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D3.7

Title: Report on identification of possible biotechnological products from the processed mesopelagic catch in terms of production possibilities and value

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Executive Summary

Biotechnology approaches including hydrolysis and precision fermentation can transform and recover speciality ingredients and compounds from fish proteins, oils and carbohydrates.
Report D3.7 focuses on the bioactivity of hydrolysates and concentrates (termed biotechnological products) generated in MEESO to date and discusses potential applications of these products in the functional foods, companion animal and small molecule/pharmaceutical sector as well as their potential applications as biostimulants in agriculture or use in biotechnology for development of environmental products such as surfactants.

The therapeutic effects of several drugs and functional foods are often a direct consequence of enzyme inhibition in mammalian bodies. The majority of catalysing biological process is a result of enzymes, which are a large category of bio-molecules.

The report provides examples of functional food products and small molecules with similar target markets that are presently commercially available but which were developed from other fish resources (sardines, Blue whiting and white fish) as well as alternative protein sources (dairy and meat). These products have similar enzyme inhibition activities or mechanisms of action and are commercially available in countries including Canada, Japan and South-East Asia as supplements or functional foods.

Specific areas the reports examines are assays performed within Meeso related to heart health, type 2 diabetes and metabolic syndrome. Additionally, assays concerning anxiolytic, anti-emetic, anti-nocicpetive and anti-inflammatory benefits.

Sections on assessment of generated hydrolysates with different methods, market applications and value, relevant regulations, and potential to upscale are also included.

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1. Introduction

Biotechnology approaches including hydrolysis and precision fermentation can transform and recover speciality ingredients and compounds from fish proteins, oils and carbohydrates. Products that may result include techno-functional ingredients like emulsifiers, flavours and palatants that find use in the human food, companion animal and functional foods markets (Tugiyono et al., 2020). Fish protein may also find use in the production of surfactants found in everyday products like laundry detergents and used in environmental oil spill clean-up operations. For example, fish oil and protein are proving to be a viable option to grow microbes (xenobiotic strains) for industrial-scale biosurfactant production and furthermore fish may be a potential resource of pharmaceuticals, enzymes, amino acids, hormones and antifreeze proteins (Yulu et al., 2022)

Report D3.8 focuses on development of possible products from processing, contaminants and bioactivity testing in MEESO, reporting on market possibilities and environmental impact. Report D3.7 focuses further on the bioactivity of hydrolysates and concentrates (termed biotechnological products) generated in MEESO to date and discusses potential applications of these products in the functional foods, companion animal and small molecule/pharmaceutical sector as well as their potential applications as biostimulants in agriculture or use in biotechnology for development of environmental products such as surfactants. Bioactivities identified for mesopelagic fisheries products to date in MEESO relate largely to their potential use as functional food/nutraceutical ingredients based on the ability of these hydrolysates and permeates to inhibit enzymes key to maintenance of health in humans and companion animals. It describes assays carried out to identify hydrolysates and permeates with potential for development as functional foods/nutraceuticals and characterisation of these products. Enzymes discussed include those relevant to maintenance of heart health and cholesterol including Angiotensin Converting Enzyme I (ACE; EC 3.4.15.1); Renin (EC 3.4.23.15) and acetylcholinesterase (EC 3.1.1.7). It also examines enzyme targets for hydrolysates developed from mesopelagic products that can prevent development of type 2 diabetes including Amylase (EC 3.2.1.1) and Dipeptidyl peptidase IV (EC 3.4.14.5). It looks at enzymes that are targets for prevention of inflammation including Cyclooxygenases (EC 1.14) and Monoacylglycerol lipase (MAGL) (EC 3.1. 1.23), and enzymes linked with maintenance of mental health such as prolyl endopeptidase enzymes (EC 3.4.21.26). An issue often raised concerning development of bioactive ingredients as foods/pharmaceuticals is the enormous potential of bioactives to treat and prevent disease but the prohibitive costs and stringent legislation necessary to get bioactive products to market which, can be prohibitive or minimally increase the duration of time required before products are available in relevant markets (Días et al., 2020).

D3.7 provides examples of functional food products and small molecules with similar target markets that are presently commercially available but which were developed from other fish resources (sardines, Blue whiting and white fish) as well as alternative protein sources (dairy and meat). These products have similar enzyme inhibition activities or mechanisms of action and are commercially available in countries including Canada, Japan and South-East Asia as supplements or functional foods. D3.7 looks at the timelines for delivery of functional foods and bioactives for food or pharmaceutical use to market along with the steps required to obtain EFSA/EMA or FDA approval for functional food products/small molecule pharmaceuticals.

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Table 1: List of abbreviations

Abbreviation	Definition
2-AG	2-arachidonocylglycerol
7-HCA	7-Hydroxycoumarinyl-arachidonate
AA	Arachidonic acid
ACE	Angiotensin converting enzyme
AMP	Antimicrobial peptide
AMR	Antimicrobial resistance
Ang1	Angiotensin 1
BMI	body mass index
COX	Cyclooxygenase
CPNP	Cosmetic Products Notification Portal
DPP IV	Dipeptidyl peptidase IV
EFSA	European food safety authority
EMA	European medicines agency
FDA	Food and drug administration
FOSHU	Food for specified health use
FPH	fish protein hydrolysate
GLP-1	Glucagon like peptide 1
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
MAGL	Monoacylglycerol lipase
MDR	Multidrug resistant
MHLW	the janapnese ministry of health, labour and welfare
NCD	Non-communicable disease
NDA	Scientific panel on dietetic products, Nutrition and Allergies
NLEA	Nutritional Labelling and Education Act
NSAID	NonSteroidal Antiinflammatory drug
OD	Optical density
PGHS	prostagalndin H Synthase
PIF	product information file
T2D	Type 2 diabetes
WHO	World health organization

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2. Enzyme inhibition

The therapeutic effects of several drugs and functional foods are often a direct consequence of enzyme inhibition in mammalian bodies. The majority of catalysing biological process is a result of enzymes, which are a large category of bio-molecules. Enzymes serve a wide range of functions inside living organisms. They are crucial for metabolic processes, cellular signalling and regulation, cell division and apoptosis, and so on. In addition, enzymatic reactions play important roles in chemical modifications and post-translational modifications of molecules or proteins. These modifications include phosphorylation, myristoylation, glycosylation, acylation and glycolytic degradation as well as reductive processes. The World Health Organization (WHO) reports that greater than 36 million deaths result each year from non-communicable diseases (NCDs),(Global NCD Compact 2020-2030 (who.int)). NCDs include cardiovascular diseases, diabetes, cancers and chronic respiratory diseases. Bioactive protein hydrolysates and peptides derived from fish or any food protein have a broad scope of functions related to human health if consumed in sufficient quantities. Hydrolysates containing bioactive peptides have less potent effects than pharmaceutical drugs, but are less likely to accumulate in body tissues or to confer serious side effects as they can be metabolised following use in the body and subsequently excreted. Within MEESO, the potential health benefits of several hydrolysates and permeates were explored with a view to developing products from this resource with the ability to impact positively on human or animal health. The target enzymes explored to date in MEESO include enzymes that play a role in maintenance of heart health including ACE-1 and renin inhibitors, enzymes like Dipeptidyl peptidase IV (DPP-IV) that has an impact on development of type 2 diabetes (T2D) and others outlined below. The rational for selection of these enzyme targets relates to the significant health and healthcare cost impacts of the relevant disease to society and healthcare systems and subsequently potential markets for alternatives to drugs that exist already that help to treat these diseases ordinarily in society.

2.1. Angiotensin-1-converting enzyme (ACE-1; EC3.4.15.1) inhibition

High blood pressure is the major independent risk factor for cardiovascular diseases. ACE-1 regulates blood pressure by converting angiotensin I (a vasodilator) to angiotensin II (a vasoconstrictor). Inhibition of ACE-1 activity is a major target in the prevention of hypertension (Razo et al., 2022). Over 10 different synthetic drugs to prevent hypertension by ACE-1 inhibition exist today but these synthetic drugs have several side effects and a number of novel compounds such as bioactive peptides are of interest as natural, functional foods for use in normotensive, blood pressure maintenance. These ACE-1 inhibitors can be developed as nutraceuticals and pharmaceuticals with potential to prevent hypertension. Several marine-derived ACE-1 inhibitor-containing hydrolysates already exist and some have been trialled to date in animal and human clinical trials (Table 1). However, none has European Food Safety Authority (EFSA) approved human health claims but some have novel food claims (https://www.efsa.europa.eu/el/efsajournal/pub/5267) Indeed, EFSA rejected health claims proposed for the C12-peptide (FFVAPFPDVFGK) derived from bonito hydrolysate as well as the milk tri-peptides IPP and VPP citing inadequate human studies and/or ‘major methodological limitations’ in the reported studies, and a lack of convincing evidence for the mechanism responsible for the claimed effect at the proposed dose. Despite this ACE-1,

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inhibitory hydrolysates are made and sold globally. Examples of marine derived ACE-1 inhibitory hydrolysates shown to maintain blood pressure in humans are in Table 1.

Commercially available ACE-1 inhibitory proteins/peptide products

BIOZATE® WHEY PROTEIN ISOLATE

Commercially available products from other non-fish protein sources include the BioZate ® whey protein isolate produced by Davisco Foods (Minnesota, USA). Davisco's proprietary process generates bioactive peptides with unique nutritional and functional characteristics. In experiments with hypertensive rats performed at the Hypertension Research Unit, Laval University, Quebec, Canada, BioZate 1 was compared with another whey protein isolate and a control. The work showed that a single optimal dose of BioZate 1 significantly reduced mean arterial blood pressure, within one to seven hours of administration. A trial with human participants was also completed (www.daviscofoods.com/HWP/clinical.php3) Results indicate that the whey protein isolate reduces blood pressure in borderline hypertensive patients (Gauthier S. F., Pouliot, Y. 2003). In addition to its effect on blood pressure, BioZate 1 is a high quality source of protein that includes essential amino acids that can be formulated into food products. Examples of formulations include dark chocolate and mocha protein beverage mixes. In this application, it is suggested that 30g of the mix be dissolved in 8-oz. of cold milk or water to deliver a 20g dose of BioZate 1. The powdered protein dissolves readily in aqueous systems. It has a slightly bitter taste that is masked by chocolate, mocha or other flavours.

