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Metabolites in fish and humans as a response to different food ingredients

A metabolomics approach

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Metabolites in fish and humans as a response to different food ingredients; a metabolomics approach

Abstract

The main objective of this thesis was to evaluate metabolomics changes in humans and fish as a response to food/feed consumption.

To alleviate the environmental impact of animal production and maximize the use of resources, the valorization of meat by-products might be an attractive alternative. A meat product containing heart and aorta tissue from pork was designed and analyzed for fatty acid and metabolite composition. In comparison with a control of similar qualities, the designed meat product (or test product) showed higher monounsaturated fatty acid and tyramine levels and lower levels of sugars. The test meat product was used in a randomized controlled clinical trial to test for potential health effects in patients showing atherosclerosis symptoms. Patients receiving the test product showed a decrease in blood levels of low-density lipoproteins, total cholesterol, atherogenic index and triacylglycerols.

To reduce the impact of animal production on ecosystems, the replacement of feed ingredients by a microbial alternative was realized. In this study, vegetable oils included in the feed of Arctic char (*Salvelinus alpinus*) were replaced by biomass of the oleaginous yeast (*Rhodotorula toruloides*). The analysis of the yeast biomass showed safe levels of pollutants and heavy metals. Fish growth and muscle fatty acid profile were similar to the control. A higher liver weight and hepatosomatic index were observed in fish fed including the yeast biomass, albeit no significant difference in liver fat content or in hepatic enzyme activity was observed. Quantification of plasma metabolites revealed higher levels of metabolites involved in energy pathways such as one-carbon metabolism and gluconeogenesis.

In conclusion, this thesis showed that metabolomics can be applied to evaluate effects of food/feed at the molecular level in complex systems. It adds knowledge on the effects of meat by-product consumption in the particular case of atherosclerosis symptoms. The fish feed trial showed the possibility of feed modification with a specific yeast.

Keywords: lipid analyses, heart and aorta-based food, atherosclerosis, fish feed trial, yeast biomass, fish performance, TCA cycle, gluconeogenesis, one-carbon metabolism, *Rhodotorula toruloides*

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Metaboliter i fisk och människa som respons på olika matingredienser; utvärdering med metabolomisk metodik

Abstract

Huvudsyftet med denna avhandling var att utvärdera metabola förändringar hos människa och fisk som ett svar på mat-/foderkonsumtion.

För att minska miljöpåverkan från animalieproduktion och maximera resursanvändningen kan värdehöjning av köttbiprodukter vara ett attraktivt alternativ. En köttprodukt innehållande hjärt- och aortavävnad från gris designades och analyserades med avseende på fettsyra- och metabolitsammansättning. I jämförelse med en kontrollprodukt av liknande kvalitet, visade den designade köttprodukten (eller testprodukten) högre andel enkelomättade fettsyror och tyraminnivåer och lägre nivåer av sockerarter. Testköttprodukten ingick i en randomiserad kontrollerad klinisk prövning för att testa potentiella hälsoeffekter hos patienter som visar åderförkalkningssymtom. Patienter som fick testprodukten visade en minskning av lågdensitetslipoproteiner i blod, totalt kolesterol, aterogent index och triacylglyceroler, som kan anses positivt.

För att minska animalieproduktionens påverkan på ekosystemen genomfördes en förändring av foderingredienser med ett mikrobiellt alternativ. Vegetabiliska oljor som ingår i fodret till röding (*Salvelinus alpinus*) ersattes i denna studie med biomassa av den oljehaltiga jästen (*Rhodotorula toruloides*). Analysen av jästbiomassan visade på ofarliga halter av föroreningar och tungmetaller. Fisktillväxt och muskelfettsyraprofil var jämförbart med kontrollen. Högre levervikt och hepatosomatisk index observerades hos fiskar som utfodrats med jästbiomassa i fodret, även om ingen signifikant skillnad i leverfettinnehåll eller i leverenzymaktivitet erhölls. Kvantifiering av plasmametaboliter avslöjade högre nivåer av metaboliter involverade i energivägar såsom en-kolsmetabolism och glukoneogenes.

Sammanfattningsvis visar denna avhandling att metabolomik kan användas för att utvärdera effekter av mat/foder på molekylär nivå i komplexa system. Det tillför kunskap om effekterna av konsumtion av köttbiprodukter i det specialfallet med symtom på åderförkalkning. Fiskfoderförsöket visade möjligheten till fodermodifiering med en specifik jäst.

Nyckelord: lipidanalys, hjärt- och aorta innehållande produkt, fiskfoderförsök, jästbiomassa, fiskftillväxt, TCA-cykel, glukoneogenes, en-kols metabolism, *Rhodutorula toruloides*

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Les métabolites chez l´homme et le poisson en réponse à différents aliments : une approche métabolomique

Résumé

L´objectif principal de cette thèse était d´évaluer les changements métabolomiques chez l´homme et le poisson en réponse à la consommation de nourriture.

Pour atténuer l´impact environnemental de la production animale et maximiser l´utilisation de ressources, la valorisation de coproduits animaux tels que les abats est une alternative intéressante. Dans cette étude, un produit à base de cœur et d´aorte de porc (produit test) a été élaboré et sa composition en acides gras et en métabolites analysée. En comparaison avec un produit de qualité similaire (control), le produit test a montré une plus grande quantité d´acides gras monoinsaturés, tyramine et moins de sucres. Le produit test fut inclus dans un essai randomisé contrôlé pour tester ses potentiels effets sur la santé chez des patients montrant des symptômes d´athérosclérose. Les patients recevant le produit test ont montré une baisse de leurs niveaux en lipoprotéines de basse densité, cholestérol total, indice athérogène et en triglycérides dans le sang.

Pour réduire l´impact de la production animale sur les écosystèmes, les huiles végétales contenues dans la nourriture pour l´Omble chevalier (*Salvelinus alpinus*) ont été remplacées par de la biomasse de levure oléagineuse (*Rhodotorula toruloides*). L´analyse de la biomasse de levure a montré des niveaux de polluants et métaux lourds sans dangers. La croissance du poisson nourri à la biomasse de levure et son profil musculaire en acides gras se sont révélés similaires à un groupe de poissons nourris aux huiles végétales. Une augmentation du poids du foie et de l´indice hépato-somatique ont été observés chez le poisson nourri avec la biomasse de levure bien qu´il n´y ait pas de différence dans la teneur en lipides dans le foie ou dans l´activité des enzymes hépatiques. La quantification de métabolites dans le plasma sanguin a révélé des niveaux plus importants de métabolites impliqués dans des voies énergétiques telles que le métabolisme monocarboné et de la gluconéogenèse.

En conclusion, cette thèse a montré que la métabolomique peut être appliquée pour évaluer les effets de la nourriture à l´échelle moléculaire et ajoute des connaissances sur les effets de la consommation de nourriture de manière plus durable.

Mots-clés : analyses de lipides, nourriture à base de cœur et d´aorte, athérosclérose, essai alimentaire sur le poisson, biomasse de levure, performance du poisson, cycle de Krebs, gluconéogenèse, métabolisme à un carbone, *Rhodotorula toruloides*

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Dedication

À ma famille.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Brunel, M., Chernukha, I. M., Kotenkova, E. A., Fedulova, L. V., Moazzami, A. A., Sampels, S., Pickova, J., & Müllner, E. Pork by-product characterization via metabolites and fatty acid profiling (Manuscript as a short communication).

II. Brunel, M., Chernukha, I. M., Kotenkova, E. A., Derbeneva , S. A., Fedulova, L. V., Moazzami, A. A., Sampels, S., Pickova, J., & Müllner, E. A pilot study to investigate the effect of a porcine cardiac tissue-based product in a randomized clinical trial on patients with atherosclerosis symptoms using ^{1} H-NMR (Manuscript).

III. Brunel, M., Burkina, V., Sampels, S., Dahlberg, A. K., Passoth, V., & Pickova, J. Addition of the oleaginous yeast *Rhodotorula toruloides* as a feed ingredient in the diet of Arctic char (*Salvelinus alpinus*); effect on performance (Manuscript).

IV. Brunel, M.*, Burkina, V., Pickova, J., Sampels, S., & Moazzami, A. A. (2022). Oleaginous yeast *Rhodotorula toruloides* biomass effect on the metabolism of Arctic char (*Salvelinus alpinus*). *Frontiers in Molecular Biosciences*. 9:931946. https://doi.org/10.3389/fmolb.2022.931946

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The contribution of Mathilde Brunel to the papers included in this thesis was as follows:

I. Performed the fatty acid analysis of the meat products. Processed the fatty acid analysis data. Interpreted the findings and was mainly responsible for writing the manuscript.

II. Performed the fatty acid analysis of the meat products. Analyzed the plasma metabolites and blood lipids data using statistical methods. Interpreted the findings and was mainly responsible for writing the manuscript.

III. Participated in the field work, the fish feed preparation and participated in the sensory test. Performed the statistical analysis of the data. Interpreted the findings and wrote most of the manuscript.

IV. Participated in the field work and the fish feed preparation. Performed the metabolomics part of the laboratory work. Performed the statistical analysis of the data. Interpreted the findings and was mainly responsible for writing the manuscript.

The following papers were published during the timeframe of the doctoral education, but are not part of this thesis.

V. Ahi, E. P., Brunel, M., Tsakoumis, E., & Schmitz, M. (2019). Transcriptional study of appetite regulating genes in the brain of zebrafish (*Danio rerio*) with impaired leptin signalling. *Scientific Reports*, 9, 20166 https://doi.org/10.1038/s41598-019-56779-z

VI. Ahi, E. P., Tsakoumis, E., Brunel, M., & Schmitz, M. (2021). Transcriptional study reveals a potential leptin-dependent gene regulatory network in zebrafish brain. *Fish Physiology and Biochemistry*, 47, 1283-1298 https://doi.org/10.1007/s10695-021-00967-0

VII. Ahi, E. P., Brunel, M., Tsakoumis, E., Chen, J., & Schmitz, M. (2022). Appetite regulating genes in zebrafish gut; a gene expression study. *PloS ONE*, 17: e0255201. https://doi.org/10.1371/journal.pone.0255201

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Abbreviations

^{13}C	Carbon 13
^{19}F	Fluoride 19
1C	One carbon
1D	One-dimensional
^{1}H	Proton
^{1}H-NMR	Proton nuclear magnetic resonance
2D	Two-dimensional
^{31}P	Phosphorus 31
AhR	Aryl hydrocarbon receptor
AI	Atherogenic index
ALA	α-linolenic acid
ANOVA	Analysis of variance
AQuA	Automated Quantification Algorithm
ARA	Arachidonic acid
ASICS	Automatic Statistical Identification in Complex Spectra
BATMAN	Bayesian automated metabolite analyzer for NMR spectra
BCAAs	Branched-chain amino acids
BMI	Body mass index
BQuant	Bayesian Quantification

CE-MS	Capillary electrophoresis mass spectrometry
CI	Confidence interval
CL	Cholesterol
CoA	Coenzyme A
CV	Cross-validation
CV-ANOVA	Cross-validated analysis of variance
CYP1A	Cytochrome P450 1A
D_2O	Deuterium oxide
Da	Daltons
DA	Discriminant analysis
DAG	Diacylglycerols
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EPA	Eicosapentaenoic acid
EROD	7-ethoxyresorufin-O-deethylase
FA	Fatty acid
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization of the United Nations
FFA	Free fatty acids
FID	Free induction decay
FM	Fish meal
FO	Fish oil
GC-MS	Gas chromatography mass spectrometry
GHG	Greenhouse gas
GLM	General linear model
GLMM	General linear mixed model

HCB	Hexachlorobenzene
HDL	High-density lipoprotein
HMDB	Human metabolome database
HSI	Hepatosomatic index
Hz	Hertz
I	Spin quantum
IARC	International Agency for Research on Cancer
J	Scalar coupling constant
LA	Linoleic acid
LC	Liquid chromatography
LCD	Low-calorie diet
LC-MS	Liquid chromatography mass spectrometry
LC-PUFA	Long-chain polyunsaturated fatty acids
LDL	Low-density lipoproteins
LOD	Limit of detection
MAG	Monoacylglycerols
MHz	Mega Hertz
MS	Mass spectrometry
MUFA	Monounsaturated fatty acids
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHST	Null hypothesis significance testing
NL	Neutral lipids
NMR	Nuclear magnetic resonance
OECD	Organisation for Economic Co-operation and Development
OPLS	Orthogonal projections to latent structures
PAHs	Polycyclic aromatic hydrocarbons

PCA	Principal component analysis
PCBs	Polychlorinated biphenyls
PL	Phospholipids
PLS	Partial least squares projection to latent structures
ppm	Parts per million
PUFA	Polyunsaturated fatty acids
RCT	Randomized controlled trial
RF	Radio frequency
SFA	Saturated fatty acids
SGR	Specific growth rate
TAG	Triacylglycerol
TCA	Tricarboxylic acid
TCL	Total cholesterol
TLC	Thin-layer chromatography
TSP-d_4	3-trimethylsilylpropionic acid-d4
Tw	Total weight
UV	Unit variance
VIP	Variable importance in the projection
VLDL	Very-low density lipoproteins
VOs	Vegetable oils
WHO	World Health Organization
Wl	Weight liver
YPD	Yeast extract-peptone-dextrose

1. Introduction

The search for alternative food and feed sources is a focus of research to improve sustainability of the food system and maintain the integrity of ecosystems (El Bilali *et al*., 2019). The study of suitable food/feed ingredients to alter conventional diets is important to reduce the carbon footprint and minimize the risk of non-communicable diseases.

Various food/feed trials have to be conducted to understand the effects of new dietary ingredients on the metabolism of any organism, which eventually add knowledge for nutritional recommendations.

Dietary intervention trials provide the opportunity to investigate the effect of a specific food product on individuals in a controlled environment (Papers I and II). For example, meat by-products are an important source of essential nutrients and have shown hypolipidemic effects in rat and human trials (Chernukha *et al*., 2018; Chernukha *et al*., 2021) (Papers I and II).

The search for more sustainable food systems extends to the question of animal feed production as well as animal production and welfare. Global fish production by aquaculture is expected to increase by 14% in 2030 (FAO, 2022). The long-established fish feed ingredients, fish meal and fish oil, are available in limited amounts and have been partially replaced with sources of plant origin (FAO, 2022). The use of microbial ingredients such as yeast biomass could alleviate this problem of resource limitation, depending on the fish response to the feed, the method of feed production and other factors. In this study, oleaginous yeast was included in the feed of Arctic char (Carlberg et *al*., 2018; Blomqvist et *al*., 2018) (Papers III and IV).

Physiological changes related to nutrient intake in animals (fish) or in humans can be studied at different levels. Regulatory mechanisms of lipids and metabolites are relatively conserved within vertebrates (Peregrín-

Alvarez et *al*., 2009), and allow the application of established analytical methods. This thesis investigated the metabolic and lipid responses in humans and fish related to diet in different trials, using nuclear magnetic resonance spectroscopy and gas chromatography as the main methods.

2. Background

2.1 Metabolomics field

Over the last decades, technological advances in medicine and molecular biology have led to the development of the "multi-omics" fields described as genomics, transcriptomics, proteomics and metabolomics (Figure 1). The study of molecules at different levels allows a holistic approach of physiological and pathophysiological processes happening in a sample or in an organism (Wishart, 2019). The use of technologies such as DNA sequencing and elaborate computational tools to analyze large datasets generated, provides the possibility for a more precise or personalized medicine related to human health and diseases (Chen & Snyder, 2013).

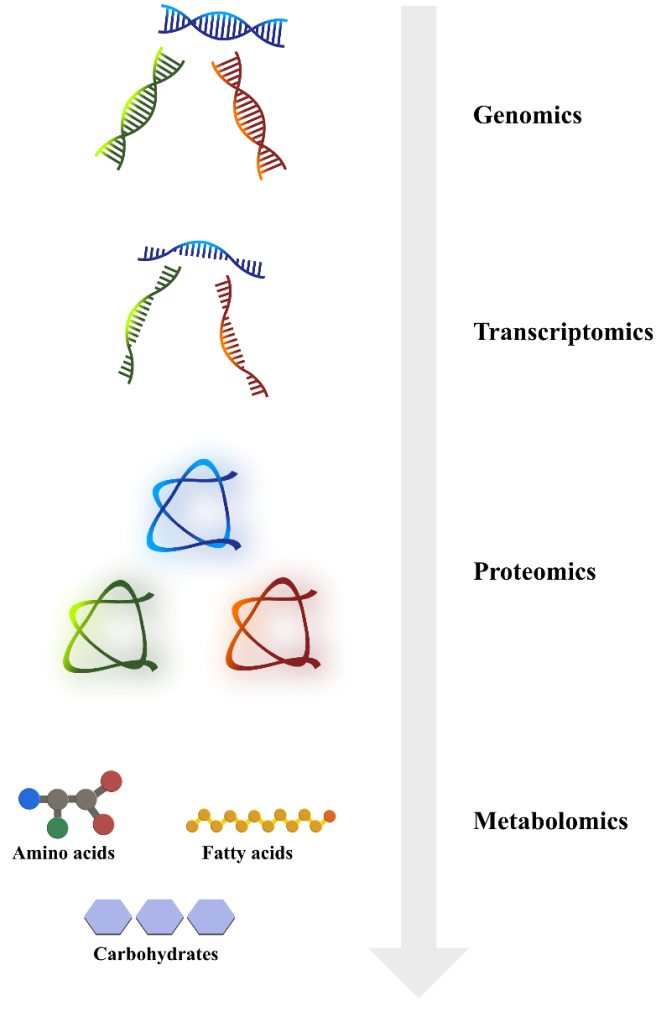

Figure 1. Representation of multi-omics and their associated molecules.

Metabolomics can be defined as the study of small molecules called metabolites of low weight (< 1,500 Da) in an organelle, cell, organism, tissue or biological fluid (Markley *et al*., 2017; Wishart, 2019). The metabolome represents all types of metabolites including fatty acids (FA), amino acids, carbohydrates, nucleotides, vitamins, alcohols and others found in an organism (Wishart, 2019).

Metabolites are substrates, intermediates or end-products of inter- and intra-cellular regulatory processes (Rattray *et al*., 2018). For that reason, metabolomics analyses provide an overview of the physiological reactions occurring at a specific time in a sample, tissue or fluid. The metabolome is the closest level to the phenotype and contributes to the understanding of the gene-environment interaction (Fiehn, 2002; Guijas *et al*., 2018). Internal and external factors such as genes, age, diet, gender, time of the day, geographical location and environment exist and affect the metabolome to different degrees (Wishart, 2019).

The comprehensive identification of metabolic pathways in an organism helps in the effective prevention of modern diseases (Subramanian *et al*., 2020). Classification of diseases into groups could facilitate the identification of biomarkers and understanding of disease etiology (Subramanian *et al*., 2020). Various multi-omics data repositories exist and can be classified by fields: genes with the Human Genome Project, metabolites with the Human Metabolome Project (Human Metabolome Database, HMDB) and proteins with the Human Protein Atlas. Other classifications of multi-omics data repositories include by disease, such as the Cancer Genome Atlas and Clinical Proteomic Tumor analysis (Ren *et al*., 2015; Subramanian *et al*., 2020).

Advanced analytical chemistry techniques such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are applied for the characterization and quantification of metabolites. Common MS methods for metabolomics analyses are liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS) and capillary electrophoresis-mass spectrometry (CE-MS) (Peng *et al*., 2015). As the most widely used NMR approach in metabolomics (Markley *et al*., 2017), one-dimensional (1D) ^{1}H-NMR and basic knowledge of NMR will be presented in the following part.

2.2 Nuclear magnetic resonance spectroscopy

2.2.1 Principles

Nuclear magnetic resonance (NMR) spectroscopy is an analytical tool used to determine the conformation and function of compounds in addition to the molecular composition of a substance. This advanced method allows the study of organisms at the molecular level in diverse types of samples (plasma, cerebrospinal fluid, liver) and in different states of matter (liquid, solid and gas). NMR principles rely on the application of an external magnetic field on nuclei with a nuclear spin quantum (I) equal to or above zero and multiples of ½ (Claridge, 2016). Nuclei with $I = 0$ are called NMR silent and are unable to exhibit NMR signals. Specific nuclei called NMR-active nuclei (^{1}H, ^{13}C, ^{19}F, ^{31}P) with $I \neq 0$, have an angular momentum P, a charge and spin on an axis, creating a nuclear magnetic field or magnetic moments (μ) with the following formula:

$$\mu = \gamma P \qquad \text{(Eq. 1)}$$

with γ the *magnetogyric ratio* (constant)

When an external and static magnetic field (B0) is applied to the nuclei, the magnetic moments of the nuclei become aligned with the external magnetic field with $2I + 1$ possible directions. The movement of rotation induced by the static field on the magnetic moment is referred to as the Larmor precession. The rate of the Larmor precession, defined as the Larmor frequency of the nucleus, refers to the resonance frequency of a spin. For proton nuclei, the orientation of the magnetic moment is parallel (the α state) to or anti-parallel (the β state) to the static field with different energy levels. The parallel alignment of the magnetic moments to the applied field has a lower energy compared to the anti-parallel orientation. At the equilibrium, there is an excess of spin nuclei at the lower energy of spin state (α) resulting in a *bulk magnetization vector* M (Figure 2). This magnetization or NMR moment is observed when there is a difference in distribution of magnetic moments between the possible spin states. The *vector model* of NMR or Bloch vector model is a simplified theory on the collection of nuclear spins (Claridge, 2016).

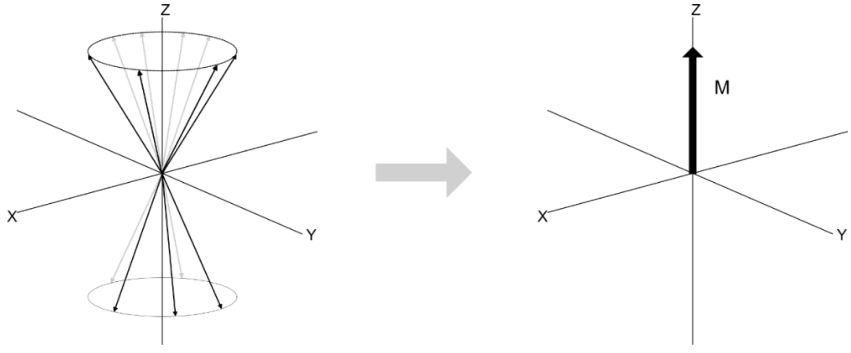

Figure 2. Illustration of Bulk magnetization (Bloch vector model). Adapted from Claridge (2016).

To create resonance, a radio frequency (RF) pulse is applied along the x-axis (Figure 3) for a specific amount of time. The frequency of the applied pulse (B1) must exactly match the spin Larmor frequency to meet magnetic resonance conditions (Claridge, 2016). During the pulse application, the RF electromagnetic field will move the bulk magnetization vector M from the z-axis towards the y-axis. The application of a pulse at 90 degrees (90^{o}_{x} or until the vector reaches y-axis) equalizes the populations of the α and β states. There is no net magnetization along the z-axis and the spins possess a phase coherence. The magnetization along the x-y plane will induce a signal intensity detected by a coil surrounding the sample. Over time, the bulk magnetization vector will move back to its initial position (equilibrium state) along the z-axis while rotating (Claridge, 2016). The rotations of the bulk magnetization while returning to the z-axis will induce a weak oscillating voltage recorded as the observed NMR signal (Figure 3). NMR signal decays with time during the return to equilibrium (relaxation) and produces free induction decay (FID).

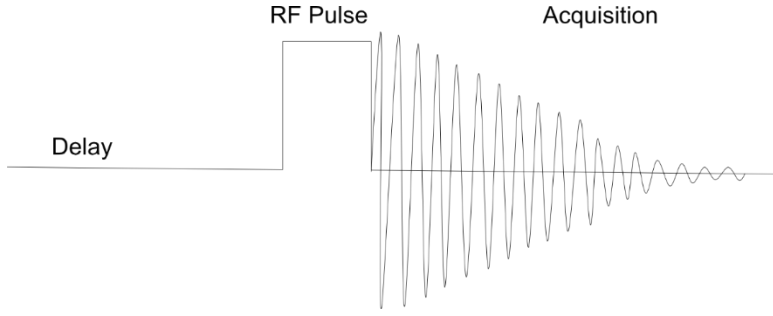

Figure 3. Schematic representation of a single pulse sequence. Adapted from Mardor & Cohen (2002).

The data produced as FID is expressed in the time domain data and needs to be processed into frequency data using the function Fourier transformation. Fourier transformation changes the FID to a resonance frequency spectrum with signals along a chemical shift scale expressed in parts per million (ppm). The chemical shift is defined as the difference in resonance frequency between experimental nuclei in a different chemical environment and nuclei from a reference compound (Jameson, 1996).

In the case of the ^{1}H-NMR spectrum, signals represent a proton or group of protons within a molecule in its specific chemical environment (Jameson, 1996). The interaction of a proton within a molecule will create additional resonance, translated into multiple signals on a spectrum. The interaction is known as spin-spin *J*-coupling with a magnitude expressed as the coupling constant *J* in Hertz (Sternhell, 1969). The splitting pattern of nuclei is formulated as $2nI + 1$ with n the number of neighboring protons and *I* the nuclear spin quantum number (Hatzakis, 2019). Multiplicity of signals and their overlapping are important points to consider when quantifying metabolites (Figure 4).

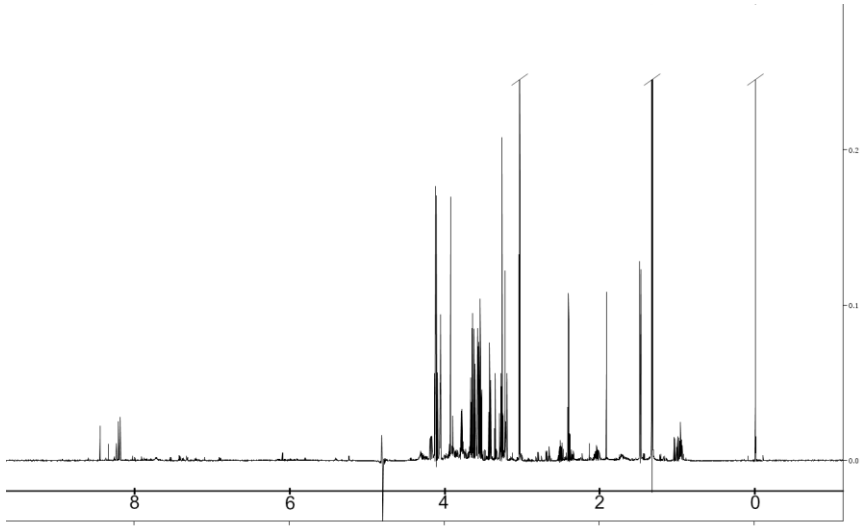

Figure 4. Example of a ^{1}H-NMR spectrum of meat product analysis with NMR.

Advantages of NMR are its non-destructive nature, high reproducibility and a minimal sample preparation with very few steps, allowing a high-throughput screening. The NMR technique can be qualitative or quantitative and applied to a diverse range of substances such as biological fluids (urine, plasma, serum) and solids (biopsy). NMR is highly quantitative and allows the detection of hundreds of metabolites per measurement (Nicholson & Lindon, 2008; Nagana Gowda & Raftery, 2015; Markley *et al*., 2017).

