate blood sugar and alleviate diseases such as metabolic syndrome (Mets) (Tang & Hazen, 2014) (**Table 3, Fig 2**).

Table 3. Effects of marine polysaccharides on gut microbiota (GM)

Source	Marine polysaccharides	Methods	Study	Microbiota analysis	Daily dosage	Effects on GM	Ref.
Algae	Alginates	alginates and gut microbiota were studied by using an in vitro batch fermentation system	human	Fecal samples 16S rRNA sequencing		↑the relative abundance of *Bacteroides* ↑the concentration of SCFAs	(Bai et al., 2017)
	Alginates	collagen peptides were supplemented to the diet	mice	Liver and spleen samples HT-29-Luc cell model	2 % (*w/v*)	↓the infection of mice liver and spleen of mice was reduced, it plays a role in immunomodulatory	(Kuda et al., 2017)
	Alginate Laminaran	alginate/ laminaran were supplemented to the diet	mice	Cecal samples 16S rRNA sequencing	2 % (*w/v*)	↑the abundance of *Bacteroidetes*	(Takei et al., 2020)
	Alginate Laminaran	alginate/ laminaran were supplemented to the diet	mice	Cecal samples	2 % (*w/w*)	↑the diversity of bacterial bands,	(An et al., 2013)

				16S rRNA sequencing		GM and its fermentable capacity were changed by adding soluble fermentable fiber to the diet.	
	Laminarin Fucoidan	laminarin/fucoidan were used as feed	boars	Colon samples analysis of volatile fatty acids	300 ppm Laminarin 240 ppm Fucoidan	↓the abundance of *Enterobacter spp.* ↑the abundance of *Lactobacillus*, improved intestinal health.	(Lynch et al., 2010)
	Fucoidan	fucoidan were supplemented to the diet	mice	Cecal samples 16S rRNA sequencing	10/60 % kcal fat	↓the weight of mice and blood sugar ↑the abundance of *Akkermansia* and SCFAs	(Shang et al., 2017)
	Fucoidan	fucoidan were supplemented to the diet	mice	Cecal samples 16S rRNA sequencing	100 mg/kg	↑the abundance of *Bacteroidetes* ↓the abundance of *Firmicutes*, serum total cholesterol was regulated.	(Chen et al., 2019)
Algae	Alginates	alginates and gut microbiota were studied by using an in vitro	human	Fecal samples 16S rRNA sequencing		↑the relative abundance of *Bacteroides* ↑the concentration of SCFAs	(Bai et al., 2017)

	batch fermentation system					
Alginates	collagen peptides were supplemented to the diet	mice	Liver and spleen samples HT-29-Luc cell model	2 % (*w/v*)	↓the infection of mice liver and spleen of mice was reduced, it plays a role in immunomodulatory	(Kuda et al., 2017)
Alginate Laminaran	alginate/ laminaran were supplemented to the diet	mice	Cecal samples 16S rRNA sequencing	2 % (*w/v*)	↑the abundance of *Bacteroidetes*	(Takei et al., 2020)
Alginate Laminaran	alginate/ laminaran were supplemented to the diet	mice	Cecal samples 16S rRNA sequencing	2 % (*w/w*)	↑the diversity of bacterial bands, GM and its fermentable capacity were changed by adding soluble fermentable fiber to the diet.	(An et al., 2013)
Laminarin Fucoidan	laminarin/fucoidan were used as feed	boars	Colon samples analysis of volatile fatty acids	300 ppm Laminarin 240 ppm Fucoidan	↓the abundance of *Enterobacter spp.* ↑the abundance of *Lactobacillus*, improved intestinal health.	(Lynch et al., 2010)

	Fucoidan	fucoidan were supplemented to the diet	mice	Cecal samples 16S rRNA sequencing	10/60 % kcal fat	↓the weight of mice and blood sugar ↑the abundance of *Akkermansia* and SCFAs	(Shang et al., 2017)
	Fucoidan	fucoidan were supplemented to the diet	mice	Cecal samples 16S rRNA sequencing	100 mg/kg	↑the abundance of *Bacteroidetes* ↓the abundance of *Firmicutes*, serum total cholesterol was regulated.	(Chen et al., 2019)
Animal	Fucosylated chondroitin sulfate	administered subcutaneously	mice	Kidney samples real-times qPCR	8 mg/kg administered subcutaneously	reduce cell damage	(Gomes et al., 2014)
	Fucosylated chondroitin sulfate	high-fat diets were supplemented with fucosylated chondroitin sulfate	mice	Colon samples 16S rRNA sequencing	1 mg/kg/day 40 mg/kg/day	↑the abundance of *Bacteroides* hyperglycemia, hyperlipidemia and inflammation were relieved	(Li et al., 2019)

	Squid ink polysaccharide	squid ink polysaccharide was supplemented to the diet	mice	Fecal samples 16S rRNA sequencing	200 mg/kg 50 mg/kg	↓the abundance of *Ruminococcus*, *Bilophila*, *Oscillospira*, *Dorea* and *Mucispirillum*, inflammatory diseases were alleviated.	(S. Lu et al., 2016)
Others	Sulfated polysaccharides of abalone gonad	sulfated polysaccharides were supplemented to the diet	mice	Fecal and cecum samples relative quantitative PCR	2 mg/mL	↑the level of *Bacteroidetes* ↓the level of *Firmicutes*	(Liu et al., 2019)
	α-D-glucan YCP	induction of acute colitis model and administration of YCP	mice	Fecal samples 16S rRNA sequencing		↑the abundance of *Bacteroidetes* ↓the abundance of *Firmicutes* and *Proteobacteria*	(Liu et al., 2020)
	polysaccharide from Spirulina platensis	mouse model of constipation was supplemented with polysaccharide	mice	Intestinal contents 16S rRNA sequencing	50 mg/kg 100 mg/kg	↑the abundance of *Akkermansia*, *Lactobacillus*, *Butyricimonas*, *Candidatus Arthromitus* and *Prevotella*	(Ma et al., 2019)

*TNF: Tumor necrosis factor, IL: interleukine, GM: gut microbiota, SCFAs: short-chain fatty acids

4.1 Algae-derived polysaccharides

Seaweeds, which are widely distributed around the oceans, constitute an important source of polysaccharides, some of them similar to those from terrestrial plants (Gullón et al., 2020; Roohinejad et al., 2017). The content of polysaccharide in seaweed is about 20~75 % and its type varies also among the different seaweeds (Wells et al., 2017). For example, brown algae contain alginates, laminarin and fucoidan, while red algae contain agarose and porphyran. Some of them have been shown to resist enzymatic degradation in the stomach and small intestine, to promote the growth of some components of GM and to improve intestinal health (Jesumani et al., 2019).

Alginate, which is a structural polysaccharide, is a common component of the cell wall and it is commonly used in the food and pharmaceutical industries (Taemeh et al., 2020) due to its good physical and chemical properties as well as its beneficial effects on the intestinal environment. Different studies have shown that alginate can improve Mets in many ways. As a dietary supplement, it can increase satiety and reduce energy intake (Wang et al., 2018). Other benefits have also been reported including the regulation of postprandial blood glucose levels by delaying gastric emptying and interfering with digestion (Georg Jensen et al., 2013). Furthermore, it can be also fermented by specific GM to increase the concentration of SCFAs (Hoebler et al., 2000).

Bai et al. (2017) obtained alginate from *Pseudomonas aeruginosa* PAO1 mutant and alginate was used as the sole carbon source of the culture medium for batch fermentation *in vitro* to evaluate the degradation and utilization of alginate by human fecal samples. After *in vitro* fermentation, SCFAs yield in bacterial alginate was similar and higher than

that in a starch medium. The results showed that both algae and bacterial-source alginate could be degraded by human GM.

Interestingly, the potential use of alginate against pathogen infections are getting interest (Kuda et al., 2017). Different studies have explored the effects of alginate on the adhesion and invasion of *Salmonella Typhimurium* in human enterocyte-like HT-29-Luc cells and also, in BALB/c mice model (Bai et al., 2017), observing that supplementation of high and low concentrations of alginate could alleviate liver and spleen infection in mice and had immunomodulatory effects. Moreover, different researches have explored the effect of polymannuronic acid, one of the alginates, on the body weight and inflammation caused by high sucrose diets in mice as well as the effect of GM. Polymannuric was observed to increase the bacterial richness and has potential as a personalized therapy (Liu et al., 2017).

On the other hand, fucoidan is a type of sulfated polysaccharide also present in brown seaweed whose biological activities are determined by their different sources (Zheng et al., 2021). As a highly sulfated molecule, it cannot be degraded by human digestive enzymes or used by GM. But it can still play a prebiotic-like role to regulate intestinal ecology (Wang et al., 2018). In this regard, the inclusion of fucoidan in the diet has been reported to increase the concentration of gut SCFAs (Zaporozhets et al., 2014) and has been suggested to prevent and treat a variety of diseases (Shang et al., 2018). Kelp and sarcoidum on diet-induced Mets were investigated and the results showed that both types of seaweed polysaccharides reduced body weight, blood sugar and systemic inflammation. Moreover, fucoidan can enrich the GM in beneficial microorganisms to human host health, such as *Akkermansia muciniphila* and SCFAs producers, and can be used as a

functional food to Mets (Shang, Song, et al., 2017). It is worth noting that not all fucoidan would act on GM, and further research should be carried out to investigate the different effects of fucoidan on GM.

Laminarin is also one of the most studied seaweed polysaccharides. As a linear polysaccharide, it is easier to ferment than alginate. Researchers evaluated the effects of alginate, fucoidan and laminarin on GM in rats (An et al., 2013). Laminarin has been described to increase the diversity of GM and may regulate intestinal metabolism by influencing mucus production (Y. Cui et al., 2020). Indeed, laminarin in the diet could stimulate the growth of *Lactobacillus* spp. in the gut, possibly improving intestinal health.

In addition to the algae polysaccharides mentioned above, some aquatic carbohydrates have also been studied (Wang et al., 2018). Polysaccharides extracted from green algae have also been used as drugs to treat diseases such as obesity (Tang, Gao, Wang, Wen, & Qin, 2013; Zhang et al., 2017). Agarose and porphyrins found in red seaweed that cannot be digested by human body enzymes. Some studies have shown that agarose oligosaccharides could have a prebiotic effect promoting the growth of *Bifidobacterium* and *Lactobacillus* genus, both considered beneficial bacteria (Suwapat et al., 2018). Regarding this, the presence of carrageenan in the diet significantly reduced the number of anti-inflammatory bacteria in the gut, suggesting that further research into the potential adverse effects of carrageenan (Shang, Sun, et al., 2017).However, some algae polysaccharides have also been reported to have adverse effects on GM (Sun et al., 2019).

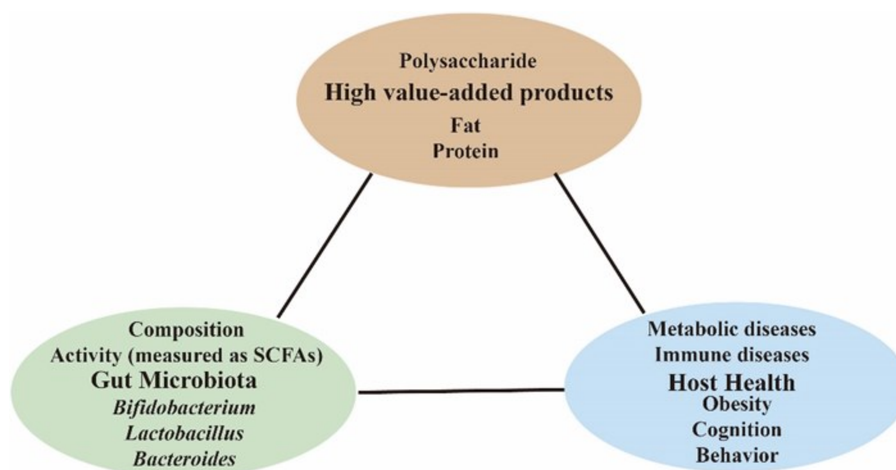

Figure 2. The interaction amongst high value-added products, gut microbiota, and host health.

4.2 Animal-derived polysaccharides

Polysaccharides, including chitin, chitosan, chondroitin, etc. are found in many marine animals, such as fish, mollusks and shellfish. They have been observed to confer some protection to obesity and cardiovascular diseases (Shang et al., 2018). Chitin is a structural polysaccharide found in crustaceans and consists of linear β-1,4-linked *N*-acetylglucosamine. It is produced after partial or complete deacetylation of chitin, which is the most abundant polysaccharide in clams and shrimps, among others (Tao et al., 2020). Both chitin and chitosan cannot be metabolized by human digestive enzymes, but they can be used as potential GM regulators triggering the increment of SCFAs concentration. Chitin has been used in aquaculture and is considered a fish health promoter (Askarian et al., 2012). On the other hand, chitosan is widely used because of its beneficial effect on GM, which differs according to its molecular weight. Moreover, chitosan has been used as an antibacterial agent, as well as to reduce the release of tumor necrosis factor and interleukin and lowering blood sugar and improving glucose tolerance (Liu et al., 2015).

