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Insights from a zebrafish model to combat dyslipidemia using microbe-derived bioactive compounds

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FACULTY OF BIOSCIENCES AND AQUACULTURE

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A thesis for the degree of
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Preface

This thesis is submitted in fulfilment of the requirements for the degree of Philosophiae Doctor (PhD) at the Faculty of Biosciences and Aquaculture (FBA), Nord University, Bodø, Norway. The studies included in this dissertation represent original research that was carried out over a period from 01.11.2018 to 24.05.2023 at Nord University, Bodø.

Adnan Hussain Gora was financially supported by Netaji-Subhas International Fellowship provided by the Indian Council of Agricultural Research, India.

The core project team consisted of the following members:

Adnan Hussain Gora, MFSc, FBA Nord University: PhD Candidate

Kiron Viswanath, Professor, FBA, Nord University: Main supervisor

Jorge M.O. Fernandes, Professor, FBA, Nord University: Co-supervisor

Pål A. Olsvik, Professor, FBA, Nord University: Co-supervisor

Adnan H. Gora

Bodø, 5th June 2023

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Finally, I want to thank Papa, Mum, Arika, Saima, Owais, and little Sireen, who have been a constant support throughout this PhD journey.

All of you have made the journey a happy and memorable one.

Adnan H. Gora

Bodø, Norway

Contents

List of abbreviations

ABCA1	-	ATP binding cassette subfamily A member 1
abca1a	-	*ATP-binding cassette, sub-family A (ABC1), member 1A*
ABCG1	-	ATP-binding cassette sub-family G member 1
ABCG5	-	ATP-binding cassette sub-family G member 5
ABCG8	-	ATP-binding cassette sub-family G member 8
ACAT	-	*acyl-coenzyme A: cholesterol acyltransferase*
acot22	-	acyl-CoA thioesterase 22
apoa1a	-	*apolipoprotein A-Ia*
apoa1b	-	*apolipoprotein A-Ib*
apoa4a	-	*apolipoprotein A4-IV a*
apoa4b.1	-	*apolipoprotein A-IV b, tandem duplicate 1*
apoa4b.2	-	*apolipoprotein A-IV b, tandem duplicate 2*
apoa4b.3	-	*apolipoprotein A-IV b, tandem duplicate 3*
APOA-I	-	apolipoprotein A-I
APOB	-	apolipoprotein B
APOB-100	-	apolipoprotein B-100
APOB-48	-	apolipoprotein B-48
apoc2	-	*apolipoprotein C 2*
apodb	-	*apolipoprotein Db*
apoe	-	*apolipoprotein E*
apoeb	-	*apolipoprotein Eb*

ATP	-	adenosine triphosphate
BiP	-	binding protein
CD36	-	cluster of differentiation 36
CDP	-	cytidine diphosphate
CE	-	cholesterol ester
CER	-	ceramide
CETP	-	cholesteryl ester transfer protein
cpt1aa	-	*carnitine palmitoyltransferase 1Aa*
CVD	-	cardiovascular disease
DEG	-	differentially expressed gene
DHA	-	docosahexaenoic acid
EPA	-	eicosapentaenoic acid
ER	-	endoplasmic reticulum
ERAD	-	ER-associated degradation
FATP4	-	fatty acid transporter protein 4
GRP94	-	glucose-regulated protein 94
GST	-	glutathione-S-transferases
HDL	-	high density lipoprotein
HL	-	hepatic lipase
HMGCR	-	3-hydroxy-3-methylglutaryl-CoA reductase
IFABP	-	intestinal fatty acid binding protein
Insig-1	-	insulin-induced gene 1

LCAT	-	lecithin-cholesterol acyltransferase
LCPUFA	-	long chain polyunsaturated fatty acid
LDL	-	low density lipoprotein
LDLR	-	low density lipoprotein receptor
ldlra	-	*low density lipoprotein receptor a*
ldlrb	-	*low density lipoprotein receptor b*
LFABP	-	liver fatty acid binding protein
LPC	-	lysophosphatidylcholine
LPCO	-	alkyl lysophosphatidylcholine
LRP-1	-	ldl receptor related protein 1
lrp2b	-	*low density lipoprotein receptor-related protein 2b*
MTTP	-	microsomal triglyceride transfer protein
NPC1L1	-	Niemann-Pick C1-like 1
PC	-	phosphatidylcholine
PCSK9	-	proprotein convertase subtilisin/kexin type 9
PEMT	-	phosphatidylethanolamine N-methyltransferase
PI	-	phosphatidylinositol
PLIN	-	perilipin
PPAR	-	peroxisome proliferator-activated receptors
PUFA	-	polyunsaturated fatty acid
ROS	-	reactive oxygen species
scarb1	-	scavenger receptor class B, member 1