DMV C12 PEPTIDE

Dutch ingredient group DMV International also make a milk peptide for blood pressure lowering called C12 peptide. DMV International is part of Dutch co-operative Campina. It works directly with the body's natural blood pressure mechanism and claims to provide safe and natural support for healthy blood pressure (Cadee et al., 2007). A human study carried out by the group found that a dose of 3.4 grams per day reduced both diastolic and systolic blood pressure.

CALPIS® AND AMEALPEPTIDE®

AmealPeptide® is known also as Lactotripeptides. AmealPeptide® was discovered over the course of 40 years of continuous research into the physiological functions of fermented milk, produced during the process of manufacturing the lactic acid drink CALPIS®. Calpis® is a carbonated soft drink produced in Japan and consumed there for over 100 years. Key features include its blood pressure-lowering effects and ability to improve cardiovascular functions. AmealPeptide® has an inhibitory activity against ACE-1. It works on blood vessels by inhibiting enzymes that cause elevated blood pressure, thereby minimizing increases in blood pressure, and by stimulating production of nitric oxide (NO), which helps to protect blood vessels. Lactotripeptides (AmealPeptide®) is certified as a "food for specified health use" (FOSHU) in Japan and has been granted GRAS approval in the US.

AmealPeptide® is an Informed-Choice, Informed-Sport certified raw material, each batch undergoes banned substance testing, which reassures elite athletes, and drug tested consumers that the AmealPeptide ingredient does not lead to failed anti-doping tests (Takano et al., 2002)

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Marine derived commercially available ACE-1 inhibitory hydrolysates

KATSUOBUSHI OLIGOPEPTIDE

This is a hydrolysate of dried bonito fish and contains a penta-peptide (peptide containing five amino acids). It has demonstrated antihypertensive benefits in human clinical trials and sells widely as a nutraceutical/functional food outside of Europe. Several natural ACE-1 inhibitor products have been commercialized as dietary supplements, which promote healthy blood pressure levels, including Bonito peptide, VasotensinR, and PeptAce™ fish peptides derived from fish bonito (*Sarda orientalis*). The main active bonito peptide called LKPNM (penta-peptide), based on its amino acid sequence, is converted by ACE-1 to LKP, the active peptide shown to significantly support healthy blood pressure level. Following 5 weeks of administration of the bonito hydrolysate, the systolic blood pressure (SBP) of subjects was reduced by 11.7 ± 1.3 mmHg. This clearly demonstrates it effectively reduces the SBP of hypertensive and borderline hypertensive subjects *in vivo (*Abachi et al., 2022).

2.2. Dipeptidyl peptidase IV (DPP-IV; EC 3.4.14.5) inhibitors

Diabetes is accepted as a global epidemic, with 415 million people suffering type 2 diabetes worldwide. Europe has 60 million diabetics, that account for 13% of the EU healthcare budget, or €290bn. (Horizon magazine - Diabetes – the preventable epidemic | Research and Innovation (europa.eu). Dipeptidyl peptidase IV (DPP-IV) inhibitors are a group of anti-hyperglycaemic medications used to manage type 2 diabetes (T2D) mellitus, which is a significant risk factor for coronary disease, heart failure, stroke, and many other cardiovascular conditions. The insulinotropic hormone, glucagon-like peptide 1 (GLP-1), is metabolized extremely rapidly by the ubiquitous enzyme, dipeptidyl peptidase IV (DPP-IV), resulting in the formation of a metabolite, which may act as an antagonist at the GLP-1 receptor. Inhibitors of DPP-IV may completely protect exogenous GLP-1 from DPP-IV-mediated degradation, thereby greatly enhancing its insulinotropic effect. Also known as, gliptins, DPP-IV inhibitors are oral drugs approved by the Food and Drug Administration (FDA) to treat T2D in adults (Makrakalikis et al., 2019). These drugs act through incretin hormones, which are gut hormones responsible for glucose homeostasis after oral food intake. Peptides derived from food protein sources have demonstrated DPP-IV inhibitory activities previously. In order to exert their action on the enzyme present on the surface of the intestinal epithelium, to prevent the degradation of the incretin hormones, and consequently improve glycaemic regulation, pro-peptides or their degradation products, must reach the endothelium of the capillary bed within the intestinal wall where DPP-IV is present in near proximity to the cells secreting the incretins. In relation to functional foods for DPP-IV inhibition, EFSA recently stated, “A reduction, in post-prandial glucose response may be considered a beneficial physiological effect as long as insulin responses are not disproportionally increased” (Sharkey et al.,2020) Several fish derived protein hydrolysates have demonstrated DPP-IV inhibition but many also increase insulin production.

PEPTIFORCE

Nuritas (Dublin, Ireland) has developed PeptiForce – this peptide-containing product regulates blood sugar, which is key in the battle with T2D. Nuritas is expected to generate significant

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global sales by 2024. The source of the peptide is secret but is likely to be of dairy or pea protein origin.

2.3. Antimicrobial hydrolysates and peptides

Worldwide, the re-emergence of infectious diseases and increases in multidrug-resistant (MDR) bacteria, resistant to commercially available antibiotics is a serious problem. The search for novel molecules with antibacterial activity that could overcome the resistance phenomenon is a priority. Antimicrobial peptides (AMP) and AMP-containing hydrolysates are an attractive option for the development of alternatives to conventional antibiotics. AMPs have been isolated from a variety of marine resources. It is estimated that antimicrobial resistance (AMR) may cost between $300 billion to more than $1 trillion annually by 2050 worldwide. High costs associated with expensive and intensive treatments and escalation in resource utilisation are the direct monetary effects of AMR on health care. The global antimicrobial peptides market size was valued at USD 228.61 million in 2021 and is expected to expand at a CAGR of 15.38% during the forecast period, reaching USD 539.32 million by 2027. The target markets for AMPs include animal feed, pharmaceutical, cosmetics and agriculture.

Previously identified fish-derived antimicrobial peptides

Fish live in an environment where saprophytic and pathogenic microbes flourish putting them in constant direct contact with potential pathogens. Fish skin acts a physical barrier by providing immediate protection from the environment and as a chemical barrier through several innate immune factors such as AMPs. AMPS are low MW peptides that have a net positive (cationic) charge and are amphiphilic. They are involved in the natural defense mechanism against pathogens (innate immunity); however, their main role is modulation of mammalian cell functions. AMPS include defensin, parasin, cathelicidin and hepcidin, and piscidin. These AMP families are species-specific, with piscidin being unique to teleost fish (Masso-Silva et al., 2014). The antimicrobial peptide with sequence SJGAP has been identified from the skin of Skipjack tuna (*Katsuwonus pelamis*) previously (Seo et al., 2014). YFGAP identified from Yellowfin tuna (*Thunnus albacares*); GKLNLFLSRLEILKLFVGA identified from Yellow catfish (*Pelteobagrus fulvidraco*) and GWGSFFKKAAHVGKHVGKAALTHYL from Winter Flounder (*Pleuronectes americanus*) previously (Cole et al., 1997). All identified peptides had antimicrobial activities against pathogens like *Escherichia coli*, *Staphylococcus aureus* and *Micrococcus luteus*.

2.4. Monoacylglycerol lipase (MAGL; EC 3.1.1.23) inhibition

Mood and anxiety disorders are chronic, disabling conditions that impose enormous cost on both individuals and society. Monoacylglycerol lipase (MAGL) plays a crucial role catalysing the hydrolysis of monoglycerides. MAGL inhibitors are important agents in many therapeutic fields, including anti-nociceptive, anti-inflammatory and anti-cancer (Deng et al., 2020). Nociceptive pain is one of the two main types of physical pain (the other being neuropathic pain). Nociceptive pain is potentially harmful stimuli detection by nociceptors around the body. Nociceptive pain covers most leg, arm, and back pain. It is categorised as either radicular or somatic. Recent studies have demonstrated anxiolytic potential of pharmacological

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endocannabinoid (eCB) augmentation approaches in a variety of preclinical models (Armstrong et al., 2022). Pharmacological inhibition of endocannabinoid-degrading enzymes, such as fatty acid amide hydrolase (FAAH) and mono-acylglycerol lipase (MAGL), elicit promising anxiolytic effects in rodent models with limited adverse behavioural effects.

GABOLYSAT PC60 - GABOLYSAT® PC60

Gabolysat ® PC60 is a Mackerel fish protein hydrolysate with anxiolytic properties commonly used as a nutritional supplement. It has demonstrated diazepam-like effects on stress responsiveness in a rat pituitary-adrenal system study. This anti-stress/pain effect was demonstrated in human studies also (Dinel et al., 2021 Freret et al., 2022;)

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Table 1: Commercially availalbe fish protein hydrolysate products and companies that produce them.

Company	**FPH /oil/associated products**	**Product applications**						**Species**
		Feed	**Pet Ingredients**	**Nutri-cosmetics**	**Functional foods/nutraceuticals/ supplements**	**Other**	**Pharma**	Whole Blue whiting fish
Bio-marine Ingredients Ireland (BII Ltd.), Monaghan, Ireland.	***FPH***	***YES***	**YES**	**YES**	**YES**	**YES**	**YES***	Whole Blue whiting fish
	ProAtlantic				Fish Protein Isolate - muscular health; satiety; glycaemic control; weight management			Whole Blue whiting fish
	ProShore		Fish protein isolate 55-90% protein pet food applications		ProShore - digestive health in cats			Whole Blue whiting fish
	Ishca	Fish protein hydrolysate for hatchery and fish farming -						Whole Blue whiting fish

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		boosts growth						
	ProGlas					Soil health		Whole Blue whiting fish
	Mineral complex (WhiteCal)				Bone health; Dental health			Whole Blue whiting fish
	Lipids							Whole Blue whiting fish
	OmegaBlue				Omega-3 fish oil powder that guarantees 80 mg EPA/g; 55 g DHA/g			Whole Blue whiting fish
Copalis Sea Solutions, France	***Fish protein hydrolysates***	***YES***	**YES**	**YES**	**YES**	**YES**	**NO**	salmon & whitefish
	CPSP®	Optimum palatability and contribution to animal well-being	Optimum palatability and contribution to animal well-being					salmon & whitefish

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	PHOSCAL™	Fish meal - ideal protein source rich in bioavailable calcium				Fertiliser		salmon & whitefish
	FISHMEAL	Protein source mono-gastric animals and use in aquaculture						salmon & whitefish
	FISHOIL	Use in ruminants, mono-gastric and aquaculture	Use in pet feeds as a palatant					salmon & whitefish
	Seanov(R)			Brand of marine ingredients used in functional foods, nutraceuticals, nutri-cosmetics	Brand of marine ingredients used in functional foods, nutraceuticals, nutri-cosmetics			salmon & whitefish
	Collactive®			Skin anti-aging				salmon & whitefish
	Collactive® HM™			Skin anti-aging				salmon & whitefish