Guan et al. fed mice with chitosan for 14 consecutive days, chitosan was also reported to have the ability to shape the mice GM, towards an increase in the Bacteroidetes phylum and a decrease in the relative proportion of Firmicutes phylum, (Guan et al., 2016). Chitosan has also been found to inhibit oxidative stress by the regulation of GM structure (Zhu et al., 2020). Besides, chitosan has been shown to improve diabetes and suppress obesity by the reduction of weight gain in mice, the increase of serum leptin levels also influencing the inflammation associated with a high-fat diet (Tang et al., 2020)

On the other hand, chondroitin sulfate (CS) is a sulfated glycosaminoglycan that can be obtained from shark cartilage. Because of its special structure, four different types have been described, namely CSA, CSC, CSD and CSE (Abdallah et al., 2020). As a dietary supplement, CS can be degraded by GM (Shang et al., 2016). CS and its oligosaccharides were found to regulate the GM in mice, increasing the relative abundance of *Lactobacillus*, *Odoribacter* and *Prevotellaceae*. In addition, CSA has been reported to be metabolized by various microorganisms in the intestinal tract with different degradation rates among them (Shang et al., 2016).

The fucosylated chondroitin sulfate (FCS) extracted from the wall of sea cucumber is similar to the core skeleton of CS. Studies have shown that FCS, as a biologically active substance, plays a significant role in preventing obesity and diabetes. FCS has been reported to inhibit the deposition of mesangial matrix and the expansion of interstitial tubules, relieving cell damage in diabetic rats, and thus, it has been proposed as a potential functional drug (Gomes et al., 2014). Similarly, FCS has been administered to mice on a high-fat, high-protein diet, and the results showed that it can effectively relieve obesity, hyperglycemia, and inflammation, reducing the proportion of Bacteroidetes and

increasing the abundance of *Porphyromonadaceae* and *Barnesiella* genus. Therefore, it has been suggested to be further developed to prevent GM alterations and metabolic syndrome (Mets) (Li et al., 2019).

In addition, other animal-derived polysaccharides have also been studied, such as squid ink polysaccharides (SIP) isolated from squid ink as well as abalone polysaccharides contained in abalone. The biological activities of SIP have been confirmed, such as anti-tumor, antioxidant, anti-coagulant activity, etc. (Gao et al., 2014). SIP has been observed to reduce the relative abundance of *Ruminococcus*, *Bilophila* and *Mucispirillum* genus, which may cause intestinal inflammation (S. Lu et al., 2016) and to enhance intestinal mucosal immunity (Zuo et al., 2016). Sulfated polysaccharides contained in abalone have also been shown to affect the GM, reduce the ratio of Firmicutes phylum/Bacteroides phylum and change the overall GM structure, which also have an impact on nutrient absorption and energy metabolism (Ai et al., 2018; Liu et al., 2019). Compared with algae-derived polysaccharides, animal-derived polysaccharides may be more at risk of contamination and deserve attention.

4.3 Other microorganisms

In addition to animals and algae, there are many microorganisms in the ocean water, including fungi, bacteria, viruses and phages, which have been found to contain biologically active compounds. It has been proven that α-D-glucan extracted from *Phoma herbarum YS4108* (a marine fungus) can increase the content of butyric and isovaleric acid produced by GM, and can be used as a potential adjuvant therapy in the ulcerative colitis treatment (Liu et al., 2020).

Spirulina polysaccharides were reported to have a significant effect on the GM, showing an increased relative abundance of beneficial bacteria, such as *Akkermansia*, *Lactobacillus*, *Butyricimonas genera*, thus they could be considered as a useful strategy for the treatment of constipation (Ma et al., 2019). Moreover, other aquatic polysaccharides may play a role in regulating GM composition and have a positive effect on the treatment and prevention of obesity, diabetes, and hyperlipidemia (Ma et al., 2019).

Comparing these three compounds, most polysaccharides cannot be directly digested and absorbed by the human body to reach the distal colon because the human body lacks carbohydrate active enzymes (Chen et al., 2018). Polysaccharides are converted into fermentable polysaccharides in the intestine and produce SCFAs and other metabolites, which have a positive impact on the host (Song et al., 2020). Then, dietary fat is also important for regulating the composition of GM, being saturated and unsaturated fatty acids the opposite effect on GM. For instance, saturated fatty acids reduce the content of probiotic, while PUFA can increase the relative abundance of *Bifidobacterium* and *Lactobacillus* genus (Yang et al., 2020). After the undigested endogenous protein is degraded by GM, in addition to generating SCFAs, a series of other compounds were also produced (such as indoles and amines). In the intestine, they can be combined with macromolecules in the colon to disrupt the balance of GM (Ramos & Martín, 2021).

In addition, the effects of other active compounds on GM have also been suggested. Sun et al. studied the effect of fucoxanthin in brown algae on high-fat diet mice (Sun et al., 2020) and they showed that the addition of fucoxanthin could inhibit the growth of the microbial families *Lachnospiraceae* and *Erysipelotrichaceae*, which are related to inflammation, and promote the growth of potential beneficial bacteria as *Lactobacillus*

and *Bifidobacterium* genus. Therefore, dietary fucoxanthin could have the potential to reduce obesity and inflammation by its effect on GM. The impact of other high-added-value components from aquatic sources on GM has been less studied.

5. Perspectives

Marine life is rich in high-added-value components, which would shape and modulate the GM. Furthermore, the dietary supplementation of high-added-value compounds may reduce the risk of specific diseases linked to GM dysbiosis such as Mets, obesity, diabetes, etc., and at same time, would have an impact on the immune regulation and intestinal homeostasis. Evidence is showing that diet with fish oil rich in ω-3 PUFA can improve intestinal health and alleviate the metabolic dysfunction associated with obesity. In addition to providing energy for the human body, protein and carbohydrate supplementation can also increase the relative abundance of beneficial bacteria (*Lactobacillus*, *Bifidobacterium*, etc.) and the concentration of SCFAs in the intestine. The intake of polysaccharides can increase the beneficial bacteria in the intestine, thereby reducing diseases related to sugar metabolism. Although it has been proved that the high-added-value components have a positive effect on GM, the results currently obtained mostly use animals as experimental models, and there are few experiments to test its efficacy through human clinical trials. Due to the complexity of the GM, the structure of high-added-value components and the complexity of their physiological functions, etc., may affect the results. In addition, most studies have been focused on the effects of polysaccharides, fats and proteins on GM, and the effects of other high-added-value components from aquatic products, such as polyphenols, on GM are rarely studied. This review also focuses on the impact of these three compounds on GM and host health, there

are few other compounds introduced. In the future, exploring the digestive behavior of high-added-value components of aquatic life in the intestine and the exact molecular mechanism of their interaction with GM needs to be more clarified. The effect of the interaction between high-added-value components on human health needs to be further studied. It is foreseeable that the application of high-added-value components as functional ingredients in food and medicine can be used as a new perspective to regulate GM, which can improve health while reducing the waste of high-added-value components. However, the available information about them and the possible impact on human health is still scarce and further research is needed.

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4.9 Applications of algae to obtain healthier meat products: a critical review on nutrients, acceptability and quality

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Applications of algae to obtain healthier meat products: A critical review on nutrients, acceptability and quality

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Abstract:

Meat constitutes one the main protein sources worldwide. However, ethical and health concerns have limited its consumption over the last years. To overcome this negative impact, new ingredients from natural sources are being applied to meat products to obtain healthier proteinaceous meat products. Algae is a good source of unsaturated fatty acids, proteins, essential amino acids, and vitamins, which can nutritionally enrich several foods. On this basis, algae have been applied to meat products as a functional ingredient to obtain healthier meat-based products. This paper mainly reviews the bioactive compounds in algae and their application in meat products. The bioactive ingredients present in algae can give meat products functional properties such as antioxidant, neuroprotective, antigenotoxic, resulting in healthier foods. At the same time, algae addition to foods can also contribute to delay microbial spoilage extending shelf-life. Additionally, other algae-based applications such as for packaging materials for meat products are being explored. However, consumers' acceptance for new products (particularly in Western countries), namely those containing algae, not only depends on their knowledge, but also on their eating habits. Therefore, it is necessary to further explore the nutritional properties of algae-containing meat products to overcome the gap between new meat products and traditional products, so that healthier algae-containing meat can occupy a significant place in the market.

Keywords: algae; bioactive compounds; healthier meat product; nutritional

1. Introduction

As people's understanding of the relationship between dietary functional components and health has deepened, consumers are paying more attention to dietary balance and physical health. Several healthy foods with functional ingredients that can mitigate diseases have aroused people's interest (Olmedilla-Alonso, Jiménez-Colmenero, and Sánchez-Muniz 2013), which include not only natural foods, but also products processed by the food industry. They have the potential to improve health and reduce the risk of diseases, thus being considered functional (Granato et al. 2020; Nystrand and Olsen 2021). Among the many dietary foods for human beings, meat and meat products are one of the foods with high nutritional value. Meat products can provide high-value nutrients including proteins, essential amino acids, lipids, minerals (such as selenium, iron and zinc) and vitamins, which have a positive effect on human health (Hygreeva, Pandey, and Radhakrishna 2014; Yousefi, Khorshidian, and Hosseini 2018). At the same time, there are many types of meat and meat products, which are mainly divided into two groups, red meat (such as beef and pork) and white meat (such as fish, chicken, duck, among others). These raw meats can be processed by different technologies to obtain some cooked or processed meat products such as meat patties, ham, sausage, among others, which have more popular/familiar sensory properties (Lu et al. 2022).

However, the production and consumption of meat at this stage does not follow a sustainable development model, due to several reasons. The breeding and production of a large number of livestock and poultry products puts a burden on the environment and has negative impacts in ecosystems in terms of water consumption and significant

emissions of methane and CO_2 to the atmosphere (Lemken, Spiller, and Schulze-Ehlers 2019; Onwezen et al. 2021). Then, meat is low in dietary fiber but is a rich source of fats and proteins. Therefore, the processing of meat products can promote Maillard reactions, protein degradation, lipid oxidation, among others, resulting in the production of advanced glycation end products (AGEs), nitrosamines (NAs), and other harmful substances (Akyüz, Ata, and Dinç 2016). Consequently, the accumulation of these substances in the body contributes to increase the occurrence of various diseases, such as diabetes (Illippangama et al. 2022; Trevisan et al. 2016). Compared with white meat, red meat and its processed products are more related to the occurrence of some chronic diseases (Ekmekcioglu et al. 2018). In addition, some vegan or flexible vegetarians demand other plant-derived foods instead of meat. Based on the above shortcomings and limitations, a reduction on the consumption of meat and meat-based products is now foreseen as an increasing tendency, with consumers looking for meat substitutes and non-traditional protein products and sources (Apostolidis & McLeay, 2016).

Nowadays, some meat substitutes based on plants and algae are being explored, which can replace some livestock and poultry products to a certain extension and improve the quality of traditional meat products. These new protein sources are widely available, cheap and easy to obtain, and contribute for a more sustainable development model, causing lower negative pressure for the environment (Saerens et al., 2021; Zhang et al., 2021). Among them, algae are considered a promising source to be investigated due to their unique properties. Algae can be divided into macroalgae (seaweed) and microalgae, having the former a long history of consumption in several Asian countries (Cofrades et

al. 2017). Although the consumption of algae in Europe is not as high as in Asia, in spite of being considered an excellent source of natural bioactive compounds due to the content of a great variety of these compounds. Algae are rich in polysaccharides, proteins, bioactive peptides, polyunsaturated fatty acids (PUFAs), vitamins, among others (Holdt and Kraan 2011). Some of these algae-derived compounds exhibit biological activities and health properties, such as anti-viral, anti-hypertension, anti-coagulation and so on, which provides a basis for algae to be used to improve food quality by adding functionality to foods (Jimenez-Lopez et al. 2021; Zheng, Chen, and Cheong 2020). Algae have some advantages over plants, as they grow faster and do not occupy arable land. Moreover, compared with some foods that use insects derived products as functional supplements, algae are more easily accepted by consumers (Verbeke 2015). These algae particularities have promoted the quest for healthier meat products based on algae. This review summarizes the potential applications of algae derived bioactive compounds, as well as their application status to improve the quality of meat and meat products.