In metabolomics studies, NMR methods can be considered versatile as the combination of 1D and 2D NMR experiments allows the structure identification of unknown metabolites in complex biological samples (Nagana Gowda *et al*., 2015). Dynamic changes in the metabolome related to diseases and treatments can be observed with NMR, leading to the development of new therapeutic approaches. Drawbacks of the NMR methods compared to LC-MS are low sensitivity, resolution and limit of detection. The sensitivity of NMR can be improved with the use of a cryoprobe (Nicholson & Lindon, 2008; Nagana Gowda & Raftery, 2015; Markley *et al*., 2017).

The NMR technique can be applied to foods to provide information on the complexity of the food products in relation to their composition or environmental impact such as process, transportation and storage (Hatzakis,

2019). The versatility of the NMR technique in food studies has been shown with, for example, the quantification of compounds related to lipid oxidation in mayonnaise (Merkx *et al*., 2018), quantification of carbohydrates in honey (Schievano *et al*., 2017), or the detection of biogenic amines in fish fillets (Shumilina *et al*., 2015).

2.2.2 Spectral processing

Once a ^{1}H-NMR spectrum is produced after Fourier transformation of the FID, adjustments are needed before the identification of metabolites. Common pre-processing steps required are baseline correction, signal alignment, data normalization and scaling (Smolinska *et al*., 2012).

Baseline correction should be performed as distortions can occur due to spectral artifacts. The correction can be manual or automated using polynomial-fitting or algorithms (Smolinska *et al*., 2012).

Signals from the same metabolite can shift their position on each spectrum from one sample to another due to a change of pH, temperature, ionic strength, salt concentrations, instrumental variation and other factors. Spectral alignment is corrected by setting the internal standard at 0 ppm for all spectra. Further correction alignment can be performed by adjusting the signal from the database with the experimental one for each metabolite in each sample (*J*-coupling information). One way to minimize this shift effect is to use a buffered solution, usually phosphate buffer for a desired pH of 7 (Weljie *et al*., 2006; Smolinska *et al*., 2012).

Data normalization is carried out to adjust differences in overall concentrations of metabolites (Smolinska *et al*., 2012). The spectral intensities are adjusted according to the dilutions realized during extraction or filtration steps. For example, urine samples usually require data normalization due to large variations in urine volume as a result of a drug or treatment factor (Craig *et al*., 2006).

Scaling represents an adjustment according to the samples in order to equalize the variances using different techniques such as autoscaling, mean centering, pareto scaling and unit variance scaling, logarithmic scaling (Craig *et al*., 2006; Smolinska *et al*., 2012).

After pre-processing the data into comparable spectra among samples and compounds, metabolomics analyses can be applied. Diverse approaches

(targeted, untargeted) and methods (manual or automated) of metabolomics analyses are available and will be introduced in the next part.

2.3 Metabolomics analyses

2.3.1 Definitions

An untargeted approach is defined as the analysis of all detectable known and unknown compounds (Cajka & Fiehn, 2016; Gorrochategui *et al*., 2016). This approach allows the analysis of a large number of metabolites and gives the opportunity to identify new compounds or candidate biomarkers (Gorrochategui *et al*., 2016). Untargeted metabolomics relies on the comparison of peaks between groups of samples (control/treatment) with the identification limited to compounds with discriminative features.

Targeted quantification of metabolites focuses on the accurate measurement of selected metabolites from a database and uses internal standards or calibration curves as references (Cajka & Fiehn, 2016; Gorrochategui *et al*., 2016). Common internal standards include 3-trimethylsilylpropionic acid-d4 (TSP-d4), 2,2-dimethyl-2- silapentane-5-sulfonic acid sodium salt and tetramethylsilane (Smolinska *et al*., 2012).

2.3.2 Targeted and untargeted metabolomics

The selection of targeted or untargeted methods applied to a study depends on different factors including the type of analytical instruments and the concentration of the metabolites of interest.

When differentiating the two approaches, untargeted metabolomics would typically require minimal sample treatment to maintain a broad range of metabolites in the samples and allow a fast processing. With targeted quantification, extraction or filtration steps are needed for accurate and sensitive measurements (Cajka & Fiehn, 2016).

Removing proteins is recommended for quantitative studies using ultrafiltration with low molecular weight cut-off filters or protein precipitation with organic solvents to improve the quantification (Nagana Gowda *et al*., 2015).

2.3.3 Untargeted approach or metabolic fingerprinting application

Untargeted NMR metabolomics or metabolic fingerprinting is commonly carried out with spectral binning or bucketing characterized as the division of spectra into segments called bins or buckets of equal width (usually between 0.04 and 0.05 ppm per bin). For each bin, the peak intensity or area under the curve is measured and analyzed with multivariate statistical analysis. Binning process reduces the dimensionality of data by transforming the raw data into a matrix of X-variables and allows the removal of unwanted spectral regions such as the water region. Limitations of the binning process are a reduction of spectral resolution and potential signals detected across multiple bins (Smolinska *et al*., 2012; Gorrochategui *et al*., 2016).

2.3.4 Targeted approach or metabolic profiling application

After the pre-processing of data, manual quantification can be performed using deconvolution. Metabolites to quantify are selected from a database such as in the Chenomx NMR Suite professional software (Edmonton, Canada). For each metabolite of interest available in the database, the area of each signal representing a specific metabolite from the database is fitted to the height of the experimental peak at the highest point (Weljie *et al*., 2006; Smolinska *et al*., 2012). In addition, the same signal is compared between samples and adjusted for a similar position of the chemical shift at a specific pH set up for the experiment (commonly a pH of 7).

Targeted quantification of metabolites performed manually is time-consuming when a large number of metabolites are measured (Weljie *et al*., 2006; Gorrochategui *et al*., 2016). Moreover, the detection of metabolites with 1D ^{1}H-NMR results in a large amount of signals. With high number of signals, overlapping peaks appear in the spectrum and the quantification becomes less reliable.

The quantification process can be automated with computational methods such as automated quantification algorithms AQuA (Röhnisch *et al*., 2018) or Dolphin (Gómez *et al*., 2014), the statistical linear model ASICS (Tardivel *et al*., 2017), Bayesian model selection BATMAN (Hao *et al*., 2012; Hao *et al*., 2014) or BQuant (Zheng *et al*., 2011) or BAYESIL (Ravanbakhsh *et al*., 2015).

The AQuA algorithm was selected in this thesis for an automated quantification of plasma metabolites in human. AQuA uses spectral data obtained from a database including one metabolite per spectrum library to

quantify metabolites in experimental spectra while accounting for interferences (Röhnisch *et al*., 2018; Röhnisch *et al*., 2021). This method includes manual spectral pre-processing, identification of metabolites using previous literature (Nagana Gowda *et al*., 2015) and databases ChenomX and HMDB. The detection and quantification of metabolites using advanced computational techniques allow a faster and more precise metabolic study of an organism, which can be implemented in the clinical research field.

Clinical research is characterized as all investigations related to diseases, prevention, treatment, diagnosis, health enhancement or maintenance including health services research, epidemiologic studies and clinical trials (Ioannidis, 2016). Clinical trials are complex human studies conducted in order to explain a disease diagnosis, prognosis, and treatment. Broad information on human trials is described in the following paragraph.

2.4 Clinical trials

A clinical trial is defined as "*a prospective study comparing the effects and value of intervention (s) against a control in human beings*" (Friedman *et al*., 2015). Clinical trials are performed to determine the applicability of clinical research (Friedman *et al*., 2015). These trials should ideally be followed forward in time (prospective) with intervention techniques conducted on participants. The intervention techniques include diagnosis, prevention or educational training, the application of therapeutic drugs, medical devices, diet and more (Friedman *et al*., 2015). Clinical trials are designed following a study protocol including objectives of the study, hypotheses, inclusion and exclusion criteria, sample size estimation, details on participant enrollment, informed consent, interventions, follow up and adverse effects.

An ideal clinical trial contains randomization of the participants, the application of an intervention to a treatment group against a control group and a double-blind process (Friedman *et al*., 2015). However, each clinical trial is specific to a population, a disease or a research question, requiring compromises in the design. Different types of designs exist with, for example, parallel or cross-over designs (Friedman *et al*., 2015). A parallel design is characterized as control and treatment groups of individuals followed at the same time and remaining in the same group throughout the study. In a cross-over trial, each group of patients received the control

conditions and the treatment conditions at separate times with washing out periods in between (Friedman *et al*., 2015).

One major advantage of conducting clinical trials is the ability to test and understand the environmental effects on participants when the genetics factors are already studied (Friedman *et al*., 2015). Moreover, clinical trials examine the incidence of adverse effects or complications from an intervention (Friedman *et al*., 2015). Examples of limitations in these studies include participant drop out, human bias, presence of confounding factors and length of a study as diseases might require decades to develop (Friedman *et al*., 2015).

2.5 Lipids and lipid metabolism

2.5.1 Definition, structure and biochemistry

The word lipid comes from the Greek word "*lipos*" and means fat (Jones, 2008). Lipids are broadly described as a heterogeneous group of hydrophobic or amphipathic molecules. Lipids are compounds found ubiquitously in living organisms and their functions are diverse. Lipids can act as structural components of cell membranes, energy storage sources and as signaling molecules (Fahy *et al*., 2011). Lipids include true fats (esters of FA and glycerol), lipids (phospholipids, waxes), sterols (for example, cholesterol (CL) and ergosterol) and hydrocarbons (squalene, terpene, carotene) (Jones, 2008; Fahy *et al*., 2011; Gunstone *et al*., 2007). Based on polarity, lipids are divided into two groups, polar lipids and nonpolar or neutral lipids (NL). Polar lipids include phospholipids (PL), sphingolipids and glycolipids. NL contain free fatty acids (FFA), monoacylglycerols (MAG), diacylglycerols (DAG), triacylglycerols (TAG), steryl esters and wax esters (Jones, 2008; Vance & Vance, 1996).

Based on their complexity, lipids can be further differentiated into simple lipids, compound lipids and derived groups lipids. Simple lipids are esters of FA with alcohols groups and act as energy stores in the adipose tissue such as FFA, MAG, DAG and TAG. Compound lipids are FA with at least an alcohol group and a different group often presented as phosphorus or nitrogen. These lipids can be found in the central nervous system as phospholipids and glycolipids. Derived lipids are other FA produced from

the hydrolysis of simple and compound lipids. Common derived lipids are CL, steroids, terpene and bile acids (Jones, 2008; Fahy *et al*., 2011).

Lipids have diverse functions in an organism as sterols, including CL and PL, are found in cell membranes and play an important role in the regulation of cell membrane fluidity (Harayama & Riezman, 2018). TAG act as storage of lipids as droplets while DAG regulate diverse cellular functions when binding to domains of protein kinase C (Murphy & Vance, 1999; Sakane *et al*., 2007). Sphingolipids can modify the order of the lipids in a cell membrane as well as its curvature and thickness. In addition to their role as structural components, sphingolipids are able to form specialized membrane domains called lipid rafts. Lipid rafts are able to interact with membrane proteins and modify membrane signaling and intracellular trafficking (Breslow & Weissman, 2010).

Lipids are mainly composed of FA and glycerol (Gunstone *et al*., 2007). FA are characterized by a carboxyl group (COOH), an aliphatic chain of variable length (CH_2), and a methyl group (CH_3). FA are commonly found in vertebrates with an even number of carbon atoms and in the range of 12-22 carbon atoms in the *cis*-configuration. Saturated FA (SFA) are FA with a saturated carbon chain whereas unsaturated FA show at least one double bonds (Figure 5). The total number of double bonds in FA can vary from zero and six. Monounsaturated FA (MUFA) are characterized by a single double bond. FA with at least two double bonds are called polyunsaturated FA (PUFA). Delta (Δ) nomenclature is a system used to describe the location of a double bond in relation to the carboxyl group (Cook, 1991). For example, the oleic fatty acid (Figure 5) holds a double bond between carbons 9 and 10 or in the Δ9 position introduced by the enzyme Δ9 desaturase (Cook, 1991).

The length and level of unsaturation of FA allow a classification of FA according to their chemical characteristics. The FA classes are divided into three groups: SFA, MUFA and PUFA (Gunstone *et al*., 2007). An additional subdivision exist in the PUFA class according to the position of the first double bond from the methyl group. PUFA with a first double bond at position 3 from the methyl end are called n-3 FA while PUFA with a first double bond at position 6 from the methyl end are called n-6 FA. Long-chain PUFA (LC-PUFA) represent a PUFA with a chain length of at least 18-20 carbon atoms.

This FA classification is broadly applied in nutritional studies to understand the composition and structure of food as well as the relation between health and food consumption.

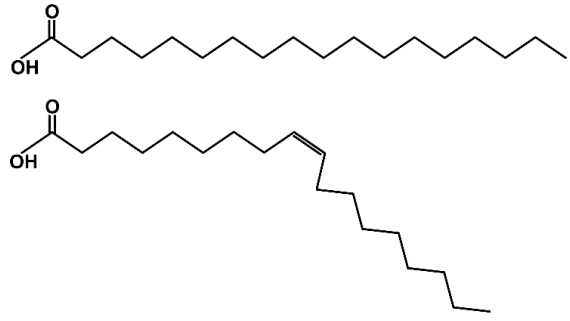

Figure 5. Configuration of saturated stearic acid (above) and unsaturated oleic acid (below). Adapted from Cook (1991).

2.5.2 Regulation in humans and fish

Human and fish satisfy their daily lipid requirements via dietary intake or via lipid biosynthesis in the liver and adipose tissue. Biosynthesis of FA *de novo* occurs from non-lipid carbon sources with first the conversion of acetyl-CoA to malonyl-CoA using acetyl-CoA carboxylase and secondly with the conversion of malonyl-CoA to palmitic acid and small amounts of stearic acid using FA synthase complexes (Cook, 1991; Sargent *et al*., 2003). Vertebrates have a conserved mechanism of desaturation and elongation of FA (Cook, 1991). MUFA are synthesized in mammals and fish by the oxidative desaturation of SFA with Δ9 desaturase as the main enzyme (Cook, 1991; Tocher, 2003). Vertebrates are unable to introduce the second double bond beyond the Δ9 position. Plants contain additional enzymes capable of desaturation at position Δ12 and Δ15, producing n-3 and n-6 FA. Human and fish are reliant on dietary intake of essential C-18 carbon PUFA linoleic acid (C18:2 (n-6); LA) and α-linolenic acid (ALA; C18:3 (n-3)). From LA and ALA to LC-PUFA, diverse reactions of desaturation and elongation are required in human and fish (Figure 6) (Cook, 1991; Voss *et al*., 1991; Tocher, 2003).

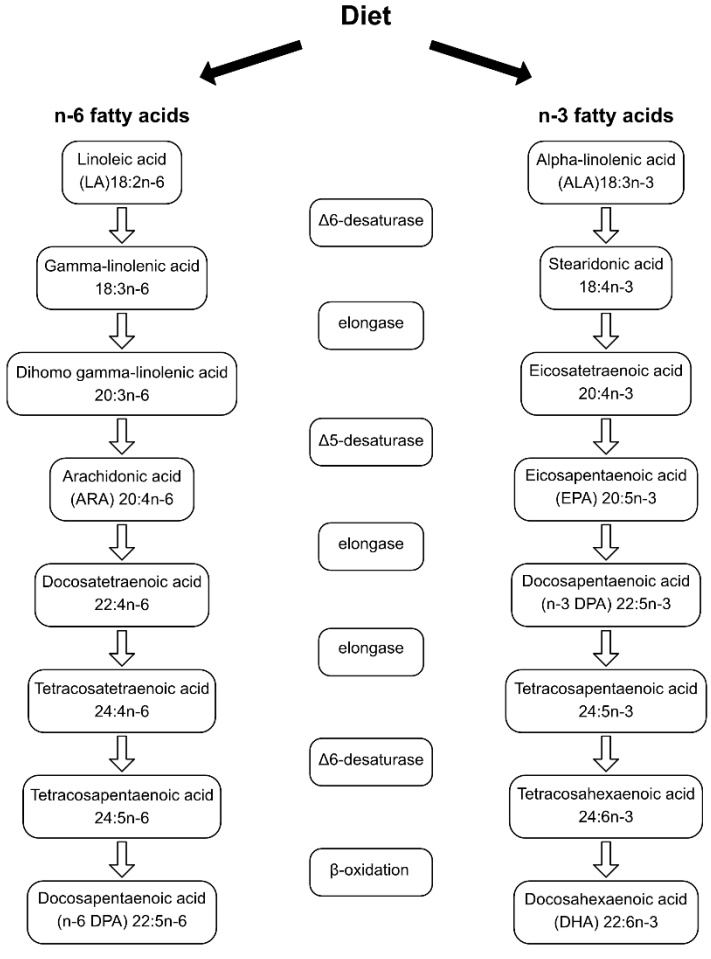

Figure 6. Fatty acids n-6 and n-3 elongation and desaturation pathway. Adapted from Voss *et al*. (1991) and Schiller Vestergren (2014).

Among the LC-PUFA, eicosapentaenoic acid (EPA), n-3 docosapentaenoic acid (n-3 DPA) and docosahexaenoic acid (DHA) are synthesized in low quantities and their conversion competes with n-6 PUFA for the same elongation and desaturation enzymes (Sargent *et al*., 2003). To ensure proper physiological functions in human and in fish, these FA must be acquired from the diet (Sargent *et al*., 2003).

Hydrophobic molecules such as lipids are transported from one organ (mainly liver) to another organ via plasma as FFA or lipoproteins.

Lipoproteins are constituted of lipids and proteins and divided into four groups: chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Eisenberg & Levy, 1975; Tocher, 2003).

These macromolecular complexes from endogenous or exogenous sources transport TAG, CL, PL and proteins. Chylomicrons and VLDL are mainly composed of TAG and transport dietary fats, CL and liposoluble nutrients. LDL are mostly formed with CL while HDL contain CL and PL (Eisenberg & Levy, 1975; Ginsberg, 1998).

Remnants of triglyceride-rich lipoproteins such as chylomicrons and VLDL have been associated with pro-inflammatory effects and pro-atherogenic effects, obesity and type 2 diabetes, whereas HDL have been shown to exert anti-atherogenic effects (Ginsberg *et al*., 2021). Structural modification of LDL, for example, by oxidation or glycation has been linked to inflammation (Lee *et al*., 2021). Nevertheless, the regulation of intestinal lipoproteins is highly complex and their effects on atherosclerotic cardiovascular disease is not fully understood (Ginsberg *et al*., 2021).

Other factors related to lipids and health are the types and quantities of FA provided by the diet. FA are heterogeneous in their chain length and levels of saturation/unsaturation, leading to diverse outcomes on health.

As mentioned earlier, FA can be divided into the groups SFA, MUFA and PUFA. SFA such as lauric acid and palmitic acid have been correlated with inflammatory responses in the body as a result of activation of pro-inflammatory cytokines. SFA have been associated with an increase of total CL, LDL and insulin resistance and might be involved in higher incidence of cardiometabolic diseases (Calder, 2015). Nevertheless, the effect of SFA on health depends on the type of SFA as pentadecanoic acid, heptadecanoic acid and some very long chain SFA such as arachidic acid or lignoceric acid have shown an opposite association with the incidence of type 2 diabetes (Calder, 2015).

Among MUFA, oleic acid is the FA predominantly represented in foods and organisms (Calder, 2015; Mozaffarian, 2016). Oleic acid and other MUFA have been shown to lower blood pressure, improve glucose and insulin sensitivities when replacing SFA. The cardioprotective effect of oleic acid and other MUFA from the diet is unclear since they are often introduced in the diet via vegetable oils (olive oil) containing antioxidants such as polyphenols (Calder, 2015; Mozaffarian, 2016).

LA and ALA are essential FA, the most common PUFA and are produced in plants (Mozaffarian, 2016). The n-3 FA hold anti-inflammatory properties while n-6 FA are pro-inflammatory. The effects of LA on health are unclear as pro-inflammatory effects have been shown in rodents but not in humans (Mozaffarian, 2016). Nevertheless, no direct association between LA intake and pro-inflammatory response has been demonstrated. Instead, lower LDL concentrations have been observed when replacing SFA with LA (Calder, 2015; Mozaffarian, 2016). Arachidonic acid (ARA), a product of LA (Figure 6), promotes inflammatory responses via NF-κB pathway and is an eicosanoids precursor. Prostaglandins, thromboxanes and leukotrienes are examples of eicosanoids and have essential roles in the regulation of pain and inflammation, immune response, cancer progression and more (Calder, 2015).

In addition to the type of PUFA, the ratio between n-6 and n-3 is important to reduce adverse effects on human health. ALA obtained from plant sources can be converted to EPA and then n-3 DPA and DHA (Figure 6). This elongation and desaturation pathway shares the same enzymes used for the conversion of LA to n-6 DPA. This competition for the metabolism of n-3 FA and n-6 FA is affected by the n-6/n-3 ratio as the LA is a dominant FA of the diet.

The current recommendation for n-6/ n-3 ratio is 4 to 1 (Simopoulos, 2002) and the actual ratio in the human diet is deficient in n-3 FA with an n-6/n-3 ratio varying between 20:7 (Calder, 2015) and 20:1 (Simopoulos, 2016). An increase in n-3 FA in the diet is required to improve the ratio to the recommended value.

As previously mentioned, LC-PUFA are metabolized in low concentrations in humans and fish from ALA. An increase of EPA, n-3 DPA and DHA dietary intake have demonstrated important health benefits with improved cardiovascular risk factor, inflammatory response, brain and visual functions (Calder, 2015). Seafood and fatty fish are adequate sources of EPA, n-3 DPA and DHA (Sargent *et al*., 2003; Calder, 2015). To provide fish and fish products to a growing population, aquaculture has developed rapidly during the past decades (FAO, 2022).

2.5.3 Aquaculture

Aquaculture has increased to provide essential nutrients of high quality such as proteins and LC-PUFA to the growing human population at a time

when wild capture fisheries are stagnating (FAO, 2022). With an increasing human population, there is a need to find more efficient food production systems while ensuring their sustainability. In 2020, aquaculture supplied 49% of the fish production worldwide, representing 88 million tonnes.

Several areas of aquaculture require improvements to expand production in a long-term perspective. One challenge is the eutrophication in coastal waters due to the release of an excessive amount of nutrients (often nitrogen and phosphorus) leading to algal blooms, hypoxia and loss of diversity (Carpenter *et al*., 1998; Streicher *et al*., 2021). Another challenge in aquaculture is the monitoring of fish health and welfare. Fish feed composition is of importance for animal welfare and for the nutritional quality of the fish related to human consumption.

Fish nutrition is a major challenge in the sustainability of aquaculture. Carnivorous fish are of high trophic level and require fish-based ingredients such as fish meal (FM) and fish oil (FO) to meet their protein and lipid needs. FM and FO are obtained from forage fisheries in limited amounts in a growing fish production system. The use of FO and FM is no longer sustainable and efforts are made for their replacements (Tocher, 2015). Studies have been conducted to include oils and proteins from plants to the fish diet at different percentages (Thomassen & Røsjø, 1989; Bell *et al*., 2005; Pickova & Mørkøre, 2007; Torstensen *et al*., 2008; Pettersson *et al*., 2009). The partial replacement of fish-based ingredients such as FO with plant ingredients has led to beneficial effects such as a reduction of contaminant levels in salmon including polychlorinated biphenyls (PCBs) and dioxin (Bell *et al*., 2005; Berntssen *et al*., 2005). Nevertheless, complete replacement of FM and FO from the feed of carnivorous fish is not attainable and the partial substitution by plants has shown its own limitations with a reduction of LC-PUFA levels in fish fillets (Bell *et al*., 2005; Berntssen *et al*., 2005; Sprague et al., 2016; Monge-Ortiz *et al*., 2018).

Microalgae, macroalgae, bacteria, yeast and insects as well as marine invertebrates are potential candidates as a new source of nutrients due to their diversity in macro- and micronutrient composition in addition to their fast growth. These alternatives are capable of producing large amount of nutrients on diverse substrates, often by-products, resulting in a fast biomass production (Sprague *et al*., 2017; Gamboa-Delgado & Márquez-Reyes, 2018; Liland *et al*., 2021). Advantages of using microbial organisms for feed production are an important oil production, a fast growth (within days), and

the possibility to grow on substrates of low-value (Abeln & Chuck, 2021). The suitability of a new fish feed ingredient is commonly investigated by characterizing the analytical composition of the novel candidate, the feed digestibility and the effects on fish performance (Roques *et al*., 2020). The fish feed content is estimated to ensure appropriate percentages of nutrients for fish health. Similar to terrestrial animals, fish require essential amino acids and FA, vitamins and minerals to maintain health, avoid stress and diseases (Oliva-Teles, 2012). Fish performance is evaluated using specific indexes such as condition factor, hepatosomatic index (HSI), specific growth rate (SGR) and others (Blomqvist *et al*., 2018; Roques *et al*., 2020). The evaluation of fish performance indicates the overall response of the fish to a new feed. Nevertheless, information on fish physiology at the molecular level in relation to a new feed is not provided with performance indexes.

The application of omics-based methods for fish production adds new knowledge to aquaculture research by providing qualitative and quantitative information on genes, proteins and metabolites in fish (Lulijwa *et al*., 2022). Studies on metabolomics are developing in aquaculture to understand the relationships between gene expression and biochemical phenotype. A metabolomics approach can be combined with conventional methods used in fish feeding trials to explore the effect of a new feed ingredient on fish performance and health in a more comprehensive manner (Alfaro & Young, 2018; Roques *et al*., 2020).

2.6 Statistical analyses

The analysis of metabolites produces a complex and large amount of information requiring different statistical strategies to account for multiple factors. Metabolomics data can be analyzed with univariate and multivariate techniques, depending on the experimental design of the study, the hypotheses, the number of variables and observations. Some statistical methods applied in metabolomics will be introduced in the following part.

Null hypothesis significance testing (NHST) is a commonly used approach in metabolomics. NHST refers to the application of statistical tests to compare groups, using a null hypothesis (absence of difference between groups) tested against the alternative hypothesis (presence of a difference between groups) with a pre-defined level of significance (Saccenti *et al*.,

2014). A significance level of 5% is typically used to reject the null hypothesis (p-value < 0.05).

The application of relevant statistical tests is essential for a correct interpretation of metabolomics data. The choice of statistical test depends on the design of the study and on the data dimension (Rattray *et al*., 2018). High-dimensional data is defined by a larger number of variables compared to the number of observations (Bühlmann & van de Geer, 2011). Low-dimensional data shows the opposite with a larger number of observations for fewer variables.

Univariate methods are suitable in metabolomics analyses for the interpretation of low-dimensional data. Mean and variance of a single variable are commonly used in univariate methods (Saccenti *et al*., 2014). Analysis of variance (ANOVA) and t-test are two available options to compare differences between groups (Ren *et al*., 2015). In cross-over studies, repeated measure ANOVA or paired t-test can be performed (Westerhuis *et al*., 2010). For all these univariate methods, normal distribution of the data is assumed (Ren *et al*., 2015).

To test hypotheses on a non-normally distributed data, non-parametric or "distribution-free" methods are possible to implement. Non-parametric methods are based on the ranking or classification of the observations and on the comparison between the sum of ranks between groups (Siegel, 1957). For instance, the Wilcoxon rank sum test may be used to compare two independent samples based on the median difference (Wilcoxon, 1945; Mann & Whitney, 1947).