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	Marine cartilage powder				Bone health; Dental health			salmon & whitefish
	Nutripeptin TM				Weight control			salmon & whitefish
	Phoscalim®				Bone health; Dental health			salmon & whitefish
	Prolastin®			Anti-age cosmetic (hydrolysed elastin)	Joint well being			salmon & whitefish
	Protensin TM			Inhibits ACE enzyme - heart health				salmon & whitefish
	PROTEIN M+TM			soluble marine cartilage - rich in chondroitin sulphate	Bone health; Dental health			salmon & whitefish
	Protizen TM				Anti-stress peptide - well-being; relaxation - mood food			salmon & whitefish
	Collagen HM SOL			Pale liquid - moisturises; stimulates cell regeneration				salmon & whitefish

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	Glycosann ® sol			Patented chondroitin sulphate with low molecular weight; stimulates the proliferation of fibroblasts and the biosynthesis of collagen and elastin				salmon & whitefish
	Aromatic extracts							salmon & whitefish
	Prolys		100% soluble aromatic fish extract - high protein content					salmon & whitefish
	Profish		Soluble aromatic fish extract					salmon & whitefish
BASF SE, Brattvåg (Now Marine Ingredients – sold 2014),	Highly concentrated Omega-3 fish oils. Products include Omacor®				Heart health		Omacor ® is approved for the treatment of	Salmon

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Norway & Ludwigshafen, Germany. BASF also acquired Pronova in 2013.	(introduced in 1994 as the world's first prescription omega-3 drug). Pharma products based on Omega-3 from fish oil developed include K85EE Omega-3-acid Ethyl Esters; Omega-3-Acid Ethyl Ester Capsules; Maxomega EPA 96/97 EE; CN 600 TG Omega-3-acid triglycerides; Maxomega DHA 95 EE AS						hypertriglyceridemia in approximately 70 countries worldwide	

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	PronovaPure® 360:240 EE (ethyl ester)				Heart health			Salmon
	PronovaPure®360:240 TG (triglyceride)				Heart, Pregnancy, cognitive, eye health			Salmon
	PronovaPure® 150:500 EE				Heart			Salmon
	PronovaPure® 46:38 EE				Heart health			Salmon
Croda Healthcare, UK	Incromega™ DHA – Active Pharma Ingredients (API)				Vision maintenance		Pharma ingredient for vision maintenance	Norwegian sourced fish (likely Salmon)
	Incromega™ E1070				Eye and brain health		Pharma grade ingredient	Norwegian sourced fish (likely Salmon)
	Incromega™ E550220				Eye and brain health		Pharma grade ingredient	Norwegian sourced fish (likely Salmon)
	Incromega TM TG3322				General well being		Pharma grade	Norwegian sourced fish (likely Salmon)

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							ingredient	
Janatha Fish meal and oil products, Kota, India	**Fish protein hydrolysates**	**YES**	**YES**	**YES**		**YES**	**NO**	Varies
	Fish amino acid liquid	Aquaculture applications				Boosts yield of vegetables, fruits and crops		Varies
	Fish amino acid powder					Bio-stimulant		Varies
	Bone meal					Organic fertiliser		Varies
	Fish meal	Aquaculture applications						Varies
	Fish oil	Animal health - improved immunity against disease, reduced incidences of deformities, higher survival and growth						Varies

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	Fish protein hydrolysate liquid (40 % hydrolysed protein)	Animal health - improved immunity against disease, reduced incidences of deformities, higher survival and growth				feed attractant		Varies
	Fish protein hydrolysate powder (40 % hydrolysed protein)	Animal health - improved immunity against disease, reduced incidences of deformities, higher survival and growth				feed attractant		Varies
	Sulphited fish oil					Used in leather tanning industry		Varies

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	Fat liquors					Used in the tanning process		Varies
	Refined fish oil				Used for cardiovascular health		To make soft gel capsules, hard gelatin capsules,	Varies
Scanbio Marine Group AS, Trondheim, Norway	**Fish protein hydrolysate**	**YES**	**YES**	**YES**	**YES**	**YES**	**NO**	Salmon, Pelagic, White fish
	ScanPro® (Salmon, White, Pelagic)	Fish meal replacer - hypoallergenic properties - autolysis process						Salmon, Pelagic, White fish
	ScanOil®	Animal nutrition						Salmon, Pelagic, White fish
	ScanHydro®	Animal nutrition						Salmon, Pelagic, White fish
	Aquaculture fish silage					Bioenergy		Salmon, Pelagic, White fish
Sopropêche,	**Fish protein hydrolysate**							Salmon, Pelagic, White fish

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Wimille, France	CPSP® 90/CPSP® G	Animal nutrition	Pet Feeds/ingredient					Salmon, Tuna, Trout, Herring
	Fishmeal	Animal nutrition	Pet Feeds/ingredient					Salmon, Tuna, Trout, Herring
	Fish oils	Animal nutrition	Pet Feeds/ingredient					Salmon, Tuna, Trout, Herring
Biomega, Bergen, Norway	***Fish protein hydrolysates***							
	Biomega® peptides				Nutraceutical applications- weight control; satiety			Salmon
	Biomega® salmon oil				Nutraceutical applications- heart, BMI control			Salmon
	Salmigo® salmon oil		nutritional source of protein					Salmon
	Salmigo® active		nutritional source of protein					Salmon
Royal DSM (The Netherlands)	MEG-3 fish oils				Provides a source of EPA and DHA			Omega-3 rich fish species
	EPA Epax Ultra						Pharma grade	Herring; Mackerel;

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	concentrates and other customised oils						Omega-3 products – guarantee 700 mg/g EPA/DHA (EPAX Ultra concentrates)	
	Enviro fish oil; Fish	Aqua and animal diets						
Epax AS/Pelagia AS, Bergen, Norway	Group 1 Fishmeal	Good amino acid profile – produced from whole meal pelagic protein (fresh & chilled) – animal/aqua diets						Pelagic fish
	Group 2 Fishmeal	High quality protein source – wild caught fish						Wild caught fish

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	Group 3 Fishmeal	High quality protein produced with low drying temperature – aqua feeds and animal diets						
	Enviro Group 1 Fishmeal	Organic (according to DeBio and Naturland regulations in Norway) – aqua feed and animal diets						Organic fish
	Enviro Group 2 Fishmeal	Animal diets/aqua diets						Organic fish

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3. Overview Of Assays Performed In Meeso

3.1. Assays Concerning Heart Health, Type 2 Diabetes And Metabolic Syndrome

Angiotensin-I-converting enzyme (ACE-I, EC 3.4.15.1), renin (EC 3.4.23.15), and dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5) play key roles in the control of hypertension and the development of type-2 diabetes and other diseases associated with metabolic syndrome.

3.1.1 ACE-1 inhibition assay

Angiotensin I-converting enzyme (ACE-1), plays a key role in blood pressure (BP) regulation, as it catalyses the cleavage of the C-terminal His-Leu dipeptide of angiotensin-1, a vasodilator peptide, into angiotensin II, a potent octapeptide vasopressor. ACE-1 also extends its catalytic impact towards the inactivation of bradykinin and kallidin, both considered vasodilator peptides. Water and lipid extracts as well as hydrolysates, permeates, concentrates and isolates generated from mesopelagic species by MEESO partners (Nofima, AZTI and Teagasc) as well as chemically synthesised peptides were tested in vitro for ACE-I inhibition. The ability of test inhibitors and controls to inhibit *in vitro* ACE-1 activity was determined using a spectrophotometric method with FAPGG as the substrate. The method uses the ability of active ACE-1 enzyme to hydrolyse the synthetic substrate (FAPGG), which results in the decrease in OD (optical density) at 450 nm in the presence of an inhibitor. In the presence of Captopril, an ACE-1 specific inhibitor, the ACE-1 enzymatic activity is greatly reduced and there is a decrease in the OD at 450 nm and a colour change from yellow to clear. The bioassay was carried out as follows: 20 µl of each sample inhibitor at a concentration of 1 mg/mL ddH_2O was added to 20-µl substrate and 20-µl enzyme working solution in triplicate. Samples were incubated at 37 °C for 1 h. Each well then had 200 µl indicator working solution added, followed by a further incubation at room temperature for 10 min. Absorbance at 450 nm was read using a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Offenburg, Germany). All fractions were assayed at a concentration of 1 mg/mL HPLC grade water in triplicate, and means and SD were calculated. The known ACE-I inhibitor Captopril© was used as a positive control at a concentration of 1 mg/mL. The percentage inhibition was calculated using the following equation:

% ACE-I inhibition = 100% Initial activity − Inhibitor × 100/100% Initial activity

ACE-I IC_{50} values were determined for active hydrolysates and peptides by plotting the percentage of inhibition as a function of the concentration of test compound.

3.1.2 Renin inhibition assay

Renin catalyses the rate determining step in the renin-angiotensin-aldosterone system that regulates mammalian blood pressure by converting angiotensinogen to angiotensin I (Ang I). Excessive plasma levels of Ang I is a causative factor in hypertension development. Therefore, inhibition of renin activity can lower blood pressure and provide relief from clinical symptoms associated with hypertension. Synthetic compounds are currently the most used group of renin inhibitors; however, only aliskiren is approved as a drug for hypertension treatment. Some negative side effects are associated with aliskiren therapy, which have necessitated the search for alternative natural compounds such as food protein-derived renin-inhibitory peptides with

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blood pressure-reducing effects. *In vitro* assay of human recombinant renin activity was conducted using the Renin Inhibitor Screening Assay Kit according to the manufacturers. Briefly, sample was diluted in DMSO and pre-warmed to 37°C prior to initiating the reaction. Before the reaction, 1) 20 µL substrate, 160 µL assay buffer, and 10 µL water (DDW) were added to the background wells; 2) 20 µL substrate, 150 µL assay buffer, and 10 µL DDW were added to the control wells; and 3) 20 µL substrate, 150 µL assay buffer, and 10 µL sample were added to the inhibitor (sample) wells. The reaction was initiated by adding 10 µL renin to the control and sample wells. The microplate was shaken for 10 s for proper mixing and incubated at 37°C for 15 min; fluorescence intensity (FI) was then recorded at excitation and emission wavelengths of 340 and 490 nm, respectively, using a Fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA, USA). The percentage renin inhibition was calculated as follows:

Renin inhibition (%) = [1 - $\Delta FIUmin^{-1}_{(sample)}/\Delta FIUmin^{-1}_{(blank)}$] × 100

Where $\Delta FIU\ min^{-1}_{(sample)}$ and $\Delta FIU\ min^{-1}_{(blank)}$ are renin activity in the presence and absence of inhibitory peptides, respectively. IC_{50} was calculated by non-linear regression from a plot of percentage renin inhibition versus peptide concentrations (0.125, 0.25, 0.5, and 1.0 mg/mL). The renin inhibition kinetics was conducted using 0.625, 1.25, 2.5, 5, and 10 µmol/L substrate concentrations in the absence and presence of samples. The known renin inhibitor Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe was used as a positive control and renin IC_{50} values were determined in triplicate for tests and hydrolysates by plotting the percentage of renin inhibition as a function of the concentration of test compound.