SER	-	smooth endoplasmic reticulum
SERCA	-	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SLC25A11	-	solute carrier family 25 member 11
SM	-	sphingomyelin
SR-B1	-	scavenger receptor class B type 1
STARD	-	steroidogenic acute regulatory related lipid transfer domain
TG	-	triacylglycerol
tmem	-	*transmembrane protein 70*
UPR	-	unfolded protein response
VLDL	-	very low density lipoprotein
WHO	-	World Health Organization

List of Figures and Tables

List of Papers

Paper I

Gora, A. H., Rehman, S., Kiron, V., Dias, J., Fernandes, J. M., Olsvik, P. A., ... & Cardoso, M. (2022). Management of hypercholesterolemia through dietary ß-glucans–insights from a zebrafish model. Front Nutr 8, 1209. doi.org/10.3389/fnut.2021.797452

Paper II

Gora, A. H., Rehman, S., Dias, J., Fernandes, J. M., Olsvik, P. A., Sørensen, M., & Kiron, V. (2023). Protective mechanisms of a microbial oil against hypercholesterolemia: evidence from a zebrafish model. Front Nutr 10, 1161119. doi.org/10.3389/fnut.2023.1161119

Paper III

Gora, A. H., Rehman, S., Dias, J., Fernandes, J. M., Olsvik, P. A., Sørensen, M., & Kiron, V. (2023). Microbial oil, alone or paired with β-glucans, can control hypercholesterolemia in a zebrafish model.

(Manuscript)

SUMMARY

Dyslipidemia, characterized by an altered blood lipid profile, is a risk factor that triggers the development of cardiovascular diseases in humans. Zebrafish is an emerging model species that can be used to investigate diet-based therapeutic strategies for preventing dyslipidemia. This thesis presents new knowledge about how diet-induced dyslipidemia affects molecular responses in the gut and liver of zebrafish. This PhD project focused on how β-glucans and n-3 PUFAs from new microbial sources affect plasma lipids and gene expression in the intestine and liver of zebrafish.

The impact of dietary cholesterol, β-glucans, and Simvastatin (cholesterol-lowering medication) on the blood lipoprotein levels, histological architecture of the tissues, and intestinal transcriptome of zebrafish were explored in the first study. The results indicated that feeding a cholesterol-rich diet leads to a dyslipidemic blood profile and hepatic lipid accumulation in zebrafish, and dietary β-glucan and simvastatin could establish the normal phenotype. Intestinal transcriptome sequencing revealed the downregulation of genes connected to cholesterol biosynthesis and the upregulation of genes associated with organellar dysfunction by high-cholesterol diets. β-glucans and simvastatin in zebrafish diet helped to restore the expression pattern of several intestinal genes altered by high-cholesterol diets.

Next, the effect of dietary n-3 PUFA-rich microbial oil from *Schizochytrium* sp. on the blood lipoprotein and total triacylglycerols levels, liver histological and gene expression profiles, and intestinal transcriptome was investigated in the zebrafish model of dyslipidemia. The study revealed that dietary *Schizochytrium* oil increased HDL cholesterol while lowering the total cholesterol, LDL cholesterol, and total triacylglycerol contents in the blood. The intestinal transcriptome sequencing revealed that dietary *Schizochytrium* oil increased the expression of genes connected to cholesterol biosynthesis and glutathione metabolism. The effect of dietary microbial oil on the plasma lipidomic landscape of the zebrafish model was also explored in the study. The oil increased the abundance of long-chain polyunsaturated fatty acid-rich

triacylglycerols and reduced diacylglycerols containing monounsaturated and saturated fatty acids in the plasma. The effect of a combination of dietary microbial oil with β-glucans from two different microalgae (*Euglena gracilis* and *Phaeodactylum tricornutum*) on the blood lipoprotein and total triacylglycerol levels, hepatic transcriptome, and plasma lipidome of the zebrafish model was elucidated in the third study. The results indicated that microbial oil reduced the expression of genes and the abundance of lipids connected to the steroid biosynthesis pathway. Combining the microbial oil with β-glucans from different sources impacted the hepatic transcriptome and plasma lipidome of the zebrafish model differently, compared to the sole effect of the microbial oil. Dietary microbial oil with *E. gracilis* β-glucan significantly reduced the plasma total triacylglycerol levels, downregulated genes connected to the PPAR signaling pathway, and increased the abundance of lysophosphatidylcholine species in the plasma of the zebrafish. On the other hand, combining microbial oil with *P. tricornutum* β-glucan increased the abundance of plasma phosphatidylcholines rich in long-chain polyunsaturated fatty acids and reduced the abundance of triacylglycerols with mono- and unsaturated fatty acids.