A broader statistical method called general linear model (GLM) includes ANOVA and linear regression. The term "general" of GLM refers to the capacity of the model to integrate multiple variables. These variables can be continuous or categorical (Rutherford, 2011). In a GLM, variables are considered fixed when all experimental conditions during the sampling are included and variables are assumed to be measured without error. A general linear mixed model (GLMM) relies on the same principle as a GLM with the addition of random variables. Variables are defined as random when the experimental conditions are applied on a sample of the population and the outcome of the analysis can be generalized to the population (Rutherford, 2011). For GLM and GLMM, normal distribution of the residuals is assumed (Christensen, 2002). A generalized linear model is extensive of the GLM and includes other types of regression such as binomial or Poisson regressions.

A generalized linear mixed model is a more flexible approach to a generalized linear model by including random factors (Bolker *et al*., 2009).

Limitations exist with the application of univariate statistics in metabolomics analyses. A considerable number of metabolites can be detected and tested for significance individually. With a large number of metabolites detected, the number of tests increases. When an increasing number of tests are performed, a higher probability of false positives occurs. Correction for multiple testing is needed to reduce the false positives numbers. For example, the Benjamini and Hochberg procedure or the more conservative Bonferroni correction can be used to correct for multiple testing (Dunn, 1961; Benjamini & Hochberg, 1995; Saccenti *et al*., 2014).

For high-dimensional data, multivariate methods can be a more appropriate choice. Multivariate statistical analysis provides information on the relationship between several variables X (such as metabolites) or between variables X and one or several variables Y (outcome) (Saccenti *et al*., 2014; Ren *et al*., 2015). Multivariate methods can be divided into two groups, the unsupervised methods and supervised methods. Unsupervised methods are solely explained by X-variables and allow a summary of the data. Supervised methods include both X-variables and Y-variables to classify and predict associations between variables (Ren *et al*., 2015).

Principal component analysis (PCA) is an unsupervised method used as an explanatory tool to reduce complexity in a dataset and detect trends (Rattray *et al*., 2018). PCA is achieved with the linear projection of X-variables (metabolites measured) into several principal components of lower dimensional space at orthogonal positions (Wold, 1987; Abdi & Williams, 2010; Saccenti *et al*., 2014). The first two components are the most explanatory. Within the projected data, the two principal components will form a plane in K-dimensional variable space (Figure 7). The coordinates of projected variables are represented as vectors along the plane and called scores (t, for *i* observations). Other vectors explaining the correlations between the principal component and the original variables are called loadings (p, for *j* variables) (Wold, 1987; Sartorius, 2020).

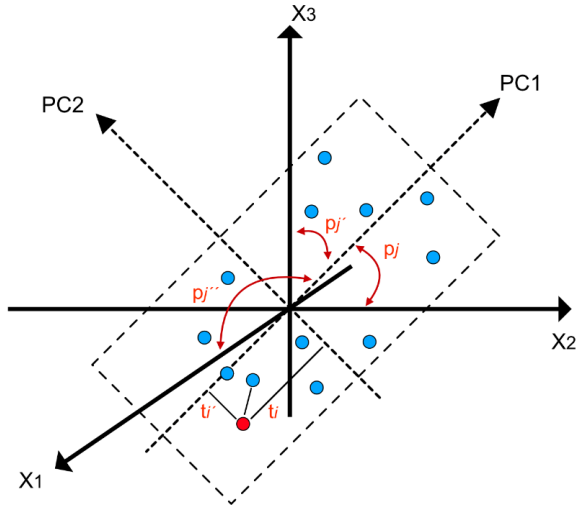

Figure 7. Principal component theory. Projection of observation *i* (red dot), distance to PC1 and PC2 (scores), orientation (red arrow, loadings for *j* variables). Adapted from Sartorius ® (2020).

Supervised methods include the partial least squares projection to latent structures (PLS) (Wold *et al*., 2001). PLS is based on multivariate regression analysis with variables X and Y. Similar to PCA, PLS describes a projection of the data into components with X-variables creating scores and loadings to interpret the correlation between variables. The linear combination of the original variables X and Y creates new variables called latent variables (Wold *et al*., 2001; Bylesjö *et al*., 2006; Ren *et al*., 2015).

PLS can be further developed into other models such as non-linear modelling, hierarchical modelling or PLS-discriminant analysis (PLS-DA). PLS-DA is differentiated from PLS by the response variables Y characterized as categorical variables (Bylesjö *et al*., 2006; Westerhuis *et al*., 2008).

PLS can be extended to orthogonal projection to latent structures (OPLS) (Trygg & Wold, 2002; Bylesjö *et al*., 2006; Westerhuis *et al*., 2010). With OPLS, the systematic variation in the X-variables not correlated to variables Y (or orthogonal to Y) is separated from the X-variables correlated to Y-variables to provide an easier interpretation than regular PLS. The removal of orthogonal variables or "orthogonal correction" is achieved with preprocessing and filtering methods including transformation, centering and

scaling of the X-variables (Trygg & Wold, 2002). Similar to PLS, the Y-variables in OPLS can be categorical (OPLS-DA).

Using an appropriate number of orthogonal components to fit a model reduces the risk of overfitting. Cross-validation (CV) and response permutation testing methods can be used to test the significance of the principal components (Wold *et al*., 2001; Eriksson *et al*., 2008). CV estimates the predictive significance of a model using Y variables. Cross-validated analysis of variance (CV-ANOVA) compares the size of residuals of two models created within the same dataset. Permutation tests are based on the random permutation of the order of elements from Y-variables with X-variables fixed. CV-ANOVA and permutation tests are indicators of the reliability of PLS or OPLS models (Eriksson *et al*., 2008).

Multivariate methods can be improved with several steps of data pre-processing/transformation such as normalization, scaling and centering (Wold *et al*., 2001). Normalization of data is achieved with a logarithmic transformation to obtain a more symmetrical distribution of the X- and Y variables (Wold *et al*., 2001). Scaling of the data gives more weight to informative X-variables and thereby highlights important Y-variables. In the absence of information about the importance of variables, the common approach is scaling to unit variance (UV). UV is calculated by the division of the scaling weight of each variable by their standard deviation. Data mean centering is performed with the subtraction of the averages from all variables included in X and Y (Wold *et al*., 2001).

The identification and removal of outliers is an important part of statistical analyses. In multivariate statistics, a PC scores plot can be used for the visual representation of outliers via Hotelling´s t-squared statistics (t^2) (Hotelling, 1931). Hotelling´s t^2 statistic is a generalization of Student´s t-distribution and is used as a detection tool to identify strong outliers. Another approach applied in multivariate and univariate statistics consists of investigating the normal probability plot of the residuals from the response variables and identifying data points deviating from the line representing the theoretical distribution (Wold *et al*., 2001).

3. Objectives and hypotheses

The main objective of this thesis was to evaluate metabolomics changes occurring in complex systems as a response to food/feed consumption. To identify these changes, the following sub-objectives were developed:

- To compare the nutritional composition of a meat product containing aorta and heart tissue formulated in a laboratory and a commercially available product of comparable category (Paper I).

- To evaluate the effect of a meat product based on aorta and heart tissue on the metabolite levels of patients during a clinical trial (Paper II). The hypothesis was a change in plasma metabolite levels and in blood lipids towards a hypolipidemic effect after the consumption of a pork-based product in individuals showing atherosclerosis symptoms.

- To evaluate the effect of fish feed composition change due to a novel ingredient on fish health, using toxicological, lipid and metabolomics analyses (Papers III and IV). The hypothesis was the possibility to replace the lipid content of fish feed partly with oleaginous yeast (*Rhodotorula toruloides*) biomass without detrimental effects on the fish.

4. Methodology

4.1 Study design

4.1.1 Clinical trial (Papers I and II)

A randomized controlled trial (RCT) was performed in 2019 at the Clinic of the Federal Research Centre of Nutrition, Biotechnology and Food Safety as described previously (Chernukha *et al*., 2021). The RCT included 36 participants in total and plasma samples from 20 patients were sent to the Swedish University of Agricultural Sciences for analysis. From the 20 samples sent to the Swedish University of Agricultural Sciences , the patients were adults of both genders between 61-66 years old and with a body mass index (BMI) between 22.53- 60.65 kg/m^2. The trial lasted 30 days with a patient stay at the clinic under observation for the first 9 days. Plasma was sampled from each patient on the first day of the experiment, on the 9th day and on the last day as described in Chernukha *et al*. (2021). The recruitment was based on voluntary measures and on the patients' wish to change their food consumption behavior and to improve their health. The inclusion criteria were defined as showing atherosclerosis signs of the heart and blood vessels. Atherosclerosis diagnosis was confirmed with the combination of clinical checks for symptoms, physical exercise tests, anamnesis and coronary angiography data. Patients having other health related issues such as diabetes mellitus, fever, chronic renal failure, anemia, and exacerbation of a chronic disease were excluded. Other criteria of exclusion were pregnancy, breastfeeding and use of medications or dietary supplements to maintain or lose weight (Chernukha *et al*., 2021).

The BMI was calculated as follows:

$$BMI = \frac{body\ weight}{height * height} \qquad \text{(Eq. 2)}$$

with body weight (kg) and height (m).

Atherogenic index (AI) was estimated with the formula:

$$AI = \frac{TCL - HDL}{HDL} \qquad \text{(Eq. 3)}$$

with TCL: total cholesterol and HDL: high-density lipoproteins.

At the beginning of the study, patients were randomly divided into control and treatment groups. The control group of patients received a low-calorie diet (LCD) of around 1700 kcal/day for 30 days. The treatment group received the same LCD and 100 g of meat product in addition every day for 30 days. The meat product consisted of pork aorta and heart pieces homogenized into a pâté and processed as previously explained in Kotenkova & Chernukha (2019) and Chernukha *et al*. (2021). Macronutrient composition of the meat product was 17.5 ± 0.95% of protein, 3.82 ± 0.13% of fat, 2.35 ± 0.25% of starch and 0.305 ± 0.015% of sodium chloride (Kotenkova & Chernukha, 2019; Chernukha *et al*., 2021).

The study was carried out in accordance with the Good Clinical Practice program with a signed written consent form from each participant. The protocol of the study was approved at the profile commission meeting of the Health Care Expert Council of the Ministry of Health of Russia, Protocol No. 5 of June 14, 2018 and approved by the Ethics Committee of the Centre (Protocol No. 7 of 03.12.2018), following the Russian Federation's rules and the Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects, 2013.

4.1.2 Fish feeding study (Papers III and IV)

Wheat straw hydrolysate obtained from agricultural waste was selected as a substrate for cultivation of the oleaginous yeast *Rhodotorula toruloides*. To release the carbon sources from the complex structure of wheat straw, the hydrolysate was pre-treated using steam explosion and enzymatic hydrolysis

methods at the Department of Chemical Engineering, Lund University, Sweden (Blomqvist *et al*., 2018).

Cultivation of the *R. toruloides* CBS 14 strain (Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands) was performed in a similar manner as in Blomqvist *et al*. (2018), Brandenburg *et al*. (2021) and Nagaraj *et al*. (2022). Briefly, the yeast was retrieved from glycerol stocks at –80°C (50% *v/v*), pre-cultivated in YPD-medium, harvested by centrifugation, washed with saline solution and cultivated in Dolly fermenters (Belach Bioteknik, Stockholm, Sweden) with a mix of 60% sterile filtered cellulose and 40% hemicellulose hydrolysate. Cells of *R. toruloides* were harvested by centrifugation, washed with deionized water and kept at –20°C. To minimize potential digestibility issues in fish, yeast cell walls were disrupted using a French press (Constant systems LTD, Daventry, United Kingdom).

Table 1. *Composition of control and treatment feeds (% of dry matter). "Vitamin mix" and "Mineral mix" ingredients were provided by NOFIMA (Norway) and "astaxanthin & vitamin mix" ingredients were provided by Aller Aqua A/S (Denmark); Table from Brunel et al. (2022).*

Ingredients	Control feed	Treatment feed
Fish meal	52.8	53.5
Fish oil	12.5	12.6
Mineral mix	0.48	0.49
Vitamin mix	0.96	0.97
Astaxanthin & vitamin mix	0.14	0.15
Gelatine	0.48	0.49
Wheat meal	18.7	14.1
Ca_2PO_4	2.40	2.43
Casein	5.76	-
Vegetable oil	5.80	-
Yeast biomass (*Rhodotorula toruloides*)	-	15.3 (5.8% oil)

Two fish feeds were prepared manually by mixing ingredients listed in Table 1. The treatment feed differed from the control feed in containing yeast biomass to replace the vegetable oil mix and casein included in the control feed.

To obtain a comparable FA composition between the two feeds, the FA profile of *R. toruloides* was analyzed and revealed a majority of oleic and palmitic acid (Paper III and IV: Table 2). The FA profile of the control feed was adjusted to FA profile of the yeast biomass by adding rapeseed and palm oil in equal quantities. All mixed ingredients in each feed were pressed using a kitchen meat grinder, manually cut into pellets and left to dry overnight at room temperature (Figure 8). Each feed was packed and stored at –20°C until the beginning of the feeding trial.

The amino acid and FA compositions of the two feeds were evaluated (Papers III and IV: Tables 2 and 3).

Figure 8. Fish feed prepared by hand at SLU. Source: Sabine Sampels.

The fish feeding trial took place at the Aquaculture Centre North, Kälarne, Sweden (Figure 9). The Arctic Superior fish strain was obtained from the Swedish breeding program for Arctic char (Nilsson *et al*., 2010).

Figure 9. Landscape, Aquaculture Center North, Kälarne, Sweden.

A total of 126 Arctic char fish were randomly distributed into six 1 m x 1 m tanks provided with a flow-through water system of freshwater from Lake Ansjön (in natural photoperiod, 10 $L.min^{-1}$ at around 7 ºC) (Figure 10). Commercial feed was distributed to all fish during the period of acclimation until the fish gained an approximate weight of 165 g (fish number, n = 6). During the feeding trial, fish were fed their respective feed by band feeders 4 times a day for 74 days with a pre-sampling at day 19 (fish number, n = 24) and a final sampling at day 74 (fish number, n = 96).

Figure 10. Fish tanks, Aquaculture Center North, Kälarne, Sweden.

At each sampling date, the growth parameters total body weight (g), total length (cm) and liver weight (g) were measured to estimate fish performance and to compare these parameters in fish between the two feeds. HSI was calculated as follows:

$$HSI = \frac{Wl}{Tw} * 100 \qquad \text{(Eq. 4)}$$

with Wl: weight liver (g), Tw: total body weight (g).

Fish were maintained in adequate conditions with no mortalities observed. The study was in accordance with the European legislation (i.e., Directive 2010/63/EU), and approved by the Ethical Committee for Animal Experiments in Umeå, Sweden.

4.2 Lipid analyses

4.2.1 Total lipid quantification

Lipids from the meat product, the fish feed and fish muscle, liver were extracted from samples according to Hara & Radin (1978) as well as Mráz & Pickova (2009). Samples were homogenized with an Ultra-Turrax in a mix of hexane and isopropanol solvents (3:2, *v/v*). Liposoluble molecules were separated from hydrosolubles in each sample with the addition of sodium sulphate (Na_2SO_4, 6 ml, 6.67%, *w/v*) followed by mixing and a resting time at 4˚C. The lipid phase was transferred to a new tube and dried under nitrogen gas before gravimetric quantification. Lipids from the yeast biomass were extracted according to a modified method of Folch (Folch *et al*., 1957; Brandenburg *et al*., 2016).

4.2.2 Lipid classes

Paper IV included a lipid class analysis of fish liver. Total lipids were separated into TAG and PL using thin-layer chromatography (TLC) according to Mráz & Pickova (2009). The solvents hexane, diethylether, and acetic acid (85:15:1, *v/v/v*) were selected as mobile phase. The stationary phase was TLC pre-coated with silica gel plates (20 cm × 20 cm; 0.20-mm thickness from Merck, Darmstadt, Germany). The PL class was extracted

with different chloroform:methanol mixes while the TAG class was individually extracted with successive steps of chloroform.

4.2.3 Fatty acid profiling

Extracted lipids were methylated into fatty acid methyl esthers (FAME) using boron trifluoride (14%) and methanol according to Appelqvist (1968). FAME were separated and quantified using a gas chromatography instrument equipped with a flame ionization detector and a split-mode injector. Identification of FAME was carried out by comparing retention times of FA in each sample with retention times of a standard mixture GLC 68A (Nu-check Prep, Elysian, USA).

4.3 Metabolomics analyses

4.3.1 Sample preparation

Both meat products (Papers I and II) and fish liver tissue (Paper IV) underwent a similar liquid-liquid extraction for metabolomics analysis (Moazzami *et al*., 2011; Wagner *et al*., 2014; Cheng *et al*., 2017; Wagner *et al*., 2019). The first step was the homogenization of samples in ice-cold methanol-chloroform solution followed by a sonication step for 30 minutes. Ice-cold water and ice-cold chloroform were added to the samples and samples were centrifuged at 1,800 × g for 35 min at 4 ˚C to obtain a clear phase separation. The aqueous supernatant was collected, dried using a speedvac (Savan, SVC 100H, Techtum Instrument AB, Umeå, Sweden) and reconstituted with sodium phosphate buffer (520 µl, 0.135 M, pH 7.0).

Before NMR analysis, reconstituted samples were filtered in pre-washed Nanosep centrifugal filters (3-kDa cut-off, Pall Life Science, Port Washington, NY) and centrifuged at 10,000 × g or 12,000 × g for at least 1 hour at 4˚C (Röhnisch *et al*., 2018). Ultrafiltration was a necessary step to remove macromolecules interfering with the analysis such as plasma proteins and heparin (Nagana Gowda *et al*., 2015; Röhnisch *et al*., 2018).

No extraction step was performed for human and fish plasma samples (Papers II and IV). Plasma samples were directly filtered using the same protocol as meat products and fish liver tissue (Röhnisch *et al*., 2018).

4.3.2 NMR experiment

Human and fish plasma samples (Papers II and IV) were prepared in 3-mm outer diameter NMR tubes due to the limited amount of sample. Similar to Röhnisch *et al*. (2018), plasma filtrate (40 µl) was mixed with a solution containing sodium phosphate buffer (50 µl, pH 7), deuterium oxide (D_2O, 15 µl), Millipore water (55 µl), and the internal standard sodium-3-(trimethylsilyl)-2,2,3,3,-tetradeuteriopropionate (TSP-d_4,10 µl, 5.8 mmol/l, Cambridge Isotope Laboratories, Andover, MA, United States).

Extracted meat products and fish liver samples (Papers I, II and IV) were analyzed in 5-mm outer NMR tubes with the following mix: sample filtrate (350 µl), sodium phosphate buffer (170 µl, pH 7), D_2O (50 µl), and TSP-d_4 (30 µl, 5.8 mmol/l, Cambridge Isotope Laboratories, Andover, MA, United States).

All metabolomics analyses were performed on a 600 MHz NMR Bruker spectrometer including a cryogenically cooled probe for higher sensitivity. Pulse sequence zgesgp was used for all samples to obtain ^{1}H-NMR spectra with 512 scans for samples in 3-mm NMR tubes and 128 scans for samples in 5-mm NMR tubes. The software Bruker TopSpin (versions 3.5 pl7 and 4.1.1) was used for spectral processing. All data was Fourier-transformed after multiplication by a line broadening of 0.3 Hz. The experimental spectrum was defined as the spectrum obtained for each sample after the NMR experiment.

4.3.3 Targeted metabolomics profiling

Manual quantification of metabolites was carried out for meat products, fish plasma and liver (Papers I, II and IV). The baseline and internal standard signals were adjusted manually in each experimental spectrum and a line broadening of 0.30 Hz was automatically applied. A number m of metabolites in the spectra of meat products (m = 26) were identified using the databases from the ChenomX version 7.1 profiler and the HMDB. Metabolites in spectra of fish plasma (m = 57) and fish liver (m = 48) were identified using the same databases and additional literature (Cheng et al., 2017) (Figure 11).

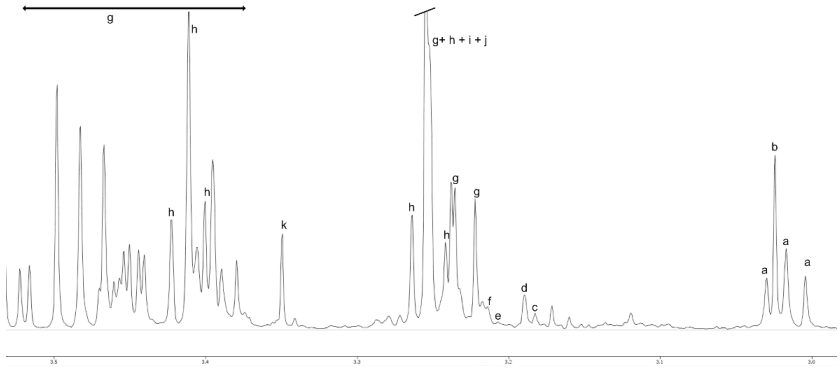

Figure 11. Example of metabolites assignation in a ^{1}H-NMR spectrum (fish plasma). Metabolites: a = lysine; b = creatine; c = o-acetylcarnitine; d = choline; e = o-phosphocholine; f = carnitine; g = glucose; h = taurine; i = betaine; j = trimethylamine N-oxide; k = methanol.

For each identified metabolite, a specific signal was selected considering the least interferences with other signals and other factors. The database spectra for the identified metabolites were superimposed on each experimental spectrum and the selected signals were successively adjusted to fit the experimental spectrum. The manual quantification of metabolites was considered complete when the sum of all database signals (profiler line) fitted the experimental spectrum at the specific positions of selected signals.

A different method was considered for the quantification of metabolites in human plasma collected from the clinical trial (Paper II). An automated quantification algorithm (AQuA) from the study of Röhnisch *et al*. (2018) was applied. In a similar way as the manual approach, AQuA uses one signal per metabolite for the quantification. In this case, the same signals as in the study by Röhnisch *et al*. (2018) were used. A spectral library (with a total of 57 metabolites) was created *in silico* from ChenomX software. The library spectrum for each metabolite was obtained by the alignment of library signals to the experimental data, followed by adjustment of the internal standard (TSP-d_4) signal.

To automate the quantification of metabolites, MATLAB software (version R2018b, Math Works Inc) was used. Library and experimental spectra were subjected to spectral binning of 0.0002 ppm from -0.5 to 8.5 ppm, with the water region (4.5-5.1 ppm) excluded before the import of binned data into the software.

To quantify the m number of metabolites in each sample, the experimental spectrum n was converted to vectors $\bar{y}_n$ using data reduction relying on automated peak-picking of the selected signals (intensities). Library spectra were converted to matrix $\bar{\bar{\mathbf{m}}}$ (*m* × *m*) using data reduction and normalization.

To remove interferences from the individual metabolite signals, vector $\bar{x}_n$ was computed with the following formula:

$$\bar{y}_n = \bar{\bar{\mathbf{m}}} * \bar{x}_n \qquad \text{(Eq. 5)}$$

Vector $\bar{x}_n$ was converted to the metabolite concentrations in sample n using calibration factors (ratio between concentration and intensity of each library signal). Final metabolite concentrations were obtained after accounting for the dilutions during the sample preparation.

4.4 Organic pollutants and EROD measurements

Treatment feed included in the fish feeding trial contained yeast biomass grown on agricultural side streams, and could potentially show increased levels of pollutants from soil. Therefore, we measured the content of polycyclic aromatic hydrocarbons (PAHs), hexachlorobenzene (HCB) and PCBs, all lipophilic compounds in the used yeast biomass and in its hydrolysate (Paper III).

Hydrophobic organic contaminants such as PAHs are becoming ubiquitous in soil and water with their major source from the results of anthropogenic origins such as oil spills, incomplete combustion of fossil fuels and coal, as well as industrial effluents (Whyte *et al*., 2000; Santana *et al*., 2018). PAHs have been described as carcinogenic compounds difficult to degrade and accumulating in food chains (Whyte *et al*., 2000). PCBs are organic chlorine compounds that can be absorbed by the soil through organic matter and accumulate in the food chains (Valizadeh *et al*., 2021).

In the same way, HCB used previously as a selective fungicide for agricultural purposes has shown carcinogenesis properties and bioaccumulation in living organisms (Starek-Świechowicz *et al*., 2017).

Organic pollutant PAHs in the yeast biomass and its hydrolysate were extracted using different steps of liquid-liquid partitioning, saponification and clean-up by solid phase extraction using a silica column. PAH levels were measured GC-MS in selected ion monitoring mode (Paper III; Arp *et al*., 2014; Lundstedt *et al*., 2014; Sess-Tchotch *et al*., 2018).

PCBs and HCB were extracted in a similar manner as PAHs with a liquid-liquid extraction with hexane and a clean-up step with a silica column. GC-MS technique was applied with a triple quadrupole mass spectrometer and in multiple reaction monitoring mode (Paper III; Dahlberg *et al*., 2020; Dahlberg *et al*., 2021). Authentic reference standards were used for the identification and quantification of all organic compounds.

To understand potential toxicological effects of the new feed at the organism level and to cover a broad range of pollutants, the catalytic activity of 7-ethoxyresorufin-O-deethylase (EROD) was measured in livers from the fish of the feeding trial (Paper III: Figure 1) as a biomarker of exposure to contaminants (Whyte *et al*., 2000). EROD activity reflects the enzymatic activity of cytochrome P450-dependent monooxygenases (CYP1A subfamily) (Whyte *et al*., 2000). CYP1A1 is often used as a marker of PAH exposure and halogenated aromatic hydrocarbons (Whyte *et al*., 2000). PAH

binds to aryl hydrocarbon receptor (AhR) involved in complex regulation of enzymes responsible of xenobiotic metabolism, leading to a change in gene expression of AhR-dependent genes and an upregulation of CYP450 enzymes, in particular CYP1A (Whyte *et al*., 2000; Franco & Lavado, 2019). CYP1A proteins are able to catalyse deethylation of the substrate 7-ethoxyresorufin to the fluorescent product resorufin detected and quantified by spectroscopy (Whyte *et al*., 2000).

EROD activity was measured in microsomes of both fish groups after a common procedure of isolation and purification using centrifugation and calcium aggregation (Rasmussen *et al*., 2011). Hepatic microsomes were selected as samples for EROD analyses as CYP1A proteins are mainly located in the membrane of the endoplasmic reticulum (Whyte *et al*., 2000). Resorufin was quantified with high-performance liquid chromatography using a standard and florescence detection at 530 nm and 582 nm (Zamaratskaia & Zlabek 2009).

4.5 Sensory analysis

Fish fed with control and experimental feeds were evaluated for their sensory difference in taste, texture or smell using the Triangle Test method of Sinkinson (2017). As described in Paper III, fish fillets were divided into pieces of approximately 3 cm x 3 cm and cooked in a water bath at 70˚C until the core of the fillet reached the temperature of 60˚C. Volunteers at the Swedish University of Agricultural Sciences were served three samples of fillets of each fish group randomly and were asked to identify the different samples.

4.6 Histology

Liver histology was performed in control and treatment groups of fish to further understand the increased liver weight observed in the group of fish fed with feed containing yeast biomass (Paper IV). Liver tissue was fixed in neutral buffered formaldehyde solution and processed at the Pathology Department of the Swedish University of Agricultural Sciences, Uppsala, Sweden. Livers were embedded in paraffin blocks, sliced with a microtome and stained with Mayer´s hematoxylin and eosin staining after several dehydration and cleaning steps (Brunel *et al*., 2022). Signs of liver

pathologies such as inflammation, cholestasis and steatosis were investigated.