3.1.3 Dipeptidyl peptidase IV inhibition assay

A number of therapies are available for the treatment of diabetes including sulphonyl ureas, biguanides, α-glucosidase inhibitors, thiazolidine diones, non-sulphonylureas secretogogues (Rapaglinide and Nateglinide) and direct insulin therapy. However, these therapies are known to be associated with various side effects including obesity and insulin resistance. Incretin based therapies are emerging as drug candidates for T2D treatment and consist of glucagon like peptide-1 (GLP-1) agonist treatments and dipeptidyl peptidase (CD26; DPP-IV) inhibitors. Dipeptidyl peptidase (DPP-IV) inhibitors suppress the degradation of many peptides, including GLP-1, thereby extending their bioactivity. Samples from mesopelagic species, as described above were tested in vitro for DPP-IV inhibition. The assay uses a fluorogenic substrate, Gly-Pro-aminomethylcoumarin (AMC) to measure DPP-IV activity in the presence and absence of an inhibitor. Cleavage of the peptide bond by DPP releases the free AMC group resulting in fluorescence that can be measured at an excitation wavelength of between 350-360 nm and an emission wavelength of 450-465 nm. Initially, 10 µl of each sample inhibitor/test inhibitor at a concentration of 1 mg/mL assay buffer was added to 30 µl diluted assay buffer, 10 µl diluted DPP-IV, and 50-µl substrate solution, in triplicate. Samples were incubated at 37 °C for 30 min. Fluorescence was read with excitation wavelengths of 355 nm and emission wavelengths of 460 nm using a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Offenburg, Germany). All hydrolysates were assayed in triplicate, and means and SD were calculated. The known DPP-IV inhibitor Sitagliptin was used as a positive control. The percentage inhibition for each test sample and control was calculated using the following equation:

% DPP-IV inhibition = 100% Initial activity − Inhibitor × 100/100% Initial activity

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DPP-IV IC_{50} values were determined for active hydrolysates by plotting the percentage of inhibition as a function of the concentration of test compound.

3.2. Anxiolytic, Anti-Emetic, Anti-Nociceptive and Anti-Inflammatory assays

3.2.1 MAGL inhibition assay

Aspirin suppresses pro-inflammatory eicosanoid production and is an anti-pain agent. It is a COX inhibitor but has untoward effects that discourages chronic usage, including gastrointestinal toxicity. Marijuana has anti-inflammatory action through stimulating cannabinoid receptors but can results in cognitive deficit if used chronically. The enzyme serine hydrolase Monoacylglycerol lipase (MAGL) links the endocannabinoid and eicosanoid systems together through hydrolysis of the endocannabinoid 2-arachidonoylglycerol (2-AG) to provide the major arachidonic acid (AA) precursor pools for pro-inflammatory eicosanoid synthesis in specific tissues. MAGL inhibitors elicit anti-nociceptive, anxiolytic, and anti-emetic responses. They also have anti-inflammatory action in the brain and protect against neurodegeneration through lowering eicosanoid production. MEESO generated samples/extracts were screened for their ability to inhibit MAGL using a fluorescence-based assay of Monoacylglycerol lipase (MAGL) activity that is simple, sensitive, and amenable to the screening of small molecule inhibitors. Purified recombinant human MAGL protein and 7-hydroxycoumarinyl-arachidonate (7-HCA), a fluorogenic substrate for MAGL, were employed in the assay. MAGL protein catalyses the hydrolysis of 7-HCA to generate arachidonic acid and the highly fluorescent 7-hydroxyl coumarin (7-HC). Release of 7-HC is measured using a fluorometer.

3.2.2 Cyclooxygenase inhibition assay

Cyclooxygenases (COX), or Prostaglandin H Synthases (PGHS), are the target enzymes for nonsteroidal anti-inflammatory drugs (NSAIDS). Selective inhibitors of COX-1 and COX-2 could provide potential benefit over existing nonselective NSAIDS. COX-1 and COX-2 inhibitors from natural sources have found application in veterinary products previously.

3.3. Assays concerning food safety and gut health

3.3.1 Antimicrobial screening assay

The antibacterial activity of synthesised peptides was determined using an overnight culture of pathogenic bacteria in a modified agar well diffusion assay. A zone of inhibition around wells containing the peptides indicates anti-bacterial activity. *Escherichia coli* DSM 301 and *Bacillus cereus* DSM 31 were used as indicator pathogen strains. Ampicillin (Merck, Dublin, Ireland) and Cecropin P1 (Merck, Dublin Ireland) were used as positive control antibiotics and antimicrobial peptides. Positive controls or test peptides were incubated (concentration 1 mg/mL) in a well (30 µl capacity) on the agar plate which was seeded with the pathogenic strain at an overnight culture to media ratio of 100 µl: 500 mL (v:v). Plates were left at 4 °C for 4 h and subsequently incubated at the optimum culture temperature and conditions for the pathogen in question. After 24 h, zones of inhibition indicated the presence of antibacterial activity.

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4. Assessment hydrolysates and extracts for enzyme inhibitory bioactivities

4.1. Biomass used to generate hydrolysates and extracts – Norwegian hauls (2019-2020)

A number of hydrolysate samples were generated at AZTI, Teagasc and Nofima during the course of MEESO. Various protein extracts from *Maurolicus muelleri* and *Meganyctiphanes norvegica* (Northern Krill) and combinations of proteins from these species were generated using hydrolysis methods. Protein Hydrolysates were generated using four different enzymes including Alcalase, endocut-01, endogenous enzymes and FoodPro PNL at NOFIMA. Hydrolysates were characterized and assessed for their ability to inhibit enzymes important in diseases associated with metabolic syndrome. The ability of generated Hydrolysates to inhibit enzymes including Angiotensin-1-converting enzyme (ACE-1; EC. 3.4.15.1) associated with blood pressure regulation, Acetylcholinesterase (AChE; EC 3.1.1.7) associated with maintenance of the nervous system, and Dipeptidyl peptidase IV (DPP-IV; EC 3.4.14.5) linked with development of type-2-diabetes, was determined. In a separate process, the same mesopelagic fish species were transformed into fishmeal, hydrolysates, fish-silage, and aqueous extracts and screened for bioactivities using the same bioassays. The Hydrolysates contained greater than 60% protein (dry weight basis) when analysed using the DUMAS method.

4.1.1 Silage, Fishmeal, hydrolysate and aqueous extract generation

Silage Preparation

In the preparation of the silage biomass, 2.5 g of Grindox 1032 (tocopherol blend) and 25 g of 99% formic acid were added to 1,000 g of ground raw material. The mixture was stirred at 22°C for 47 h and then heated to 90°C in a microwave oven and kept at 90°C for 10 min. After centrifugation at 20,000 × *g* for 30 min, the liquid phase was decanted into a separator funnel, and the oil and water phases were separated.

Fishmeal Process

To 1,000 g of ground raw material were added 2.5 g of Grindox 1032 and 500 g of water. The mixture was heated with stirring to 85°C over 56 min, kept at 85°C for 10 min, and then pressed in a tincture press. The liquid phase was then centrifuged and poured into a separator funnel where the oil phase and the water phase were separated. The sediment after centrifugation was mixed with the press cake and homogenized.

Hydrolysis

Mesopelagic protein hydrolysates generated using proteolytic enzymes and aqueous extracts were generated using processes developed at Nofima in Tromsø and Bergen, Norway. Briefly, enzymatic hydrolysis was performed using the three commercial enzymes Alkalse 2.4L (Al2.4), Endocut 01-L (01-L), Foodpro PNL (PNL) at optimum temperatures ranging from 50 to 55°C. Additionally, endogenous enzymes (i.e., no additional enzymes at reaction start) were used at 30°C. All enzyme-reactions had identical setups: 1:1 w:v mix with water (~500 g of each), 1 h hydrolysis time and 15 min deactivation at 90°C. After deactivation, the hydrolysates were

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separated by centrifugation (30 min, 7,000 g) into an oil-phase, hydrolysate, and sediment fraction. Weight measurements were performed on all the separate fractions. In a separate method, biomass was processed for fishmeal, hydrolysates and silage. Aqueous extracts were separated from the solid and lipid fraction of each preparation, freeze-dried and supplied to Teagasc, Ireland for bioactivity assessments *in vitro*.
Mesopelagic Hydrolysates were abbreviated as Krill (K), *M. muelleri* (M) and a combination of Krill and *M. muelleri* (C) and 1–4 based on the enzyme used for hydrolysis i.e., Alcalase, endocut-01, endogenous M/K enzymes, and FoodPro PNL. Further AQ 1–8 were generated. The first three aqueous samples (AQ1–AQ3) were aqueous phase from fishmeal process derived from H4, H6, and H7 trawls, AQ4–AQ6 was aqueous phase from enzymatic process derived from H4, H6, and H7 trawls and AQ7–AQ9 was aqueous phase from Silage process obtained from H4, H6, and H7 trawls.