This PhD project has revealed insights into the tissue-specific processes associated with dyslipidemia in zebrafish and helped elucidate the beneficial effects of microbial oil and β-glucans as agents to counter the disorder.

SAMMENDRAG

Dyslipidemi, betegnet ved en endret blodlipidprofil, er en risikofaktor som utløser utviklingen av kardiovaskulære sykdommer hos mennesker. Sebrafisk er en modellart som brukes til å undersøke hvordan endringer i dietten kan forebygge dyslipidemi. I denne avhandlingen presenteres ny kunnskap om hvordan diett-indusert dyslipidemi påvirker molekylære responser i tarm og lever hos sebrafisk. Dette prosjektet fokuserte på hvordan β-glukaner og n-3 PUFA fra nye mikrobielle kilder påvirker plasmalipider og genuttrykk i tarm- og lever hos sebrafisk.

Virkningen av kolesterol, β-glukaner og simvastatin (kolesterolsenkende medikament) i dietten på blodlipoproteiner, vevshistologi og genuttrykk i tarm hos sebrafisk ble undersøkt i den første studien. Resultatene indikerte at fôring med en kolesterolrik diett fører til en dyslipidemisk blodprofil og hepatisk lipidakkumulering hos sebrafisk, og at tilsetning av β-glukan og simvastatin i dietten kan motvirke denne effekten. Sekvensering av tarmtranskriptomet viste at en høykolesteroldiett førte til en nedregulering av gener knyttet til kolesterolbiosyntese og en oppregulering av gener assosiert med organell-dysfunksjon. β-glukaner og simvastatin i sebrafiskdietten gjenopprettet uttrykksmønsteret til flere tarmgener endret av høykolesteroldietter.

Den andre studien fokuserte på effekten av en diett rik på n-3 PUFA mikrobiell olje fra *Schizochytrium* sp. på blodlipoprotein og totale triacylglycerolsnivåer, leverhistologi og genuttrykk i lever og tarm hos sebrafisk. Studien viste at en diett med *Schizochytrium*-olje økte HDL-kolesterolet mens den senket det totale kolesterolet, LDL-kolesterolet og det totale triacylglycerolinnholdet i blodet. Sekvensering av tarmtranskriptomet viste at *Schizochytrium*-olje i dietten økte uttrykket av gener knyttet til kolesterolbiosyntese og glutationmetabolisme. Effekten mikrobiell olje i dietten hadde på plasmalipider ble også undersøkt i studien. Vi observerte at denne dietten med økte mengden av langkjedede flerumettede fettsyrerrike triacylglyceroler og reduserte mengden av diacylglyceroler som inneholder enumettede og mettede fettsyrer i plasma. Effekten av en kombinasjon av mikrobiell olje i dietten med β-glukaner fra to

forskjellige mikroalger (*Euglena gracilis* og *Phaeodactylum tricornutum*) på lipoprotein- og totalt triacylglycerolnivå, levertranskriptom og plasmalipidom hos sebrafisk ble undersøkt i den tredje studien. Resultatene indikerte at mikrobiell olje reduserte uttrykket av gener og mengden av lipider knyttet til steroidbiosyntese. Når den mikrobielle oljen ble gitt sammen med β-glukaner fra forskjellige kilder ble effekten på levertranskriptomet og plasmalipidomet forskjellig. Mikrobiell olje kombinert med β-glukan fra *E. gracilis* reduserte signifikant de totale triacylglycerolnivåene i plasma, nedregulerte gener koblet til PPAR-signalveien og økte forekomsten av lysofosfatidylkolinarter i plasmaet hos sebrafisk. Derimot økte kombinasjonen av mikrobiell olje med β-glukan fra *P. tricornutum* plasmamengden av fosfatidylkoliner rike på langkjedede flerumettede fettsyrer og reduserte mengden av triacylglyceroler med mono- og umettede fettsyrer.

Dette doktorgradsprosjektet har gitt ny innsikt om vevsspesifikke prosesser assosiert med dyslipidemi hos sebrafisk og bidratt til å belyse de gunstige effektene av mikrobiell olje og β-glukaner som midler for å motvirke denne sykdommen.

1. INTRODUCTION

1.1 Cardiovascular diseases in the modern world

Non-communicable diseases are causing most of the global deaths and every minute 28 people die prematurely from a non-communicable disease. In May 2013, World Health Assembly approved the 'World Health Organization (WHO) Global Action Plan for the Prevention and Control of NCDs 2013-2020' that aimed to reduce 25% of the premature mortalities by four main non-communicable diseases by 2025. These diseases are cancer, diabetes mellitus and chronic respiratory and cardiovascular disorders (CVDs). Of these diseases, CVDs account for more than 18 million deaths every year (Figure 1). WHO has listed hypertensive heart disease, ischemic heart disease, cerebrovascular disease and inflammatory heart disease as the major forms of CVDs.