4.7 Statistical analyses

Paper I

Multivariate data analysis was applied to the two meat products data to evaluate differences in metabolites levels using the SIMCA software (version 17; Umetrics, Suite of Data Analytics Solutions, Sartorius) according to Wagner *et al*. (2019). All data was UV-scaled and potential outliers were investigated with visual interpretation of PC scores including an ellipse (confidence interval, CI 95%) generated from Hotelling´s t^2. The OPLS-DA model was performed on the metabolites data to search for discriminative variables. Variables with a variable importance in the projection (VIP) above 1 and with a VIP confidence interval (CI 95%) equal or above zero were considered discriminative. Reliability of the OPLS-DA model was confirmed using CV-ANOVA as a diagnostic tool.

Differences in the proportions of FA were investigated using SAS software with PROC MIXED and NPAR1WAY models. Variability in FA and metabolites levels with a p-value < 0.05 was considered significant.

Paper II

Variability in BMI, TAG, TCL, LDL, HDL and AI were investigated with PROC MIXED model in SAS 9.4 software (SAS Institute Inc, Cary, NC, USA) using the Least Squares Means method. Different GLMM were performed taking into consideration the fixed factors treatment (control or treatment group), time points (day 0, day 9 and day 30), sex, the interaction time points * treatment and the factor patient as a random factor.

Changes in the concentration of metabolites from plasma samples were analyzed between treatment/control and between the two time points day 9 and day 30. A GLMM was applied to the data with the fixed factors treatment, time points, and interaction time point * treatment. The time point day 0 was integrated to the model as a cofactor (baseline) and patient was included as a random factor. The effects of BMI and sex were adjusted individually.

Normal distribution was determined with visual interpretation of the residual plot and a logarithm transformation was applied to the non-normally

distributed variables. Statistical significance was estimated at p-value $p < 0.05$. Two outliers were detected in the variable α-ketoglutarate from the same patient and no missing values were reported.

Paper III

To evaluate the effect of the new fish feed, growth parameters and EROD activity were separately compared between the two groups of fish using GLMM with the PROC MIXED model in SAS 9.4 software with the Least Squares Means method and by including fish tank as a random factor.

Results of the sensory analysis were interpreted using a similar procedure as in Sinkinson (2017) with the number of participants N = 34 and a statistical significance set at $\alpha \leq 0.05$.

Paper IV

Differences in total fat contents and FA in total lipids (liver and muscle tissues), FA in lipid classes (liver tissue), lipid droplets (liver tissue) as well as in growth parameters between the two fish groups were estimated using the PROC MIXED model in the SAS 9.4 software. Least Squares means of fixed effect and the random factor fish tank were included to the models.

The effects of the new feed on fish plasma and liver were investigated at the metabolites level with the SIMCA software (version 17; Umetrics, Suite of Data Analytics Solutions, Sartorius) on UV-scaled data similar to Wagner *et al*. (2019). Two outliers in the plasma of the fish group receiving the treatment feed were identified with a PC score plot. Plasma data was interpreted with and without the outliers for the conclusion of the study. No outliers were revealed in the liver data. OPLS-DA models were carried out on metabolite levels in plasma and liver to classify metabolites using the feed (control and treatment) as a discriminative variable. Reliability of OPLS-DA models was verified with CV-ANOVA. Similar to the other papers, metabolites were considered discriminative with a VIP > 1 and with confidence interval of VIP (95%) above or equal to zero. Metabolites found significantly altered by the feed in SIMCA were further evaluated in SAS 9.4 using PROC MIXED model with GLMM including the fish tank factor as a random variable. Normal distribution of the selected metabolites was verified using the PROC UNIVARIATE model in SAS 9.4. The non-parametric method was applied to metabolites non-normally distributed using the NPAR1WAY model with Wilcoxon scores in SAS 9.4.

Additional information was generated for the interpretation of histological data with a PC score plot and a loading plot of the number of lipid droplets and liver fat content (%).

5. Results and discussion

5.1 Meat products and clinical trial

The composition of the test product made of porcine heart and aorta tissue was compared with the composition of a control product of similar quality containing porcine heart tissue (Papers I and II).

The metabolite profile of the two products revealed differences in their composition (Table 2). The control product showed significantly higher levels of sugars (glucose, sucrose, fructose and maltose) while no sucrose, fructose and maltose were detected in the test product. Several metabolites involved in protein biosynthesis were detected at different levels in the control and test products, including significantly higher glycine and tryptophan levels in the test product. No significant differences were found in succinate and valine levels.

The FA composition of the two meat products revealed higher percentages of SFA and PUFA in the control, while the test product showed higher percentages of MUFA.

Table 2. *Metabolites present in significantly higher levels ($p < 0.05$) in the control and test products.*

Product	Metabolites in higher concentrations (p-value < 0.05)
Control	2-Hydroxybutyrate Beta-alanine Inosine Niacinamide Pyruvate *Protein metabolism*: arginine, asparagine, glutamate, glutamine, proline, tyrosine *Carbohydrates*: fructose, glucose, maltose, sucrose
Test	2-Aminobutyrate Aspartate O-phosphocholine Tyramine *Protein metabolism*: glycine, tryptophan
	Fatty acids in higher proportions (p-value < 0.05)
Control	SFA: C14:0; C17:0; C18:0 MUFA: C16:1(n-9) PUFA: C18:2(n-6); C18:3(n-3); C20:4(n-6); C22:4(n-6); C22:5(n-3) *In general*: higher SFA, PUFA, n-3, n-6
Test	MUFA: C18:1(n-9); C20:1(n-9) *In general*: higher MUFA

The consumption of heart and aorta tissues from cattle and pigs previously showed improvements in the condition of rats presenting hyperlipidemic and atherosclerotic traits (Chernukha *et al*., 2018). In this thesis, it was hypothesized that this effect can apply to atherosclerosis in humans. We evaluated the effect of a porcine heart and aorta tissue (test product) on the metabolites levels of patients in a clinical trial (Paper II). The hypothesis was a change in plasma metabolites levels towards a hypolipidemic effect in individuals showing atherosclerosis symptoms after a daily consumption for 30 days of a pork-based product containing heart and aorta.

In this thesis work with 20 patients, the analysis of BMI and blood TCL profiles revealed a decrease of the BMI in both control and treatment groups between day 0 and day 9 as well as between day 0 and day 30 (Paper II: Figure 2 or thesis Figure 12). Interestingly, only the treatment group showed an effect in CL indexes with a reduction of AI, LDL and TCL between day 0 and day 9 as well as between day 0 and day 30. In addition, a reduction of TAG between day 0 and day 30 was observed only for treatment group. HDL, a marker inversely proportional with cardiovascular risk (Rader & Hovingh, 2014), was found at comparable levels between the two groups of patients and over time.

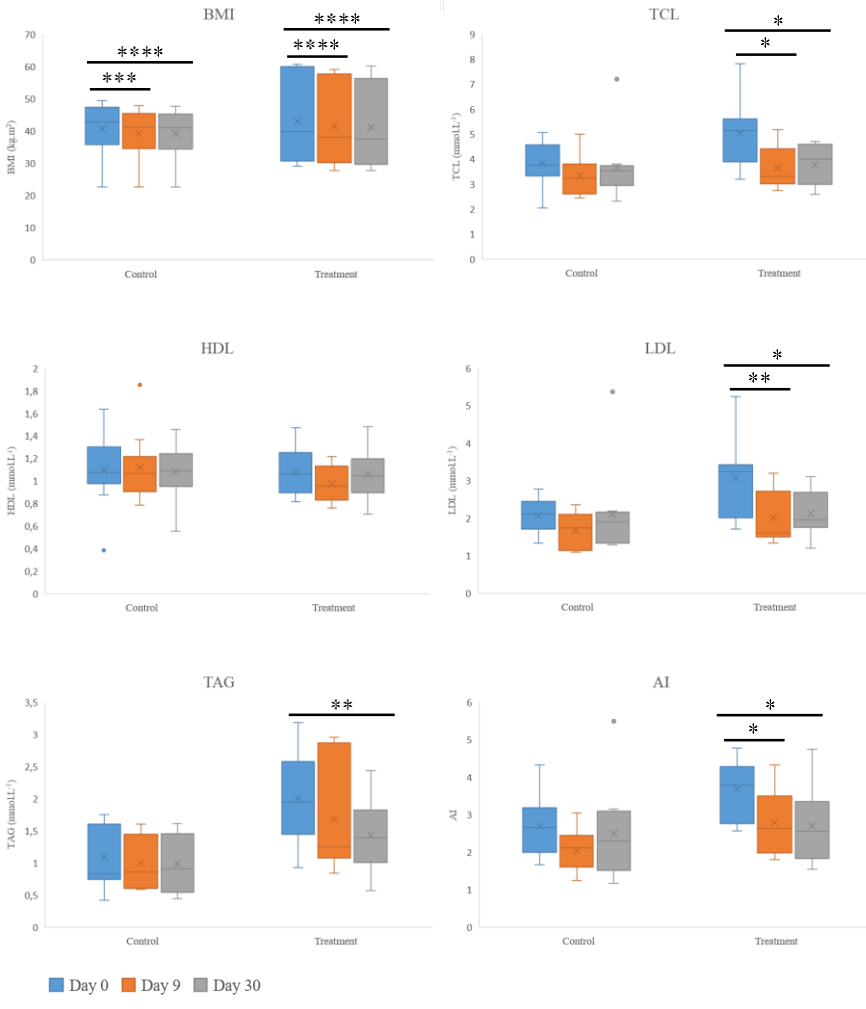

Figure 12. Blood lipid parameters in clinical trial of patients with atherosclerosis symptoms receiving a test product containing porcine heart and aorta (treatment group) compared to a control group with no particular supplementation.

The automated quantification of plasma metabolites with AQuA has led to the profiling of 46 metabolites. Carnitine levels in the treatment group were significantly reduced between day 9 and day 30. Levels of the same

metabolite in the control group followed a similar trend as the treatment group in a non-significant way (Paper II: Figure 1).

Carnitine is an important metabolite synthesized by the body and can be found in meat and dairy products. This metabolite is mainly localized in the heart and skeletal muscle tissue in humans and stimulates FA oxidation in the liver when transferring long-chain FA to the mitochondria for β-oxidation (Fritz and McEwen, 1959; Bremer, 1983; Longo *et al*., 2016). A reduction of plasma carnitine levels in the treatment group may indicate a higher use of carnitine for transportation of FA and for β-oxidation. The amino acids methionine and lysine are precursors of carnitine endogenous synthesis (Longo *et al*., 2016). In this study, methionine and lysine levels were comparable between treatments and time points.

Related to the tricarboxylic acid cycle (TCA cycle or Krebs cycle, Figure 13), a significant increase in α-ketoglutarate levels in the control group was shown between day 9 and day 30. A similar trend was observed in the treatment group at the same time points and for the same metabolite. The precursor of α-ketoglutarate, citrate, had a non-significant decrease of levels for the same time points. No significant changes in the levels of glucose were observed across the different treatments and time points. Glucose and pyruvate levels showed similar trends within each group with stable levels between day 0 and day 9 for control group followed by an increase in levels between day 9 and day 30. The treatment group had decreasing levels of pyruvate and glucose between day 0 and day 9 and increasing levels between day 9 and day 30.

Another intermediate of the TCA cycle, succinate, remained at similar levels between and within groups at all time points. Glycerol, an intermediate in the conversion of glucose to pyruvate, had decreasing levels between day 0 and day 9 and increasing levels between day 9 and day 30 for both groups of patients. Lactate and alanine, both precursors of gluconeogenesis, showed no significant differences but some trends. Gluconeogenesis corresponds to the endogenous production of glucose from pyruvate, lactate, glycerol, alanine and other amino acids in the liver in absence of glucose (Ruderman, 1975; Hers and Hue, 1983). Lactate increased over time in the control group while decreasing at the same time in the treatment group. Levels of alanine showed opposite trends between groups with an increase in the control group between day 0 and day 30, and a decrease between day 9 and day 30. The treatment group had decreasing levels of alanine between day 0 and day 30

as well as increasing levels between day 9 and day 30. Levels of glutamine, another substrate for gluconeogenesis, were slightly increased for control and treatment groups between day 0 and day 9 and decreased for the same groups between day 9 and day 30.

Interestingly, metabolites involved in the urea cycle (or ornithine cycle) such as glutamate, α-ketoglutarate and ornithine had all increased levels in treatment and control groups between day 9 and day 30.

Additionally, BCAA, isoleucine, leucine and valine had no significant differences across the study and between treatments. BCAA are essential amino acids supplied by the diet and required for protein biosynthesis (Harper *et al*., 1984). The levels of ketone metabolites (3-hydroxybutyrate, acetoacetate, acetone) were expected to increase in the two patient groups as a result of the LCD. Nevertheless, no significant differences were observed. Former studies have shown a relation between fasting and higher hepatic gluconeogenesis in addition to higher enzyme activity related to the urea cycle (Klain *et al*., 1977; Korenfeld *et al*., 2021).

During fasting, the organism requires glucose from non-carbohydrates or more precisely from proteins. Muscles are degraded into amino acids and these amino acids are transported to the liver for the production of glucose by gluconeogenesis. The excess of plasma amino groups from amino acids are converted into urea and eliminated (Schimke, 1962; Schutz, 2011; Korenfeld *et al*., 2021).

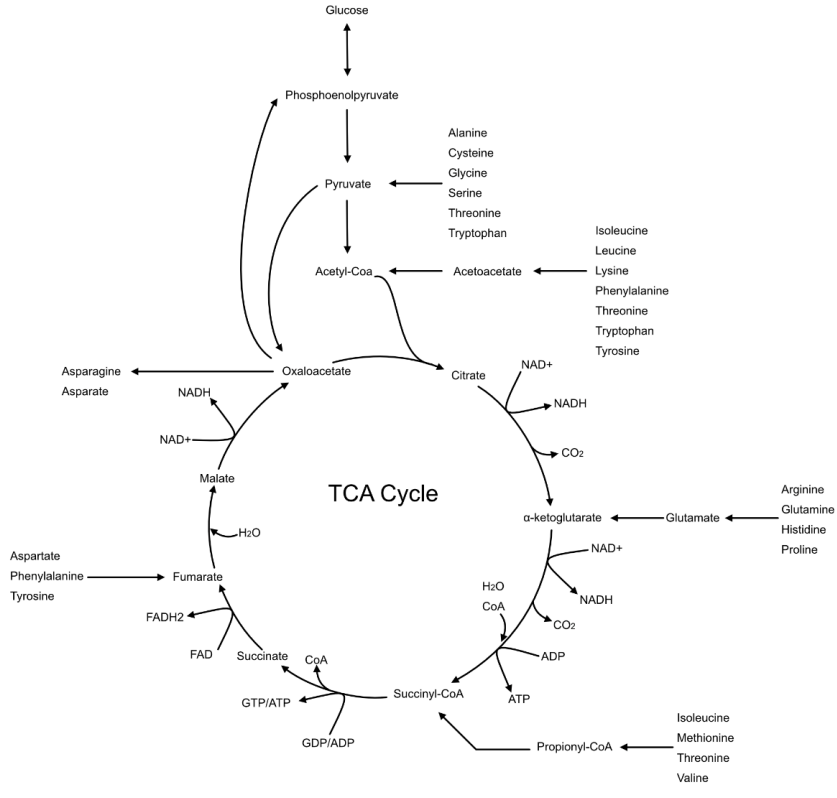

Figure 13. Krebs cycle (TCA cycle or citric acid cycle). Adapted from Cheng (2017).

Taken together, the main effects observed in plasma metabolites levels were in relation to energy production with the involvement of TCA and urea cycles, β-oxidation of FA and the gluconeogenesis pathway. Some metabolites involved in the TCA cycle seemed to increase slightly for both groups of patients between day 9 and day 30 (glucose, glycerol, pyruvate, α-ketoglutarate) or decrease (citrate) while other metabolites such as succinate showed no difference. To understand further the effects seen on plasma metabolites and obtain more specific information on metabolic pathways, clinical trials set up with a longer intervention time and/or the use of isotope labeling might be potential options (Chokkathukalam *et al*., 2014) while respecting ethical standards.

The goal of the meat product studies (Papers I and II) was to characterize the composition of a porcine cardiac product defined as organ meat and

investigate its effects in a particular group of individuals. Organ meat products contain nutrients of high values such as vitamin A (liver), vitamin B1 and niacin (liver, kidney, heart and brain), vitamin B2 (liver, kidney, heart), vitamin B12 (all organ meats), pantothenic acid, vitamin B6 (liver kidney), folate (liver), vitamin E (liver), n-3 (kidney, liver and brain), and essential and non-essential amino acids (Mullen and Álvarez, 2016; Fayemi *et al*., 2018). Higher proportions of vitamins are found in organ meat compared to lean meat (Rahman *et al* 2014). Organ meat has been shown to have multiple health properties related to bioactive peptides such as antioxidant, anti-bacterial, anti-inflammatory, anti-thrombotic, anti-hypertensive, hypocholesterolemic, and immunomodulatory (Arihara, 2006; Toldrá *et al*., 2012; Lafarga and Hayes, 2014; Lafarga *et al*., 2017; Chernukha *et al*., 2021).

Nevertheless, before consumption organ meat should be controlled for hygiene considerations and for diseases such as Bovine spongiform encephalopathy (Toldrá *et al*., 2012). In addition, the nutritional quality of an organ meat product depends on the type of tissue, with, for example, fat/tallow products having approximately 80% of fat while the fat content of heart products is between 4-9% (Mullen and Álvarez, 2016). Furthermore, the meat quality is impacted by the type of animal including breed, age, sex, and nutrition (Lebret and Čandek-Potokar, 2022).

In 2015, the World Health Organization (WHO) defined red meat as "*all mammalian muscle meat, including beef, veal, pork, lamb, mutton, horse and goat*" and processed meat as "*meat that has been transformed through salting, curing, fermentation, smoking, or other processes to enhance flavour or improve preservation*" (WHO, 2015). A review panel from the International Agency for Research on Cancer (IARC, 2015) and WHO experts classified red meat in Group 2A as "probably carcinogenic to humans" and processed meat in Group 1 as "carcinogenic to humans" (WHO, 2015) (Table 3). Nevertheless, no specific amount of meat was included in the review panel as a guideline for meat consumption.

Table 3. *Summary of the identification of carcinogenic hazards to humans. Information retrieved from IARC Monographs (IARC, 2015).*

Groups	**Effects**
Group 1	Carcinogenic to humans
Group 2A	Probably carcinogenic to humans
Group 2B	Possibly carcinogenic to humans
Group 3	Not classifiable as to its carcinogenicity to humans

The detrimental or beneficial effects of meat products are a topic of debate. High meat consumption has been associated with increases in total mortality according to some studies, e.g., Sinha *et al*. (2009) and Larsson & Orsini (2014).

Red meat and processed meat have often been associated with increased risks of developing cardiovascular diseases, colon cancer, stroke, obesity and diabetes (Abete *et al*., 2014; Johnson *et al*., 2013; Chen *et al*., 2013; Babio *et al*., 2012; Pan *et al*., 2011; Wolk, 2017; Al-Shaar *et al*., 2020). These associations have been shown as weak in other studies (Nöthlings *et al*., 2009; Alexander *et al*., 2015; Bernstein *et al*., 2015; Saliba et al., 2019; Lescinsky *et al*., 2022) or have shown no effect (O´Connor *et al*., 2017) or even beneficial effects in the case of lean red meat (McAfee *et al*., 2010).

Overall, the association between meat consumption and the development of metabolic diseases seems inconsistent. One reason could be the nutritional content of the diet associated with the meat consumption. A study investigated the quality of the diet related to the consumption of red and processed meat consumption (Fogelholm *et al*., 2015). The results showed a high consumption of red and processed meat was inversely associated with consumption of fruits and whole grain, while positively associated with potatoes, oil and coffee consumption. In addition, high meat consumption was positively associated with higher BMI (Fogelholm *et al*., 2015). Another factor could be the difficulty in comparing health outcomes between the different studies with an unclear classification of the types of meat from different animals or the classification of the different animal parts. The distinctions between red and white meat vary as well (Oostindjer *et al*., 2014). Some consider white meat as the light colored meat before cooking (poultry, fish) and red meat as dark colored meat before cooking (beef, lamb)

(Oostindjer *et al*., 2014). WHO considers red meat as any "*mammalian muscle meat*" (WHO, 2015). Regarding the classification of the different animal parts, there is no clear definition on the word meat (Zhang *et al*., 2017). A broad definition exists from the Merriam Webster dictionary as "*animal tissue considered especially as food*" (Merriam-Webster, 2023). The American Meat Science Association provides a more specific definition of meat as "*skeletal muscle and its associated tissues derived from mammalian, avian, reptilian, amphibian, and aquatic species commonly harvested for human consumption*". Meat products include edible offal such as organs and non-skeletal muscle tissues and exclude milk and eggs (Boler & Woerner, 2017). To provide more reliable scientific evidence between meat consumption and health outcome, the standardization of the different wording related to meat as well as the discovery of more specific biomarkers of meat consumption would be desirable for nutritional recommendations.

In 2019, the EAT-Lancet Commission set up recommendations based on healthy diet and environmental sustainability (Willett *et al*., 2019). From the protein sources, the EAT-Lancet Commission recommends 7 g/day of beef and lamb, 7 g/day of pork, 29 g/day of chicken and other poultry, 13 g/day of eggs and 28 g/day of fish (Willett *et al*., 2019). The Swedish recommendations on the consumption of red meat is a maximum of 500 g of cooked weight/person/week (Swedish Food Agency, 2023).

Concerning the consumption, the total consumption of meat in Sweden has decreased from 88.3 kg per capita in 2016 to 80.0 kg per capita in 2021. The reduction in meat consumption was observed for lamb, pork and beef, while poultry consumption increased (Swedish Agency for Agriculture, 2022). At the global level, meat consumption is expected to increase by 14% in 2030 due to an increasing average of individual incomes and as a result of population growth (Godfray *et al*., 2018; OECD-FAO, 2021). To face this demand, global meat production is expected to continue rising to 373 million tonnes by 2030 (OECD-FAO, 2021) or to 455 million tonnes by 2050 (Alexandratos & Bruinsma, 2012). Meat production contributes to emission of greenhouse gas (GHG), constituting 54% of the total emissions from agriculture between 2018-2020 and representing 14.5% of all human-induced emissions in 2007 (Gerber *et al*., 2013; OECD-FAO, 2021). In addition, meat production requires finite resources as water and arable land (Gerber *et al*., 2013; Godfray *et al*., 2018; OECD-FAO, 2021) and creates waste. The largest proportion of waste produced in the meat processing takes

place during slaughter and carcass processing depending on the type of animal and on the specific waste index for slaughterhouses (Anzani *et al*., 2020). Meat waste is estimated to be on average 60% during processing for Swedish conditions with meat losses from slaughter to fork adding up to 70% (Swedish Agency for Agriculture, 2022). From the expected global increase in meat consumption over the next ten years, 33% will represent pork meat (OECD-FAO, 2021). Therefore, efforts should be made to improve the sustainability of pork meat production and consumption with, for example, a higher use of organ meat.

5.2 Fish feed trial

Two fish feeds were formulated using ingredients fulfilling nutritional needs of the fish (Papers III and IV: Table 1). Fatty and amino acid profiles of the two feeds were evaluated to ensure comparable nourishment between the two fish groups (Papers III and IV: Tables 2 and 3).

Levels of pollutants and heavy metals were measured in oleaginous yeast (*Rhodotorula toruloides*) biomass and its hydrolysate to account for potential contaminants in the process from raw wheat straw to fermented yeast. Analyses for toxicants were divided into two parts: concentrations in yeast biomass and in the wheat straw hydrolysate (yeast substrate). The analysis of the yeast biomass revealed a concentration of the sum of PAHs at 200 $ng.g^{-1}$ of lipid weight, no detection of PCBs and a concentration of 0.21 $ng.g^{-1}$ of HCB (Paper III: Table 4). From the sum of PAHs measured, acenapthalene, fluoranthene and pyrene were detected (Figure 14). The hydrolysate has shown no detected levels of pollutants (Paper III: Table 4).

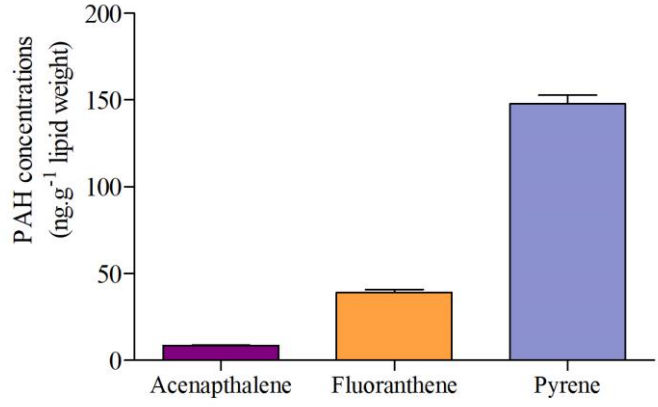

Figure 14. Sum of PAHs in yeast (*Rhodotorula toruloides*) biomass per ng.g^{-1} of lipid weight.

Contaminants such as persistent organic pollutants and derivatives are the by-products of human activities including pesticide and plastic productions (Mozaffarian & Rimm, 2006). Their productions have been restricted or prohibited by the Stockholm Convention since 2001 (The Stockholm Convention on Persistent Organic Pollutants, 2001; Mozaffarian & Rimm, 2006). Nevertheless, these compounds are very stable, accumulate in the food chain and are detected in many foods (Mozaffarian & Rimm, 2006). For example, PCBs can be detected in beef, salmon, butter and chicken at levels above 300 pg.g^{-1} of wet weight (Saktrakulkla *et al*., 2020).

Another approach for investigating the potential contamination of the treatment fish feed was the evaluation of heavy metals in both the yeast biomass and the hydrolysate. The detected levels of heavy metals were compared with the European and WHO regulation (FAO & WHO, 1995; Commission Regulation (EC), 2006; European Food Safety Authority, 2008) to assess the safety of the fish feed. All heavy metals measured showed concentrations below the European limits for food and foodstuff and no mercury levels were detected (Paper III: Table 5).

The partial replacement of traditional fish feed ingredients with alternatives containing low or no levels of contaminant could improve the ratio between risks and benefits of consuming fish. The protective effects of LC-PUFA obtained from fish on the cardiovascular system and on health have been considered higher than the risk associated with consumption of

fish containing contaminants, with the exception of some fish species for women of childbearing age (Mozaffarian & Rimm, 2006).

Another approach to study exposure to contaminants is to measure the hepatic activity of specific enzymes. The enzyme CYP1A1 in fish has received considerable attention recently and has been frequently studied at the levels of mRNA, protein, and enzyme activity in controlled laboratory and field experiments. CYP1A1 activities in fish are highly variable and depend on species, reproductive status, adequate nutrition, overall health status, and the presence of contaminants in feed and aquatic environments. CYP1A1 is readily inducible in fish by many contaminants, including PAH and halogenated aromatic hydrocarbons (Whyte et al., 2000). The activity of CYP1A1 is usually estimated as a rate of the O-dealkylation of 7-ethoxyresorufin. Thus, increased EROD activity might indicate exposure to contaminants or other xenobiotics. EROD activities observed in this thesis were similar in both groups and within previously reported physiological levels (Oris & Roberts, 2007) (Paper III: Figure 1 and thesis Figure 15), indicating no exposure to contaminants via feeding with yeast biomass.