4.2. Proximate composition of hydrolysates and aqueous extracts

The total protein content of the mesopelagic hydrolysates and AQ was estimated using two different methods. Initially protein was determined using a LECO FP628 Protein analyser (LECO Corp., St. Joseph, MI, United States) based on the Dumas method and according to the Association of Official Analytical Chemists (AOAC) method 955.04 (Association of Official Analytical (AOAC), 2000) (Simone et al.,1997). A nitrogen conversion factor of 6.25 was used to calculate protein content in the samples. The Bicinchoninic Acid (BCA) method was also used in accordance with the manufacturers' instructions (Sigma-Aldrich, Dublin, Ireland) and according to the method of Walker (1996). The lipid content of samples was quantified using AOAC Method 2008.06 with an Oracle rapid NMR fat analyser. Samples were prepared according to standard procedures used for these analyses as described by Naik and colleagues (Naik et al., 2020)

5. Bioactivities determined for 1st haul samples (generated by Nofima)

A hydrolysate from *M. muelleri* generated using FoodPro PNL (M1) inhibited the ACE-1 enzyme by 89.56% when assayed at a concentration of 1 mg/ml compared to the positive control Captopril©. Aqueous extract two (AQ2) inhibited ACE-1 by 95.28% when assayed at 1 mg/ml compared to the control. Sample M1 inhibited DPP-IV by 100% and aqueous extract 1 (AQ1) inhibited the same enzyme by 90.08% when assayed at a concentration of 1 mg/ml compared to Sitagliptin used as the positive control. All samples assayed did not significantly inhibit the enzyme AChE–fraction C3 (combined hydrolysate 3: Krill and *M. muelleri*) inhibited AChE by 27.48% only. Based on these results samples M1, C3, and AQ1 were selected for further characterization and the IC_{50} values for each were determined in relation to ACE-1 and DPP-IV inhibition as well as their amino acid composition. Glutamate and aspartate were the most abundant amino acids in the selected samples. IC_{50} values of <0.2 mg/ml and distinct terminal amino acids were identified in each of the three fractions. The study shows that targeting processing of mesopelagic fish have potential to generate Hydrolysates for use in the prevention of type-2-diabetes and hypertension.
Active hydrolysates and aqueous extracts were further characterised and their bioactive peptide compositions identified. *In silico,* analysis was applied to peptides and those predicted to be

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most active were chemically synthesised and re-tested using the enzyme inhibition assays described previously.

Peptides were characterised in active fractions using mass spectrometry. One hundred and thirty five peptides were identified in fraction A1; 83 peptides in fraction Q1 and > 500 in fraction C3. Following peptide identification, *in silico* analysis (Figure 1) was applied to peptide sequences to identify potential "hit" peptides. Following identification, peptides were chemically synthesised and re-tested using the relevant bioassay as outlined above (Section 3).

Figure 1: In silico, strategy applied to MS identified peptides from mesopelagic fractions (Lafarga and Hayes, M, et al., (2015).

Three peptides identified from different hydrolysate samples as having potential to be bioactive according to Peptide Ranker were chemically synthesised. These peptides were; GPLGPLGPLGPLGPLGPLGP - a peptide consisting of 20 amino acid, derived from AQ1 sample with a peptide ranker score of 0.95. It was derived during hydrolysis from an early growth response protein; peptide FPGPFGPLGTPGPFG, 15 amino acids long, derived from C3 sample with a peptide ranker score of 0.950 peptide isolated from an uncharacterised protein and peptide ALLVADFGLQVSYDWNWR, 18 amino acids long, derived from M1 sample with a peptide ranker score of 0.728 derived from protein (Fc fragment of IgG binding protein OS=Pan troglodytes OX=9598 GN=FCGBP PE=4 SV=2; Fc fragment of IgG binding protein OS=Pongo abelii OX=9601).

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Table 2: Bioactive peptides identified from mesopelagic fractions M1, AQ1 and C3; associated bioactivities of peptide fragments derived from the same peptides following simulated gastrointestinal digestion predicted by selection of the enzymes with Pepsin and Trypsin

Peptide single amino acid sequence	**Peptide Ranker value[1]**	**Novelty (found in database [1,2])**	**Observed bioactivity *in vitro***	**Simulated digestion using PeptideCutter[3]/Peptide digestion fragments**	**Associated predicted bioactivities of peptide fragments resulting from simulated GI digestion**	**References**
ALLVADFGLQVSYDW NWR (from fraction M1)	0.728	Novel	Not tested *in vitro*	AL, VADF, GL, VSY, DW, NWR	AL – DPP-IV inhibitor; GL – ACE-1 inhibitor, WR – dipeptidyl peptidase IV inhibitor	Nongonierma A. B., Mooney C., Shields D. C., FitzGerald R. J. (2013) Food Chemistry, 141, 644-653 (AL); Cheung H.-S., Wang F.-L., Ondetti M. A., Sabo E. F., Cushman D. W.(1980), J. Biol. Chem., 255, 401-407 GL); Nongonierma A. B., Mooney C., Shields D. C., FitzGerald R. J. (2014). Peptides 57, 43-51 (WR).
GPLGPLGPLGPLGPLG P (from fraction AQ1)	0.95	Novel	Antimicrobial against *E.coli* and *Staphylococcus aureus*; ACE-1 inhibition	GP, GPL	GP ~ noted in BIOPEP as a Prolyl endopeptidase inhibitor, an ACE inhibitor, Dipeptidyl peptidase IV inhibitor, peptide regulating the stomach mucosal membrane. Potential impact on mental, heart health & hypertension, diabetes type 2 and the stomach. GPL~ ACE inhibitor – Potential impact on heart health and hypertension.	Ashmarin I. P., Karazeeva E. P., Lyapina L. A., Samonina G. E. (1998). Biochemistry-Moscow, 63, 119-124 (GP); Byun H.-G., Kim S.-K.(2002) Bioch. Mol. Biol., 35, 2, 239-243 (GPL – ACE inhibitor from Alaskan Pollock skin).

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FPGPFGPLGTPGPEG	0.95	Novel	Not tested in vitro	GP, PGP	GP ~ noted in BIOPEP as a Prolyl endopeptidase inhibitor, an ACE inhibitor, Dipeptidyl peptidase IV inhibitor, peptide regulating the stomach mucosal membrane. Potential impact on mental, heart health & hypertension, diabetes type 2 and the stomach. PGP~ Chemotactic peptide which, is elevated in chronic obstructive pulmonary disease (COPD), it's an inhibitor of insulin secretion, It's antithrombotic, and stomach regulating. It prevents an increase in vascular permeability in Inflammation. Matrikine - peptides originating from the fragmentation of extracellular matrix proteins.	GP – as above; PGP - Ashmarin I. P., Karazeeva E. P., Lyapina L. A., Samonina G. E. (1998). Biochemistry-Moscow, 63, 119-124 (PGP – antithrombotic peptide);

1 https://biochemia.uwm.edu.pl/biopep-uwm/
2 http://pepbank.mgh.harvard.edu
3 https://web.expasy.org/peptide_cutter

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6. Spanish and Irish haul sample preparations and bioactivities

Additional hydrolysates were generated from Irish and Spanish hauls and resulting biomass (Table 3) using different proteolytic enzymes. Their proximate compositions were also determined.

Table 3: Hydrolysates screened for enzyme inhibitory activities in MEESO.

Biomass & Enzymes used to generate hydrolysates	**Product code**
Alcalase 2.4 L FG	MMD02
	MME02
Papain	MMD06
	MME06
Bromelain	MMD10
	MME06
Papain & Bromelain	MMA058
	MMD14
Protamex	MMB010
	MMD18
Endogenous enzymes	MMB034
	MMC019
Hydrolysates generated from Norwegian mesopelagic hauls (2022)	
Corolase hydrolysate	n/d
Roholase hydrolysate	n/d
Bromelain	n/d
Maxi Pro	n/d
Hydrolysates generated from Irish mesopelagic hauls (30.11.2021)	
CE21009 Haul 23 hydrolysis with Alcalase®	23
CE21004 Haul4BHG hydrolysis with Alcalase®	14
CE21004 Haul 13 MAV hydrolysis with Alcalase ®	13
CE21004 Haul 02 Lyd hydrolysis with Alcalase®	2

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6.1. Bioactivities associated with Spanish and Irish hydrolysates

Hydrolysates listed in Table 3 were assessed using assays described in section 3. Results are shown in the following figures.

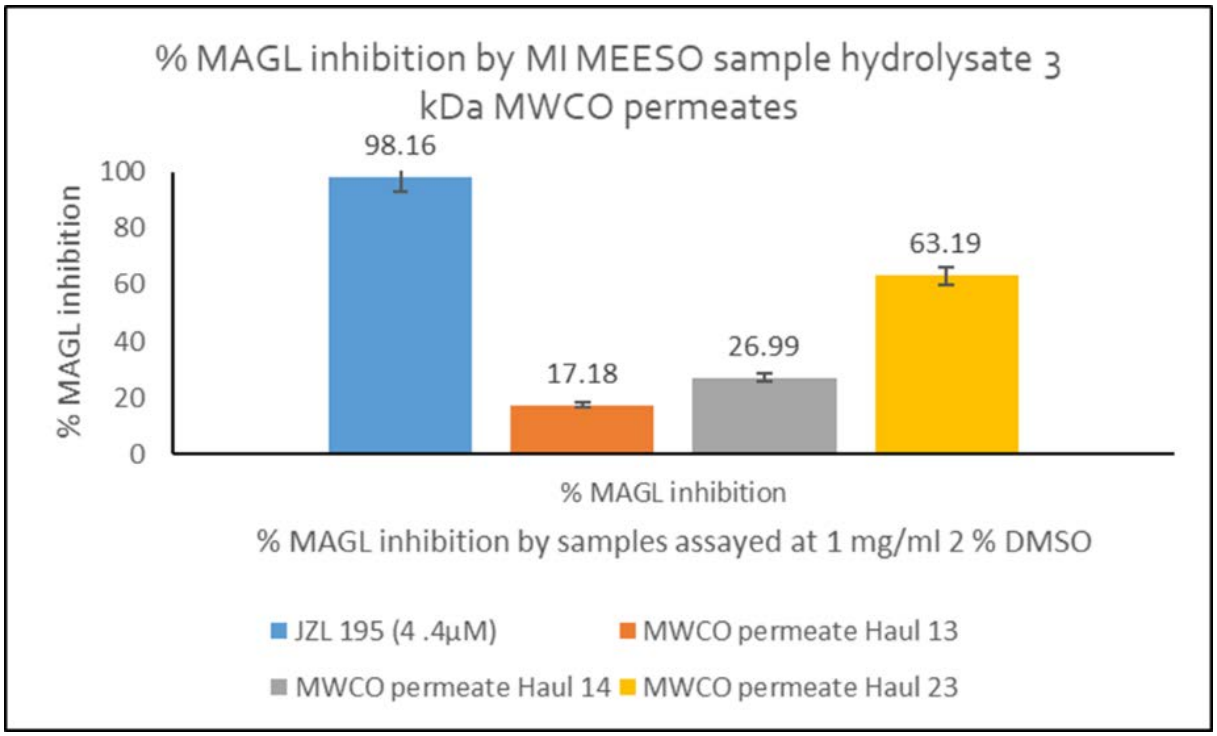

Figure 2: MAGL inhibition by Irish haul (Marine Institute supplied samples) hydrolysed with Alcalase and permeate production (Teagasc).