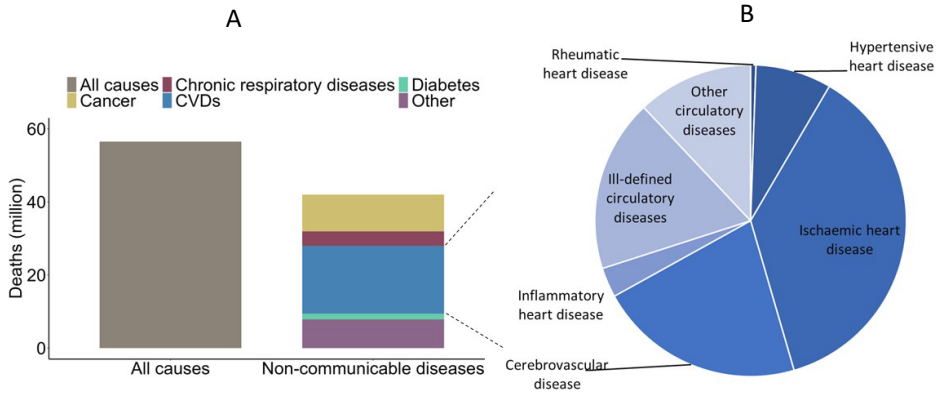


Figure 1. Non-communicable diseases-associated deaths in 2019 and percentage of deaths caused by each type of CVD. (A) More than 42 million people died of non-communicable diseases, i.e., 74.3% of all deaths recorded in 2019 (Data were obtained from Global Burden of Disease Study 2019 (GBD 2019) Results provided by Institute for Health Metrics and Evaluation, Seattle, United States (https://vizhub.healthdata.org/gbd-results/). (B) The pie chart is created based on cardiovascular disease mortality registered in 58 countries (Data obtained from the WHO mortality database, https://platform.who.int/mortality).

The last three decades have witnessed year-on-year increase in CVD-related deaths, from 12 million in 1990 to 18.5 million in 2019 (Figure 2).

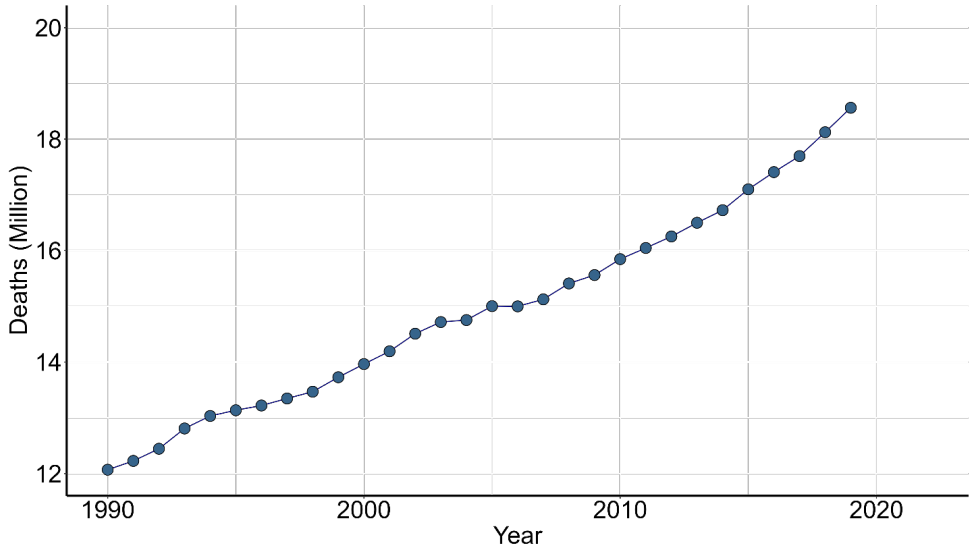


Figure 2. Global year-on-year increase in CVD-linked mortality. The total number of deaths by CVDs has increased from 12.06 million to 18.56 million during the period 1990-2019. Raw data were obtained from Global Burden of Disease Study 2019 (GBD 2019) Results provided by Institute for Health Metrics and Evaluation, Seattle, United States, https://vizhub.healthdata.org/gbd-results/.