2. Bioactive compounds in algae: as a nutritional and functional supplements

2.1 Proteins and bioactive peptides

The protein content of algae varies between species and changes with the seasons and surrounding environment. In terms of general production, red algae such as *Porphyra yezoensis* has a higher protein content, followed by green algae, while brown algae protein content is slightly lower (Kadam, Tiwari, and O'Donnell 2013). The main algae proteins can be divided into four categories according to wavelength (λ) of absorption which are, phycoerythrin (PE, λ= 490 to 570 nm), phycocyanin (PC, λ= 610 to 625 nm),

allophycocyanin (APC, λ= 650 to 660 nm) and phycoerythrocyanin (PEC, λ= 560 to 600 nm), collectively referred to as phycobiliproteins (PBP). PE is located in the outermost layer of PBP, where it absorbs light energy and transfers it to PC and APC, with an efficiency close to 95 % (**Figure 1**) (Li et al. 2019). The types of PBP vary, depending on the algae, i.e., the red and blue algae are mainly composed by PE, PC and APC. As a water-soluble pigment protein, it is a chromophore group that exists in algae, which can participate in the photosynthesis of algae, and is used for coloring foods, pharmaceuticals, and other fields such as clinical medicine (Kissoudi, Sarakatsianos, and Samanidou 2018; Rotan et al. 2017). **Table 1** (Christaki, Florou-Paneri, and Bonos 2011) shows the protein content of the most protein-rich foods, where it can be seen that the protein content (% dry weight, DW) of algae is higher than other traditional protein-rich foods in the human diet. At the same time, the amino acid types of algae proteins are similar to other high-protein foods, and in fact, aspartic acid (Asp) and glutamic acid (Glu) contents are higher in most algae. In some algae, the content of essential amino acids (EAA), such as isoleucine (Ile) and threonine (Thr), is even closer to that of soybean and egg proteins compared to meat (Becker 2007; Pimentel and Pimentel 2003).

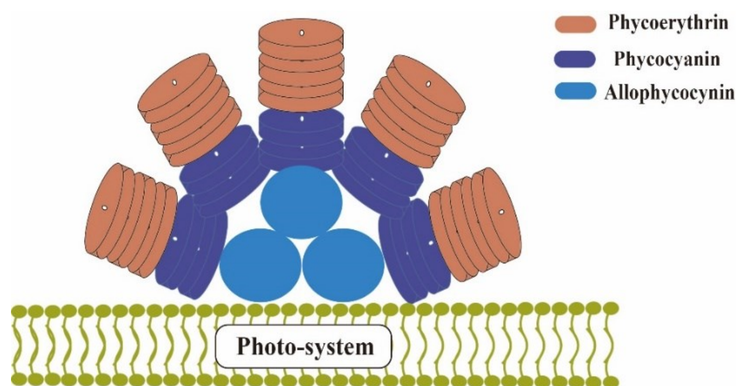

Figure 1. Structure of phycobiliprotein in *Arthrospira platensis*

As photosynthetic organisms, algae proteins have biological functions, such as anti-oxidation, anti-hypertension, anti-coagulation, cancer suppression, immune stimulation and so on (Chen et al. 2019), albeit proper extraction methodologies are to be used and optimized to maximize either the extraction yield and bioactivity of the extraction products. For example, Robin et al. (2018) used pulsed electric field (PEF) technique to extract proteins from *Ulva* sp. and determine its antioxidant activity. The results showed that the concentration of the extracted proteins increased after PEF treatment, and its antioxidant activity was 10~20 times higher than that of β-lactoglobulin (bovine serum albumin and potato protein, used as standard), being a potential candidate antioxidant for food applications. Researchers also used algae as a source to find substances that can inhibit angiotensin-converting enzyme (ACE) as components of anti-hypertensive drugs. The studies have shown that peptide chains with aromatic or aliphatic amino acid (such as proline (Pro), tyrosine (Tyr) and phenylalanine (Phe)) at the C-terminal or peptide chains with valine/isoleucine (Val/Ile) amino acids at the N-terminal have anti-hypertensive effects (Wijesekara et al. 2011). Cao et al. (2017) observed that *Gracilariopsis lemaneiformis* proteins were hydrolyzed by a variety of proteases, and it was found that in the presence of trypsin, the hydrolyzed product showed high ACE inhibitory activity (78.15±1.56 %) resulting in the amino acid sequence of glutamine-valine-glutamic acid-tyrosine (i.e., Gln-Val-Glu-Tyr). Furthermore, the anticoagulant activity of algae proteins has been confirmed, since, for example, Indumathi and Mehta (2016) isolated a new type of anticoagulant peptide from *Porphyra yezoensis* (Nori), with the identified sequence as NMEKGSSSVVSSRM (+15.99) KQ, which was confirmed by

in vitro anticoagulation experiments. It was also confirmed that this peptide plays a role in the coagulation process and may be used to prepare new drugs or functional foods in the future. Therefore, algae can be used as a good source of protein from the nutritional point of view, but also as functional protein/peptides with positive impact in human health (**Table 2, Figure 2**).

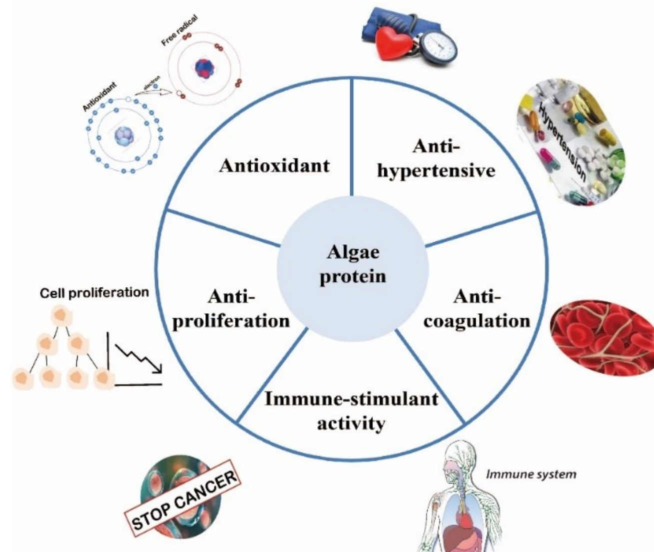

Figure 2. Biological activities of algae proteins.

Table 1. Protein content in different foods. Adapted from Christaki et al. (2011).

Food		Protein content (% DW)
Meat (Beef, chicken, fish)		17 ~ 24
Plants (Peanut, wheat, soybean)		26 ~ 36
Milk		36
Egg		47
Algae	*Chlorella* sp.	50~60
	Arthrospira sp.	60~70
	Dunaliella sp.	49~57
	Chlamydomonas sp.	50

Table 2. Algae proteins and their biological activities.

Algae (*Scientific name*)	**Possible bioactivity**	**Bioactive amino acid or peptide sequence**	**Evaluation of activity**	**Reference**
Ulva sp.	Antioxidant	Cys, Met, Tyr, Phe, Trp	10-20 times higher antioxidant capacity than β-lactoglobulin	Robin et al. (2018)
Macrocystis pyrifera	Antioxidant ACE inhibitory		83 µmol TE g^{-1}*	Vásquez, Martínez, and Bernal (2019)
Chondracanthus chamissoi	Antioxidant		35 µmol TE g^{-1}	
Navicula incerta	Antioxidant, hepatic fibrosis inhibitory effect	Pro-Gly-Trp-Asn-Gln-Trp-Phe-Leu Val-Glu-Val-Leu-Pro-Ala-Glu-Leu	IC_{50}> 196.0 µg/mL**	Kang et al. (2011)
Ascophyllum nodosum	Antioxidant		IC_{50}= 0.013 mg/mL	Liu et al. (2019)
Ulva sp. *Gracilaria* sp.	Antioxidant	Trp, Tyr, Met		Kazir et al. (2019)
Syngnathus schlegeli	Anti-hypertensive (ACE-I inhibitory activity)	Thr-Phe-Pro-His-Gly-Pro His-Trp-Thr-Thr-Gln-Arg	IC_{50}= 0.62 mg/mL	Wijesekara et al. (2011)

Gracilariopsis lemaneiformis	ACE inhibitory activity	Gln-Val-Glu-Tyr	IC_{50}= 0.255 mg/mL	Cao et al. (2017)
Enteromorpha clathrata	ACE inhibitory activity	Pro-Ala-Phe-Gly	IC_{50}= 35.9 µM	Pan et al. (2016)
Caulerpa lentillifera	ACE inhibitory activity	short-chain peptides	IC_{50}= 58.89 ± 0.68 µM 65.76 ± 0.92 µM	Joel et al. (2018)
Porphyra yezoensis	Anti-coagulant	NMEKGSSSVVSSRM (+15.99) KQ	IC_{50}=0 .3 µM	Indumathi and Mehta (2016)
Spirulina platensis	Anti-proliferation (prevent carcinogenic pneumonia)		IC50= 26.82 µg/mL	Deniz, Ozen, and Yesil-Celiktas (2016)
Spirulina platensis	Anti-cancer		inhibition rate: 75.4%	Liu et al. (2018)

TE g-1*: Trolox Equivalent; IC50**: The concentration of peptide required to inhibit 50% of the activity.

2.2 Lipids

Algae have a lower lipid content when compared to other marine organisms, such as some fish species, however, due to its higher content unsaturated fatty acids (UFAs), unique characteristics of growth and reproduction, being also considered a high-quality lipid source. Algae lipids can be divided into membrane lipids represented by glycosylglycerides and phosphoglycerides, storage lipids in the form of triglycerides (TG), and a small amount of fat-soluble pigments, sphingolipids, sterols and so on (Li-Beisson et al. 2019). Furthermore, the lipids of algae also can be divided into polar lipids (phospholipids and glycolipids) and neutral lipids (glycerol and UFAs) (Schnurr and Allen 2015). Similarly to proteins, the type of algae and the growth environment will affect the lipid content, for example, low temperature and cold water can promote the accumulation of UFAs in the algae, and some algae have a higher total lipid amount when they are collected during the summer season (Miyashita, Mikami, and Hosokawa 2013). But overall, algae are an important source of UFAs, especially long-chain UFAs with a high content, such as omega-3 polyunsaturated fatty acids (PUFAs) and eicosapentaenoic acid (EPA), as well as high levels of omega-6 fatty acids, oleic acids and so on (Quilodrán et al. 2020; Castejón and Señoráns 2019). These fatty acids have also been proven to have antioxidant, hypolipidemic and immune regulation effects (Nascimento et al. 2020; Freitas 2017). A variety of algae-derived lipids have been shown to have significant inhibitory effects on a variety of bacteria. For example, the linolenic acid contained in *Fischerella* sp. has been found to have obvious inhibition effects on *Staphylococcus aureus*, *Escherichia coli*, and in other microorganisms. Additionally, lipids extracted

from *Phaeodactylum tricornutum* and *Anabaena* spp. also showed anti-bacterial properties against a variety of bacteria, including *Salmonella typhimurium* (Asthana et al. 2006; Desbois, Mearns-spragg, and Smith 2009). The anti-inflammatory ability of omega-3 PUFAs has also been confirmed. For instance, the inhibitory effect of the lipid extracts from *Chlorella* sp. on the tumor necrosis factor-α (TNF-α) produced by human peripheral blood monocytes has been explored, and the inhibitory range of different kinds of *Chlorella* sp. lipid extracts on TNF-α can reach 58.39 to 78.67 % (Sibi 2015). In addition, the lipid extracts of algae also showed advantages in preventing cardiovascular diseases and modulating gut microbiota. It can be used as a substitute for fish oil and has the potential to become a dietary supplement (Koukouraki et al. 2020; Wan et al. 2019). Besides fatty acids, algae also contain 10 to 20 % phospholipids, which are the main carriers for stability of cell membrane structure and the transportation of nutrients. They can act as emulsifiers in the diet and have strong antioxidant capacity (Akyüz, Ata, and Dinç 2016).

2.3 Polysaccharides

Polysaccharides are regarded as high-quality biologically active ingredients because they are degradable, easily soluble in water, and have a wide range of bioactivity spectrum and are widely present in plants, microorganisms, marine sources, among others. As one of the most abundant resources in the ocean, algae contain a large number of polysaccharides (present in the cell wall structure, storage polysaccharides, fungal polysaccharides and others) and their content varies between 4 to 75 % of the dry weight (Zheng, Chen, and Cheong 2020; Usman et al. 2017). According to the evaluated species, the amounts of

polysaccharides differ, similarly to that observed for proteins and lipids. For instance, kelp polysaccharides, alginate and fucoidan are mainly found in brown algae; carrageenan, agar, xylan and porphyrin are the predominant in red algae; and ulvans are mainly found in green algae – being the later one the only source of agar, carrageenan, among others (Lim et al. 2021). Algae polysaccharides have been widely used in food, medicine, textile, and other industries, and have positive effects on human health such as antibacterial, anti-inflammatory, and low-caloric index, as some of these polysaccharides are considered dietary fiber (**Table 3**, **Figure 3**).