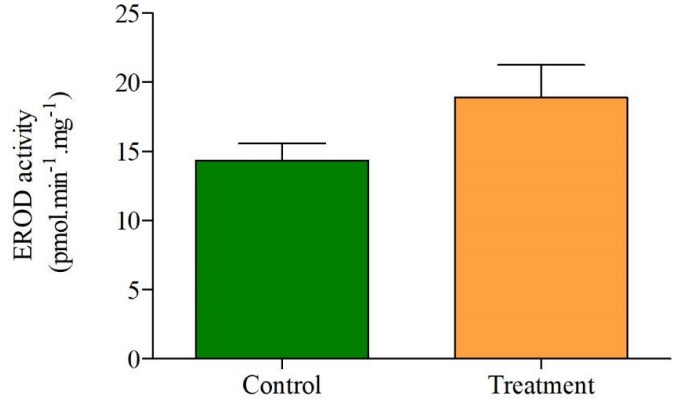

Figure 15. EROD activity in microsomes of Arctic char per pmol.min^{-1}.mg^{-1}. Control group of fish fed with vegetable oils, treatment group of fish fed partly with yeast biomass (*Rhodotorula toruloides*).

Other parameters related to fish health, growth and welfare were investigated in this study. Fish performance was evaluated during the fish feed trial and compared between the two fish groups to measure the overall

effect of the feed on the fish growth and health. All performance parameters were comparable between the fish groups with the exception of liver weight and HSI, significantly higher in the treatment group (Paper III: Table 6 or Paper IV: Table 4).

The reasons explaining the difference in liver weight were further investigated by measuring the fat content and FA composition of the liver tissue and by comparing between the fish groups (Paper IV: Table 6). The liver is the main organ for lipid and carbohydrate storages and previous studies have linked a higher liver weight and higher HSI with an increase in dietary lipid or starch levels (Hemre *et al*., 2002; Moreira *et al*., 2008; Enes *et al*., 2009; Polakof *et al*., 2012; Monge-Ortiz *et al*., 2018).

The liver fat content was numerically higher in the fish group fed with yeast. Nevertheless, these differences were not statistically different. Related to the FA profile, livers of control fish showed higher percentages of PUFA and LC-PUFA whereas the livers of treatment fish showed higher percentages of MUFA. SFA percentages in the livers were similar between the two fish groups. LA and ALA found abundantly in plant lipids were in higher percentages in the liver of control fish group fed with vegetable oils. Levels of EPA and DHA were higher in the total lipids class of the livers in control group while DPA was higher in the PL class of livers from the treatment group (Table 4).

Further analyses of liver were conducted using histology. No major differences were observed between the fish fed control feed and the fish fed yeast biomass-based feed.

Table 4. *Fatty acids detected in significantly higher percentages in livers of control and treatment fish groups (p < 0.05). Control group of fish fed with vegetable oils, treatment group of fish fed partly with yeast biomass (*Rhodotorula toruloides*).*

Fish groups	Fatty acids in higher proportions (p-value <0.05)
Control	*Total lipids*: C18:2(n-6); C18:3(n-3); C20:4(n-6); C20:5(n-3); C22:6(n-3); PUFA; n-3; n-6 *TAG*: C18:1(n-7); C18:2(n-6); C18:3(n-3); C20:2(n-6); C20:5(n-3); C24:1; PUFA; n-3 *PL*: n.s. *In general*: higher PUFA, n-3 and n-6
Treatment	*Total lipids*: C16:1(n-9); C18:1(n-9); MUFA *TAG*: C16:1(n-9); C18:1(n-9); MUFA *PL*: C22:5(n-3) *In general*: higher MUFA

n.s.: non-significant

In addition, the muscle fat content and FA composition were measured in both fish groups to assess the nutritional quality of fish fillet. The lipid quality of fillets was similar as no differences were observed for the fat content and FA profile between the two fish groups. The sensory analysis of fish fillets revealed that the majority of volunteers could not differentiate between the fish fed control feed or the fish fed treatment feed.

To understand the higher liver weight and higher HSI observed in treatment group of fish, metabolomics analyses were conducted. Levels of 48 metabolites detected in fish livers were comparable between the two fish groups. The validity of the multivariate model OPLS-DA could not be confirmed with low predictive values and a non-significant CV-ANOVA test. Nevertheless, trends were observed with very low levels of fumarate

and succinate in the treatment group of fish. As the TCA cycle has evolved into an important pathway in eukaryotes for providing energy (Martínez-Reyes & Chandel, 2020), differences in these metabolites involved in the TCA cycle might indicate a disruption in fat oxidation and energy metabolism.

With the inconclusive results from the metabolomics analyses of fish liver, 57 metabolites in plasma were quantified from the two fish groups to elucidate the hepatic effects. The plotting of PC scores revealed two outliers and the OPLS-DA model of plasma data showed better predictability than the model computed for the metabolites in livers. Most metabolites with significantly different levels were measured in higher levels in the treatment fish group with the exception of tyrosine (Table 5). The highest difference observed in metabolite levels between the two fish groups was in the following order: propylene glycol, alanine and 3-hydroxybutyrate. The higher concentration of propylene glycol could be explained by the addition of polypropylene glycol as an anti-foaming agent during the yeast culture. The yeast biomass produced was processed with a French press and added to the feed without purification or extraction steps. Nevertheless, previous studies have shown no detrimental effects of propylene glycol on salmonid health (Hilton *et al*., 1986; Aru *et al*., 2021), no effect on aquatic health and no bioaccumulation in aquatic and terrestrial organisms (West *et al*., 2014).

Table 5. *Plasma metabolites in control and treatment fish groups with a significant difference (p < 0.05). Control group of fish fed with vegetable oils, treatment group of fish fed partly with yeast (*Rhodotorula toruloides*) biomass.*

Fish groups	**Metabolites in higher proportions (p-value <0.05)**
Control	*Protein metabolism:* tyrosine
Treatment	Propylene glycol *Fatty acid oxidation*: 3-hydroxybutyrate *Gluconeogenesis:* alanine, lactate *One-carbon metabolism:* betaine, choline, glycine, N-N dimethylglycine, serine *Protein metabolism:* creatinine

Other metabolites with significantly higher levels in treatment fish plasma were metabolites involved in the one-carbon (1C) metabolism (Figure 16). Folate cycle, methionine remethylation and transsulfuration pathways shape the 1C metabolism (Figure 16).

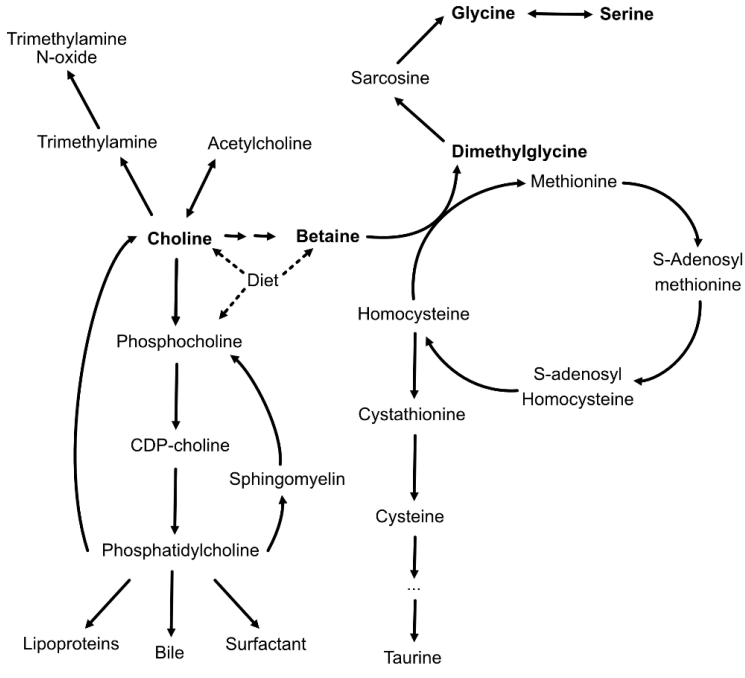

Figure 16. Partial representation of the one-carbon metabolism (choline and methionine-homocysteine pathways). Adapted from Friesen *et al*. (2007), Li & Vance (2008), Wiedeman *et al*. (2018).

In this thesis, choline, was found in higher levels in plasma of fish fed with treatment feed. This metabolite, an essential nutrient for fish and humans, was most probably provided by the diet, however we did not analyze metabolites in the feed. Choline can be synthesized *de novo* in insufficient amounts via the methylation of phosphatidylethanolamine to phosphatidylcholine, later converted to choline by phospholipases (Li & Vance, 2008; Onono & Morris, 2020). Choline is characterized by four main functions: the synthesis of the neurotransmitter acetylcholine (brain), production of trimethylamine (intestine), synthesis of phosphatidylcholine (cells) and production of betaine though different steps of oxidation (liver and kidney) (Wiedeman *et al*., 2018). Low levels of choline in human and fish have been associated with increased risk of liver damages due to TAG accumulation (hepatic steatosis) and lipid malabsorption syndrome (Sherriff *et al*., 2016; Wiedeman *et al*., 2018; Hansen *et al*., 2020). In our study with fish, the higher levels of choline in the treatment group cannot explain the

higher liver weight and HSI as liver fat content was not significantly different. Imbalanced levels of choline can be reversed via hepatic choline metabolism (Li & Vance, 2008). Nevertheless, no differences in the levels of choline were found in the liver between the two fish groups. Choline can be irreversibly converted to betaine using different dehydrogenases (Lever & Slow, 2010).

Betaine was similar to choline and shown in higher levels in plasma from the treatment group. Betaine can be provided by the diet and has shown to have appetite enhancing properties in fish (Lim *et al*., 2016). Betaine levels in the fish feeds were not measured but fish fed with the yeast biomass showed lower appetite than fish fed with control feed (data not shown). Betaine can act as a methyl donor group for the methylation of homocysteine to methionine, producing N,N-dimethylglycine as a by-product (Li & Vance, 2008; Wiedeman *et al*., 2018). Dimethylglycine can be further metabolized into sarcosine by dimethylglycine dehydrogenase in the liver mitochondrion. Sarcosine, a glycine transporter can be converted to glycine by sarcosine dehydrogenase. Glycine can be reversibly converted to serine using serine hydroxymethyl transferase 1 and 2 (Lever & Slow, 2010; Labuschagne *et al*., 2014). Taken together, significant changes in the 1C metabolism could lead to modification of functions related to 1C metabolism such as cell proliferation, methylation reactions and indirectly glucose metabolism (Ducker & Rabinowitz, 2017).

A disruption of some metabolites related to energy metabolism was observed in fish fed yeast biomass with increased levels of plasma alanine, lactate, glycine and serine involved in gluconeogenesis. In salmonids, alanine function has been described as a transporter of amino acid carbon from muscle to liver, heart and other organs for metabolism (Mommsen *et al*., 1980). High activity of gluconeogenesis has been shown in isolated salmonid hepatocytes with high rate of CO_2 production from lactate and glucose with lactate as a preferred substrate (French *et al*., 1981; Mommsen, 1986). Nevertheless, no differences were observed in hepatic levels of lactate and alanine in our study.

Gluconeogenesis or the endogenous production of glucose from lactate or alanine occurs when glucose is not provided by the diet and when glycolysis is not sufficient to provide glucose. Another metabolic pathway exists when glucose is required and is called ketogenesis. The liver produces ketone bodies such as acetoacetate and 3-hydroxybutyrate from acetyl-CoA

during incomplete β-oxidation of FA (Drackley, 2000). Among ketone bodies, only 3-hydroxybutyrate showed significant differences with higher levels in treatment group of fish. An increase of plasma ketone bodies suggests an effect of food deprivation in the treatment group of fish despite no significant differences in levels of ketone bodies in the livers.

Interestingly, tyrosine was the only one metabolite found significantly higher in the control group of fish. Similar observations were seen in phenylalanine levels in a non-significant way. Phenylalanine is an essential amino acid and can be converted to tyrosine in liver and kidney (Wilson & Halver, 1986; Li *et al*., 2009). Low plasma levels of these aromatic amino acids in fish have been explained by lower levels of these amino acids from the feed (Cheng *et al*., 2017). In our study, slightly lower levels of tyrosine and phenylalanine were measured in the treatment feed compared to the control feed (Paper III and IV: Table 3).

Ensuring appropriate amounts of tyrosine and phenylalanine in the fish diet is important as tyrosine is a common precursor for the synthesis of hormones and neurotransmitters involved in immune system, skin pigmentation and more (Borlongan, 1992; Li *et al*., 2009).

The careful consideration of the fish feed composition is essential for ensuring proper growth, health and welfare (Oliva-Teles, 2012). The amino acid composition of a new fish feed formulated should be analyzed, as fish require ten amino acids considered essential: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Wilson & Halver, 1986; Oliva-Teles, 2012). Other than amino acids, the composition of the dietary lipids is essential to investigate as they have important effect on growth, immune response and body composition reflecting the fish fillet nutritional quality (Oliva-Teles, 2012). Additionally, the inclusion of vitamins and minerals to fish feed is needed to avoid deficiencies, improve immune response and reduce lipid oxidation (Oliva-Teles, 2012). As opposed to vitamins and minerals, proteins and lipids, carbohydrates are not found in high levels in fish feed and the carbohydrate utilization varies between carnivorous, omnivorous and herbivorous fish (Oliva-Teles, 2012). Herbivorous fish are more efficient in carbohydrate utilization and carnivorous fish tolerate low levels of carbohydrates. More knowledge on the metabolism of carbohydrates in fish is required to improve the fish nutrition and therefore welfare and health.

To summarize, the partial replacement of fish feed ingredients with a yeast biomass resulted in a similar growth between the two groups of fish, no mortalities during the trial and comparable nutritional qualities in fish fillets. Our findings of increased liver weight and HSI should be further examined to further develop alternative fish feed ingredients with microorganisms.

Concluding remarks and future perspectives

Developing sustainable alternatives to produce protein is essential to reduce human impact on the environment. Related to food sustainability, the valorization of meat by-products can change the meat production system by reducing meat loss and waste. Meat loss and waste globally account for at least 20% of the meat produced from farm to plate (FAO, 2011).

The strategy of the meat consumption to "less but better" in Western or high-income countries was suggested in different studies (de Boer *et al*., 2014; Hyland *et al*., 2017; Apostolidis & McLeay, 2019; Resare Sahlin *et al*., 2022). The idea of "less but better" constitutes a reduction in meat production and consumption while integrating livestock within the complex of agricultural practices and ecosystems. In practice, the strategy applies a transition to agroecology for developing sustainable farming with, for example, the maximization of biomass production (inedible to humans) to feed livestock instead of crops (Øverland *et al*., 2010; Resare Sahlin *et al*., 2022).

When considering the complexity of food consumption such as health, climate, economy and animal welfare, the responsibility of eating "less but better" should not fall on the consumers' shoulders. Developing a sustainable and healthy diet is a matter of political and scientific strategies (Resare Sahlin & Trewern, 2022).

Related to scientific strategies to produce food in a sustainable manner, the investigation of the novel ingredients in fish feed and its effects on fish health, welfare and growth are relevant ideas. The replacement of vegetable oils with an oleaginous yeast biomass in the diet of Arctic char has shown promising results in our study. Nevertheless, additional investigations should be conducted to understand the higher liver weight and HSI observed in fish.

These investigations could include for example, the analysis of extracted lipid phase from the livers with NMR or the comparison between control and treatment fish groups for protein efficiency ratio, digestibility and immune system response. Histology of intestinal tissues could provide additional information on the digestibility of the yeast biomass. Other future perspectives include the genetic selection of fish capable of digesting new ingredients. Genomics and transcriptomics studies related to fish feed would provide new knowledge on lipid and energy metabolisms and could guide us on how to improve animal welfare.

An additional focus not included in this thesis is the reproducibility of trials involving fish feeds with microorganisms, as the yeast biomass composition varies with different culture conditions (Shen *et al*., 2017; Glencross *et al*., 2020). Measurements of sterols such as CL and ergosterol or other fat-soluble metabolites in the yeast biomass would add information on the composition. Ergosterol is the main type of sterol in yeasts (Daum *et al*., 2007; Dupont *et al*., 2012). Furthermore, evaluating the levels of sterols in the feed could facilitate the interpretation of fish performance. CL has important metabolic functions such as the regulation of steroid hormones (Pickova & Mørkøre, 2007; Hu *et al*., 2010). The ratio of CL/ergosterol for fish health in predatory fish should be evaluated in further feeding trials involving microorganisms.

Related to microbial feed, the evaluation of carbohydrate composition of yeast biomass is complex. Cell walls of yeast contain high amounts of mannan-oligosaccharides, β-glucans, and chitin, varying with the growing conditions and with the yeast species (Nalage *et al*., 2016; Glencross *et al*., 2020). In addition, the regulation of fish metabolism related to glucose and other carbohydrates is not fully understood, in particular among all different fish species (Polakof *et al*., 2012) and the metabolism of complex carbohydrate compounds in fish could be another focus of future research.

In this thesis, yeast biomass was produced from agricultural by-products (hydrolysate of lignocellulosic biomass from wheat straw). A different area of research could focus on other potential by-products for yeast biomass production, such as lignocellulose from the forest industry. With the largest wooded area in the European Union, Sweden produces the largest quantity of roundwood partially used for pulp and paper production (European Commission, Eurostat, Cook, 2019). In addition, between 6-7% of the land in Sweden is used as agricultural land (European Commission, Eurostat,

Cook, 2019). There is an opportunity to develop biomass production for biofuels and food purposes with 680,000 ton/year of straw resources as net supply for energy purposes and 910,000 ton/year of currently harvested forest biomass (as logging residues) in Sweden (Börjesson *et al*., 2013).

Limitations in the utilization of by-products from forest and agricultural industries for fish feed production are the risk of contamination, the competition with other use of wood by-products such as heating, products manufacturing, production of organic fertilizers and biofuels (Gupta *et al*., 2022). Lignocellulosic biomass is characterized by a complex structure of polysaccharides (cellulose and hemicellulose) and lignin, difficult to degrade (Maity, 2015; Gupta *et al*., 2016; Passoth & Sandgren, 2019). The sugars found in lignocellulose need to be made available for microbial fermentation. Pretreatment of the lignocellulose includes thermochemical and enzymatic steps such as acid steam explosion and enzyme hydrolysis, requiring energy and the synthesis of enzymes.

Further development of fish feeds could involve other microorganisms, ideally producing the FA essential to fish and humans. One example is the use of heterotrophic microalgae-like unicellular organisms providing proteins and DHA rich lipids (Kousoulaki *et al*., 2020). The combination of different microorganisms for their specific nutritional profile such as high lipid content from oleaginous yeast, high protein content from Brewer´s yeast or from bacteria (Glencross *et al*., 2020) and LC-PUFA content from microalgae could cover a large range of nutritional needs in fish.

Main findings

The main aim of this thesis was to investigate changes in physiology of humans and fish by metabolomics. The first part evaluated the nutritional composition of a meat by-product (test product containing porcine heart and aorta) and included the test product in a clinical trial. The second part of this thesis replaced traditional fish feed ingredients with a microbial alternative and investigated the change of feed on fish growth and performance.

- The test meat product contained more monounsaturated fatty acids and tyramine than a control product of similar quality

- The consumption of the test meat product in a clinical trial with patients presenting atherosclerosis symptoms reduced levels of blood lipids associated with increased cardiovascular risks compared to the control

- The replacement of vegetable oils with oleaginous yeast (*Rhodotorula toruloides*) in fish feed did not affect the growth and performance of Arctic char (*Salvelinus alpinus*) with the exception of a higher liver weight and hepatosomatic index than in the control

- Toxicological investigation of the yeast biomass as a fish feed ingredient revealed safe levels of pollutants and heavy metals

- Metabolomics analysis of plasma and liver from fish fed with the yeast biomass showed higher levels of metabolites involved in energy pathways such as tricarboxylic acid cycle and gluconeogenesis compared to the control group

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Popular science summary

Food and feed are essential constituents of life. Nutritional recommendations exist to guide individuals in the consumption of food in suitable proportions and to avoid diseases related to food. Insufficient intake of important micronutrients such as vitamins D and B12, iron, selenium and essential amino acids leads to severe or life-threatening conditions. Similarly, the overconsumption of nutrients can results in toxicity or serious health outcomes such as obesity, type 2 diabetes and cancer.

To understand the effect of the food composition on health, the study of food constituents at the molecular level is a suitable approach.

Metabolomics is defined as the large-scale study of small molecules called metabolites and includes lipids, amino acids and sugars. Metabolites can be obtained from cells, biofluids, tissues or organisms. The study of metabolites within a cell or organism provides a broad view of the physiological functions occurring at a specific time (sampling, before and after a disease). Metabolomics can be applied to the field of nutrition and food science to understand the connection between food and health.

The thesis was conducted in two parts using similar analytical methods, nuclear magnetic resonance and gas chromatography. The first part evaluated the nutritional composition of a meat product and its effects on patients during a clinical trial. The second part concerned the sustainability of fish feed ingredients and microbial alternatives.

Organ meats are defined as edible organs such as liver, heart, lungs, tongue, ears and contain a large set of vitamins and amino acids. The production of animal and animal feed has been associated with detrimental effects of the environment with higher greenhouse gas emissions and the use of finite resources such as water and land. The meat consumption is expected to increase as a result of a growing population and an increased average

income. One approach to reduce the impact of our food production on the carbon footprint and limit the use of resources could be the valorization of meat by-products to avoid an increase animal production. In this study, the test meat product made of pork heart and aorta contained fatty and amino acids commonly found in animal products such as saturated, unsaturated and polyunsaturated fatty acids, valine, proline, glycine and tryptophan. Previous studies with this product have demonstrated lowering effects of blood lipids, a decrease in body weight and a reduction of inflammation, in rats and humans showing atherosclerosis symptoms. The clinical trial conducted in this thesis showed similar results with a decrease of triglycerides and other blood lipids in the group of patients consuming the test meat product and not in the control group.

Another approach to reduce the utilization of resources for animal production is the search for sustainable ingredients to feed. Aquaculture has developed for the last decades to respond to the increasing demand for fish and seafood. Traditional ingredients in fish feed include limited fish meal and oil sources from fisheries. Fish and seafood are a rich source of proteins, vitamins, minerals and long-chain omega-3 fatty acids. Finding alternatives to traditional fish feed ingredients is necessary to improve food security and to reduce our impact on ecosystems. Fish oil can be replaced with vegetable oils in moderate amounts without affecting the fish growth and welfare. Nevertheless, vegetable oils are widely used for other food purposes and the production requires considerable land, water, fertilizers and pesticides. One potential alternative is to replace vegetable oils by microbial oils. For example, oleaginous yeasts are capable of producing up to 60% of their cell weight in lipids and have a fast growth. Our study including yeast biomass in fish feed during a trial showed comparable growth between the fish fed with the new feed based on yeasts and a control group. The fish fed partially with the yeast biomass showed a higher liver weight compared to the control group and more research is needed to understand this effect. Additionally, the production costs of microbial ingredients should be further investigated to be able to implement the idea at a larger scale.

The findings in this thesis provide additional information on a more sustainable formulation of animal feed and use of animal products to valorize existing products and decrease the impact on ecosystems.

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IV

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Oleaginous yeast *Rhodotorula toruloides* biomass effect on the metabolism of Arctic char (*Salvelinus alpinus*)

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Sustainability issues arise when using fish oil and vegetable oils in fish feed production for aquaculture purposes. Microbial production of single cell oil is a potential alternative as a lipid ingredient in the production of fish feed. In this study, we replaced the vegetable oils with the oleaginous yeast *R. toruloides* biomass in the diet of Arctic char (*S. alpinus*) and investigated the effects on health and composition. Measurement of fish growth parameters showed a higher liver weight and hepatosomatic index in the experimental group of fish fed partly with yeast biomass compared to a control group fed a diet with vegetable oils. No significant differences in the lipid content of muscle and liver tissues were found. The fatty acid profiles in the muscle of both fish groups were similar while the experimental fish group had a higher amount of monounsaturated fatty acids in the liver. Histology of livers showed no significant difference in the number of lipid droplets. The size of hepatic lipid droplets seemed to be related to liver fat content. Quantification of metabolites in the liver revealed no differences between the fish groups while plasma metabolites involved in energy pathways such as alanine, 3-hydroxybutyrate, creatinine, serine, betaine, and choline were significantly higher in the experimental fish group.

KEYWORDS

metabolomics, fish feed replacement, fatty acids, metabolites, gluconeogenesis, plasma, liver, oleaginous yeast

1 Introduction

Over the past decades, aquaculture has steadily expanded when wild capture fisheries have stagnated. As the world's fastest-growing food production industry, aquaculture has developed and contributes to providing animal protein, n-3 long-chain polyunsaturated fatty acids (LC-PUFAs). Aquaculture represents an essential source of income, livelihood, and nutrition for millions of people (FAO 2020). Marine products contain important

high-quality lipids, proteins, and minerals required for human health (FAO, 2020). Different health agencies worldwide recommend weekly fish and fish products consumption as essential sources of LC-PUFAs, in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) whose synthesis *de novo* is not possible in humans (Bradbury, 2011; de Roos et al., 2017).

One of the limitations in the growth of aquaculture is the sustainability of all fish feed ingredients. Fish feeds are traditionally formulated with fishmeal (FM) and fish oil (FO) to fulfill protein and lipid requirements for optimal fish growth (Sprague, Betancor & Tocher, 2017). Finding appropriate alternatives to the lipid portion of fish feed is imperative as oils used for fish feed formulation are at present sourced from limited and unsustainable wild fisheries or from terrestrial plants whose current production is shared with other food products. In addition, the growing use of plant oils for fish feed might lead to increased water footprint, expansion of agricultural land, excess of nitrogen and phosphorus fertilizer leading to eutrophication, and additional use of chemical pesticides (Fry et al., 2016).

Potential alternatives and sustainable lipid sources are microbial organisms such as yeasts, bacteria, and microalgae (Sahlmann et al., 2019), with high nutritional values (Sprague, Betancor & Tocher, 2017; Gamboa-Delgado & Márquez-Reyes, 2018). Microalgae and other microorganisms are the main producer of LC-PUFAs and therefore the main provider of EPA and DHA in marine organisms (Morales-Sánchez et al., 2017; Sprague, Betancor & Tocher, 2017). An interest in cultivating heterotrophic single cell organisms (SCOs) as fish feed ingredients is growing in aquaculture (Shah et al., 2018). Heterotrophic SCOs have interesting protein and lipid productions and less demanding cultivation requirements such as lower light, and thereby volume, compared to autotrophic organisms. Moreover, heterotrophic organisms could potentially consume biomass from other biological processes as an organic carbon source for growth (Morales-Sánchez et al., 2017). SCOs have been shown to be suitable protein alternatives to FM protein in aquafeeds without detrimental effects on fish growth (Shah et al., 2018). Protein content and quality of yeasts have been previously evaluated (Sahlmann et al., 2019), specifically, proteins from baker's yeast, showing no adverse effect on fish health, growth performance, digestibility, and nutrient retention, when incorporated in moderate amounts (Øverland et al., 2013; Øverland & Skrede, 2017; Vidakovic et al., 2020). Information on yeast as a sustainable protein source in aquaculture is available, while knowledge on yeasts' suitability as a lipid source (oil) is limited (Øverland & Skrede, 2017; Blomqvist et al., 2018).