1.2 Diet as a risk factor for CVDs

Risk factor(s) predispose(s) an individual to a disease. Genetic, habitual and environmental factors can be determinants of a condition that causes a disease. For instance, according to the pioneering Framingham Heart Study, serum cholesterol levels and elevated systolic blood pressure promote the development of several forms of CVDs, i.e., these factors can independently increase the likelihood of CVD events (Tan et al., 2018). Several genome-wide association studies have helped to identify the genetic basis for the development of CVDs (Johnson et al., 2011). These studies have revealed important variants at several loci within the human genome, which are associated with increased systolic blood pressure and increased serum cholesterol

levels (Kathiresan et al., 2007, Newton-Cheh et al., 2009). However, the development of CVDs is not purely a consequence of the genetic makeup but can also be the outcome of other habitual and environmental factors, which are often modifiable and, if addressed, can help assuage the sufferings of the CVD patients. Unhealthy diet represents a crucial deciding factor in the development of CVDs (Figure 3).

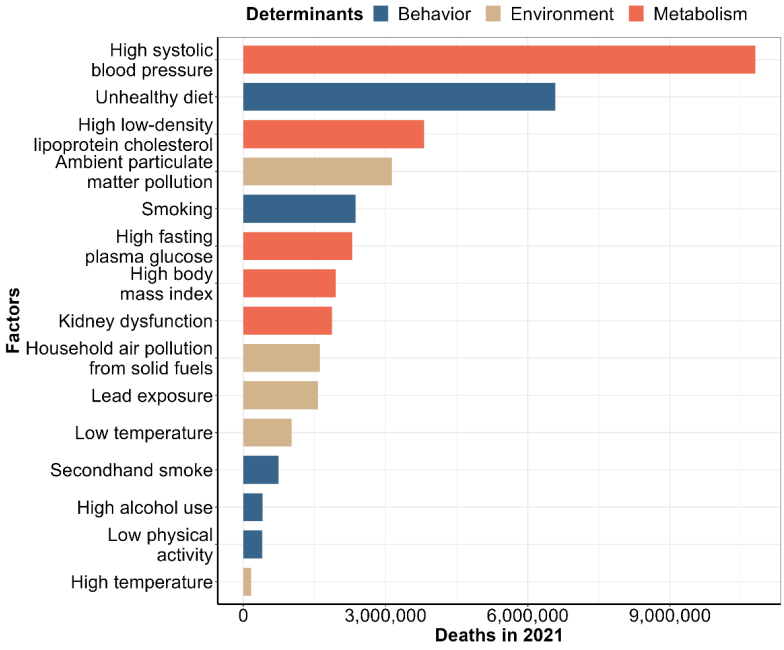
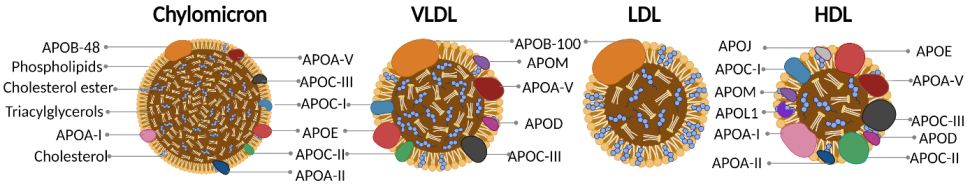


Figure 3. Contribution of different determinants of CVDs to global deaths. Behavior, environment and metabolism are the main determinants that trigger the development of CVDs. Among the behavioral determinants unhealthy diet causes the greatest number of deaths. The graph was generated using the data from Vaduganathan et al. (2022).

More than six million deaths are attributed to unhealthy diet-induced CVDs (Vaduganathan et al., 2022), indicating that such diets adversely affect metabolism. The development of several metabolic diseases has been linked to a dietary pattern that is at odds with the evolutionarily-determined genetic makeup (Knight, 2011). The shift towards a Western diet (Kopp, 2019) that is rich in processed red meat, butter, high-fat dairy products (Hu, 2002) and low in soluble and non-soluble fibers (Wilson et al., 2016) is known to be the main cause of many noncommunicable diseases. A recent analysis revealed that low intake of whole grains, vegetables and fruits, and high intake