Compared to the positive control (JZL 195) assayed at a concentration of 4.4 µM the hydrolysate generated using Alcalase from biomass gathered in Haul 23 (by the Irish Marine Institute) and subsequently enriched using 3-kDa MWCO filtration inhibited MAGL by 63.19 % when assayed at a concentration of 1 mg/ml in DMSO. This holds potential for further development but characterisation of the peptides within the fraction using MS is first required.

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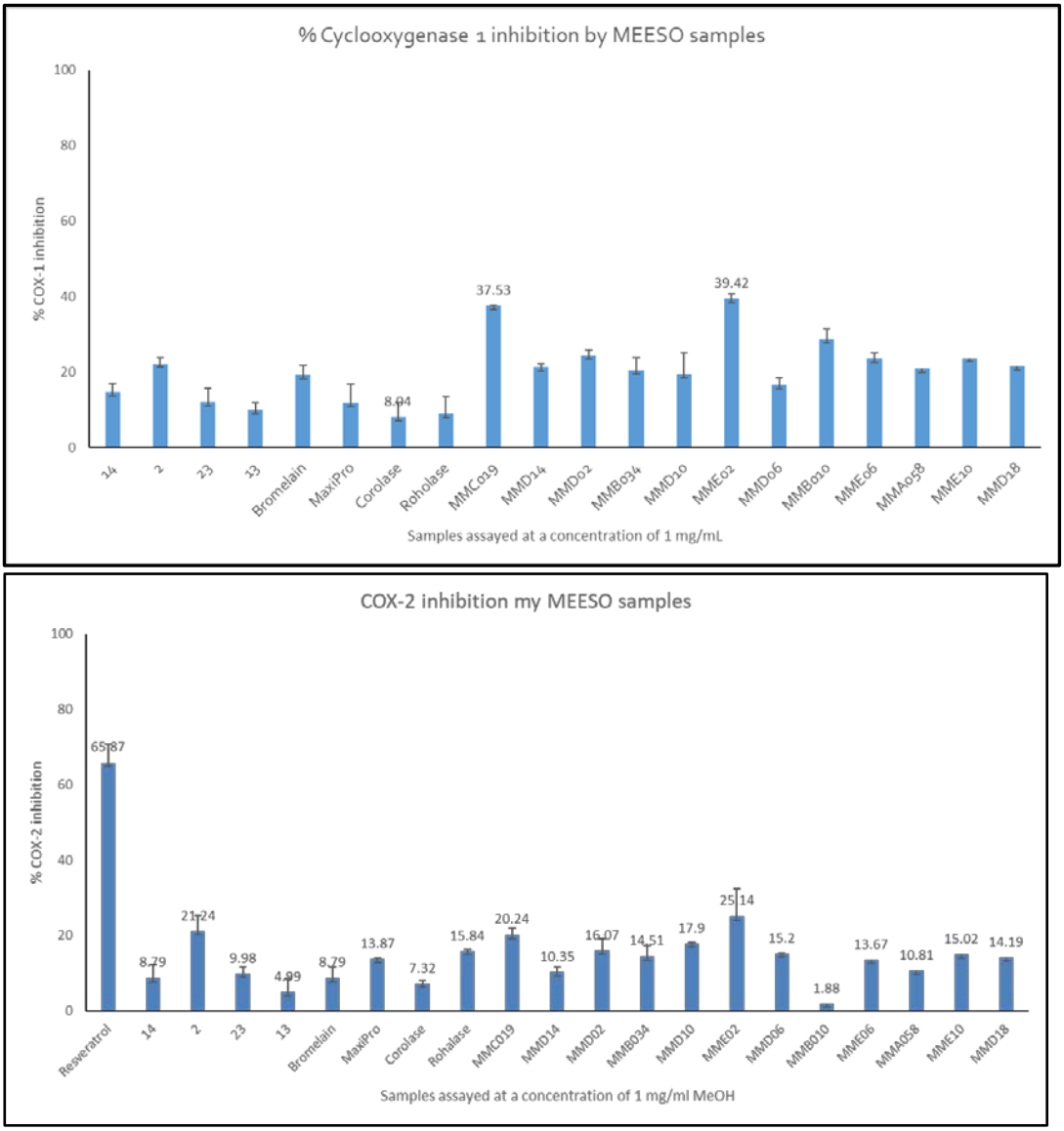

Figure 3: COX 1 (top) and COX 2 (bottom) inhibition by hydrolysates shown in Table 2

The Spanish hydrolysate labelled (MME02) inhibited COX-1 by 39.42 % relative to the positive control Resveratrol. This was the only sample that did inhibit COX-1. It is likely that these hydrolysates do not possess COX-1 inhibitory bioactivities.

The Spanish hydrolysate labelled (MME02) inhibited COX-2 by 25.14 % relative to the positive control Resveratrol. This was the only sample that did inhibit COX-2. It is likely that these hydrolysates do not possess COX-2 inhibitory bioactivities.

ABTS antioxidants have become scientifically interesting compounds due to their many benefits such as anti-aging and anti-inflammatory action. In food technology, antioxidants are added to many foodstuffs in order to enrich the foods and extend shelf life. We identified one (Haul 2 Marine Institute hydrolysed with Alcalase®) potential antioxidant from Irish haul hydrolysates and three from Spanish hydrolysates (MME02; MMC019; MMD06).

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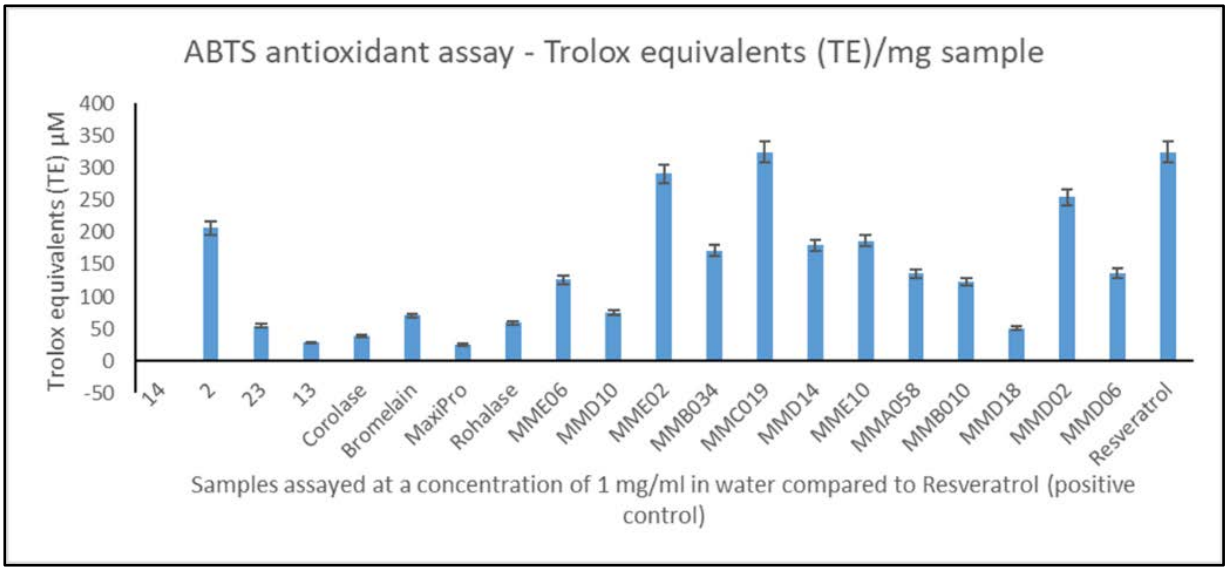

Figure 4: Antioxidant activities of hydrolysates measured using the ABTS assay

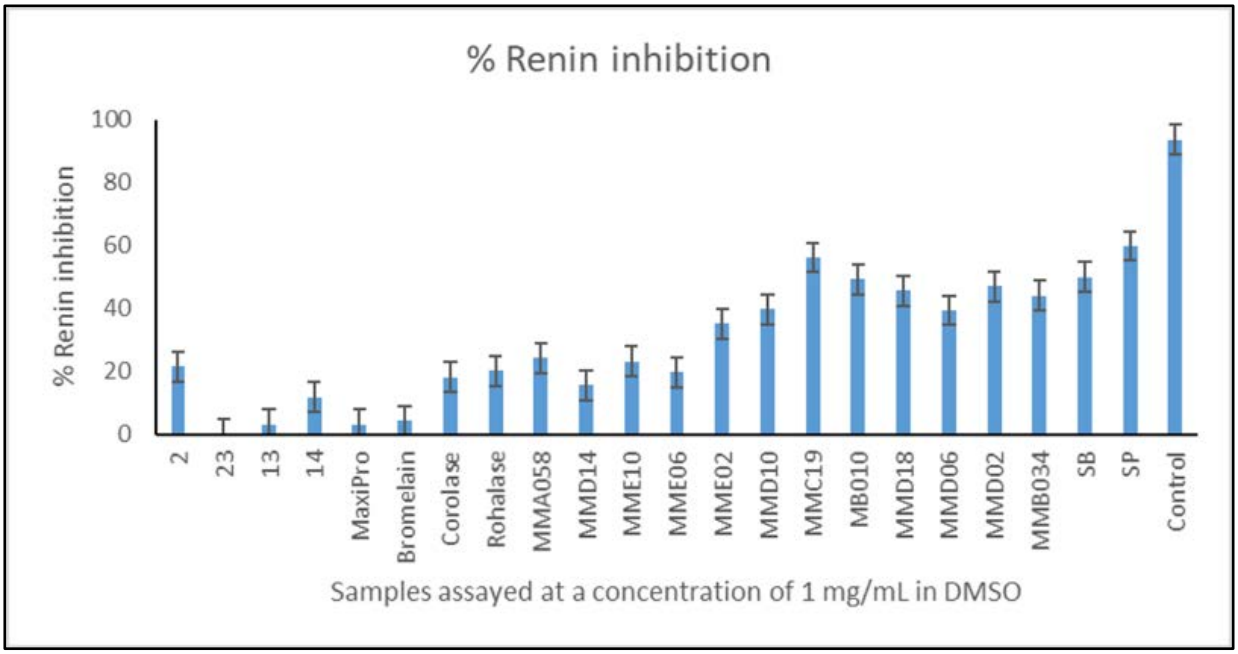

Figure 5: Renin inhibition of hydrolysates

Several of the hydrolysates generated from Spanish hauls had Renin inhibitory activity compared to the positive control. Selected hydrolysates (MMC19) were selected for further purification and peptide characterisation work.