Oleaginous yeasts, being heterotrophic, have been considered a sustainable alternative lipid source due to their capacity to accumulate 20% and more of their dry matter mainly as triacylglycerols (TAG), their fast growth, and their ability to grow on low-value substrates such as lignocellulose hydrolysate (Abeln & Chuck, 2021). The recovery of lignocellulosic biomass to grow alternative ingredients included in fish feed production would limit the use of unsustainable resources needed for aquaculture and would repurpose by-products generated by agricultural and forestry areas (Øverland & Skrede, 2017). The costs related to the production of oleaginous yeast biomass remain more expensive than cheap plant oil such as palm oil. Nevertheless, the production costs could be limited with the use of low-cost substrates such as lignocellulosic biomass (Abeln & Chuck, 2021). With climate change, the resources and energy needed to grow plants will change drastically while the production of oleaginous yeast should not be affected (Abeln & Chuck, 2021).

Oleaginous yeast biomass has been previously included as an ingredient in fish feed with no effect on fish as demonstrated by Blomqvist et al. (2018). In that study, the oleaginous yeast *Lipomyces starkeyi* biomass was added to the diet of Arctic char (*S. alpinus*) and revealed no difference in the hepatosomatic index, condition factor, and specific growth factor.

Similar to *L. starkeyi,* the red yeast *R. toruloides* can consume lignocellulose as carbon and nitrogen sources to grow and produce lipids. The yeast *R. toruloides* was selected in our study for its higher content of polyunsaturated fatty acids compared to *L. starkeyi* strains (Brandenburg et al., 2021). In addition, *R. toruloides* yeast is capable of producing carotenoids including torulene, torularhodin, and β-carotene (Pinheiro et al., 2020; Nagaraj et al., 2022), potentially leading to a higher antioxidant activity when preserving the fish feed.

To obtain a better understanding of the effect of alternative fish feed ingredients on fish physiology, metabolomics tools can be used. Metabolomics can be defined as the non-selective study of metabolites through relative or absolute quantification (Roques et al., 2020) using nuclear resonance spectroscopy (NMR) or mass spectroscopy. The metabolites identified in an organism can be an indicator of a biological process or exposure to contaminants (xenobiotics). Fish nutrition metabolomics is a new field, as recently reviewed by Roques et al. (2020) and Lulijwa, Alfaro & Young (2022), identifying biomolecules in response to different feeds. As suggested by Roques et al. (2020), the application of metabolomics techniques in fish nutrition provides essential information on the effect of a diet on fish metabolism from a nutrient perspective and at the molecular level, which is not possible with the use of more traditional techniques such as measurement of growth parameters, digestibility, and feed conversion.

Several studies exist on the effect of diet change on fish using metabolomics when searching for more sustainable fish feed ingredients (Casu et al., 2017; Cheng et al., 2017; Wagner et al., 2019). Publications on including an oleaginous yeast biomass as a fish feed ingredient can be found (Hatlen et al., 2012; Blomqvist et al., 2018) but not from a metabolomics perspective. Therefore this study aimed to evaluate the oleaginous yeast *R. toluroides* grown on lignocellulosic biomass as a sustainable alternative ingredient in the fish feed of Arctic char and to assess the metabolic effects of this ingredient alteration in Arctic char. For that purpose, we

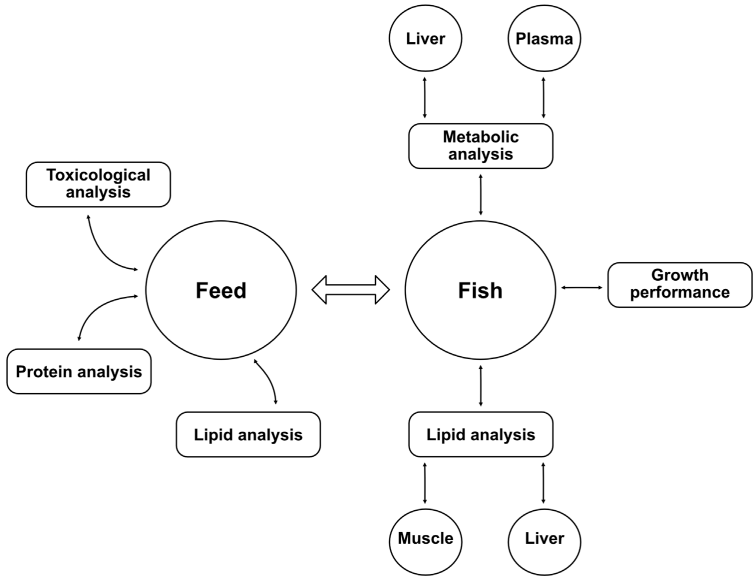

FIGURE 1
Diagram of the different analyses performed in the study. Lipid analyses were achieved with gas chromatography, metabolic analyses were conducted using nuclear magnetic resonance spectroscopy. The growth performance of fish was measured with total weight, length, and other parameters shown in Table 4. Protein analysis (crude protein content and amino acid profile) was carried out by Eurofins Food & Feed Testing Sweden AB. Toxicological analyses of the yeast biomass included measurement of organic pollutants (polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and hexachlorobenzene) and heavy metals (aluminum, arsenic, cadmium, mercury, and lead) (manuscript in preparation).

TABLE 1 Composition of control and experimental yeast feeds (g kg^{-1}) for fish in duplicates. "Vitamin mix" and "Mineral mix" ingredients were provided by NOFIMA (Norway) and "astaxanthin and vitamin mix" ingredients were provided by Aller Aqua A/S (Denmark).

Ingredients	Control feed	Experimental feed
Fish meal	4,950	4,950
Fish oil	1,170	1,170
Vegetable oil	540	–
Mineral mix	45	45
Vitamin mix	90	90
Astaxanthin and vitamin mix	13.5	13.5
Gelatine	45	45
Wheat meal	1.755	1.305
Casein	540	–
Ca_2PO_4	225	225
Yeast	-	1,413 (540 g oil)

investigated the lipid composition of the fish by measuring fatty acid profiles of muscle and liver tissues with gas chromatography, including different lipid classes of the liver. Plasma and liver metabolites compositions were studied using one proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. Histological analysis was performed on livers using hematoxylin and eosin staining.

2 Materials and methods

A summary of the different chemical analyses conducted in this study on feed and fish can be found as a diagram in Figure 1.

2.1 Formulation of control and experimental feeds

Control and experimental fish feeds were formulated to contain similar protein and fat contents to meet the nutritional requirements for salmonids (Sánchez-Vázquez et al., 1999; Pettersson et al., 2009). Control feed was differentiated from the experimental feed in consisting of vegetable oils and casein while experimental feed contained no VO and no casein, and was partially made of yeast oil and proteins from the yeast biomass (Table 1).

TABLE 2 Total fat content (g kg^{-1} wet weight), fatty acid composition (% of total identified FA) of the two fish feeds (duplicate analyses), and yeast biomass separately.

Fatty acids	Control feed	Experimental feed	Yeast biomass (raw)
Total fat content	19.8	18.0	29.6
14:0	4.62	4.86	
15:0	0.53	0.34	
16:0	17.6	18.7	29.4
16:1 (n-7)	4.62	4.59	
16:2 (n-4)	0.31	0.21	
17:1	0.00	0.24	
18:0	2.43	2.68	4.40
18:1 (n-9)	23.7	23.0	43.4
18:1 (n-7)	2.56	2.06	
18:2 (n-6)	6.21	5.92	16.0
18:3 (n-3)	1.86	1.47	3.50
20:1 (n-11)	1.62	1.59	
20:1 (n-9)	8.65	8.54	
18:4 (n-3)	0.32	0.34	
20:4 (n-6)	0.39	0.33	
22:1 (n-9)	11.2	11.2	
20:4 (n-3)	1.02	1.03	
20:5 (n-3)	4.95	5.12	
24:1	0.51	0.54	
22:5 (n-3)	0.65	0.50	
22:6 (n-3)	6.26	6.71	
SFA	25.2	26.6	
MUFA	52.8	51.8	
PUFA	22.0	21.6	
n-3	15.1	15.2	
n-6	6.60	6.25	
n-3/n-6	2.28	2.42	

Abbreviation: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

To obtain yeast biomass, the yeast strain *R. toruloides* CBS 14 (Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands) was cultivated according to Blomqvist et al. (2018), Nagaraj et al. (2022) methods before its incorporation into the experimental feed. *R. toruloides* yeast cells were retrieved from glycerol stocks at −80°C (50% v/v) and kept on YM-agar plates at 25°C. A pre-culture of the yeast was performed before cultivation after adding a loopful of yeast cells to a 100 ml YPD-medium (Blomqvist et al., 2018). A second inoculation step was performed for 3 days under the same conditions after transferring 100 ml of yeast culture from the first inoculation to a new YPD medium. Yeast precultures were harvested by centrifugation (4,000 × g, 10 min) and washed with a saline solution (NaCl, 9 g L^{-1}). *R. toruloides* was cultivated in 8 L Dolly fermentors (Belach Bioteknik, Stockholm, Sweden) at 25°C with a mix of 60% sterile filtered cellulose and 40% hemicellulose hydrolysate for 3 days (Blomqvist et al., 2018). Yeast cells were harvested by centrifugation (5,400 × g, 10 min), washed with deionized water and their cell walls were disrupted after applying a French press (Constant systems LTD, Daventry, United Kingdom). The product of the yeast cultivation was stored at −20°C until the preparation of the fish feed.

Similar composition of fatty acids was achieved between the two feeds. Fatty acids found in yeast oil were adjusted by mixing palm and rapeseed oils (1:1) in the vegetable oil mix added to control feed. All ingredients in each fish feed were added simultaneously and mixed by hand to form a homogenous paste consistency. Each fish feed was placed into a kitchen meat grinder, pressed, cut manually into pellets (2–4 mm length), and left to dry at room temperature overnight. Finally, the fish feed pellets were packed in airtight plastic bags and stored at −20°C until further use. Fatty and amino acid compositions of both feeds were analyzed (Tables 2, 3).

Crude protein content and amino acid composition of both fish feeds were analyzed by Eurofins Food & Feed Testing Sweden

TABLE 3 Amino acid composition (%) of the two fish feeds. The confidence interval of all values is below 15%. Data are presented as a proportion of total determined amino acids [% of dry matter] for both feeds. Amino acid analysis was performed by Eurofins Food & Feed Testing Sweden AB in addition to crude protein content analysis (ISO 16634-1 2008; ISO 16634-2 2016).

Amino acids	Control feed	Experimental feed
Crude protein (%)	44.8	42.1
Alanine	2.7	2.5
Arginine	2.7	2.5
Aspartic acid	4.2	3.8
Cysteine + Cystine	0.5	0.5
Glutamic acid	7.3	5.8
Glycine	2.8	2.7
Histidine	1.0	0.8
Isoleucine	1.9	1.6
Leucine	3.4	2.9
Lysine	3.5	2.8
Methionine	1.2	1.2
Phenylalanine	1.9	1.6
Proline	2.4	1.9
Serine	2.2	1.8
Threonine	1.9	1.7
Tryptophan	0.5	0.4
Tyrosine	1.6	1.3
Valine	2.2	1.9

AB (Lidköping, Sweden), following the method SS-EN ISO 13903:2005 for amino acid analysis and ISO 16634-1 2008, ISO 16634-2 2016, respectively, for crude protein content analysis.

2.2 Feeding trial and sample collection

Arctic char (n = 126, both genders, juveniles) were randomly assigned to six 1 m × 1 m water tanks (triplicate tanks per feed with 21 fish per tank) with access to a flowthrough system of freshwater (10 L min^{-1} with a water depth of 20 cm) from Lake Ansjön at Kälarne Aquaculture Center North, Sweden. Fish were acclimated for 7 days in the tanks. A natural photoperiod was kept and water temperature averaged at 7.1 ± 1.8°C. Before the feeding trial, fish were weighed, measured, and fed with a commercial feed appropriate for juvenile Arctic char. During the trial, the fish feed was distributed by band feeders 4 times a day with a feeding ratio of 2% of the actual biomass in the tanks. After 53 days of the feeding experiment, all fish were weighed and measured after a 24 h starvation period. The total body length (cm), and body and liver weights (g) were recorded for all fish. The specific growth rate (SGR) was calculated from day 19 of the trial due to technical problems occurring at the rearing station during the start of the trial.

Indicators of fish growth performance such as specific growth rate (SGR), condition factor (CF) and hepatosomatic index (HSI) were calculated as follows:

$$SGR = [(\ln Wt - \ln W0)/t] \times 100$$
$$CF = Wt/T_L^3 \times 100$$
$$HSI = (Wl/Tw) \times 100$$

Where Wt = final weight of fish in g; W0 = initial weight of fish in g; t = time (days); T_L = total length in cm; Wl = weight of liver in g; Tw = total weight in g.

After the starvation period, 36 fish (6 from each tank) were anaesthetized using tricaine methanesulfonate (MS-222, 30 mg L^{-1}, Sigma Chemicals Co., St. Louis, MO, United States) to overdose in a standardized way and in a well-oxygenated water, followed by a blow to the head before sampling for chemical analyses (Cheng et al., 2016b; Cheng et al., 2017) while the remaining fish were sampled for sensory analysis (manuscript in preparation).

Blood plasma was collected using venipuncture with a heparinized syringe (from 36 fish in total, with plasma quantity too low for four fish to be analyzed). Blood samples were stabilized with an aqueous solution of heparin sodium salt in Eppendorf tubes and stored at −80°C. Both liver (36 fish) and muscle tissues (dark and light fillet areas, 36 fish) were collected from the same fish according to the Cheng et al. method (2016a; 2016b) and stored at −80°C until analyses. During the chemical analysis, 32 plasma samples and 12 liver samples (two from each tank) were analyzed for the quantification of metabolites. From a total of 36 muscle samples, 12 samples (two from each tank) were analyzed for lipid content and fatty acid profiles with technical replicates.

The survival rate of the fish during the feeding trial was at its maximum with no death recorded. The experiment was approved by the Ethical Committee for Animal Experiments (Umeå, Sweden) and carried out in compliance with the European legislation (i.e., Directive 2010/63/EU).

2.3 Lipid extraction and fatty acid analyses

2.3.1 Lipid extraction

Total fat content and FA composition of muscle and liver tissues as well as of the two feeds were determined using a lipid extraction method with gas-liquid chromatography. Total lipids were extracted from fillets (n = 12), liver (n = 36), and the fish feed with approx. 2 g suspended in 8 ml H_2O, in technical duplicates (Hara & Radin, 1978; Mráz & Pickova, 2009). Briefly, 1 g of sample was homogenized in the hexane-isopropanol mix (HIP; 3:2, v:v) three times for 30 s with an Ultra-Turrax (Janke and Kunkel, IKA Werke, Germany). Lipids were separated from non-lipids after adding 6 ml of Na_2SO_4

solution (6.67%, *w:v*) and mixing with a vortex. The samples were left for separation at 4°C for 15 min and the transferred lipid phase was dried under nitrogen gas. The total lipid content was quantified gravimetrically and lipids were stored in hexane −80°C until further analysis.

2.3.2 Lipid classes

In addition to total fat content and FA composition analyses, total extracted lipids (TL) from fish liver were separated into phospholipids (PL) and triacylglycerols (TAG) classes using thin layer chromatography (TLC) as described earlier by Pickova et al. (1997) and Mráz & Pickova (2009). TLC plates (20 cm × 20 cm; pre-coated Silica gel 60; 0.20-mm thickness from Merck, Darmstadt, Germany) were selected as stationary phase and solvents (hexane, diethylether, and acetic acid; 85:15:1, v/v/v) were added to TLC chamber. PL and TAG sample areas were identified by comparison with a reference standard separately revealed in iodine. Areas of interest were scraped off the TLC plates. The PL class was extracted successively with chloroform:methanol (2:1, 2 ml, v:v); chloroform: methanol (1:1, 3 ml, v:v) and chloroform (2 ml), while TAG was extracted three times in succession with chloroform (2 ml). Samples were evaporated under nitrogen gas, recovered in 0.5 ml hexane, and stored at −20°C until further analysis.

2.3.3 Methylation and gas chromatography

From the total extracted lipids of fish feed, fish muscles, and liver, 2 mg of each sample was taken for identification of FA composition after a methylation step using boron trifluoride (BF3, 14%) and methanol reagents according to the method described by Appelqvist (1968). Briefly, methylation was performed by adding NaOH (2 ml, 0.01 M) in dry methanol in each sample followed by an incubation time of 10 min at 60°C. A similar next step was completed with each sample by adding BF3 (3 ml, 14%) with another incubation time of 10 min at 60°C. After cooling, NaCl (2 ml, 20%) and hexane (2 ml) were added simultaneously to each sample and left to separate. The upper phase containing hexane and FA methyl esters (FAME) was transferred to a new tube and evaporated under nitrogen gas. The same methylation method was applied to the different liver lipid classes (PL and TAG) using the entire sample. FAME generated after methylation were stored in hexane at −80°C until analyses.

The FAME were analyzed by GC with a CP 3800 instrument (Varian AB, Stockholm, Sweden) equipped with a flame ionization detector, and a split-mode injector, and separated on a 50 m fused silica capillary column BPX 70 (SGE, Austin, Tex) with 0.22 mm i.d. × 0.25 µm film thickness. The injector and detector temperatures were 230°C and 250°C, respectively. Helium at a flow rate of 0.8 ml min^{-1} was the carrier gas while nitrogen was the make-up gas. Identification of FA peaks was achieved by comparing retention times of each FA peak with those of the standard mixture GLC-68A (Nu-check Prep, Elysian, United States). Peak areas were integrated using the Galaxie chromatography data system software version 1.9 (Varian AB, Stockholm, Sweden).

2.4 Liver and plasma metabolomics using ^{1}H-NMR spectroscopy

Fish liver tissue was extracted according to previous studies (Moazzami, Andersson & Kamal-Eldin, 2011; Wagner et al., 2019) with minor modifications. Briefly, the frozen liver tissue sample (100 mg) was homogenized with an Ultra-Turrax (Janke and Kunkel, IKA Werke, Germany) in an ice-cold methanol-chloroform mix (2:1, *v:v*, 3 ml) for 1 min and sonicated for 30 min. After adding 1 ml of ice-cold chloroform and 1 ml of ice-cold water, each sample was vortexed for 1 min before centrifugation at 1,800 g × for 35 min at 4°C. The aqueous supernatant (polar phase) was collected, dried using a speedvac (Savan, SVC 100H, Techtum Instrument AB, Umeå, Sweden), and redissolved in 520 µl sodium phosphate buffer (0.135 M, pH 7.0). Reconstituted samples were filtered using pre-washed Nanosep centrifugal filters with a 3-kDa cutoff (Pall Life Science, Port Washington, NY) by centrifugation at 12,000 × g at 4°C for at least 2 h. For each sample, fish liver filtrate (350 µl), sodium phosphate buffer (170 µl, 0.135 M, pH 7), deuterium oxide (D_2O, 50 µl), and the internal standard sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate solution (TSP-d4, 30 µl, 5.8 mmol/L, Cambridge Isotope Laboratories, Andover, MA, United States) were mixed and added to a 5-mm outer diameter NMR tube (Bruker Spectrospin Ltd., BioSpin, Karlsruhe, Germany) before analysis.

Plasma samples were filtered following a similar method to Röhnisch et al. (2018), using pre-washed Nanosep centrifugal filters with a 3-kDa cutoff (Pall Life Science, Port Washington, NY). Samples were added to filters and centrifuged at 10,000 × g, 4°C for at least 1 h 30 min. For quantification of metabolites, a mix containing filtrate (40 µl), sodium phosphate buffer (50 µl, 0.4 M, pH 7), D_2O (15 µl), Millipore water (55 µl), and TSP-d_4 (10 µl, 5.8 mmol/L, Cambridge Isotope Laboratories, Andover, MA, United States) was prepared for each sample and added to a 3-mm outer diameter NMR tube.

Both plasma and liver analyses were performed on the same Bruker spectrometer instrument operating at 600 MHz equipped with a cryogenically cooled probe and autosampler. ^{1}H-NMR spectra were acquired using a zgesgp pulse sequence (Bruker Spectrospin Ltd.) at 25°C, over a spectral width of 17,942.58 Hz (acquisition time: 1.83 s, relaxation delay 4 s) at 65,536 data points with 512 scans for plasma samples and 128 scans for liver samples. Data were processed using Bruker Topspin 4.1.1 software and Fourier-transformed after multiplication by line broadening of 0.3 Hz. NMR spectra had their baseline adjusted manually and were calibrated using TSP-d_4 at 0.0 ppm. ChenomX NMR suite version 7.1 profiler (ChenomX Inc., Edmonton, AB, Canada), the Human Metabolome database as well as previous literature (Cheng et al., 2017) were used to identify and quantify metabolites in plasma and liver samples.

2.5 Histological preparation of fish livers

Fish livers (n = 8) were prepared for histological analysis using hematoxylin (Mayers HTX, HistoLab) and eosin (Eosin Y 0.2%, HistoLab) staining.

The samples kept at −80°C were first thawed and fixed in neutral buffered formaldehyde solution (10%) for 24 h. The livers were sliced (size 4 mm) and placed in cassettes for embedding. The sliced livers were prepared for dehydration and paraffin infiltration using a tissue processing machine (Thermo Scientific Excelsior AS) for 13–14 h. The samples were embedded in paraffin blocks, sectioned with a microtome (Thermo Scientific HM 355S, section size = 4 µm), and placed on glass slides. The slides were dried overnight at 37°C and later incubated at 60°C for 40 min. Several rehydration steps were performed on the slides with xylene solution and with two concentrations of ethanol (95 % and 70%). The samples were stained with Mayer's hematoxylin (5 min) and with eosin (30–60 s). Dehydration steps were performed with ethanol (95%) followed by cleaning steps with xylene solution before mounting the slides with coverslips.

Histological slides were analyzed using a light microscope (Nikon Eclipse Ni) mounted with a camera (Nikon Digital sight DS-Fi2). Images were captured with NIS-Elements Basic Research (Nikon Imaging Software).

Lipid droplets were counted at the same area of the same size of an image (1.44 cm × 1.44 cm) using the grid lines option in ImageJ with 60,000 pixels2 in area per point (Focus 33.3%).

2.6 Statistics

Differences in growth parameters were estimated using a PROC MIXED function in SAS software (version 9.4, SAS Institute Inc, Cary, NC, United States), taking into consideration the different fish tanks as a random factor. The same statistical method was applied to total fat content, FA in lipid classes, and FA in total lipids from liver and muscle samples.

Multivariate data analysis was performed on liver and plasma metabolites using the SIMCA software (version 17; Umetrics, Suite of Data Analytics Solutions, Sartorius). Data were UV-scaled and principal component analysis (PCA-X) scores were plotted to detect strong outliers (Eriksson et al., 2013) using Hotelling's T2 (CI 95-99%). No strong or moderate outliers were observed in a dataset of liver samples. Two outliers (strong or moderate) were identified in the dataset of plasma samples: fish samples 1 and 6. Interpretation of data was performed with and without the outliers in the plasma dataset.

After a search for outliers, liver and plasma data were classified using control/experimental feeds as a discriminative variable. The data classification to assess the difference between the two groups of fed fish was performed using an orthogonal partial least-squares discriminant analysis (OPLS-DA) model.

The reliability of the OPLS-DA models was verified with analysis of variance testing of cross-validated predictive residuals (CV-ANOVA), determined on an ANOVA assessment of the cross-validatory predictive residuals of some types of the models such as PLS and OPLS models. CV-ANOVA is based on significance testing using an F-distribution by comparing two models fitted to the same dataset by their fitted residuals (Eriksson et al., 2008). In addition, to confirm the validity and predictability of the different multivariate models, R^2X (proportion of variation modeled in the component, using the X-model) and Q^2 (proportion of variation predicted by the model X in that component according to predicted residuals) parameters were reported.

Discriminative metabolites were classified in OPLS-DA models based on their variable influence on projection (VIP) value. Metabolites with VIP values > 1 and with the corresponding jackknife-based 95% confidence intervals (CIs) above or including zero were considered discriminative. All metabolites were evaluated by the PROC MIXED function in SAS 9.4 with tank factor as a random factor but only metabolites meeting criteria in SIMCA were considered to have a significant difference between the two fish groups in this study.

Metabolites appearing significantly different between the fish groups in SIMCA were checked for normality with the PROC MIXED function in SAS 9.4 including tank factor. Metabolites not normally distributed were tested for significance in SAS 9.4 using NPAR1WAY with Wilcoxon score.

Regarding histology data (n = 8), the statistical difference in the numbers of lipid droplets between the two fish groups was evaluated in SAS 9.4 with the PROC MIXED function, taking into consideration the different fish tanks as a random factor. In addition, a multivariate analysis with the PCA-X score plot (SIMCA software, version 17; Umetrics, Umeå, Sweden) was added to the interpretation of histological data. Fat content (%) and number of lipid droplets were included in the PCA model as variables with UV-scaled data.

3 Results

3.1 Fish performance

Fish showed a similar growth during the feeding trial with no differences observed in the final weight, final length, and specific growth factor (Table 4). Nevertheless, fish fed with the experimental feed depicted a significantly higher liver weight and higher hepatosomatic index.

3.2 Fatty acid profiles in muscle and liver tissues

Total fat content and fatty acid profiles were of the same levels in muscle tissue of the fish between control and

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TABLE 4 Growth parameters for fish fed with control feed or experimental yeast-based feed. Data are presented as the mean ± standard deviation. Statistical significance was set at p-value < 0.05.

Growth parameters	Control group	Experimental group	p-value
Weight (g), day 19 (n = 12 per group)	209 ± 65.4	200 ± 62.9	0.7360
Final length (cm) (n = 48 per group)	29.4 ± 2.20	29.2 ± 2.54	0.7417
Final weight (g) (n = 48 per group)	314 ± 99.1	303 ± 94.3	0.6108
Liver weight (g) (n = 48 per group)	**3.75 ± 2.07**	**4.61 ± 1.91**	**0.0069**
SGR (%) (53 days)	0.68 ± 0.59	0.70 ± 0.56	0.8455
CF (%) (n = 48 per group)	1.20 ± 0.14	1.19 ± 0.22	0.9078
HSI (%) (n = 48 per group)	**1.14 ± 0.27**	**1.51 ± 0.34**	**<0.0001**

Significant results are shown in bold letters. SGR, specific growth rate; CF, condition factor; HSI, hepatosomatic index.

TABLE 5 Total fat content (%) and fatty acid profile in the fillet (dark and light muscle; % of total identified FA) from Arctic char fed with control feed and experimental yeast feed. Data are presented as mean ± standard deviation, (n = 12 in total, 3 technical replicates). Statistical significance was set at p-value < 0.05.