Alginate is mostly extracted from the cell wall of brown algae and is a linear polymer composed by (1→4)-β-D-mannuronic acid (M) and (1→4)-α-L-guluronic acid (G) (Pascaline et al. 2013). Studies have found that alginate exhibits a variety of bioactivities, including antibacterial and anti-inflammatory activity, and helps reducing high blood pressure (Table 4). For example, the alginate extracts from *Laminaria japonica* can effectively mitigate the allergic reaction of mice which is caused by ovalbumin protein (Yu et al. 2020). Sodium alginate is also an effective anti-bacterial component, with obvious inhibitory effects on *Staphylococcus aureus* (Deveau et al. 2016). Furthermore, alginate oligosaccharides with lower molecular weight and viscosity can be obtained by chemical/enzymatic methods due to their higher bioavailability (Wang, Chen, and Zhang 2021). Laminarin and fucoidan are also the main components of brown algae polysaccharides. Laminarin polysaccharide is a low molecular weight storage polysaccharide, with (1→3)-β-D-glucopyranose residues forming the main polysaccharide chain, variable degrees of β-(1→6)-intrachain links, and 6-*O*-branching

(Kadam, Tiwari, and O'Donnell 2015). Ji et al. (2012) found that, for example, laminarin polysaccharides have been proven to induce apoptosis of human colon cancer cells, and it also can significantly increase the release of inflammatory mediators, showing anti-inflammatory properties. Fucoidan can also be extracted from brown algae, being a sulphated polysaccharide composed of α-L-fucose and with a small amount of galactose and xylose (Kopplin et al. 2018). Similar to other polysaccharides, fucoidan has also been proven to have a variety of physiological functions including anti-oxidation and immune regulation. Fucoidan could be used to treat Alzheimer's disease in mice, and modulate the host's inflammatory response and intestinal microbiota balance (Wei and Lindell 2017).

Special types of polysaccharides such as agar and carrageenan (that are both indigestible film-forming polysaccharides) have been found in red algae, due to high species diversity. Agar includes agarose and agaropectin, both with the same backbone of alternating (1–4)-linked 3,6-anhydro-α-L-galactopyranose and (1–3)-linked β-D-galactose units, with agaropectin having many anioinic groups such as sulfate, pyruvate, and glycuronate and holding similar functional characteristics comparable to carrageenan, such as gel and emulsifying properties, and also are studied as prebiotics and antioxidant components (Carina et al. 2021). The most common carrageenan are κ-carrageenan, ι-carrageenan and λ-carrageenan, which are composed of an alternating backbone of α (1–4)-3, 6-anhydro-D-galactose and β (1–3)-D-galactose (Tanna and Mishra 2019). The different number of sulphate groups determines the different gel strength and solubility. Its biological activity is being explored, and some studies have shown that carrageenan is

an effective inhibitor of human papillomavirus *in vitro* (Laurie et al. 2021). Additionally, it has been studied to promote human health, such as immune regulation, and cholesterol level adjustment. Valado et al. (2020) found that carrageenan has the potential to lower the total cholesterol level by evaluating its levels in a population after taking carrageenan vegetable jelly; κ-carrageenan and λ-carrageenan from *Chondrus armatus*, which have been found to induce monocytes to product pro-inflammatory cytokines (IL1β, IL6, IL18, and TNF-α) and degradation products of high molecular weight carrageenan have lower cytostatic activity than low molecular weight carrageenan (Cicinskas et al. 2020); κ-carrageenan is used as a modulator of the intestinal microbiota, being their metabolism studied in mice and showing a potential prebiotic activity (Wang et al. 2021). In addition to its bioactivity, its excellent gelling properties also allow it to be used in the delivery of compounds, encapsulation of bioactive compounds and other applications.

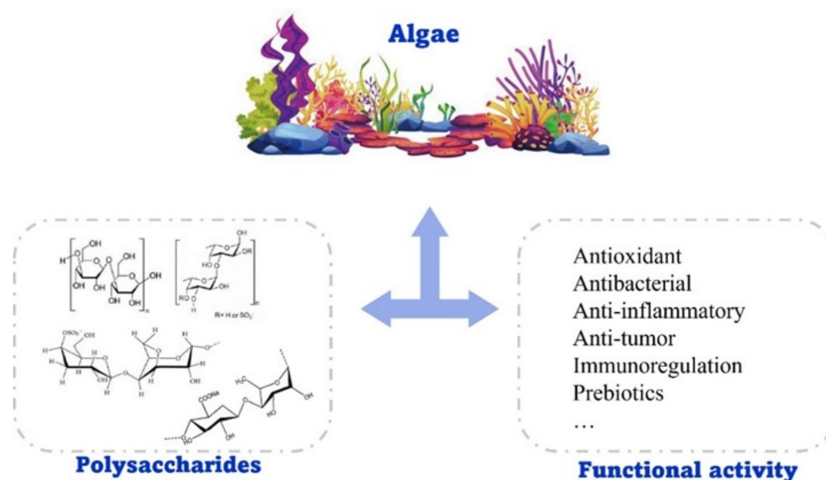

Figure 3. Structure and properties of polysaccharides from algae

Ulvan is a cell wall polysaccharide present in high level in green algae, representing 36% of the dry weight of algae. Common sources are mainly *Chaetomorpha* sp., *Cladophora* sp., *Ulva* sp., and *Monostroma* sp. species (Don et al. 2021). Ulvan is a

relatively complex sulphated polysaccharide and the main structure is composed by two repeated disaccharide units connected by β-1,4 linkages, which endows ulvan with a variety of bioactivities and functions, such as anti-inflammatory and anti-coagulation, and thus being widely used in food, medicine and other fields (Tang et al. 2021).

Table 3. Algae polysaccharides and their biological activities (n.a. stands for not applicable).

Algae		Polysaccharide	Bioactivity	Evaluation model	Reference
Brown algae	*Laminaria japonica*	Alginate	Reduced weight gain, lipid abnormality and inflammation, accompanied with the improvement of gut microbiota	Mice	Zheng et al. (2021)
	Turbinaria conoides	Fucoidan	Inhibit type 2 diabetes	n.a.	Senthil et al. (2019)
	Fucus vesiculosus	Fucoidan	Protect dendritic cells	Mice	Jeong, Ko, and Joo (2012)
	Undaria pinnatifida	Fucoidan	Antioxidant activity	n.a.	Si et al. (2019)
	Dictyota caribaea	Fucoidan	Anti-tumour, Immunostimulant	Mice	Barros et al. (2021)
	Sargassum vulgare	Sulphated polysaccharide, Fucoidan	Anticoagulant, Antithrombotic, Antioxidant and anti-inflammatory	Male Wistar rats	Maria et al. (2013)
	Padina tetrastromatica	Sulphated polysaccharide	Attenuates isoproterenol-induced oxidative damage	Adult male rats	Lekshmi, Rauf, and Kurup (2019)

	Turbinaria ornate	Sulphated polysaccharide	Inflammation inhibiting, Immunostimulant	Rats	Bhardwaj et al. (2021)
	Undaria pinnatifida	Sulphated polysaccharides	Alleviate postprandial and hyperglycaemia	Mice	Zhong et al. (2021)
Red algae	*Grlidiella acerosa*	Sulphated agaran	Anticoagulant, Antiplatelet, Antithrombotic	Female *Wistar* rats, Human blood	Diêgo et al. (2020)
	Gracilaria caudata	Sulphated polysaccharides	Relieve periodontitis	Adult female *Wistar* rats	Rodolfo et al. (2019)
	Gracilaria debilis (Forsskal)	Sulphated polysaccharides	Antioxidant, Anticoagulant	n.a.	Sudharsan, Subhapradha, and Seedevi (2015)
	Gracilaria lemaneiformis	Polysaccharide	Antioxidant, Hypoglycaemic activities	n.a.	Tang et al. (2021)
Green algae	*Caulerpa racemosa*	Sulphated polysaccharide	Antinociceptive, Anti-inflammatory	*Wistar* rats	Albuquerque et al. (2020)

Enteromorpha linza	Sulphated polysaccharide	Anticoagulant, Antioxidant	n.a.	Wang et al. (2013)
Enteromorpha prolifera	Polysaccharide	Hypolipidemic, Antioxidant	Mice	Tang et al. (2013)
Chlorella pyrenoidosa, Spirulina platensis	Polysaccharide	Modulation of lipid metabolism, alleviate obesity	Male mice	Guo et al. (2021)
Spirulina platensis	Polysaccharide	Anti-constipation	Mice	Ma et al. (2019)
Spirulina	Polysaccharide	Inhibit the proliferation of Caco-2 and HepG2 cells	n.a.	Wang et al. (2020)

Table 4. Application of seaweed in meat industry

Meat products	Algae	Additive amount	Main function	Reference
Obtain low-salt and low-fat products				
Meat emulsion	Sea Spaghetti (*Himanthalia elongata*), Wakame, Nori	5.6 %	Increase NaCl content and amino acid levels; enhance the antioxidant capacity of the model	López-lópez et al. (2009)
Ham	*Palmaria palmata* (extract)		NaCl goes down by 1 %; Show a positive effect on the senses	Barbieri et al. (2016)
Frankfurtes	*Himanthalia elongata*, *Porphyra umbilicalis*, *Palmaria palmata*	1 %	Low NaCl content; Influence the cooking loss, viscosity, etc.	Garicano et al. (2020)
Sausages	AlgySalt®	2 %	Reduce cooking losses	Triki et al. (2017)
Black puddings	Wakame	3 %	Reduce salt consumption; Reduce cooking losses	Fellendorf, O’Sullivan, and Kerry (2016a)
Pork patties	*Laminaria japonica*	1 %, 3 %	Reduce cooking losses; Obtain low energy products	Choi et al. (2012)

Frankfurt sausages	*Himanthalia elongata*	5 %	Improve the water-fat binding properties Increase the hardness and chewiness	López-lópez, Cofrades, and Jimenez-Colmenero (2009)
Frankfurt sausages	*Rhodophyceae*	0.41 %~0.70 %	Reduce cooking losses; Increase the hardness and chewiness; Improve sensory ratings	Cierach, Modzelewska-Kapituła, and Szaciło (2009)
Prevent corruption of meat products and extend shelf-life				
Minced beef meat	*Porphyridium cruentum* (polysaccharides)	0.5 %, 1 % and 2 %	Reduce psychrophilic bacteria; Extend shelf-life	Hlima et al. (2021)
Minced pork patties	*Laminaria digitata* (laminarin and fucoidan)	0.01 %, 0.1 % and 0.5 % (*w/w*)	Increased antioxidant capacity; Decreased lipid oxidation	Moroney et al. (2013)
Fish surimi restructured product	*Ulva intestinalis* (polysaccharides)	5 g/kg	Improve sensory properties; Extend shelf-life	Jannat-alipour and Rezaei (2019)
Pangasius fillets	*Padina tetrastromatica* (extract)	0.5 %~2 % (*v/v*)	Reduction in lipid oxidation; Improve the meat color;	Deepitha et al. (2021)

			Extend shelf-life	
Turkey sausages	*Cystoseira barbata* (fucoxanthin)	0.01 %, 0.02 and 0.04 %	Inhibits lipid oxidation; Keeps the color of the meat	Sellimi et al. (2017)
Pork meat	*Haematococcus pluvialis* (astaxanthin)	0.15, 0.3 and 0.45 g/kg	High dosage can delay lipid oxidation; Improved color stability	Pogorzelska et al. (2018)
Pork liver pâté	*Ascophyllum nodosum,* Fucus vesiculosus and *Bifurcaria bifurcata* (extracts)	500 mg/kg	Reduce lipid oxidation; Did not modify the microbial counts of healthy pâté.	Agregán et al. (2018)
Chinese-style pork sausage	*Arthrospira platensis* (extract)	0, 1.0, 2.5 and 5.0 % (*w/w*)	Inhibits lipid oxidation; The physical and sensory qualities were maintained	Luo et al. (2018)
Beef patties	*Spirulina, Chlorella*	1 %	Increased the concentration of total amino acids (especially flavor amino acids)	Žugčić et al. (2018)
Frankfurt sausages	Macroalgae	2~3 %	Increased mineral content; Reduced the Na/K ratio	Marçal et al. (2021)

2.4 Phenolic compounds

Phenols are constituted by hydroxyl groups directly linked to aromatic hydrocarbons and can exist in marine and terrestrial plants being considered to be an important part of the diet (Freile-pelegr and Robledo 2014). Studies have shown that phenolic components can prevent and mitigate a variety of diseases and have a positive impact on human health. Most of the phenolic components from algae show significant antioxidant activity (Plaza, Snóblová, and Lojková 2017).