of sodium and red meat is the major reason for global CVD-related deaths (Afshin et al., 2019). High intake of saturated and trans fat-based diet perturbs lipid metabolism and eventually result in atherosclerosis that enhances the progression of CVDs (Gomez-Delgado et al., 2021, Hedayatnia et al., 2020). Long-term adherence to a Western diet can increase the risk of dyslipidemia (Asadi et al., 2020, Na et al., 2019). Dyslipidemia represents an altered state of lipid metabolism, often due to excessive consumption of lipid-rich diets. Abnormal levels of total cholesterol, low-density lipoprotein (LDL) cholesterol, triacylglycerol and high-density lipoprotein (HDL) cholesterol were noted in the blood of obese (Ebbert and Jensen, 2013) and non-obese (Højland et al., 2016) individuals who were on an unhealthy diet. Dietary lipids are predominantly comprised of triacylglycerols, phospholipids, and cholesterol esters. Since most lipid species are insoluble in blood, they are transported as lipoproteins. Lipoproteins are macromolecular complexes composed of varying amounts of proteins and lipids like triacylglycerols, cholesterol esters, and phospholipids. Lipoproteins are classified according to their physical properties (density and diameter), and the content of protein and lipid classes (Francis, 2016). The major lipoproteins that transport the lipids are chylomicrons, VLDL, LDL, and HDL (Figure 4). These particles have different apolipoproteins and in the next section *only the molecules that were altered in the 3 studies performed* for this PhD project will be described in connection with the uptake and transport of cholesterol/lipids.



Physical properties				
Density (g ml^{-1})	<0.94	0.94-1.006	1.006-1.063	1.063-1.210
Diameter (Å)	6000-2000	600	250	70-120
Biochemical composition (%)				
Protein	2	10	23	55
Triacylglycerol	85	50-55	20-25	4
Free cholesterol	1	7	8	2
Cholesterol ester	3	12	37	15
Phospholipids	9	18	20	24

Figure 4. Physical and biochemical properties of the four major lipoproteins. Modified from Francis (2016). The lipoproteins chylomicrons, VDL, LDL and HDL differ in terms of their sizes and biochemical composition. The LDL particle has only one apolipoprotein, APOB-100. Conversely, other lipoproteins have many apolipoproteins on their outer membrane. APOA-I primarily synthesized by the liver and intestine is found in HDL and chylomicrons. It is an activator of lecithin-cholesterol acyltransferase and is a structural component of the lipoproteins. APOA-II that is synthesized primarily in the liver and to a lesser extent in the intestine, is found in HDL and chylomicrons. It is an activator of the hepatic lipase enzyme. APOA-V that is found in VLDL, chylomicrons and HDL particles is synthesized in the liver. It promotes the lipoprotein lipase mediated breakdown of the lipoprotein particles. APOB-48 is unique to chylomicrons and is synthesized in the intestine. It is a structural component of the chylomicrons. APOB-100 is specific to VLDL and LDL particles and is synthesized in the liver. It is a structural protein of LDL and VLDL and acts as a ligand of low-density lipoprotein receptor. APOC-I, APOC-II and APOC-III are found in chylomicrons, HDL and VLDL and are primarily synthesized in the liver. Lipoprotein lipase requires APOC-II as a cofactor for efficient lipolysis of triglyceride rich lipoproteins. Conversely, APOC-I and APOC-III prevent the binding of lipoprotein lipase to lipoproteins and inhibit lipoprotein lipase activity, respectively. APOD has a ubiquitous expression in several tissues like liver, brain, intestine, and muscles. In the plasma, APOD is present on HDL and VLDL particles. APOD is involved in lipoprotein lipase-mediated hydrolysis of VLDL and can lower the triacylglycerol content in circulation. APOM is synthesized in the liver and kidney and is associated with HDL particles. APOM provides protection against atherosclerosis by increasing the formation of pre β-HDL, thereby increasing the capacity to stimulate efflux of cholesterol. APOL-1 is a component of HDL and is synthesized in the liver, pancreas, kidney and brain. The function of APOL-1 on the HDL particle has not been deciphered yet but plasma levels of APOL-1 have been demonstrated to correlate with plasma triacylglycerol and cholesterol levels. APOJ is synthesized in several tissues including the liver, brain and gonads. It is a component of HDL particle. APOJ is associated with the protective effect of HDL and is involved in the HDL-mediated transport of cholesterol to the liver for biliary excretion. APOE is synthesized primarily in the liver and intestine and is found in chylomicron remnants, VLDL and HDL. This protein also acts as a ligand of low density lipoprotein receptor. . Note that the features in the illustration are not drawn to scale. (Created with BioRender.com)

1.3 Intestinal lipoprotein metabolism

Lipoprotein metabolism is intricately connected to digestion and absorption of lipids in the intestine. The digested lipids enter the enterocytes and form the primary raw material for the synthesis of the lipoproteins in the intestine (Figure 5). Lipases secreted in the mouth, stomach, and pancreas are required for the breakdown of the triacylglycerols, the most abundant lipid in the digestive tract. Pancreatic lipase cleaves the sn-1 and sn-3 fatty acids of the triacylglycerols, forming a 2-monoacyglycerol molecule and two free fatty acids (Ko et al., 2020). The second most abundant lipids in the digestive tract are phospholipids. Humans obtain their daily requirement of phospholipids (2-10 g) from diet. In addition, the liver also secretes about 10-20 g of phospholipids (as part of bile) into the intestinal lumen every day. The pancreatic enzyme phospholipase A2 is the major enzyme that cleaves the sn-2 fatty acid of phospholipids, producing lysophospholipids and a free fatty acid (Nilsson and Duan, 2019). Hepatic and dietary cholesterol are also absorbed in the intestine. In the intestinal lumen, cholesterol exists in two forms, as free cholesterol and as esters of different fatty acids. The cholesterol esters of fatty acids are cleaved by the pancreatic enzyme cholesterol esterase to release the fatty acid from cholesterol molecules.