Fatty acid composition (%)	Control group (n = 6)	Experimental group (n = 6)	p-value
Total fat content (%)	5.95 ± 2.06	6.46 ± 1.37	0.6335
C14:0	4.04 ± 0.13	4.07 ± 0.16	0.7665
C15:0	0.33 ± 0.16	0.29 ± 0.10	0.7185
C16:0	15.4 ± 0.60	15.2 ± 0.33	0.5582
C16:1 (n-9)	0.41 ± 0.12	0.29 ± 0.07	0.3931
C16:1 (n-7)	5.28 ± 0.44	5.81 ± 0.37	0.0663
C18:0	2.18 ± 0.13	2.13 ± 0.10	0.5287
C18:1 (n-9)	26.8 ± 1.39	28.0 ± 0.98	0.1577
C18:1 (n-7)	2.44 ± 1.19	2.33 ± 0.97	0.8783
C18:2 (n-6)	6.80 ± 0.30	6.73 ± 0.17	0.6589
C18:3 (n-3)	1.96 ± 0.21	1.79 ± 0.27	0.3988
C20:1 (n-9)	6.98 ± 0.30	7.01 ± 0.29	0.8422
C20:2 (n-6)	0.54 ± 0.09	0.43 ± 0.05	0.2310
C20:4 (n-6)	0.51 ± 0.03	0.40 ± 0.11	0.2120
C20:5 (n-3)	4.11 ± 0.31	3.94 ± 0.21	0.3049
C24:1	0.58 ± 0.11	0.51 ± 0.14	0.3955
C22:5 (n-3)	1.05 ± 0.13	1.04 ± 0.15	0.9454
C22:6 (n-3)	11.3 ± 0.96	11.0 ± 1.06	0.6134
SFA	22.0 ± 0.82	21.7 ± 0.31	0.4415
MUFA	43.6 ± 1.63	44.7 ± 1.44	0.2706
PUFA	33.2 ± 0.96	32.3 ± 1.17	0.1980
n-3	25.5 ± 1.01	25.1 ± 1.36	0.5265
n-6	7.65 ± 0.35	7.22 ± 0.38	0.0893
n-3/n-6	3.35 ± 0.24	3.49 ± 0.34	0.4579

Abbreviation: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

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TABLE 6 Total fat content of liver and TL, PL, TAG fatty acid composition (% of total identified FA) of the control fed fish and experimental (yeast) fed fish. Data are presented as mean ± standard deviation, (n = 36 in total with 18 from each feed group, 3 technical replicates). Statistical significance was set at p-value < 0.05.

	TL			TAG			PL		
	Control group	Experimental group	p-value	Control group	Experimental group	p-value	Control group<	Experimental group	p-value
	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
Fat content (%)	14.5 ± 6.60	17.2 ± 4.68	0.1785						
C14:0	2.60 ± 0.37	2.71 ± 0.27	0.5556	2.88 ± 0.23	2.96 ± 0.23	0.3294	n.d	n.d	—
C16:0	12.5 ± 1.23	12.3 ± 0.85	0.7345	10.3 ± 1.25	10.7 ± 2.77	0.5742	17.9 ± 1.21	17.3 ± 1.26	0.1981
C16:1 (n-9)	0.50 ± 0.05	0.57 ± 0.06	0.0020	0.52 ± 0.06	0.59 ± 0.07	0.0047	n.d	n.d	—
C16:1 (n-7)	8.17 ± 1.93	9.44 ± 0.98	0.0557	9.62 ± 1.24	10.4 ± 1.04	0.0669	0.93 ± 0.22	1.16 ± 0.31	0.1159
C18:0	2.15 ± 0.33	2.39 ± 0.48	0.2981	1.84 ± 0.32	2.26 ± 0.58	0.1079	5.75 ± 1.03	6.49 ± 0.91	0.1717
C18:1 (n-9)	34.1 ± 4.82	38.1 ± 2.65	0.0047	39.7 ± 1.26	41.5 ± 2.25	0.0180	13.6 ± 0.80	13.9 ± 0.85	0.2401
C18:1 (n-7)	3.93 ± 0.42	3.69 ± 0.22	0.1434	4.46 ± 0.24	3.95 ± 0.26	0.0002	2.61 ± 0.30	2.65 ± 0.35	0.7859
C18:1 (n-5)	n.d	n.d	—	0.34 ± 0.11	0.29 ± 0.02	0.5768	n.d	n.d	—
C18:2 (n-6)	3.89 ± 0.45	3.41 ± 0.47	0.0090	4.53 ± 0.92	3.67 ± 0.58	0.0013	2.25 ± 0.25	2.12 ± 0.22	0.1786
C18:3 (n-6)	n.d	n.d	—	0.35 ± 0.05	0.37 ± 0.07	0.7049	n.d	n.d	—
C18:3 (n-3)	0.79 ± 0.13	0.57 ± 0.15	<0.0001	0.94 ± 0.23	0.61 ± 0.17	<0.0001	n.d	n.d	—
C20:1 (n-9)	7.18 ± 0.85	7.56 ± 0.51	0.1234	8.65 ± 0.49	8.38 ± 0.51	0.1123	3.33 ± 0.71	3.49 ± 0.83	0.7114
C20:2 (n-6)	0.62 ± 0.07	0.59 ± 0.06	0.2492	0.68 ± 0.08	0.62 ± 0.07	0.0180	n.d	n.d	—
C20:3 (n-6)	0.63 ± 0.40	0.50 ± 0.07	0.4002	0.41 ± 0.05	0.49 ± 0.09	0.3054	n.d	n.d	—
C20:4 (n-6)	1.02 ± 0.51	0.70 ± 0.20	0.0226	n.d	n.d	—	4.51 ± 0.38	4.46 ± 0.50	0.7732
C22:1 (n-9)	0.69 ± 0.09	0.71 ± 0.05	0.6317	0.87 ± 0.07	0.85 ± 0.10	0.6650	n.d	n.d	—
C20:5 (n-3)	4.57 ± 1.26	3.54 ± 0.58	0.0037	3.75 ± 0.84	3.08 ± 0.55	0.0099	7.24 ± 0.79	7.02 ± 0.65	0.4693
C24:1	0.56 ± 0.10	0.47 ± 0.09	0.0925	0.64 ± 0.08	0.53 ± 0.09	0.0013	n.d	n.d	—
C22:5 (n-3)	1.42 ± 0.36	1.38 ± 0.19	0.8117	1.58 ± 0.27	1.36 ± 0.23	0.0630	1.18 ± 0.12	1.32 ± 0.12	0.0118
C22:6 (n-3)	15.6 ± 5.47	11.9 ± 1.78	0.0112	8.91 ± 1.47	8.10 ± 0.94	0.0582	40.6 ± 2.08	41.1 ± 1.63	0.6127
SFA	17.2 ± 1.35	17.4 ± 1.13	0.6395	15.1 ± 1.53	16.0 ± 2.79	0.5411	23.9 ± 1.89	23.8 ± 1.55	0.9334
MUFA	54.8 ± 7.86	60.4 ± 3.10	0.0087	64.4 ± 2.05	66.2 ± 2.69	0.0266	20.1 ± 1.80	20.4 ± 1.42	0.5739
PUFA	27.9 ± 7.24	22.2 ± 3.06	0.0043	20.6 ± 3.17	17.8 ± 2.45	0.0064	55.7 ± 2.56	55.7 ± 1.49	0.9827
n-3	22.3 ± 6.70	17.3 ± 2.43	0.0053	15.2 ± 2.46	13.0 ± 1.70	0.0049	48.9 ± 2.16	49.1 ± 1.33	0.8216
n-6	5.58 ± 0.79	4.90 ± 0.81	0.0157	5.40 ± 1.02	4.77 ± 0.93	0.1397	6.87 ± 0.56	6.62 ± 0.49	0.1845
n-3/n-6	3.97 ± 0.86	3.56 ± 0.40	0.0789	2.85 ± 0.42	2.78 ± 0.41	0.7099	7.14 ± 0.44	7.46 ± 0.60	0.0808

TL, total lipids; PL, phospholipids; TAG, triacylglycerols; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n.d, non-detected.

TABLE 7 Metabolites in plasma in μM L^{-1} (n = 36 in total with 4 missing values) and liver in μM g^{-1} (n = 12), (total plasma metabolites = 57 and total liver metabolites = 48). Data are presented as median (Q1-Q3) with concentrations and p-value obtained from PROC MIXED function in SAS 9.4 with tank factor. VIP values were retrieved from SIMCA. Statistical significance was set at p-value < 0.05. Plasma results are presented without outliers fish 1 and fish 6.

Metabolites	Control group	Experimental group	VIP (95% CI)	p-value
Plasma μM L^{-1}	n = 16	n = 14		
Propylene glycol	6.38 (5.10–7.44)	29.54 (25.93–32.30)	1.75	<0.0001
Alanine	480.25 (456.03–551.44)	758.41 (619.23–898.45)	1.38	0.0002
3-Hydroxybutyrate	5.53 (3.83–6.38)	11.69 (9.78–14.88)	1.28	0.0004
N,N-Dimethylglycine	1.70 (1.70–2.13)	2.55 (1.70–3.40)	1.01	0.0413
Creatinine	18.06 (15.94–21.25)	24.23 (22.53–25.50)	0.91	0.0005
Histidine	75.44 (60.14–87.13)	56.53 (51.00–65.45)	0.58	0.0765
Choline	46.11 (35.70–50.79)	58.23 (51.85–62.90)	0.44	0.0069
Taurine	1420.56 (1234.63–1814.54)	1243.76 (1115.20–1341.30)	0.41	0.0630
Lactate	5371.15 (4376.01–6001.43)	6527.79 (5001.40–7247.10)	0.17	0.0413
Inosine	101.58 (82.24–128.14)	112.20 (103.28–233.33)	0.14	0.1157
Betaine	30.60 (19.98–47.60)	47.39 (41.65–66.30)	0.13	0.0138
Tyrosine	93.93 (77.56–107.95)	76.93 (64.60–80.33)	0.09	0.0089
Serine	167.88 (151.09–198.90)	226.53 (185.30–240.98)	0.04	0.0132
Phenylalanine	97.96 (89.04–109.86)	91.38 (87.98–99.88)	0.04	0.1202
Glycine	440.51 (369.54–511.70)	544.64 (489.18–643.45)	0.03	0.0354
Liver μM g^{-1}	n = 6	n = 6		
Tryptophan	0.288 (0.222–0.505)	0.151 (0.096–0.179)	0.47	0.0669
S-Adenosylhomocysteine	0.043 (0.035–0.051)	0.031 (0.026–0.032)	0.35	0.2609
Aspartate	1.149 (0.525–1.231)	0.435 (0.284–0.860)	0.33	0.1653
Glutamate	2.674 (1.757–4.787)	2.112 (1.269–2.393)	0.32	0.4382
Glutamine	1.626 (1.153–2.351)	1.383 (0.891–1.592)	0.28	0.4426
Fumarate	0.147 (0.080–0.201)	0.067 (0.053–0.086)	0.19	0.2361
Histamine	0.708 (0.340–0.813)	0.544 (0.287–0.571)	0.07	0.3969

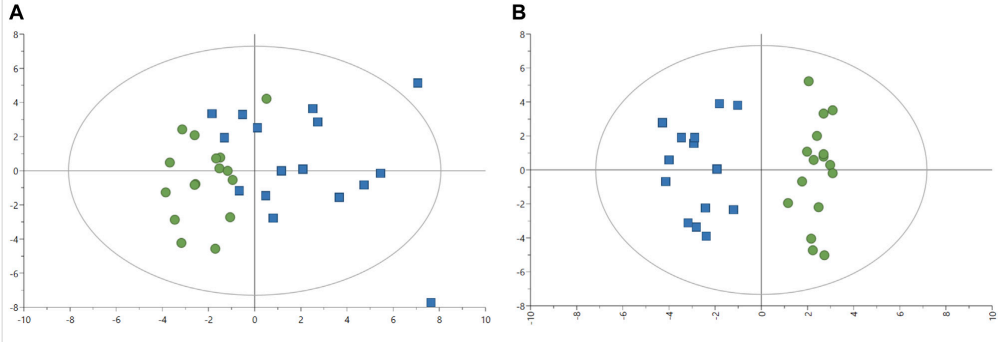

FIGURE 2
Image **(A)** Score plot of principal component analysis (PCA) model of ^{1}H-NMR data plasma (n = 32). Green circles: fish-fed control feed. Blue squares: fish fed with experimental feed. PCA score plot (all plasma data, with 2 PCs) with parameters: R^2X = 16.4%, R^2X2 = 13.4%, Q^2X = 1.74%. Image **(B)** Score plot of an orthogonal partial least squares-discriminant analysis (OPLS-DA) with one predictive (P1) and two orthogonal (O2) components) model of ^{1}H-NMR data plasma. Green circles: fish-fed control feed. Blue squares: fish fed with experimental feed. OPLS-DA score plot (plasma samples without outliers) with R^2X = 13.1%, Q^2X = 79.0%, R^2Y = 100%, CV-ANOVA = 9.02 × 10^{-07}.

TABLE 8 Histological analyses of lipid droplets count in liver tissue (n = 8 in total with 4 fish per feed group). Data are presented as mean ± standard deviation. Statistical significance was set at p-value < 0.05.

Fish group	Droplets (per 1.44 cm^2)	p-value
Control	4.45 ± 0.40	0.7032
Experimental	4.69 ± 0.85	

experimental feeds (Table 5). In the same way, the total fat content from the fish liver tissue (Table 6) was similar between the two groups of fish.

Differences were observed in the percentages of fatty acids in total lipids (TL) and triacylglycerols (TAG) of the liver between the differently fed fish (Table 6). In TL and TAG, the liver of fish fed with control feed showed higher percentages of PUFA and n-3 (in particular arachidonic, linoleic, and alpha-linolenic acids) whereas the liver of fish fed with experimental feed contained more MUFA with the exception of nervonic acid higher in TAG class of control group. Levels of EPA were significantly higher in TL and TAG lipid classes in the control group of fish while docosapentaenoic acid (DPA) was exclusively higher in PL class of the experimental group. Levels of DHA were solely higher in TL class in the control group of fish.

Levels of SFA in the liver remained similar between the two fish groups with the same level of palmitic acid in all lipid classes of the liver for both control and experimental fed fish groups (Table 6).

Fatty acid percentages in phospholipids (PL) of the liver were generally similar between the two groups of fish with the exception of DPA significantly higher in liver of the experimental fish group and ratio n-3/n-6 slightly higher in the same fish group (Table 6).

3.3 Metabolomic profiles of blood plasma and liver tissue from fish

Different metabolites were detected in fish liver and plasma samples from different classes: amino acids; lipids and lipid-like molecules; nucleosides, nucleotides and analogs; organic acids, and more to obtain a broad representation of the different activities occurring in several fish metabolic pathways.

In fish liver tissue, 48 metabolites were quantified and no strong outliers were detected according to the PCA-X score plotted (Supplementary Figure S1). The fitted PCA model was explained by two principal components (PCs), with the following model parameters R^2X = 58.3%, R^2X2 = 11.7%, Q^2 = 44.3%. The separation was not clear with the PCA score plot but became clearer with the OLPS-DA score plot (all data) with one predictive (P1) and two orthogonal (O2) components, including the model parameters: R^2X = 12.6%, Q^2X = 24.7%, R^2Y = 100%. CV-ANOVA value (p-value = 0.84) indicated that the OPLS-DA model could not be considered reliable for predictions. After classification of metabolites in a list according to VIP and CI values and verification of normality and significance using SIMCA software, no metabolite appeared significantly different in concentrations between the fish fed with control feed and the fish fed with experimental feed as seen in Table 7 (Supplementary Table S1).

The effect of the two feeds on fish was evaluated in fish plasma with 57 metabolites quantified. Two outliers were identified after plotting the PCA-X score with plasma samples from fish one and six removed for further analyses (Figure 2A). The PCA model without two outliers was estimated using two principal components with the model parameters: R^2X = 16.0%, R^2X2 = 13.8%, Q^2X = 0.66%. Similar to liver analysis, no clear pattern appeared in metabolites from plasma samples with the PCA-X score plot. A better distinction was made after plotting the OPLS-DA score without two outliers

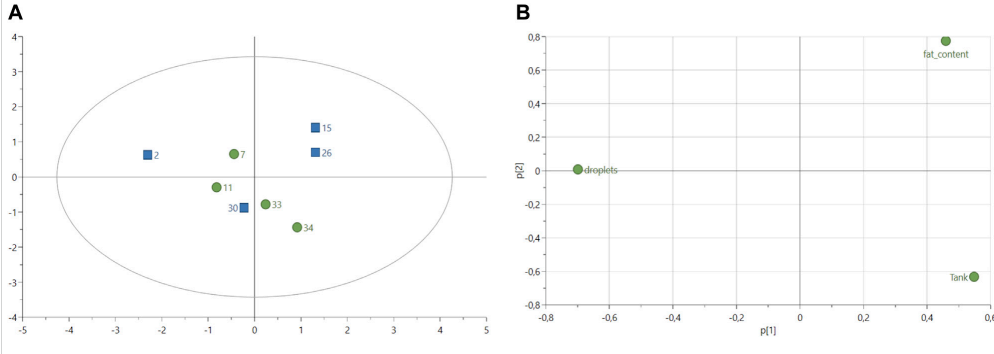

FIGURE 3
(A) Score plot of principal component analysis (PCA) model of lipid droplets calculated from histological data (n = 8 in total, four samples per treatment). Variable fish tanks and fat content (%) were included in the analysis. Green circles: fish-fed control feed. Blue squares: fish fed with experimental feed. PCA score plot (all data, with 2 PCs) with parameters: R^2X = 50.4%, R^2X2 = 32.6%, Q^2X = −36.6%. **(B)** Loadings plot from PCA model shown in **(A)**.

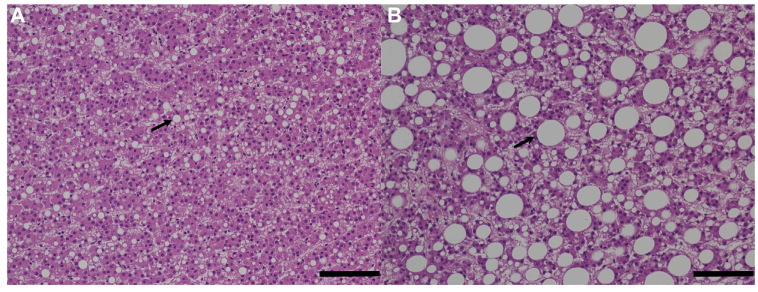

FIGURE 4
Histological analysis of fish liver tissues. The bar scale represents 100 µm. The image on the left **(A)** is control fish, and the image on the right **(B)** is experimental fish with signs of hepatic steatosis. Arrows point out lipid droplets.

(Figure 2B), with one predictive and two orthogonal components with the following model parameters: R^2X = 13.1%, Q^2X = 79.0%, R^2Y = 100%. CV-ANOVA showed better predictability of the plasma OPLS-DA model than of the liver OPLS-DA model with *p*-value = 9.02×10^{-07}. Several metabolites were identified as discriminative variables between the two groups of fish with propylene glycol, alanine, and 3-hydroxybutyrate indicating the strongest difference between the two fish groups as seen in Table 7 (Supplementary Table S2). Most plasma metabolites significantly different between the two fish groups were in higher concentrations in fish fed with experimental feed with the exception of tyrosine, and higher in control-fed fish.

3.4 Histology results

The histology analysis of fish livers (n = 8 in total, 4 in each group) from both control and experimental groups revealed no difference in the number of lipid droplets for the same size area of 1.44 cm^2 (Table 8) with on average 4.69 droplets in livers of fish fed with experimental diet and 4.45 droplets in livers of fish fed with control diet (Supplementary Table S3). The PCA score plot of the variables "number of lipid droplets," "fat content (%)" and fish "tank" showed similarities between the experimental fish number 30 and all control fish (Figure 3A). Experimental fish numbers 15 and 26 were differentiated from other samples with a higher fat content according to the loadings (Figure 3B). Fish number two from the experimental group showed the highest number of lipid droplets compared to other fish (Figure 3B).

The histological phenotypic results were consistent with the measured lipid content in the fish liver with two fish fed with experimental feed (fish number 15 and 26) showing the highest liver fat content (>20%) and the largest lipid droplets compared to any other fish from the control group (Figure 4; Supplementary Table S3).

4 Discussion

The search for alternatives to animal and vegetable lipids leads to the expansion of SCOs knowledge and broader utilization of microbial oils. The industrial application of microbial oils as lipid sources shows advantages such as the potential use for foods and feeds, biofuel production, the recovery of carbon, nitrogen, minerals, and other nutrients from under-utilized industrial by-products (Øverland & Skrede, 2017; Abeln & Chuck, 2021). Microbial production of oil remains expensive for common use as biodiesel. Nevertheless, optimizations through higher lipid yield, improved fermentation processes and the use of low cost substrates could reduce the costs (Abeln & Chuck, 2021). Microbial oil could bring substantial benefits to the environment with limited reliability on the climate as the fatty acid profile of some SCOs remains similar to the one from terrestrial plant oils (Abeln & Chuck, 2021). Oleaginous yeasts from the *Rhodotorula* genus, rich in palmitic acid, could replace palm oil in animal feed and bring other advantages as they grow rapidly and are an important source of carotenoids with antioxidant properties (Nagaraj, et al., 2022).

In the present study, *R. toruloides* was selected as a vegetable oil replacement in the diet of Arctic char, and feeds were designed to have similar fat content and fatty acid composition. The total fat content and fatty acids profile in the muscle of both the control fish fed with a vegetable oil mix and the experimental groups of fish fed partly with yeast biomass were similar, indicating that lipids from the biomass were available for uptake and similar metabolism of the feeds. These results are in agreement with Blomqvist et al. (2018) study using *L. starkeyi*. Former studies have demonstrated a mirroring effect of the fish feed on Arctic char muscle lipid composition (Pettersson, Pickova & Brännäs., 2010; Dupont-Cyr et al., 2022). Therefore, the evaluation of fatty acid profile and lipid content in fish muscle was required when evaluating fish

growth performance as a modification of the fish feed may change its lipid composition, possibly leading to effects on the fish performance such as swimming performance and total lipid content (Pettersson, Pickova & Brännäs., 2010) on growth factors (Wagner et al., 2014), or a change on fatty acid composition of fillets for human consumption (Teoh & Ng., 2016).

In the same way as muscle tissue, liver total lipid content was not significantly different between the two groups of fish, although it was slightly higher in the experimental group. As the main organ for lipid and carbohydrate storage in most fish species, the liver is commonly used as a site of biomarkers in the dysregulation of fatty acid metabolism (Monge-Ortiz et al., 2018; Roques et al., 2020). The fatty acid profile of the different lipid classes of the liver revealed differences between fish groups with higher PUFA content in the control group for TL and TAG, higher MUFA amounts in the experimental group for TL and TAG, and no difference in SFA contents between the fish groups for any lipid class. In the present study, DHA as essential FA in the PL for both fish groups with 40%, was not affected by the different feeds, indicating its importance and thereby retention. Levels of C18:2 (n-6) and n-6 were significantly higher in liver TL and TAG of fish fed with control feed. Higher levels of C18:2 (n-6) have been observed before in fish feed based on plant oils, in particular, soybean oil and rapeseed oil compared to fish feed based on fish oil (Thomassen & Røsjø, 1989; Kalogeropoulos, Alexis & Henderson, 1992; Willora et al., 2021). Higher levels of C18:2 (n-6) in fish feed have shown to be mirrored in the fatty acid composition of the fish liver as seen in Kalogeropoulos, Alexis & Henderson (1992) with a proportional increase of C18:2 (n-6) in liver phospholipids of fish fed with an increase of soybean oil quantity in the fish feed. In the current study, significantly higher levels of C18:2 (n-6) observed in the liver of the fish control group were not explained by the fatty acid profile of the feeds as C18:2 (n-6) levels were the same in both feeds. Our results suggest a potential mobilization of FA from the liver and not from muscle as an energy provider in fish-fed experimental yeast feed. The difference in FA composition observed in the liver tissue and not in the muscle tissue between the two fish groups may indicate a compensation of the liver from the experimental fish group to cover the lipid requirements in the muscle. A change of FA composition in the liver could lead to deleterious effects on the health of the fish which would bring to light differences in growth parameters, histology, and metabolic studies.

Both liver weight and HSI were significantly higher in fish fed with the experimental feed despite a non-statistical difference in liver fat content and liver n-3/n-6 ratio in the two groups of fish. Elevated HSI could indicate a disorder or injury in the liver related to an increase of glycogen or lipid storage which can be the result of the high content of lipids and carbohydrates in the feed or exposure to contaminants (Nayak et al., 1996). Related to our results, the analysis of several organic pollutants (polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and hexachlorobenzene) and heavy metals (aluminum, arsenic, cadmium, mercury, and lead) in experimental feed was carried out and values were found below the European legislation (manuscript in preparation). The histological analysis of fish livers from both control and experimental groups revealed no significant difference in the number of lipid droplets. However, the phenotypical results have shown larger lipid droplets in the fish having the highest liver fat content. Some experimental fish have shown a higher fat content than control fish but the results were not consistent and not significant.

Regarding high carbohydrate content in fish feed, several studies have shown an increase in liver glycogen content, HSI value, or lipid droplets with increased dietary starch or carbohydrate levels (Wilson, 1994; Moreira et al., 2008; Prathomya et al., 2017; Zhao et al., 2022). Carnivorous fish such as salmonids are limited in their dietary carbohydrates from their environment and have a lower tolerance for feed rich in carbohydrates compared to omnivorous or herbivorous species (Hemre, Mommsen & Krogdahl, 2002; Moreira et al., 2008). A lower tolerance for a high carbohydrate diet can lead to changes in hepatic glycolysis and gluconeogenesis regulation with for example a significant increase of glycogen content in the liver of rainbow trout fed with a high carbohydrate diet in addition to higher gene expression levels of genes encoding for glucose transporters and glycolytic enzymes (Song et al., 2018). To investigate the different energy pathways of fish in a broad range, we applied a metabolomics method using ^{1}H-NMR and quantified metabolites in the aqueous phase of fish livers from both groups.

Surprisingly, no metabolites including glucose and lactose were significantly different in the liver between the control and experimental fed fish. Differences were expected in levels of metabolites involved in carbohydrate and lipid storage metabolism to account for the elevated HSI and liver weight in fish fed with experimental yeast feed.

Metabolites involved in the citric acid cycle (TCA cycle) such as succinate and fumarate and precursors of the TCA cycle such as glycerol were slightly lower in the liver of fish fed with experimental feed while pyruvate levels were similar. These results could indicate that intermediates of the TCA cycle were not produced at similar rates in the experimental group of fish compared to the control group or mobilized from the liver to the blood flow for gluconeogenesis activity. A previous study by Geisler et al. (2016) showed an increase of hepatic phosphoenolpyruvate carboxykinase (enzyme activity and mRNA expression), another intermediate of the TCA cycle and gluconeogenic potential, with a fasting period of 8 hours in mice. In addition, serum levels of 3-hydroxybutyrate in mice were found to be elevated from 8 hours of fasting and continued to increase with longer fasting time (Geisler et al., 2016). Similar results appear in our study in fish-fed experimental feed as plasma levels of 3-hydroxybutyrate were significantly higher compared to the control group of fish.

The potential gluconeogenesis activity observed in the metabolomics analysis of the liver of experimental fed fish can be supported by the plasma metabolic profile with significantly higher plasma lactate, alanine, slightly higher but non-significant glucose and pyruvate contents, and a nearly significant increase of glutamine, all of which are important gluconeogenic substrates (deRoos et al., 1985). An early study (Mommsen, French & Hochachka, 1980) suggested that a primary function of alanine in Atlantic salmon was to act as an amino acid carrier into the blood to be metabolized in other tissues for gluconeogenesis. Kullgren et al. (2010) found elevated levels of alanine in the muscle of fasting rainbow trout and a decrease in the liver of the same fish. The authors suggested two different functions of alanine with the first role as a substrate for hepatic glycogen and glucose production and the second one as an energy provider through oxidation in the liver. In our study, alanine levels in the liver remained unchanged between the two fish groups with similar observations for glucose, glutamate, and lactate levels.