Different growth environments lead to differences between phenolics derived from algae compared to terrestrial plants, being the first to present stronger bioactivities, such as bromophenol, tichocarpol and phlorotannins. Brown algae, compared to red algae and green algae have lower phenol content, especially the phlorotannins, which are mainly found in brown algae that are also the most studied algae phenolic component (Liu, Hansen, and Lin 2011). Their structure was proved to be a ring formed by connecting phloroglucinol units, up to eight interconnected rings, which makes them have stronger oxidation resistance (Agregán et al. 2017). Rajauria et al. (2016) separated and identified eight phenolic compounds with antioxidant capacity from *Himanthalia elongate*. After purification, they showed a higher antioxidant capacity than ascorbic acid, which can be considered as a potential source of dietary antioxidants. The polyphenol extract from algae has also been confirmed to have the anti-tumoral and anti-diabetic activities. Nwosu et al. (2011) explored the anti-proliferation and anti-diabetic effects of polyphenol extracts of four edible algae (*Ascophyllum nodosum*, *Ulva lactuca*, *Palmaria palmate* and *Alaria esculenta*). The polyphenol extract from *Alaria esculenta* showed strong inhibitory

activity on human colon cancer cells (Caco-2) but the content of phenolic components in *Palmaria palmate* and *Ascophyllum nodosum* was higher, and after purification by Sephadex LH-20, it was found that the inhibitory activity of *Ascophyllum nodosum* was observed in the phlorotannins fraction. In addition, phenolic components isolated from edible algae also have the potential to inhibit the differentiation of adipocytes and mitigate obesity. In Jung et al. (2014) research, phlorotannins extracted from *Ecklonia stolonifera* were confirmed to have the ability to inhibit adipocyte differentiation and lipid accumulation. The polyphenol extract contained five types of phlorotannins, including phloroglucinol, eckol, dieckol, dioxinodehydroeckol, and phlorofucofuroeckol A, and all of them can inhibit the accumulation of lipids in 3T3-L1 cells without affecting cell viability. At the same time, they reduced the expression of adipocyte marker genes level. Moreover, algae polyphenols are also being studied in reducing cholesterol levels and mitigating cardiovascular diseases (Murray et al. 2021).

2.5 Pigments

The pigments in algae can be divided into three categories, namely carotenoids, chlorophylls and phycobiliproteins. Among them and according to the different formation methods, carotenoids can be divided into primary (such as lutein and lycopene) and secondary (like astaxanthin) (Osterrothová et al. 2019). They are present in different types of algae, such as brown algae that have more fucoxanthin, while red algae have higher carotene content (Aryee, Agyei, and Akanbi 2018). These components have been found to have applications for coloring, but as having also antioxidant properties, inducing tumor cell apoptosis, prolonging the shelf-life of food and other biological activities

(Ganesan et al. 2011). Chlorophylls are a fat-soluble pigment contained in all algae capable of photosynthesis. After being ingested, they easily lose the magnesium in the porphyrin ring and convert to pheophytin and other derivative forms. Chlorophylls exhibit a variety of bioactivities such as haematopoiesis and disease resistance (Aryee, Agyei, and Akanbi 2018). Phycobiliproteins are water-soluble natural pigments and depending on the absorption wavelength, mainly includes phycocyanin and phycoerythrin, as well as a small amount of allophycocyanin (Qiang et al. 2021). Nowadays, phycobiliproteins are mostly used as a coloring agent in many industries, being used also as antioxidant and anti-inflammatory agent, in immune regulation, among others. (Pan-utai and Iamtham 2019).

2.6 Vitamins

Vitamins are trace compounds necessary for the human body, since humans cannot synthesized them or the amount of synthesis is very limited, so they need to be obtained from the diet (Zhang et al. 2021). Algae is an important source of vitamins, including water-soluble and fat-soluble vitamins. The complex B vitamins (B_1, B_2, B_6, B_{12}, etc.) are the most important but vitamins such as vitamin A, vitamin C, pantothenic and folic are also needed, although in smaller amounts (Sona 2011). Most red and green algae have more vitamin A and vitamin B, such as c (*Undaria pinnatifida*), Nori (*Porphyra umbilicalis*) and so on.

The consumption of algae with high content in vitamin B_{12} can mitigate the symptoms of vitamin B_{12} deficiency faced by vegetarians for example (Macartain et al. 2007). Brown and green algae generally have higher content of vitamin C than red algae, such

as *Enteromorpha flexuosa* and *Ulva fasciata* (300 mg/100 g and 220 mg/100 g of DW, respectivily) (Sona 2011). Algae, such as *Ulva lactuca* and *Durvillaea antarctica* also contain high-quality fat-soluble vitamin E (tocopherol, exist as α, β, γ and δ forms), which has been proven to have antioxidant properties to protect PUFAs from oxidation (Ortiz et al. 2006). In addition, it also has been found to have a positive effect on mitigating diabetes, cardiovascular disease, controlling the occurrence of inflammation, among others (Espessailles et al. 2021; Mohammad et al. 2021).

2.7 Other compounds

In addition to the above-mentioned nutrients, there are other bioactive components in algae that can work conjointly, such as mineral and halogenated compounds. Algae have a higher mineral content than terrestrial plants, including calcium (Ca), sodium (Na), potassium (K), and magnesium (Mg) (Astorga-España et al. 2015). Moreover, algae also are an important source of iodine (I), as a dietary supplement, which has the potential to relieve goitre (Milinovic and Rodrigues 2021). Halogenated compounds also have been found in brown and red algae, in the form of metabolites in different stages, such as indole, terpenes, and volatile halogenated hydrocarbons. At the same time, these halogenated compounds have also been confirmed to have a variety of physiological functions, including antibacterial and antiproliferation activities (Vairappan 2003).

3. Product acceptability

In recent decades, the rapid increase in population has promoted increased pressure on the food chain. Meat, as a source of high-quality animal protein, lipids, and some minerals in the diet, has also increased year by year. According to Food and Agriculture

Organization of the United Nations (FAO)'s forecast, by around 2050, the demand for animal products may be twice as high as the current (Food and Agriculture Organization of the United Nations 2009). However, the excessive consumption of meat products also burdens the environment, which is not conducive to a sustainable development (Hedenus, Wirsenius, and Johansson 2014). In addition, meat products are a highly perishable food. Indeed, during meat storage, various chemical components will be affected by microorganisms and enzymes, causing the product to spoil, and makes the preservation of meat a pertinent topic for researchers (Shafiei and Mostaghim 2021). Based on this, the use of appropriate meat supplements/substitutes to reduce meat consumption and at a lower cost and with higher nutritional value is of upmost interest, as it could allow, at the same time, to reduce cardiovascular diseases and diabetes caused by excessive intake of meat products (Ferreira, Sharma, and Zannad 2021). Currently, some beans, grains and fungi have been gradually applied to the meat industry to replace and reduce the consumption of animal meat, and more new resources, including algae and insects, are also being investigated (Weinrich and Elshiewy 2019; Hartmann, Furtwaengler, and Siegrist 2022; Hashempour-Baltork et al. 2020).

Nowadays, meat products containing other ingredients represent a small proportion of the market and the main reasons can be *a)* compared with traditional meat products, the addition of other ingredients may have an impact on the flavour and taste, making the meat produces a poor fiber structure and lacking a juicy mouthfeel (Tucker 2014; Hoek et al. 2013); *b)* most consumers are reluctant to try unfamiliar foods when traditional foods can meet the demand, at the same time, there is not enough trust in the safety and

nutritional value of these new products (Krings, Dhont, and Hodson 2022); *c)* when faced with some new food resources, such as insects, consumers will be subject to psychological and cultural shocks, resulting in rejection before eating (Tan et al. 2015).

In this regard, some researchers have investigated the market acceptability of a variety of new meat products. Elzerman et al. (2021) investigated the adaptability of Dutch consumers to meat products and their substitutes. This study found that in any case, traditional meat products are more acceptable than newer alternatives. Meanwhile, gender and age are also having an impact on the adaptability of the different meat products. Considering the environmental impact of a large amount of meat consumption, Collier et al. (2021) investigated the Sweden consumers, where the demand for meat products is high, in order to explore consumers' acceptance of meat substitutes. Through a survey on the acceptance of meat substitutes, based on different genders, ages and education levels, it was found that most of the respondents were not familiar with the relationship between meat consumption and the environment, being not aware of the need to reduce meat production and consumption using meat substitutes. The doubts about the need and nutritional value are the main reasons that affect the acceptance, and the consumers pay more attention to the cooking procedure needed and function of meat substitutes than visual imitation. A closer analysis of the survey results showed that doubts regarding the nutritional value of meat substitutes and their health effects are the main reasons affecting consumer acceptability.

Researchers have also investigated the acceptability of some new proteins that can replace animal proteins. For example, fungi with high protein content and low carbon are

considered as meat substitutes. As a microbial-derived protein, it has the characteristics of rapid growth and easy cultivation, and toxicological experiments have also proved that the intake of fungal protein will not have an adverse effect on the body (Finnigan, Needham, and Abbott 2017). From the perspective of the sensory properties and consumer acceptance, the product obtained with fungi protein has better edible quality and higher acceptance compared with other meat products with vegetable protein (Hashempour-Baltork et al. 2020). In addition, consumers acceptance has also been investigated regarding the use of insects as a source of high-quality protein in the diet. It depends more on the cultural background and long-term cognition. As a new type of protein source, it has certain development limitations that needed still to be delt with (Tan et al. 2015; Caparros Megido et al. 2016).

In contrast, algae have better acceptance as a new food resource. A survey found that as far as Spain is concerned, 85 % of consumers believe that algae are a sustainable resource being also safe and healthy and this has become one of the important factors for its use as a food ingredient in food industrial production (Lafarga et al. 2021). Weinrich and Elshiewy (2019) investigated the willingness of consumers in three Western European countries (Germany, Netherlands and France) to use microalgae as a meat substitutes. In this study, 79 % consumers were found to have a positive preference for meat substitutes based on local microalgae, which is very helpful for food engineers and marketers to develop algae-based meat products.

The development of improved meat products and substitutes is able to establish a more sustainable food supply system (Hoek et al. 2013). Compared with traditional meat

products, the key to consumers' acceptance of new meat products lies in their diet habits, doubt about nutritional value and safety, the price of products, among others. As a source with high nutritional value, algae are applied to the meat industry, which can mitigate the environment pressure caused by excessive consumption of meat and obtain new products with high nutritional value (Hartmann, Furtwaengler, and Siegrist 2022; Laamanen et al. 2021). At the same time and compared with other plant-derived, fungal, or insect-derived ingredients, algae are used as a functional ingredient in the meat industry, which not only can obtain higher-quality products, but also is more easily accepted by consumers. It provides the basis and pre-requisite for the widespread launch of algae-containing meat products on the market (Weinrich and Elshiewy 2019; Lafarga et al. 2021).

4. Algae to improve quality and health characteristics of meat products

As abovementioned, algae contain a variety of bioactive components, which provides the possibility and premise for the application of algae in meat products. In addition to being added to the products as a functional supplement, the bioactive components in algae also can affect the properties of the products, such as obtaining low-salt and low-fat products, extending the shelf life, and increasing nutritional quality.

4.1 Lower-salt meat products

Meat and meat products are one of the most basic foods due to their nutritional value. However, excessive intake of meat products is also accompanied by a large amount of sodium ingestion. This is because the natural sodium content in meat is low, and thus, more sodium chloride (NaCl) is be added during the production of meat products, such as in cured ham and sausages, as a flavour intensifier and for preservation purposes

(Mariutti and Bragagnolo 2017a; Inguglia et al. 2017). However, excessive sodium intake will increase the risk of high blood pressure and coronary heart disease. The addition of NaCl plays a crucial role in meat industry, which can affect the quality and water holding capacity of the product, increase adhesion, and is also an important factor in delaying food spoilage (Inguglia et al. 2017). In fact, WHO recommends that the daily intake of NaCl for adults is 5 g (Gómez-Salazar et al. 2021). Based on these functions, reducing the NaCl content in meat products without affecting product quality and shelf life, the development of new low-salt meat products needs to be considered and explored. Currently, some NaCl substitutes are being explored for the possibility of being used in meat products, such as potassium salt (KCl), lactic acid, and so on, but some substitutes may also bring potential health problems, for example, glutamate may affect the body's metabolism (Pateiro et al. 2021; Barbieri et al. 2016).