7. Potential applications and Market values

7.1. Food & beverages for prevention of T2D in humans

Global sales of fortified/functional foods reached $292 billion in 2021, an increase from $274 billion in 2020, per Euro monitor**.** The health benefits a product offers have a significant influence on purchase decisions for six in 10 U.S. food shopper. Recent reports linking cardiovascular issues to diseases of the brain, including Alzheimer's disease and dementia, underscore the importance of preventing heart disease. Consumers are taking a more aggressive

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and preventive approach to health. Ingredients to maintain immunity and prevention of heart disease and diabetes are potential markets for functional foods as a large proportion of the population suffer from these ailments (as outlined earlier).

Hydrolysate from *M. muelleri* generated using FoodPro PNL (M1) inhibited the ACE-1 enzyme by 89.56% when assayed at a concentration of 1 mg/ml compared to the positive control Captopril©. It also inhibited DPP-IV by 100% when assayed at a concentration of 1 mg/ml relative to the control Sitagliptin. This hydrolysate has potential for use as a functional food for prevention of T2D, similar to products like ProAtlantic (BII, Ireland) which is marketed for glycaemic control or the SlimPro® product, which is a blue whiting hydrolysate for weight control. The katsobushi pentapeptide product made from bonito with thermolysin is sold in Japan and has Food of Specified health use (FOSHU) status there in relation to blood pressure control. We have identified the active peptide ALLVADFGLQVSYDWNWR in this hydrolysate and following simulated digestion we identified that peptides have ACE-1 and DPP-IV inhibitory activities demonstrated *in vitro* previously.

Consumers are more aware today that diabetes (T2D) is a lifestyle-related health issue. In the Asia Pacific region, 72% of consumers recognise the link between diet and diabetes. Type 2 diabetes patients want food products to manage blood glucose levels. A higher intake of dairy proteins has consistently shown to have a beneficial effect on blood glucose control in Type 2 diabetes patients. Research has shown that dairy proteins such as casein and whey can stimulate postprandial insulin responses, thereby increasing blood glucose disposal and lowering the postprandial rise in blood glucose concentration. The insulinotropic effect of dairy proteins thus helps to lower the GI and control blood glucose levels. Companies like Friesland Campina use dairy proteins as active ingredients in products for control of blood glucose. These are the Optimal Support Shake, a powder format based on 25g dairy protein and the Breakfast Beverage, a Ready-to-Drink concept with 15g dairy protein. Dairy proteins are seen by companies as an integral part of an effective nutritional strategy for the management of Type 2 diabetes. Several studies regarding fish protein hydrolysate use for prevention of diabetes exist. T2D control agents tested in animal models and *in vitro* were identified from fish proteins previously. For example, Pilon and colleagues (2011), identified hydrolysates from herring, mackerel, salmon, and bonito fish with the ability to control insulin sensitivity in rats; Lacroix identified DPP-IV inhibitory peptides from tuna cooking juices previously with IC_{50} values of 78.0 -116.1 µM and Wang and colleagues (2015) identified DPP-IV inhibitors from Atlantic salmon skin previously. The DPP-IV values observed regarding the mesopelagic hydrolysates compare favourably with previously published work where dairy proteins were assessed for ability to inhibit DPP-IV (Lacroix et al., 2015) and with other fish hydrolysates.

Nutripeptin™ is a marine protein hydrolysate extracted by enzymatic hydrolysis of fresh or fresh frozen fillets of codfish species. As a glycemic-index-(GI)-reducing peptide, Nutripeptin™ may help reduce fat deposition as part of weight-management products.

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Table 4: Examples of Peptide products from fish for heart and immunity and price.

Product name	Claim	Producer	Price point (B2C)
PeptACE™	Antihypertensive	Natural factors Nutritional products Ltd., Canada	£34.94 (for 90 capsules - supplement)
Vasotensin®	Antihypertensive	Metagenics, USA	$US80.00 (for 120 tablets)
Levenorm®	Antihypertensive	Ocean Nutrition Canada Ltd., Canada	N/D
Peptide ACE 3000	Antihypertensive	Nippon supplements Inc., Japan	N/D
Precardix®	Antihypertensive	Marealis Health Inc., Norway	$49.96 (for 60 tablets)
Valytron®	Antihypertensive		
Stabilium® 200	Stress relief	Yalacta, France	€38.53 (for 60 tablets/capsules)
AntiStress 24	Stress relief	Forte Pharma Laboratories, France	€11.99 (for 60 capsules)
Protizen®	Stress relief	Copalis Sea Solutions, France	€38.53 (capsules)
PeptiBal™	Immune modulatory	InnoVactiv Inc., Canada	€68.40 (30 capsules)
Seacure®	Gastrointestinal health	Proper Nutrition, USA	€89.00 (30 capsules)
Marine collagen	Joint and skin health	Seagarden Norway	€52.81 (1 jar - 300 g)
ProGo®	Weight management	Hofseth Biocare Norway	€369.99/5 kg (price of similar product)
SlimPro®	Weight management		$49.90 (60 capsules)

A supplementation of 1-2% Nutripeptin™ provides significant glycemic-index-lowering functionality (GI reduction of 30 to 50%) to ordinary foodstuffs like ice cream, chocolate, white bread, fruit juices, hamburgers. It can be purchased directly from Copalis (France) or via sites such as (mysibi.com). This ingredient is added to beverages at 0.4 % of the total volume. Mesopelagic species and the hydrolysate generated in MEESO offer potential to replace products such as Nutripeptin™ which, currently are made from non-sustainable or non-MSC certified fisheries such as cod and from species where quotas are restricted due to limited stocks.

7.2. Ingredients for companion animals for prevention of heart health issues and T2D

Several studies on health ingredients focus on humans. However, these ingredients may also exert their beneficial effects on dogs and cats but at least in some cases, have not been investigated yet. Companion animals like dogs and cats are affected by overweight and obesity

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comorbidities such as diabetes and cancers, leading to impaired health and reduced life span. Depending on breeds and the methodology used to evaluate health status, overweight and obesity, prevalence was estimated between 19.7% and 59.3% in dogs and between 7% to 63% in cats. This situation is mainly due to excessive food offer and related calorie intake, as pet owners do not follow nutritional guidance, and to a loss of physical exercise leading to the overweight-derived problems mentioned above but also to skin disorders, respiratory and locomotor diseases. Previously, French researchers (Theysgeur et al., 2020) identified a Tilapia hydrolysate and peptides with the potential to manage weight gain in companion animals, specifically dogs. They did this using a simulated dog gastrointestinal model. Studies concerning the use of fish hydrolysates as functional foods/supplements for pets are few and this area offers tremendous potential for development, not only as pet owners are more commonly feeding pets what they themselves eat but also as animal studies in dogs are often used to verify functional foods for humans in order to get EFSA approved health claims. Several companies such as Hoftseth and those listed in Table 1 offer protein hydrolysates for use in pet foods as palatants and health ingredients for health maintenance. This market is a lucrative one. Hydrolysed proteins can also be used as a palatant, which can be desired in for example performance dogs or animals with a low appetite. Cats, with their high requirement for protein, can also greatly benefit from hydrolyzed proteins, which are relatively rich in taurine and have an attractive taste (Pekel et al., 2020).

7.3. Companion animal market – pets

The functional food for health, pet treat market is estimated at €532 million in the UK (Statistica, 2020). The world pet treat market is expected to be worth €61.05 billion by 2027. Dog treats account for €51.4 billion of this market.

Competitor companies in this sector include Nestle SA, Mars Inc, Plato Pet treats, WellPet LLC and Champion pet-foods LP as well as Colgate-Palmolive and others.

Within the pet treat sector, treats for dogs in the UK outsell those for cats by more than four to one (Mars report, 2020). The number of dogs in the UK with hypertension is estimated at 7.74 million. Pets are living longer and boomers and millennials are increasingly purchasing treats for pets, especially dogs, with added health benefits. Pet parents ordinarily purchase treats from pet shops and vets as well as online. 36 % of dog owners and 31% of cat owners reported buying a brand of pet food following a recommendation made by their veterinarian.

- B2B sales could be to companies including Nestlé, Colgate-Palmolive, Mars Inc.
- B2C sales could be online, via veterinarians, pet-shops. Veterinarians and Pet-Shops normally command 50% of market price to their customers. Veterinarian distributors are the normal route to market via veterinarian clinics.

Palatants play an imperative role in improving the smell, taste, and texture of pet foods. Several top-tier and premium players involved in the palatants market are investing a considerable amount of resources in research and development activities to evaluate and determine the most effective manufacturing and processing technique. In recent times, research and development with various flavour enhancers have increased at a rapid pace– another factor that is expected to boost the growth of the global palatants market during the forecast period. The global palatants market is expected to reach a market value of US$ 3.7 Bn by the end of 2030.

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In this market, the USA holds a major share, due to increasing pets and pet food companies in the region. In the global pet food market, there are more than 1,200 pet food manufacturers, and more than 175 are in the USA, thus ensuring the growth of the palatants market in the region. Increasing pet ownership and the growing pet food industry has generated high demand for pet food, directly increasing the demand for palatants. In addition, many companies are investing in R&D to come up with innovative palatants, which will affect the palatability of the end-products. This offers an opportunity for mesopelagic fish species use.

The key players in this sector include: Kemin Industries, Nutriad International NV, Pancosma S.A., Frutarom Group, Alltech Inc., Diana Group, Yingtan WingBiotechnology Co., Ltd., Nestle S.A., BHJ A/S, AFB International, Darling Ingredients Inc.