Metabolites involved in the one-carbon (1C) metabolism, choline, betaine, N-N, dimethylglycine, glycine, and serine were all significantly higher in plasma of fish fed with experimental feed, as was methionine, but in a non-significant way. The liver is the main site of lipid and one-carbon metabolisms (da Silva, Eudy & Deminice, 2020) and low concentrations of metabolites involved in the one-carbon metabolism have been associated with fat accumulation in the liver of humans and mice, as well as a progression of liver disease such as nonalcoholic fatty liver disease (da Silva, Eudy & Deminice, 2020). Minor alterations of the one-carbon metabolism observed in our study suggest that experimental fish should not show signs of liver damage or fat accumulation. In line with this, no damaged tissue was observed in the histological results of fish livers for both groups. Lipid content was slightly higher in experimental fish although fat accumulation in regards to the number and size of lipid droplets could not be seen consistently in a specific fish group.

Plasma creatinine levels were significantly higher in fish fed with experimental feed. A higher level of creatinine was observed in the plasma of gilthead sea bream fish fed once a day with fishmeal and fish oil compared to fish fed the same meal multiple times a day. The effect observed in plasma creatinine was attributed to a faster protein metabolism (Busti et al., 2020). Another factor potentially influencing plasma creatinine levels is the disruption of the kidney function in fish fed with experimental feed as kidneys are responsible for the excretion of creatinine (Stoskopf, 1993). Exposure to contaminants or pesticides can be a cause of interferences in fish kidney function (Taheri Mirghaed et al., 2018) which would be revealed with histology studies of vital organs and quantification of plasma urea levels.

Propylene glycol (PG, 1,2-propanediol) is a synthetic diol incorporated in animal feed as an antifoaming agent or to improve plasticity, and texture and can act as an antimicrobial or antifreeze agent (Hilton, Atkinson, and Slinger, 1986; Nalawade, Bhat & Sogi 2015; Soaudy et al., 2021). In our study, PG was detected in the plasma of fish fed with experimental feed as the yeast production included the addition of polypropylene glycol as an antifoaming agent. No detrimental effect of dietary PG on fish health has been observed in rainbow trout (Hilton, Atkinson & Slinger, 1986), or in Atlantic salmon (Aru et al., 2021). An increase in survival, growth performance, feed utilization, and improved immune response were identified in Nile tilapia after dietary inclusion of PG to mitigate winter stress (Soaudy et al., 2021).

In relation to fish feed and interferences with energy pathways, Prathomya et al. (2017) demonstrated that a high-fat-high-carbohydrate diet fed to *Megalobrama amblycephala* induced the accumulation of amino acids leucine, isoleucine, valine, glutamine, histidine, methionine, and tyrosine in plasma. High levels of these amino acids in plasma were explained by a "congestion" of the TCA cycle due to an excess of products from carbohydrate and lipid metabolisms, reducing the number of metabolites entering the TCA cycle. Another type of diet included in the same study, a high carbohydrate diet, showed distinct signs of liver injuries, higher plasma levels of tyrosine and creatine, and no TCA cycle disruption. Our study indicates a disruption of the TCA cycle as well with a significant accumulation of plasma alanine, glycine, and serine involved in the one-carbon metabolic pathway and slightly lower levels of fumarate and succinate in the liver.

In addition to the interferences seen in our study with the TCA cycle, elevated plasma ketone bodies and gluconeogenesis activity observed in the liver and plasma of the fish fed with experimental feed suggest an unmet energy requirement in the experimental group of fish possibly due to indigestible compounds from the yeast biomass, which would translate into elevated levels of plasma metabolites involved in a fasting state.

The implementation of metabolomics tools to understand fish physiology with novel feeds brings new knowledge to aquaculture. The quantification of metabolites related to feeding intake provides more appropriate information about fish nutrition and leads to possibilities for fish health improvement (Lulijwa, Alfaro & Young, 2022). The welfare of fish when modifying the feed to more sustainable sources should be of importance. Among common metabolomics tissue and fluid samples, plasma samples are suited in the study of fish nutrition as plasma analysis provides a snapshot of different metabolic activities occurring in a system, since plasma is involved in the transport of nutrients and diverse signaling molecules to organs. Nevertheless, the measurement of plasma metabolites is not isolated from effects related to nutrition and other biological factors such as stress, diseases, and growth can confuse results (Roques et al., 2020).

The composition of both feeds in this study was designed to contain similar proportions of macronutrients with the only difference being the inclusion of yeast biomass as a new ingredient. Nevertheless, information on the exact composition of the yeast cell walls from *R. toruloides* is scarce (Buck & Andrews,

1999; Yockey et al., 2019). One study (Weijman, de Miranda & Van Der Walt, 1988) differentiated *Rhodotorula* from *Cryptococcus* yeasts by having high amounts of mannan and no amount of xylose in the cell wall, and a lower urease activity.

The carbohydrate composition of *R. toruloides* has been previously described with a major quantity of mannose and glucose followed by smaller amounts of galactose and very low amounts of rhamnose, fucose, ribose, arabinose, and no amount of xylose (Sugiyama et al., 1985). Cell walls of *R. toruloides* in our study were physically disrupted while applying a French press before incorporating the yeast biomass into the experimental fish feed. Physical disruption was applied in order to facilitate the uptake of yeast nutrients by the fish. This might also have resulted in increased bioavailability of the carbohydrates from the cell walls.

Most yeasts contain diverse immune-stimulating compounds, such as β-glucans, mannan-oligosaccharides, and chitin (Navarrete & Tovar-Ramírez, 2014) whose incorporation in fish feed could potentially improve fish growth performance, immune system, feed efficiency, stress tolerance and intestinal microbiota (Navarrete & Tovar-Ramírez, 2014; Rimoldi et al., 2020; Zhang et al., 2020). The beneficial or detrimental effects of yeasts on fish health as an ingredient in fish feed seem to be linked to multiple factors including the type of yeasts, the quantity added to the feed, and the fish species (Øverland et al. ., 2013; Navarrete and Tovar-Ramírez, 2014; Øverland & Skrede, 2017; Blomqvist et al., 2018; Sahlmann et al., 2019; Vidakovic et al., 2020). More research should be conducted on the composition of different yeast species and their inclusion in fish feed for aquaculture purposes.

5 Conclusion

The replacement of vegetable oils with yeast biomass in Arctic char feed did not lead to differences in the fat content of muscle and liver tissues. The fatty acid profiles were similar in muscle tissue and showed minor variations in the liver tissue. Higher liver weight and HSI were observed in fish fed with experimental feed along with increased plasma ketones and metabolites involved in the one-carbon metabolism. No significant difference in the number of lipid droplets was seen and droplet sizes were consistent with fat content at the individual level. Further investigations are required before using *R. toruloides* extensively in fish feed.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Ethical Committee for Animal Experiments (Umeå, Sweden) in compliance with the European legislation (i.e., Directive 2010/63/EU).

Author contributions

MB, Formal analysis, Investigation, Writing—original draft, Writing—review and editing. VB, Formal analysis, Investigation, Writing—original draft, Writing—review and editing. SS, Conceptualization, Investigation, Project administration, Supervision, Writing—review and editing. JP, Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing—original draft, Writing—review and editing. AM, Methodology, Supervision, Writing—review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2022.931946/full#supplementary-material

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Corrigendum: Oleaginous yeast *Rhodotorula toruloides* biomass effect on the metabolism of Arctic char (*Salvelinus alpinus*)

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KEYWORDS

metabolomics, fish feed replacement, fatty acids, metabolites, gluconeogenesis, plasma, liver, oleaginous yeast

A Corrigendum on
Oleaginous yeast *Rhodotorula toruloides* biomass effect on the metabolism of Arctic char (*Salvelinus alpinus*)

by Brunel M, Burkina V, Pickova J, Sampels S and Moazzami AA (2022). Front. Mol. Biosci. 9: 931946. doi: 10.3389/fmolb.2022.931946

In the published article, there was an error in Table 1 as published. The corrected Table 1 and its caption "Table 1 Composition of control and experimental yeast feeds (g kg^{-1}) for fish in duplicates. "Vitamin mix" and "Mineral mix" ingredients were provided by NOFIMA (Norway) and "astaxanthin and vitamin mix" ingredients were provided by Aller Aqua A/S (Denmark)" appear below.

In the published article, there was an error in the legend for Table 4 as published. The corrected Table 4 and its caption "Table 4 Growth parameters for fish fed with control feed or experimental yeast-based feed. Data are presented as the mean ± standard deviation. Statistical significance was set at p-value < 0.05" appear below.

A correction has been made to **2 Materials and methods,** *2.2 Feeding trial and sample collection*.

"Arctic char (n = 126, both genders) over 2 years old were randomly assigned to six 1 m × 1 m water tanks (triplicate tanks per feed with 21 fish per tank) with access to a flow-through system of freshwater (10 L min^{-1} with a water depth of 20 cm) from Lake Ansjön at Kälarne Aquaculture Center North, Sweden."

TABLE 1 Composition of control and experimental yeast feeds (g kg^{-1}) for fish in duplicates. "Vitamin mix" and "Mineral mix" ingredients were provided by NOFIMA (Norway) and "astaxanthin and vitamin mix" ingredients were provided by Aller Aqua A/S (Denmark) appear below.

Ingredients	Control feed	Experimental feed
Fish meal	4,950	4,950
Fish oil	1,170	1,170
Vegetable oil	540	–
Mineral mix	45	45
Vitamin mix	90	90
Astaxanthin and vitamin mix	13.5	13.5
Gelatine	45	45
Wheat meal	1.755	1.305
Casein	540	–
Ca_2PO_4	225	225
Yeast	-	1,413 (540 g oil)

The corrected sentence appears below:

"Arctic char (n = 126, both genders, juveniles) were randomly assigned to six 1 m × 1 m water tanks (triplicate tanks per feed with 21 fish per tank) with access to a flow-through system of freshwater (10 L min^{-1} with a water depth of 20 cm) from Lake Ansjön at Kälarne Aquaculture Center North, Sweden."

A correction has been made to **2 Materials and methods,** *2.2 Feeding trial and sample collection.*

"The specific growth rate (SGR) was calculated from day 19 of the trial due to technical problems occurring at the rearing station during the start of the trial."

A correction has been made to **2.6 Statistics**.

"Multivariate data analysis was performed on liver and plasma metabolites using the SIMCA software (version 17; Umetrics, Umeå, Sweden)."

The corrected sentence appears below:

"Multivariate data analysis was performed on liver and plasma metabolites using the SIMCA software (version 17; Umetrics, Suite of Data Analytics Solutions, Sartorius)."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

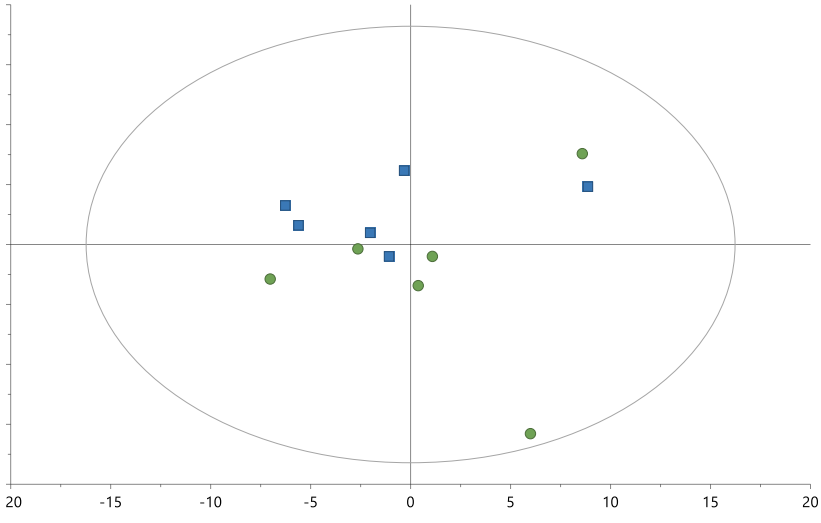
Publisher's note

TABLE 4 Growth parameters for fish fed with control feed or experimental yeast-based feed. Data are presented as the mean ± standard deviation. Statistical significance was set at p-value < 0.05.

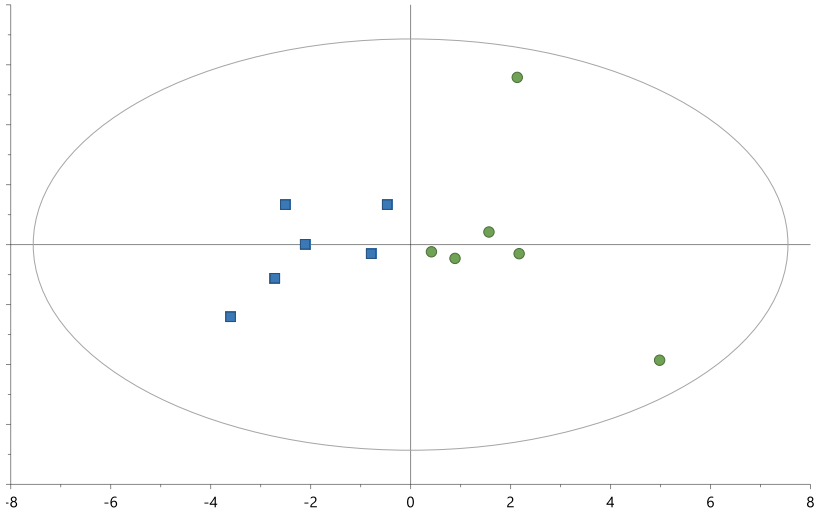
Growth parameters	Control group	Experimental group	p-value
Weight (g), day 19	209 ± 65.4	200 ± 62.9	0.7360
(n = 12 per group)			
Final length (cm)	29.4 ± 2.20	29.2 ± 2.54	0.7417
(n = 48 per group)			
Final weight (g)	314 ± 99.1	303 ± 94.3	0.6108
(n = 48 per group)			
Liver weight (g)	**3.75 ± 2.07**	**4.61 ± 1.91**	**0.0069**
(n = 48 per group)			
SGR (%)	0.68 ± 0.59	0.70 ± 0.56	0.8455
(53 days)			
CF (%)	1.20 ± 0.14	1.19 ± 0.22	0.9078
(n = 48 per group)			
HSI (%)	**1.14 ± 0.27**	**1.51 ± 0.34**	**<0.0001**
(n = 48 per group)			

Significant results are shown in bold letters. SGR, specific growth rate; CF, condition factor; HSI, hepatosomatic index.

C



D



Supplementary Figure S1. Score plots of principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) with 1 predictive (P1) and 2 orthogonal (O2) components) models of ^{1}H-NMR aqueous liver tissue (n=12) and 48 metabolites. Green circles: fish fed control feed. Blue squares: fish fed with yeast biomass based feed. **C**- liver PCA-X score plot (all data, with 2 PCs) with parameters: R^2X =58.3 % , R^2X2 = 11.7 %, Q^2X = 44.3 %. **D**- liver OPLS-DA score plot (all data) with R^2X = 12.6 % Q^2X = 24.7 % R^2Y = 100% CV-ANOVA= 0.84.

Supplementary Table S1. Metabolites in liver (aqueous tissue, n=12), µM.g^{-1} (48 metabolites). Data are presented as median (Q1-Q3) with concentrations and p-value obtained from PROC MIXED function in SAS 9.4 with tank factor. Statistical significance was set at p-value <0.05.

	Concentration µM.g^{-1}		
Metabolites	Control fish (n=6)	Yeast fish (n=6)	p-value
3-Aminoisobutyrate	0.701 (0.438-0.771)	0.833 (0.144-1.209)	0.4542
ADP	0.014 (0.012-0.018)	0.016 (0.016-0.021)	0.7300
AMP	0.014 (0.012-0.015)	0.014 (0.012-0.016)	0.6510
ATP	0.031 (0.027-0.040)	0.035 (0.029-0.036)	0.8660
Acetate	0.124 (0.100-0.170)	0.150 (0.083-0.162)	0.9357
Acetone	0.073 (0.049-0.090)	0.066 (0.049-0.081)	0.6465
Alanine	4.241 (2.825-5.267)	4.220 (2.952-4.362)	0.9444
Asparagine	0.384 (0.240-0.496)	0.255 (0.226-0.352)	0.3378
Aspartate	1.149 (0.525-1.231)	0.435 (0.284-0.860)	0.1653
Betaine	0.073 (0.047-0.078)	0.088 (0.051-0.224)	0.7936
Cholate	0.157 (0.028-0.365)	0.055 (0.032-0.170)	0.3531
Choline	0.727 (0.491-0.808)	0.458 (0.355-0.735)	0.3894
Creatine	0.094 (0.051-0.109)	0.083 (0.049-0.124)	0.9549
Formate	0.291 (0.261-0.302)	0.323 (0.265-0.353)	0.2167
Fumarate	0.147 (0.080-0.201)	0.067 (0.053-0.086)	0.2361
Glucose	14.883 (11.589-20.842)	13.215 (9.783-19.119)	0.6278
Glutamate	2.674 (1.757-4.787)	2.112 (1.269-2.393)	0.4382
Glutamine	1.626 (1.153-2.351)	1.383 (0.891-1.592)	0.4426
Glutathione	0.146 (0.052-0.220)	0.128 (0.073-0.190)	0.8247
Glycerol	1.337 (0.966-1.553)	0.744 (0.612-0.993)	0.2581

Glycine	2.254 (1.604-3.063)	1.918 (1.205-2.013)	0.4917
Histamine	0.708 (0.340-0.813)	0.544 (0.287-0.571)	0.3969
Inosine	0.115 (0.079-0.161)	0.057 (0.047-0.179)	0.5474
Isoleucine	0.594 (0.391-0.642)	0.444 (0.228-0.516)	0.4342
Lactate	4.156 (2.821-4.972)	3.177 (2.730-5.374)	0.9284
Leucine	1.280 (0.843-1.564)	0.938 (0.530-1.049)	0.2700
Lysine	0.875 (0.642-1.024)	0.672 (0.459-1.025)	0.4911
Methanol	13.983 (11.819-15.063)	14.637 (3.414-27.075)	0.5650
Methionine	0.567 (0.349-0.602)	0.390 (0.207-0.450)	0.3263
N,N-Dimethylglycine	n.d	n.d	n.d
N-Methylhydantoin	n.d	n.d	n.d
NAD+	0.062 (0.058-0.076)	0.078 (0.066-0.088)	0.2470
NADP+	0.044 (0.043-0.053)	0.058 (0.050-0.072)	0.1218
Niacinamide	0.221 (0.203-0.303)	0.193 (0.165-0.290)	0.7628
O-Phosphocholine	0.145 (0.121-0.154)	0.120 (0.086-0.160)	0.5467
Phenylalanine	0.315 (0.235-0.403)	0.209 (0.162-0.310)	0.1703
Propionate	0.025 (0.012-0.030)	0.013 (0.003-0.015)	0.4914
Pyruvate	0.012 (0.010-0.014)	0.011 (0.010-0.012)	0.6817
S-Adenosylhomocysteine	0.043 (0.035-0.051)	0.031 (0.026-0.032)	0.2609
Sarcosine	0.016 (0.012-0.026)	0.018 (0.016-0.020)	0.5034
Succinate	0.184 (0.182-0.193)	0.087 (0.079-0.119)	0.3036
Taurine	12.664 (8.924-14.799)	9.662 (8.203-11.340)	0.4763
Tryptophan	0.288 (0.222-0.505)	0.151 (0.096-0.179)	0.0669
Tyrosine	0.426 (0.305-0.631)	0.256 (0.160-0.273)	0.2288
UDP-glucose	0.083 (0.058-0.158)	0.095 (0.074-0.117)	0.8955
UDP-glucuronate	0.064 (0.053-0.092)	0.084 (0.062-0.141)	0.3152
Uridine	0.024 (0.020-0.027)	0.021 (0.015-0.023)	0.5508
Valine	1.248 (0.787-1.316)	0.875 (0.470-1.051)	0.3365
sn-Glycero-3-phosphocholine	0.226 (0.167-0.349)	0.228 (0.151-0.389)	0.8120

ß-Alanine	0.414 (0.324-0.516)	0.418 (0.326-0.497)	0.9353

Supplementary Table S2. Metabolites in plasma (n=32 in total and 2 outliers in the treatment group), $\mu M.L^{-1}$ (57 metabolites). Data are presented as median (Q1-Q3) with concentrations and p-value obtained from PROC MIXED function in SAS 9.4 with tank factor. Statistical significance was set at p-value <0.05. Results are presented without outliers fish 1 and fish 6.

	Concentrations $\mu M.L^{-1}$		
Metabolites	Control fish (n=16)	Yeast fish (n=14)	p-value
2-Aminobutyrate	33.36 (24.65-36.34)	29.96 (21.25-36.98)	0.5080
2-Hydroxybutyrate	15.51 (13.60-16.79)	16.58 (13.60-19.13)	0.2482
2-Hydroxyisovalerate	3.83 (3.19-4.25)	3.83 (2.55-4.25)	0.8802
2-Hydroxyvalerate	4.04 (3.19-4.89)	3.61 (2.98-5.10)	0.8454
2-Oxoisocaproate	2.13 (1.70-2.55)	2.34 (1.70-2.98)	0.4447
3-Hydroxybutyrate	5.53 (3.83-6.38)	11.69 (9.78-14.88)	0.0004
3-Methyl-2-oxovalerate	8.93 (7.65-10.20)	8.71 (7.65-11.05)	0.6842
Acetate	13.81 (12.54-16.36)	14.88 (12.33-17.43)	0.9478
Acetoacetate	16.79 (15.09-27.84)	15.51 (13.60-19.98)	0.1824
Acetone	3.40 (2.98-3.61)	3.40 (2.98-3.83)	0.7737
Alanine	480.25 (456.03-551.44)	758.41 (619.23-898.45)	0.0002
Arginine	214.84 (186.79-234.18)	226.31 (213.35-256.28)	0.1393
Asparagine	195.29 (169.15-208.46)	142.16 (110.93-205.70)	0.1693
Aspartate	24.23 (20.61-26.56)	24.23 (17.43-28.90)	0.7898
Betaine	30.60 (19.98-47.60)	47.39 (41.65-66.30)	0.0138
Carnitine	20.83 (18.06-34.00)	29.33 (23.38-35.28)	0.7866
Choline	46.11 (35.70-50.79)	58.23 (51.85-62.90)	0.0069
Citrate	71.61 (59.71-92.23)	65.45 (60.35-79.48)	0.4151
Creatine	326.61 (259.89-472.18)	444.98 (378.25-480.68)	0.3088
Creatinine	18.06 (15.94-21.25)	24.23 (22.53-25.50)	0.0005
Dimethyl sulfone	4.25 (3.83-4.68)	4.46 (4.25-5.10)	0.4066
Ethanol	22.31 (21.46-28.69)	28.26 (27.20-39.10)	0.2297
Formate	110.08 (106.04-114.96)	109.86 (107.95-117.73)	0.9845

Glucose	6558.39 (5947.88-7413.70)	6647.00 (6118.30-8195.28)	0.6418
Glutamate	113.05 (89.46-147.90)	105.19 (87.55-118.15)	0.3792
Glutamine	387.18 (349.78-422.66)	419.90 (362.53-450.08)	0.0631
Glycerol	351.05 (289.21-471.75)	362.95 (340.00-428.40)	0.6200
Glycine	440.51 (369.54-511.70)	544.64 (489.18-643.45)	0.0354
Hippurate	n.d.	n.d.	n.d.
Histidine	75.44 (60.14-87.13)	56.53 (51.00-65.45)	0.0765
Inosine	101.58 (82.24-128.14)	112.20 (103.28-233.33)	0.1157
Isoleucine	199.75 (177.65-214.63)	221.21 (162.78-243.95)	0.5071
Isopropanol	2.76 (2.13-42.71)	45.69 (3.40-55.68)	0.0757
Lactate	5371.15 (4376.01-6001.43)	6527.79 (5001.40-7247.10)	0.0413
Leucine	269.66 (247.56-283.05)	290.28 (227.80-314.08)	0.5939
Lysine	527.64 (443.49-574.60)	448.59 (359.13-521.05)	0.2223
Methanol	264.14 (245.44-294.74)	271.58 (249.90-285.60)	0.9372
Methionine	105.19 (84.58-116.88)	120.49 (100.30-150.88)	0.0157
N,N-Dimethylglycine	1.70 (1.70-2.13)	2.55 (1.70-3.40)	0.0413
N-Acetylcysteine	5.31 (3.19-8.71)	8.08 (5.53-11.05)	0.7394
O-Acetylcarnitine	4.46 (3.61-5.53)	5.95 (5.53-7.65)	0.0719
O-Phosphocholine	8.08 (5.95-9.35)	8.08 (7.23-10.20)	0.2573
Phenylalanine	97.96 (89.04-109.86)	91.38 (87.98-99.88)	0.1202
Proline	146.20 (131.54-157.89)	180.20 (154.28-192.53)	0.2482
Propionate	1.70 (1.28-2.13)	1.91 (0.85-2.98)	0.1809
Propylene glycol	6.38 (5.10-7.44)	29.54 (25.93-32.30)	<.0001
Pyruvate	12.96 (10.20-17.64)	17.64 (7.65-28.05)	0.4004
Sarcosine	1.28 (1.28-2.13)	1.70 (1.28-2.13)	0.2821
Serine	167.88 (151.09-198.90)	226.53 (185.30-240.98)	0.0132
Succinate	11.48 (9.35-17.43)	12.75 (9.35-17.85)	0.8491
Taurine	1420.56 (1234.63-1814.54)	1243.76 (1115.20-1341.30)	0.0630

Threonine	610.73 (426.28- 690.20)	461.76 (382.08-548.25)	0.1508
Trigonelline	n.d.	n.d.	n.d.
Trimethylamine N-oxide	400.35 (303.24- 497.89)	395.46 (314.08-535.93)	0.5229
Tyrosine	93.93 (77.56-107.95)	76.93 (64.60-80.33)	0.0089
Valine	459.21 (419.69-480.68)	453.26 (341.28-517.23)	0.5617
myo-Inositol	139.83 (132.39-168.94)	146.20 (127.50-153.85)	0.3391
trans-4-Hydroxy-L-proline	202.30 (164.26-239.49)	219.94 (177.65-260.10)	0.6920
β-Alanine	123.46 (100.30-149.60)	147.90 (124.53-162.78)	0.4989

Supplementary Table S3. Fat content (%) and number of lipid droplets in fish livers from control and experimental (yeast) groups (n= 8 in total, with 4 in each group). Lipid droplets were counted manually in ImageJ at the same area of (1.44 x 1.44) cm^2 using grid (line) option. Data are presented in the table by decreasing fat content (%).

Tank	**Feed group**	**Fish ID**	**Fat content (%)**	**Droplets number**
179	yeast	15	23.81	3.83
181	yeast	26	23.34	4.29
178	control	7	16.85	4.58
182	control	33	15.82	4.88
177	yeast	2	14.48	5.80
181	yeast	30	13.26	4.83
182	control	34	11.70	3.92
178	control	11	10.82	4.42

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This thesis addresses the dietary effect of food/feed consumption on human and fish metabolisms using a metabolomics approach. A pig meat product was analyzed for its nutritional content and included in a randomized controlled clinical trial. The partial replacement of fish feed ingredients by a sustainable alternative from oleaginous yeast was tested on the different parameters related to fish performance and health in a trial after evaluating the feed composition.

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