As a substance rich in a variety of bioactive components of different chemical nature, algae are used in meat products, providing more possibilities for meat products. Algae are an important source of marine minerals, rich in trace elements and macro elements required by the human body such as Na, Ca, Fe, P, Zn, among others (Astorga-España et al. 2015). The existence of these minerals provides a prerequisite for algae to be used as a NaCl substitute for meat products. Prior, López-lópez et al. (2009) explored the effect of edible algae (Spaghetti, Wakame and Nori) on the low-salt meat emulsion model. In the meat emulsion model with algae, the amount of Na added was one-fourth of the control group. Compared with Spaghetti and Wakame, the presence of Nori algae, increased the content of some amino acids in the products, including serine, alanine and

glycine. Moreover, the addition of edible algae also improves the antioxidant capacity and polyphenol composition of the model, which contribute to the stability of the system.

Algae are also used to reduce the salt content in cooked ham (Barbieri et al. 2016). added the extract from *Palmaria palmata* to produce cooked ham and could reduce the NaCl content (1.0 %, 1.2 % NaCl *vs* 1.8 % control group). In this way, the amount of NaCl can be reduced by 1 %, and it has a positive effect on organoleptic properties. Triki et al. (2017) also compared a commercial algae extract (AlgySalt®) with mixed salt in fresh and cooked sausages as a substitute for NaCl. Starting with sensory, physicochemical properties and microbial properties, the results showed that sausages with AlgySalt® added compared with mixed salt, showed less cooking loss, but the hardness was increased compared to mixed salt products. In addition to ham and sausages, the used of algae in pork puddings to reduce the amount of salt while ensuring the taste has also been studied. In the study of Fellendorf et al. (2016a), 22 substitutes, including Wakame algae, were added to the pudding to explore the effects of fat and salt. The results showed that the addition of Wakame can reduce the cooking loss and salt content, in addition to the stronger salty taste of the product. Similarly, they also studied the effect of algae added to low-salt and low-fat white puddings on the acceptability of the product (Fellendorf, O'Sullivan, and Kerry 2016b). The results showed that algae were considered unsuitable for increasing the puddings flavor and improving the color, which may be related to the pigment contained in the algae, because when compared with the black puddings, the pigment is more obvious in the white puddings.

Although not all studies have shown positive results, adding algae and algae-derived components to meat products to obtain healthier low-salt products is one of the positive solutions to improv the quality of meat products to a certain extent.

4.2 Low-fat meat products

As one of the most important macronutrient for humans, lipids contain a variety of essential fatty acids, fat-soluble vitamins and many bioactive components required by human organism (Gropper and Smith 2017). In addition, lipids also can provide phosphoglycerides, which are important components of cell membranes. More than 95 % of the dietary lipids are triacylglycerols (TAG) and depending on the food source, the composition of TAG in fatty acids is different, which leads to differences in their physical, chemical properties and nutrition (Zhang et al. 2015; Ye et al. 2019).

Meat and meat products are an important source of lipids (Mehta et al. 2015). However, meat and meat products derived from animal contain high levels of saturated fatty acids (SFAs), including stearic acid, palmitic acid, among others. In addition, the presence of some compounds such as nitrite, which are considered to have a negative impact on health, and excessive meat intake will increased the probability of obesity, cardiovascular and other diseases (Johns et al. 2015; Santos et al. 2020).

The purpose of preparing new meat products based on healthy foods is to reduce the risk of diseases while ensuring nutritional balance. Compared with animal lipids, plant and marine-derived lipids have higher levels of UFAs, such as oleic acid, linolenic acid, linoleic acid and so on and these fatty acids are believed to have a positive effect on health and reduce the risk of various diseases including cardiovascular diseases (Barta, Coman,

and Vodnar 2021). Seeking some plant or marine-derived lipids to partially replace animal lipids and ensure nutritional and sensory characteristics has also become promising research.

Algae are an important source of marine lipids and the high content of EPA and DHA provides the possibility for it to be developed as a functional food (Castejón and Señoráns 2019). In addition, to being used in low-salt meat products, the role of algae lipids in meat or meat products also has been studied. In the production of pork patties, *Laminaria japonica* was added directly (1 % and 3 %) to samples and the normal fat addition group was set as control group (Choi et al. 2012). Through the evaluation of the physical and sensory properties of the patties it was verified that when compared with the control group, the addition of *Laminaria japonica* can improve the cooking loss, diameter, and thickness of the low-fat patties, and can overcome the influence of low-fat on texture and sensory characteristics, resulting in lower energy products. Additionally, as the high-quality source of dietary fiber, the addition of *Laminaria japonica* was also considered as the functional bioactive components in the pork patties. In order to compare with other high-quality lipids, some authors explored the effects of olive oil and omega-3 rich algae (namely, 5 % of *Himanthalia elongata*) on the cold storage, sensory and microbial changes in low-fat (10 %) Frankfurt sausages (López-lópez, Cofrades, and Jimenez-Colmenero 2009). Compared with olive oil, the addition of algae can improve the water-fat binding properties of the product as well as increase the hardness and chewiness of Frankfurt sausage, but the sensory changes brought by olive oil are more acceptable.

Moreover, carrageenan, which is unique in *Himanthalia elongata* algae has been often studied as a fat substitute. In fact, Cierach et al. (2009) studied the effect of red algae (*Rhodophyceae* sp.) carrageenan as a fat substitute on low-fat Frankfurt sausages. The results show that the addition of carrageenan can reduce the cooking loss of the sausages, increase the hardness and chewiness and result in a higher sensory score. Similarly, carrageenan was added to pork patties, but not envisaging fat substation, and long-term storage does not cause loss of the sensory quality, physical and chemical properties of the product (Kumar and Sharma 2004). Considering the above research, it is valuable and possible to add algae as a functional or fat substitute to meat products to obtain foods with a healthier lipid composition.

4.3 Prevent degradation of meat products and extend shelf-life

As the main components of meat, lipids and proteins have an important contribution to the nutrition and quality of meat products (Li-Beisson et al. 2019; Kadam, Tiwari, and O'Donnell 2015). It is precisely due to the presence of fatty acids and protein that meat is more susceptible to oxidation, which leads to rancidity, poor quality and shortened organoleptic/sensorial shelf-life (Mariutti and Bragagnolo 2017b). The oxidation of meat products begins after slaughter, in addition to the type of animal and the position of the muscles, it is also affected by processing conditions, such as temperature, light, packaging condition and so on. Meanwhile, the metmyoglobin produced by the oxidation of meat lipids will negatively affect the color of the meat, thereby reducing the acceptability of the product (Faustman et al. 2010; Mariutti and Bragagnolo 2017a). Therefore, finding suitable antioxidants to add to meat products in order to reduce oxidation is a feasible

method to ensure product quality and extend the shelf-life, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) which are often used in the food industry (Gao et al. 2021). Although these synthetic antioxidants have great effects, their safety is also considered by consumers, and thus, it is urgent for scientists to find components with antioxidant capacity from natural plants and microorganisms.

Being faced as "super food", algae contain a variety of bioactive compounds, including antioxidants, such as polysaccharides and phenols, in which the application of these components in meat products, may allow to slow down the aging of meat and extending shelf-life (Jannat-alipour and Rezaei 2019). Hlima and co-workers (Hlima et al. 2021) discussed the effect of sulphated exopolysaccharide (0.5, 1 and 2 %) from *Porphyridium cruentum* on the shelf-life and microbial community of minced beef meat. In addition to its own antioxidant and antibacterial activities, sulphated exopolysaccharides can significantly reduce the psychrophile bacteria in minced beef meat, thereby extending the shelf-life to 14 days. Additionally, the addition of polysaccharides can also effectively avoid the generation of protein hydroxyl groups and the reduction of lipids oxidation in the sample, which has a real protective effect.

Other authors have explored the influence of laminarin, L, and fucoidan, F, (L/F extract, 0.1 % and 0.5 %) from brown algae (*Laminaria digitata*) on the shelf-life and quality of minced pork patties (Moroney et al. 2013). Through the analysis of the physical and chemical properties of the sample, lipids oxidation, microorganisms and sensorial quality, it was found that heating can enhance the antioxidant capacity of the L/F extract in the pork patties and reduce the degree of lipid oxidation in the cooked pork patties with

L/F extract, as well as has no adverse effects on the color, sensory and consumer acceptance. In addition, to polysaccharides that have been shown to have positive effect on the shelf-life of meat products, other bioactive components have also been explored for their application in shelf-life extension. Sellimi et al. (2017) explored the properties of fucoxanthin from *Cystoseira barbata* and its effect on the storage stability of chicken sausages and found that fucoxanthin showed strong antioxidant capacity and inhibitory activity on angiotensin-converting enzyme I, showing so that fucoxanthin has anti-hypertensive potential. Adding fucoxanthin as an additive to low nitrite turkey sausages (0.01, 0.02 and 0.04 %) was found to effectively inhibit lipid peroxidation and ensuring color stability during 15 days of refrigerated storage. In other study, Pogorzelska et al. (2018) used astaxanthin from *Haematoccocus pluvialis* as an antioxidant. During the preparation of meat samples, different levels of astaxanthin (0.15, 0.3 and 0.45 g/kg) were added to the minced meat and its antioxidant capacity and stability were evaluated. When the added amount was 0.3 and 0.45 g/kg, the lipid oxidation of meat products was significantly delayed, and the color stability was also improved.

In conclusion, some bioactive components derived from algae are natural antibacterial and antioxidants agents and when they are added to meat products, they can not only increase the nutritional properties of the product, but also ensure the quality of the meat products by inhibiting the growth of microorganisms and slowing down lipid oxidation, thereby extending the shelf-life.

4.4 Other applications in meat products

Algae are a high-quality source of natural bioactive components and the application of algae and its extracts to meat products has a positive impact on the quality and taste of the products, resulting in low-salt and low-fat healthy foods, which can also exhibit antibacterial and antioxidant properties, thereby extending the shelf-life of the products. In addition, algae are also being explored for other wider applications in meat products, such as supplements for protein and minerals, substitutes for animal fat, and packaging material for meat products. Algae have a high protein content, rich amino acid composition, and a protein value similar to other plant-derived proteins, all of which are a prerequisite to be used as a protein supplement (Lupatini and Colla 2016).

Žugčić et al. (2018) explored the effects of various pulses (soy, pea, lentil and bean) and algae proteins on the quality of beef patties. By analyzing the color, physical and chemical properties, as well as amino acid composition of beef patties, it was observed that the addition of beans and algae protein increased the amino acids concentration of the beef patties, especially flavour-related amino acids, glutamic acid (Glu), lysine (Lys) and aspartic acid (Asp), with the taste of the modified beef patties not changing considerably. Marçal et al. (2021) explored the application of high-pressure processing and algae mixtures' addition in fortifying the nutrition of Frankfurt sausages. Analysis of the nutritional properties in sausages revealed that, compared with traditional sausages, the addition of algae increases the content of minerals (Mg, K, Ca, etc.) and reduces the Na/K ratio, confirming the positive effect of algae as a new ingredient in modulating the quality of meat products.

In addition to being an additive to obtain high-quality products, algae can also play its physical and chemical role in the meat producing process. When exploring the influence of olive oil and konjac gel instead of animal fat on pork meat batter gelation, Fernández-Martín and collaborators (2009) added Sea Spaghetti (*Himanthalia elongata*) to it, to simultaneously explore the role of algae in the whole process. Sea Spaghetti was added to the pork meat batter gelation obtained with olive oil and konjac gel, and the differential scanning calorimetry (DSC) and dynamic rheological thermal analysis (DRTA) were used to monitor the gelation process of the batter. It was observed that addition of algae enhances the water and oil retention capacity of all formula products, and it also effectively improved the products' hardness and elastic modulus. The reason for this result may be that the alginate in Sea Spaghetti prevents thermal denaturation of proteins, and as a result of this can form a stronger batter matrix and fatty phases. The functional properties of alginate have contributed to improving the properties of these low-fat meat products.

Moreover, the potential application of algae in the packaging of meat products is also being explored. For example, in the production of dry fermented sausages, alginate coating was used as a substitute for traditional collagen casings. Traditional dry fermented sausage casing are mostly natural casings made of animal intestines and artificial casings made of collagen or plastics. However, they have some disadvantages, such as natural casings are easily contaminated by microorganisms, while artificial casings are limited in length. The alginate contained in algae easily interacts with uronic acid residues to form a three-dimensional network structure, which provides a basis for its use in packaging

materials. Marcos et al. (2020) explored the advantages of co-extruded alginate as dry fermented sausage casings and found that the alginate coating has drying kinetics similar to traditional collagen casings and has no significant effect on the senses. Therefore, the alginate film is considered to be a very good substitute for casings.