7.4. Fish derived peptides for use in cosmetics/nutricosmetics

The use of peptides for skin care dates to the 1980s. The cosmetic industry periodically launches new peptides, as they are promising and appealing active ingredients in the growing and innovative cosmetics market. The use of peptides in anti-aging cosmetics has increased by 7.2% in the last decade, while the variety and the number of peptide combinations in products has increased by 88.5%. The most used peptides in antiaging cosmetic formulations are, in descending order, Palmitoyl Tetrapeptide-7, Palmitoyl Oligopeptide and Acetyl Hexapeptide-8. In 2011, the majority of peptides were obtained from synthesis, while in 2018, biotechnology processing was the dominant source. The anti-aging market is expected to grow at an approximate 8% compound annual growth rate between 2018 and 2021, reaching a value of USD 271.0 billion by 2024. The use of peptides derived from mesopelagic species with antimicrobial activity has potential for development of skin care creams and formulations for sensitive skin. Peptides can be included in skincare formulations for the following reasons:

- Signal peptides, which stimulate matrix protein production (such as collagen and elastin) and cell growth, amongst other cell metabolic functions (e.g., Palmitoyl Tetrapeptide-7, Palmitoyl Pentapeptide-4);
- Carrier peptides, which may act as transportation facilitators for important substances or trace elements inside the cell, such as copper and magnesium (e.g., Tripeptide-1, GHK-Cu);
- Neurotransmitter-inhibiting peptides, which may target expression wrinkles by inhibiting acetylcholine release at the neuromuscular junction by acting on distinct molecular targets (e.g., Acetyl Hexapeptide-8, Acetyl Octapeptide-3);
- Enzyme-inhibiting peptides, which may reduce the activity of enzymes that participate in skin aging (e.g., soybean peptides, which inhibit serine proteases, such as matrix metalloproteinases, (MMPs), and silk peptides, which inhibit tyrosinase).

The fish collagen peptide market exceeded USD 685 Million in 2020 and is estimated to register over 5.5% CAGR between 2021 and 2027.

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8. Fish protein hydrolysates for use as functional foods: Regulation perspective

Europe

Products produced must comply with EU Food Law. Medicinal claims cannot be used for foods. Nutrition and health claims for foods (including food supplements) are regulated at the EU level (Regulation EC/1924/2006), where EFSA evaluates the scientific evidence supporting the claims. Nutrition claims are allowed if they are listed in the Annex of the Regulation. An example of an allowed nutrition claim is "high fiber" (on the condition that the product contains at a minimal level of 6-g fiber per 100 g). For vitamins and minerals, there are similar rules. The nutrition claim "source of vitamin/mineral x" or "contains vitamin/mineral x" can be used, if the product contains a significant amount of the vitamin/mineral. A significant amount is minimally 15% of the recommended daily allowance in 100 g, 100 mL, or a portion if the packaging contains only one dose portion (i.e., if the packaging contains several portions, the significant amount must be in 100 mg or 100 mL). For drinks, 7.5% of the recommended daily allowance (RDA) in 100 mL suffices for the claim "source of vitamin or mineral x." The RDAs for vitamins and minerals are defined in the Annex XIII of the Nutrition Information Regulation (EU) 1169/2011. The RDA for iodine, for example, is 150 µg. For macroalgae-derived foods, the claim "source of iodine" can thus be used if the iodine content reaches the level of 22.5 µg per 100 g, 100 mL, or per dose.

Functional foods or nutraceuticals, including supplements with health claims concerning cardiovascular, neurological or metabolic activities are relevant to fish protein hydrolysates and fish oils. EU Regulation (EC) No. 1924/2006 relates to the use of health claims on food products in Europe. The Scientific Panel on Dietetic Products, Nutrition and Allergies (NDA) of the European Food Safety Authority (EFSA) has provided guidelines to manufacturers and suppliers of food supplements and functional foods for placing health claims on their products. Guidelines include that the provided claims need to be well demonstrated (i.e. generally accepted scientific evidence). The demand for generally accepted scientific evidence is not easy to fulfil. A review of accepted claims in the EFSA journal highlights that weaknesses of some studies – such as not double-blinded, animal and *in vitro* studies – result in failure. A well-designed clinical trial is very important to secure a health claim.

The EFSA-NDA panel decided that the product Valtyron (a sardine muscle digest preparation containing angiotensin-1-converting enzyme inhibitory properties and produced by Senmi Ekisu Co., Japan) obtained novel food ingredient status, and is deemed safe for use as a novel food ingredient. Novel food ingredient is food that has not been consumed to a significant degree by humans in the EU prior to 1997 and regulated by Regulation (EU) 2015/2283, at a level of 0.6 g/serving.

Getting health claims approved in EU takes a significant amount of both time and resources from an entrepreneur. Therefore, a less demanding marketing strategy with health foods and health-enhancing food supplements is to use nutrition claims only.

Approved claims for Fish protein hydrolysates (FPH) in Europe relate to the Novel food ingredient regulation – Regulation (EU) 2015/2283. There are no fish protein hydrolysate products with approved health claims related to regulation EU Regulation (EC) No. 1924/2006, currently.

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Japan

The Japanese Ministry of Health, Labour and Welfare (MHLW) introduced the Foods for Specified Health Use (FoSHU) system, which is an approval system for the regulation of all health claims on packages of food products launched in Japan. The status is granted to food that contains ingredients with a proven benefit to health and demonstrated safety. FoSHU approved products can put physiological claims on their packages, which must be priory approved by MHLW. Several fish oil and FPH products have FoSHU status in the USA.

FPHs represent desirable functional food ingredients owing to their beneficial impact on both health and food quality. BWPH – a fish protein hydrolysate from *Micromesistius poutassou* induced CCK and GLP-1 secretion in STC-1 cells, was subsequently demonstrated to increase plasma concentrations of CCK and GLP-1, improve body composition and reduce body weight upon oral administration (1.4 g) to 120 overweight (25 kg/m2 ≤ body mass index (BMI) < 30 kg/m2) adults over 90 days. BWPH is now commercialised and marketed as Slimpro® (Nobile et al., 2016). In addition, peptides purified from dried bonito (katsuobushi) via thermolysin digestion exhibiting ACE-inhibitory activities *in vitro* were also shown to exhibit anti-hypertensive effects in spontaneously hypertensive rats and borderline (high normal) and mildly hypertensive adults (1.4 g/ day orally administrated over 5 weeks) (Fujita et al., 2001, Yokoyama et al., 1992). Katsuobushi oligopeptide received official approval as Foods for Specific Health Use (FoSHU) in 1999 by the Ministry of Health and Welfare in Japan.

USA

The US follow the Nutritional Labelling and Education Act (NLEA) to regulate health claims and food labelling. Such claims describe a relationship between food, a food component, or a supplement ingredient and the reduced risk of a disease or health-related condition. The authorization of a claim is generally triggered by the submission of a *health claim petition* and the evaluation is performed based on an extensive scientific literature review. Next to health claims, which are pre-approved by the Food and Drug Administration (FDA), structure/function claims can also be placed on the packages of food or dietary supplements in the USA. The *structure/function claims are regulated by the Dietary Supplement Health and Education Act (1994).* They define the effect of a dietary supplement on the structure or function of the body – e.g. "helps promote bone health". These claims do not need to be pre-approved by the FDA and must be accompanied by a disclaimer: "This statement has not been evaluated by the FDA. This product is not intended to diagnose, treat, cure or prevent any disease". ***Most dietary supplements containing fish hydrolysates in the USA – e.g. Stabilium 200, Vasotensin, PeptACE, have claims that fall into the structure/function class.***

Food additive claims/regulations in EU

The use of food additives in the EU is controlled by the Regulation (EC) No 1333/2008, containing a list of authorized food additives and comprises additives under codes E401-E407a. Commission Regulation (EU) No 231/2012 further specifies the origin, composition, and usage of the accepted additives.

Nutri-cosmetics claims/regulation in EU

Regulation 1223/2009/EC on cosmetic products contains the basic EU rules on cosmetics. There is a centralized notification procedure for all cosmetic products to be placed on the EU market. Manufacturers and importers notify the products via the EU portal (Cosmetic Products Notification Portal, CPNP). The legal responsibilities include product safety assessment,

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keeping product information file (PIF), and reporting serious undesirable effects. There are binding lists of ingredients that are acceptable, restricted, and prohibited. The EU's Cosmetic Ingredient database, CosIng (https://ec.europa.eu/ growth/tools-databases/cosing/index.cfm), contains the legal requirements and restrictions on each substance.

9. Potential to upscale

For a biotechnological process to be successful, it needs to scale (see Figure 6 for generic visualisation of the process). This task can be tricky as biological processes oftentimes progress differently in different reactor sizes, i.e. going from 0.5 kg of raw material to 4,000 kg of raw material is rarely a linear path – more volume takes longer to heat, longer to cool, and is generally more complicated to handle.

Several commercial facilities for scaling up the process exist; a comprehensive database can be found at https://biopilots4u.eu/. Additionally, Nofima AS in Norway owns and operates a pilot scale production facility with necessary approvals for creating food and food ingredients, called Biotep (Biotep - Nofima).

Case-study for a typical production in the research-focused pilot plant Biotep. This plant has 24-hour productions from Monday to Friday. The amount of raw material is as previously mentioned 4,000 kg, hourly rate is 3000 NOK. A production this size would demand the use of two reactors to run batch-wise and the production would most likely last approximately 70-80 hours and variable costs (steam for heating in reaction and electricity) would be approximately 80,000 NOK:

$$\mathbf{3,000\ NOK \times 80\ hours + 80,000\ NOK = 320,000\ NOK\ (30,500\ €)}$$

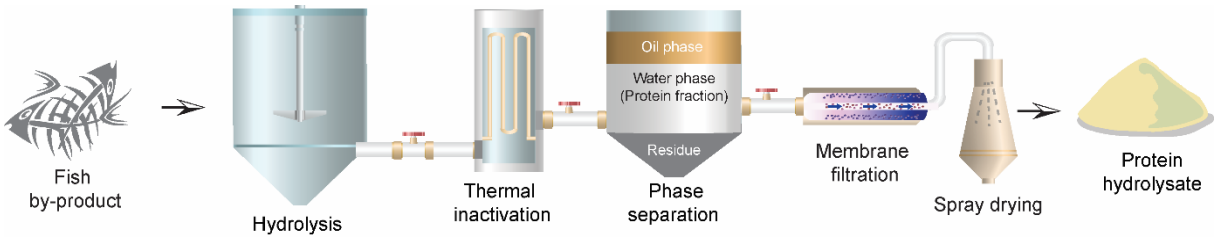

Figure 6: Visualization of the bioprocess connected to use of fishery co-products in the Biotep fascility. image credit: Sileshi Gizachew Wubshet.

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