In order to aid the process of digestion, a range of physiological surfactants like bile salts (from the gall bladder) and phosphatidylcholines (from diets/liver) act in the intestine (Monte et al., 2009). Bile salts adsorb onto the emulsified lipid particles to promote the attachment of pancreatic lipases on the lipid substrates. As the digestive enzymes hydrolyze the lipids, bile salts remove the lipolysis products from the lipid-lipase interface (Maldonado-Valderrama et al., 2011). The products of lipolysis are removed from the lipid-water interface and incorporated into mixed micelles. These mixed micelles are necessary for effective absorption of dietary lipids (Bauer et al., 2005). The net negative charge and small size (3 to 5 nm) of the mixed micelles facilitate their transport across the intestinal mucus barrier and underlying unstirred water layer (Macierzanka et al., 2019, Macierzanka et al., 2014, Wouthuyzen-Bakker et al., 2011). The acidic microclimate near the brush border membrane enterocytes, disrupts the

organization of the mixed micelles, causing the release of the constituent lipids (Verkade and Tso, 2001). The small intestine is equipped with a range of transporter proteins which are involved in the absorption of free fatty acids. These transporter proteins include fatty acid transporter protein 4 (FATP4), cluster of differentiation 36 (CD36) and different fatty acid binding proteins like liver fatty acid binding protein (LFABP) and intestinal fatty acid binding protein (IFABP) (Masson et al., 2010). Besides transporting fatty acids, LFABP is also involved in the absorption of lysophospholipids and monoacylglycerols (Storch and Corsico, 2008). The transporter proteins fatty acid binding protein 6 (FABP6) and solute carrier family 10 member 2 (SLC10A2) are involved in the transport of bile acids (Duggavathi et al., 2015). Lysophospholipids released from dietary and biliary phospholipids also cross the enterocyte membrane by passive diffusion when concentrations in the lumen exceed those inside the enterocytes. The rate of cholesterol transport is highly variable among individuals. Cholesterol is absorbed across the enterocyte membrane by the action of a glycosylated membrane transporter protein named Niemann-Pick C1-like 1 (NPC1L1) (Altmann et al., 2004). NPC1L1 is enriched in the plasma membrane microdomains called lipid rafts. The ATP-binding cassette sub-family G member 5 (ABCG5) and ATP-binding cassette sub-family G member 8 (ABCG8) transporters (known as sterolins and function as obligate heterodimers) are also involved in cholesterol homeostasis in the intestine. The action of ABCG5/G8 transporters facilitate the removal of cholesterol from the enterocytes to the lumen (Patel et al., 2018).

Once the lipids passively diffuse or are transported into the enterocytes, they are used for the formation of chylomicrons and lipoproteins. Following absorption, they are re-esterified with fatty acids through different mechanisms. For instance the fatty acids bound by FABPs target the microsome for re-esterification to triacylglycerols (Adeli and Lewis, 2008). Substantial re-esterification of the lipids (triacylglycerols, lysophospholipids and cholesterol) also takes place in the endoplasmic reticulum (ER) to produce triacylglycerols, cholesterol esters and phospholipids. Monoacylglycerols are esterified with fatty acids by monoacylglycerol acyltransferases (MGAT) to form