Briefly, algae are an important source of various nutrients and antioxidant and antibacterial components. The addition of algae and its extracts to meat and meat-based products can aid the development healthier food. Meanwhile, it also can reduce oxidation and microbial contamination during the processing and thus extending the shelf-life. It is a supplement or additive with development potential.

5. Conclusions, perspectives and challenges

Algae are rich in high-quality proteins, lipids, vitamins and minerals, being an important source of antibacterial agents and food fortifiers. Due to their properties, their application in the food industry can give food new health characteristic, such as anti-oxidant, anti-hypertensive, anti-tumour, anti-thrombotic, and anti-coagulant properties. Meanwhile, these active components can be applied to meat products as natural bacteriostatic agents and antioxidants to inhibit microorganisms and delay oxidation, ensuring the quality of the products. Moreover, based on the possible impact of over-production of meat products on the environment and human health, this review summarizes the research progress in the application of algae and their bioactive components to the meat industry, including the use of healthy and nutritious food under the premise of ensuring the taste, as well as the application of delaying spoilage and extending the shelf-life of products. These algae-containing meat products are being studied and have been proven to have a positive

impact on the product. However, due to traditional diet habits and taste constraints, the application of algae in meat products is still facing huge challenges. Further clarification on the nutritional value of these new meat products is of upmost importance to improve their taste, optimize products' formulation, etc., so that consumers have a more comprehensive and in-depth understanding of them, which will help the large-scale promotion of new products.

From the perspective of sustainable resource development, the application of algae in meat and other food industries can promote the production of products with high nutritional value. But potential negative effects also should be considered. When algae is used in large quantities as a food supplement, the possible species invasion and impact on ecosystem due to its excessive growth rate need to be considered. At the same time, the digestion and absorption of nutrients from algae in the human body also need to be explored in order to understand the bioaccessibility. More important, there are many species of algae, *in vivo* and *in vitro* experiment also need to be carried out to obtain information on the toxicity and allergy of different species of algae.

It is foreseeable that the application of algae and their bioactive components in meat products will have a considerable impact positive in the development of the meat industry, and new products are expected to be developed that can be accepted easily by consumers.

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5. GENERAL DISCUSSION

5. Summary and General Discussion

5.1 Tailored made processes for the extraction of targeted high-added-value compounds from fish side streams using pulsed electric field (PEF) and pressurized liquid extraction (PLE)

5.1.1 Pulsed Electric Fields and Pressurized Liquid Extraction for Valorization of Rainbow Trout (Oncorhynchus mykiss) and Sole (Dover sole) By-Products: Protein Content, Molecular Weight Distribution and Antioxidant Potential of the Extracts

As innovative extraction methods, the effect of PEF and PLE on protein recovery from rainbow trout and sole side streams (head, skin, and viscera) was investigated. Under PLE the protein extraction rate of rainbow trout viscera was the highest, close to ~80 %, followed by skin (~59 %) and head (~38 %), while both showed significant differences compared to control group (without PLE). For the sole side streams, the protein extraction rate of the viscera was also significantly improved, but the protein extraction rate of skin and head was not significantly ($p>0.05$) affected. For rainbow trout and sole, their skin showed different textures after freeze-drying, and the sole skin was harder, which made it difficult to mix with diatomaceous earth, the extraction rate of protein was also affected. After PEF-assisted treatment, both rainbow trout and sole skin showed higher protein extraction rates, approaching 80%, followed by viscera and head. For PEF-assisted treatment, fresh skin samples that have not been freeze-dried were used. After PEF treatment and stirring for 24 h, the tissue structure of the skin could be better destroyed, which was beneficial for protein extraction, and showed a more positive effect compared to PLE. However, it is worth noting that PEF can improve the protein extraction rate of sole skin and head, while there was not a significant effect on rainbow trout side streams.

Fish has been extensively studied as one of the main sources of marine protein. Ahmed et al. summarized the application of different extraction methods in the extraction of collagen from fish side streams (Ahmed et al., 2020), including enzymatic hydrolysis, ultrasound-assisted chemical hydrolysis and others that have been found to be effective methods for collagen recovery. Veeruraj et al. (2013) obtained 80% and 7.1% collagen from ocean eels' skin through acid extraction and pepsin extraction, respectively. Among them, the acid extraction method was more efficient, which was close to the effect of PEF-assisted treatment in this study. However, the acid extraction method takes three days, which also increases the production cost. Álvarez et al. (2018) used ultrasound-assisted acid/base extraction to improve the extraction rate of protein from mackerel side streams, and the results showed that the extraction rates of both ultrasound-assisted acid and base extraction were close to 100 and 95 %, respectively, thus showing the positive effects of ultrasound-assisted extraction process on the recovery of proteins.

Two innovative extraction techniques are involved in our study (PEF and PLE). PEF is an electricity-based treatment method that generates high-voltage short-duration electrical pulses that can keep the thermal effects at a low level and disrupt the cellular structure of the food matrix, but it still protects the sensory and nutritional properties of food. It has been shown that the application of short pulsed electric fields from 100~300 kV/cm to 20~80 kV/cm can disintegrate the cell membrane and promote the formation of membrane pores. Zhou, He, and Zhou (2017) explored the effect of PEF on the extraction of mussel protein, and the optimal processing conditions were obtained as electric field strength 20 kV/cm, pulse number 8, enzymatic hydrolysis for 2 h, while the maximum protein extraction rate could reach 77%. On the other hand, PLE reduces the strong

interaction between the solute and the sample matrix at high pressures, and reduces the viscosity of the solvent, which promotes the diffusion of the solvent into the sample matrix.

Molecular weight distributions of proteins in fish side streams extracts were obtained by SDS-PAGE. In the PLE rainbow trout side streams, the protein molecular weight distributions of head, skin and viscera extracts were 150~10 kDa (control group: 75~10 kDa), 100~10 kDa (control group: 100~75 kDa) and 50~5 kDa (control group: 25~5 kDa). It can be seen that PLE enriched the protein species in rainbow trout side stream extracts. However, PEF-assisted treatment had no significant effect.

Meanwhile, the sole side stream extracts obtained by PLE also showed similar effects compared to rainbow trout. For the sole side streams treated by PEF, the protein with a molecular weight of 150~100 kDa, 75 kDa and 50 kDa were increased in the head extract, while the protein with a molecular weight of 50~37 kDa, 25 kDa, 20~15 kDa and 15 kDa decreased. PEF-assisted treatment promoted the extraction of large molecular weight protein and inhibited the dissolution of small molecular proteins, observing different effects on the protein content and the properties of the extract according to the treatment applied.

Under PLE-assisted extraction, pressure is an important factor affecting extraction yields and extract properties. For instance, in a previous study, Gómez-Guillén et al. (2005) applied 250~400 MPa high pressure treatment to fish skin, which not only increased the production of skin collagen, but also changed the distribution of protein molecular weight. Pressurization can also promote changes in the molecular structure of protein in food matrix. Some studies have shown that pressure may lead to the disruption

of non-covalent interactions and alterations in intermolecular/intramolecular bindings as well as solvent-protein interactions, which alter the conformation of proteins (Jia et al., 2021). At a certain temperature, pressurization can increase the permeability of the solvent, making it easier to enter the food matrix and prolong the contact time, which has important implications for protein recovery (Jia et al., 2021).

For PEF, the protein molecules are polarized under the treatment of low-strength electric fields, as the strength increases, more hydrophobic amino acids are exposed to the solvent. When the electric field strength exceeds a certain limit, the special effect caused by the arc leads to the denaturation and aggregation of thermosensitive proteins. In addition, other studies have confirmed that PEF can cause damage to the secondary structure of proteins (Zhao & Yang, 2009). In this study, PEF influenced the distribution of protein molecular weight. It is speculated that the reason may be that the PEF led to the rupture of cells and accelerate the dissolution of protein. Then, the exposure of hydrophobic amino acids of proteins also leads to protein aggregation, which may cause changes in protein molecular weight.

In addition to evaluating the effects of PEF and PLE on protein extraction and molecular weight distribution, the antioxidant capacity of the extract was also evaluated by two assays (TEAC and ORAC), as an indicator of the bioactivity of the extracts. For rainbow trout, the stronger oxygen radical absorption capacity (ORAC) was observed for PLE and PEF skin extracts, followed by viscera and head. The ORAC values of rainbow trout skin and viscera were significantly improved after applying PLE, while PEF did not have a significant effect. The ORAC values of head and viscera sole side stream extracts were enhanced after PLE and PEF, having PEF a more pronounced effect on the values.

Moreover, the $ABTS^+$ scavenging capacity of the extracts was also evaluated, being PLE able to enhance the $ABTS^+$ scavenging capacity of rainbow trout and sole head extracts but obtaining the opposite effect on viscera extracts. Moreover, PEF also had a significant effect on enhancing the $ABTS^+$ scavenging capacity of the sole head and skin extracts.

5.1.2 Role of fish side stream extracts on modulation bacterial and anti-inflammatory activities

Although some previous studies have shown that fish-derived extracts/compounds have great antibacterial and anti-inflammatory potential, the biological properties of the obtained extracts after applying innovative technologies such as PEF or PLE have been rarely reported.

In this study, the effects of the extracts on the growth of four pathogen were evaluated, being their growth rates and optical densities obtained by the Gompertz equation (Zwietering et al., 1990). PEF/PLE-assisted treatment of rainbow trout side streams induced the growth of *Listeria* and *Escherichia coli* (*E. coli*), observing the most obvious effects on viscera extracts. Although the growth of *E. coli* was significantly affected, the effect on optical density was not clear. In addition, PEF head and skin extracts had no significant effect on the growth of *Staphylococcus aureus* (*S. aureus*), but the addition of viscera extracts reduced the growth rate of *S. aureus*. For rainbow trout side streams, PLE showed a different effect compared to PEF. For instance, the PLE head and skin extracts inhibited the growth of *S. aureus* but did not have any effect on the growth of *Salmonella*.

Among the sole side streams, the addition of viscera extracts promoted the growth of *Listeria*, while the head and skin extracts had no significant effect on *Listeria*, as well as PEF and PLE did not show a significant effect. In addition, the sole viscera extract in the

PEF group had an inhibitory effect on the growth of *S. aureus*, while the effect of PEF was more significant. Meanwhile, PLE head extract also inhibited the growth rate of *S. aureus*.

The effect of the extracts on the growth of two probiotics was also investigated. When PEF was explored as an assisted treatment, rainbow trout head extract showed a growth-promoting effect on *Lacticaseibacillus casei*, but there was no significant difference between PEF and control samples. In addition, the extracts had no effect on the growth of *Bifidobacterium lactis*. Meanwhile, the ASE-assisted rainbow trout extracts did not show a significant impact on the growth of *Lacticaseibacillus casei* compared to the control group.

Similarly, sole side streams were also studied. The results showed that the sole head extracts of the PEF extracts promoted the growth of *Lacticaseibacillus casei*, but there were no significant differences between the PEF and control groups. For PLE treatment, sole viscera and head extracts had no effect on the growth of *Lacticaseibacillus casei*, while the viscera extract on the control group inhibited the growth of *Lacticaseibacillus casei*. Moreover, PLE sole head and skin extracts also increased the optical density of *Bifidobacterium lactis* compared to the control group.

In recent years, researchers have explored the effects of bioactive compounds of fish side streams on the growth of pathogenic bacteria and probiotics, such as *Yersinia ruckeri* from tilapia by-product hydrolysates and Atlantic mackerel skin hydrolysates (Robert et al., 2015, Ennaas et al., 2015). In this line, they found that the by-products of Atlantic mackerel skin obtained by hydrolysis showed inhibitory effects on bacteria, and *Thunnus albacares* head hydrolysate can promote the growth of lactic acid bacteria, including

Lactobacillus acidophilus, *Lactobacillus delbrukii* and *Lacticaseibacillus casei* (Safari et al., 2012).

In the present doctoral dissertation, the effect of fish side stream extracts obtained with innovative extraction technologies on bacterial growth was also evaluated. The use of PEF/PLE to obtain the extracts not only improved the extraction rate of bioactive compounds from fish side streams, but also ensures its antibacterial properties, and had a positive effect on probiotic bacteria.

The anti-inflammatory potential of fish side streams extracts was also investigated. In the rainbow trout side stream extracts, the PEF group did not show any anti-inflammatory potential, while the extracts obtained by PLE had inhibitory effects on NF-κB, especially the viscera extracts, which could inhibit ≈40–45% of TNF-α-induced NF-κB activity. On the other hand, for sole side stream extracts, the skin extract of PEF-assisted group exhibited a significant anti-inflammatory potential, while inhibited the TNF-α activity by ≈35%. Interestingly, in the PEF group of rainbow trout extracts, the extracts from the skin and viscera (without PEF and PLE treatments) enhanced the TNF-induced NF-κB activity to levels of 150 % and 126 %, suggesting that the PEF treatment could alter some components of these extracts and reduce their intrinsic pro-inflammatory potential.

5.1.3 Effects of in vitro digestion on antioxidant capacity of fish side stream extracts and evaluation of mineral bioaccessibility

An *in vitro* static digestion model was established to explore the effect of digestion on the antioxidant capacity and mineral bioaccessibility of the extracts. Firstly, the potential contamination of fish side streams, including heavy metal (As, Cd, Hg and Pb) levels and mycotoxin were determined. In all fish side streams, the content of Cd, Hg and Pb were

below the maximum allowable limit, and no mycotoxin contamination was detected in the extracts.

In vitro digestion had different effects on the antioxidant capacity of the extracts. For rainbow trout and sole side streams extracts, the PEF group did not show a significant effect on the antioxidant capacity of the extracts. While PLE increased the antioxidant capacity of rainbow trout and sole head extracts after *in vitro* digestion compared to the control group.

It was found that *in vitro* digestion was able to affect the antioxidant capacity of the extracts. During *in vitro* digestion, protein digestion starts from stomach, and is degraded into small molecular peptides under the action of digestive enzymes, then further degraded by small intestinal epithelial cells to form amino acids and enter the body fluid circulation. The bioactive peptides in fish protein have antioxidant capacity, then the digestion and innovative technologies may change the sequence of peptides contained in the extracts, thereby affecting the antioxidant capacity.

The effects of PEF and PLE on the recovery of minerals from fish side streams were studied. The fish side streams selected in this study had relative high contents in Mg, Ca and P. Exploring the effect of PEF on mineral recovery it was found that this technique can promote the recovery of minerals in some fish side streams extracts, such as Mg and P in rainbow trout head, Fe and Ca in sole head. The effect of PLE on the recovery of minerals from fish side streams was also evaluated. Especially, PLE increased Fe and Zn contents in rainbow trout head extract, as well as Fe and Se contents in viscera extracts. Moreover, PLE had a positive effect on Zn recovery from sole head.

Based on *in vitro* digestion model, the bioaccessibility of minerals contained in fish side streams was evaluated. PEF and PLE improved the bioaccessibility of minerals in some fish side streams extracts, but not in all samples. Considering the mineral content and bioaccessibility at the same time enables a more comprehensive assessment the value of the fish side streams.

The mineral content and its bioaccessibility in fish side streams are affected by various factors such as fish species, growing environment, processing methods, etc. Moreover, interactions with other components also can have an impact on the bioaccessibility of minerals. However, little research has been done on the effects of innovative extraction technologies on the recovery of minerals from fish side streams. Therefore, it is crucial to understand the changes in the mechanism of action of the different processing techniques on minerals and other constituents as well as their impact on digestion and absorption.

5.1.4 Effects of fish side sterams extracts on gut microbiota

To more comprehensively evaluate the healthy function of the fish side stream extracts, the effect of the extracts on the gut microbiota was also explored by establishing an *in vitro* colonic fermentation model. According to the results, with the elapse of fermentation time, the number of bacteria in most of the samples increased, including total bacterial, *Enterobacteriaceae*, Bacteroides group and *Streptococcus*. Moreover, this study also measured short-chain fatty acids (SCFAs), the main metabolites of the gut microbiota, and found that the fermentation process was accompanied by the production of acetate acid and propionate acid. The elapse of fermentation time contributed to the accumulation of SCFAs. Specifically, the addition of sole viscera extract increased the

number of *Bifidobacterium* after overnight incubation. For *Enterobacteriaceae*, the number of *Enterobacteriaceae* in the sole viscera extract group was lower than in the control group at 24 h and 48 h. Likewise, the addition of rainbow trout and sole viscera extracts also reduced the number of *Streptococcus* in the control group. Among the fish side stream extracts, the sole viscera extract had a more positive effect, probably because the small molecule protein in the viscera extract is higher than that of other extracts, but more mechanisms are still needed to be explored.

For SCFAs, the acetic acid content in the sole viscera, sole head and rainbow trout viscera extracts-containing group was higher than that in the control group at 24 h, and the acetic acid content in the sole and rainbow trout viscera extracts-containing group remained higher than that in the control group at 48 h. In addition, propionic acid was lower than acetic acid. After 48 h, propionic acid was significantly increased in the group containing the rainbow trout viscera extract, while the other groups were lower than the control group (without the extract). Sole viscera and rainbow trout viscera extracts showed positive effects in promoting acetic acid and propionic acid content, respectively.

5.2 Recovery of high-added-value bioactive compounds from microalgae using innovative extraction technologies

5.2.1 Pulsed electric field assisted biomolecules and minerals recovery from Chlorella

The effect of PEF on the recovery of bioactive compounds and minerals from *Chlorella* was investigated. Firstly, the effects of PEF, solvent (100 % H_2O and 50 % H_2O+50 % DMSO) and extraction time on the extraction of bioactive compounds from *Chlorella* were explored. Compared to control group (without PEF), PEF increased the extraction of biomolecules from *Chlorella*, including protein, polyphenols, chlorophyll *a*,

chlorophyll *b* and carotenoids. Among them, 50% DMSO was beneficial to recover fat-soluble pigments, while the protein content was lower than 100% H_2O. It may be because the hydrophilic sulfinyl group and hydrophobic methyl group in DMSO can dissolve water-soluble and fat-soluble compounds, thus promoting the extraction of pigments (Mueller et al., 2019). Meanwhile the addition of DMSO resulted in protein precipitation, which affected the extraction rate. Parniakov et al. (2015) used PEF to assist the extraction of nutrients from the *Nannochloropsis spp*. and obtained a similar conclusion to that found in this study. In addition, the proportions of specific biomolecules in the extracts at different times were analyzed and it was found that the proportions between different biomolecules also changed according to the extraction time.

The antioxidant capacity of the extracts was evaluated. For ORAC, the values were higher according to following sequence: PEF-assisted-50% DMSO>control-50% DMSO>PEF-assisted-H_2O>control-H_2O, while the order of TEAC was PEF-assisted-H_2O>PEF-assisted- 50% DMSO>control-H_2O/control-50% DMSO. Principal component analysis (PCA) results showed that the contents of carotenoids and chlorophyll *a*/*b* had a strong correlation with ORAC, while TEAC was related to proteins and polyphenols, which could be used as an explanation for the different antioxidant capacity of the samples.

The content of minerals in *Chlorella* and its extracts, including Mg, P, Ca, Fe and Zn after the application of PEF and PLE was also determined. No significant differences were found on Mg content after the use of PEF, while the content of P/Zn increased, and the concentration of Ca/Fe decreased. The contents of Ca and Fe in the extracts obtained by PEF treatment were lower than those found in the control group. PEF has been

demonstrated to disrupt microalgae cells and increase the extraction rate of biomolecules. Among these minerals, Ca and Fe can be involved in cellular structure by chelating with proteins, then PEF can disrupt protein modifications, thereby changing the functional properties of proteins and causing Ca/Fe-containing protein sedimentation.

The effect of PEF on the cell structure of *Chlorella* was observed by fluorescence microscope (FM) and scanning electron microscopy (SEM). PEF resulted in some “cracks” appeared in the circular aggregates of multiple *Chlorella*. At higher magnifications, the rupture of *Chlorella* cells can be found, indicating that an “electroporation” occurs during PEF, increasing the extraction rate of biomolecules.

5.2.2 Effects of pressurized liquid extraction (PLE) with DMSO on the recovery of dietary valuable compounds from the microalgae

PLE was combined with different concentrations of DMSO to recover bioactive compounds from microalgae. Firstly, it was found that PLE and DMSO concentrations could affect the recovery of bioactive compounds in *Chlorella*, including protein, polyphenols, chlorophyll a, chlorophyll b and carotenoids. Among them, PLE+H_2O was helpful for protein recovery, while DMSO had a more significant effect on polyphenols, chlorophyll *a*, chlorophyll *b* and carotenoids.

The antioxidant capacities of the extracts and their association with bioactive compounds were also evaluated. The ORAC results showed that PLE increased the antioxidant capacity of the extracts compared to the control group, being the antioxidant capacity related to the concentration of DMSO. When the DMSO concentration was higher than 50%, the increase of the antioxidant capacity was related to the increase of polyphenol, chlorophyll and carotenoid content. From TEAC results, it can be depicted

that when the DMSO concentration exceeded 50%, the PLE and control groups showed similar antioxidant capacity, while the DMSO concentration was below 50%, the PLE extracts had higher antioxidant capacity. The reason for the analysis may be because ORAC and TEAC react with two different antioxidant mechanisms, and the principle of the former follows those antioxidants and substrate compete for thermally-generated peroxyl radicals through the decomposition of azo compounds, while the TEAC measures the ability of antioxidants to reduce oxidants (Zulueta et al., 2009).

The principal component analysis (PAC) showed that when 100% DMSO was used as the extraction solvent, the content of polyphenols, chlorophyll and carotenoids in the extract was higher, and it had a strong antioxidant capacity. Therefore, the potential of PLE+100% DMSO as a solvent to recover the high-added-value biomolecules from *Spirulina* and *Phaeodactylum tricornutum* was explored. It can be found that among the three microalgae selected in this study, the protein, polyphenols, carotenoids, and antioxidant capacity of *Spirulina* and *Phaeodactylum tricornutum* were higher than those of *Chlorella*, while *Chlorella* had higher chlorophyll content. This fact can be attributed to the different species and cell morphology. In addition, the distribution of carotenoids and minerals in the extracts were also different, having *Spirulina* a higher zeaxanthin content, and being an important source of Zn and P, while the lutein found in *Chlorella* was higher.

Thus, the PLE+DMSO combination as a solvent can be considered as a valuable method to recover high-added-value compounds from microalgae, which can significantly shorten the processing time and improving the extraction yield.

Microalgae contain a large amount of high-added-value biomolecules. Previous studies have shown that PEF and PLE are both efficient methods to recover biomolecules from microalgae, and the choice of extraction solvent can also have an impact on the content of biomolecules. On this basis, microalgae have the potential to be developed into high-added-value products, so that the biomass can be utilized very well. However, it is worth noting that there are many species of microalgae and have different cell morphologies, being the exploration of more efficient methods for target species necessary in further studies.

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6. CONCLUSIONS

6 Conclusions

From the results obtained in the present PhD thesis it can be concluded that:

1) The innovative extraction technologies (pulsed electric fields, PEF and pressurized liquid extraction, PLE) can be used to extract nutrients and bioactive compounds from fish side streams, as they can improve the recovery of proteins and antioxidant capacity of the extracts.
2) Protein molecular weight distribution in fish side stream extracts can be affected by PEF and PLE treatments.
3) The influence of fish side stream extracts on the growth of specific microorganisms (potentially pathogenic, altering and beneficial) and inflammatory activity depends on the marine source (sole, rainbow trout), the origin (viscera, skin, etc.), and the methodology used (PLE and PEF).
4) *In vitro* digestion can affect the antioxidant capacity of fish side stream extracts.
5) The recovery of minerals of fish side stream extracts was affected by PEF and PLE treatments and was related to both the specific fish species and minerals evaluated. Both PEF and PLE treatments were able to improve the bioaccessibility of some minerals, but not in all cases showed a positive effect.
6) Sole viscera extracts have been investigated to regulate the gut microbiota, as they can increase the content of *Bifidobacterium* and *Lactobacillus*, as well as promoting the accumulation of SCFAs.
7) PEF can improve the recovery rate of antioxidant bioactive compounds from *Chlorella*, which is mainly related to the "electroporation" induced by this technology.

8) PLE+DMSO-assisted extraction is also an effective method to recover high-added value compounds (i.e., carotenoids and other pigments) from microalgae.

These conclusions demonstrate that PEF and PLE can be useful tools to recover high-added-value compounds from fish side streams and microalgae as they not only improved the yield of target compounds, but also can preserve well the bioactivities of the extracts. On this basis, it is necessary to explore the mechanism of the functional activity of the target components and more nutritional properties. Meanwhile, it is also feasible to evaluate the application of more innovative approaches such as supercritical fluid extraction, microwave-assisted extraction, etc., to recover high-added-value compounds from these matrices in a green and sustainable way. Moreover, it is of a paramount importance to study the application of high-added-value compounds recovered from marine sources in food, pharmaceutical, cosmetic, and other industries, not only to achieve sustainable utilization of resources, but also to reduce waste of resources and environmental pollution.