diacylglycerols, which are converted to triacylglycerols by diacylglycerol acyltransferases (DGAT) (Hung et al., 2017). Phospholipids are also synthesized in the ER from diacylglycerol, choline, and ethanolamine (Henneberry et al., 2002). Free cholesterol taken up by the enterocytes is also esterified in the ER by membrane-bound acyl-CoA:cholesterol acyltransferases (ACAT) (Chang et al., 2009). The newly synthesized neutral lipids (cholesterol esters and triacylglycerols) can be either stored as lipid droplets in the enterocytes or used for the synthesis of lipoprotein particles. The neutral lipid core of the cytosolic lipid droplets in the enterocytes is surrounded by a phospholipid layer and several proteins that are inserted directly from the cytoplasm or via the ER (Olzmann and Carvalho, 2019). These proteins perform a variety of functions on the lipid droplets including scaffolding, lipid synthesis and lipolysis, among others (Bersuker and Olzmann, 2017). Perilipins are the scaffolding proteins found associated with the lipid droplets and they help in maintenance of membrane contact sites between lipid droplets and between lipid droplets and other organelles like mitochondria and lysosomes (Olzmann and Carvalho, 2019). Though perilipin expression is generally under the control of PPARs (Kimmel and Sztalryd, 2016) certain proteins like microsomal triglyceride transfer protein (MTTP) also regulate the expression of perilipins. In zebrafish, MTTP inhibition significantly downregulated the expression of the *plin2* in the intestine (Zeituni et al., 2016). Chylomicron formation in the enterocyte begins with splicing of the APOB-100 pre-mRNA into a smaller APOB-48 pre-mRNA which is translated to APOB-48 protein. In the rough-endoplasmic reticulum, the MTTP protein induces a configurational change in the APOB-48 protein that enables its lipidation with cholesterol and phospholipids, resulting in the formation of nascent chylomicron particles (Hussain et al., 2003). In the smooth endoplasmic reticulum (SER), these nascent particles further increase their triacylglycerol and cholesterol ester content to form prechylomicron particles (Mansbach and Siddiqi, 2010). Once prechylomicrons are formed in the SER they need to translocate to and fuse with the Golgi complex. Therefore, the LFABP mediated budding of the particle from the SER gives rise to a prechylomicron transport vesicle. The vesicle protects the

prechylomicron from cytosolic proteases before it enters cis-Golgi (Black, 2007). Inside the Golgi, protein APOA-I attaches to prechylomicrons to form mature chylomicrons which leave the enterocyte through the basolateral side (Giammanco et al., 2015).

For the transport of cholesterol, there also exists an alternate pathway from the intestine which involves the formation of HDL particles with their constituents, cholesterol, and apolipoprotein A-I (APOA-I). The cholesterol is transported out of the enterocyte with the help of the basolateral ATP binding cassette subfamily A member 1 (ABCA1) transporter and taken up by APOA1 in circulation to form a nascent HDL particle (Pan and Hussain, 2012) which takes up cholesterol from the peripheral tissues to form a mature HDL particle. The HDL and chylomicrons or their remnants eventually reach the liver tissue, where they are metabolized further to give rise to other lipoproteins.

1.4 Liver lipoprotein metabolism

The liver is another key organ that regulates several aspects of lipid metabolism (Figure 5). Apart from its role to synthesize triacylglycerols and cholesterol, the liver processes chylomicron remnants. After the secretion from the intestine, the chylomicrons enter the blood where they encounter the lipoprotein lipase in the blood capillaries. It is the major lipolytic enzyme involved in the intravascular degradation of chylomicrons (Bayly, 2014). Lipoprotein lipase catalyzes the hydrolysis of the triacylglycerols of chylomicrons to monoacylglycerols and fatty acids. Fatty acids are taken up by the tissues and either re-esterified and stored (in adipose tissues) or utilized as an energy source (in muscles) (Mead et al., 2002). Lipoprotein lipase also converts chylomicrons to chylomicron remnant particles, which are taken up by the hepatocytes and degraded in the lysosomes (Cohen and Fisher, 2013). *De novo* biosynthesis of lipids (triacylglycerols and cholesterol), uptake of circulating esterified fatty acids facilitated by hepatic lipase (HL) and non-esterified fatty acids facilitated by CD36 and FABPs (Bradbury, 2006) can also add to this pool of intracellular lipids in the hepatocytes. In the hepatocytes, lipids can be processed to i) form lipid droplets, ii) undergo β-oxidation in the mitochondria, and

iii) form VLDL particles. The VLDL particle biosynthesis begins with the lipidation of the apolipoprotein B-100 (APOB-100) protein by MTTP, leading to the formation of a nascent VLDL which is secreted by the ER (Hussain et al., 2003) and then enters the Golgi complex for further lipidation and release (Fisher and Ginsberg, 2002). After being released from the hepatocyte, the VLDL will be acted upon by lipoprotein lipase enzyme, which is localized in the blood capillaries of the muscle and adipose tissue. This facilitates the release of triacylglycerols from the VLDL particles to eventually give rise to the VLDL remnant particles (Young and Zechner, 2013). The VLDL remnant may undergo receptor-mediated endocytosis in the liver (mediated by the LDL receptor (LDLR) or the LDL-receptor-related protein 1 (LRP-1) receptor) (Vance and Vance, 2002) or continue to lose more triacylglycerols in the blood capillaries by interacting with lipoprotein lipase to give rise to LDL particles (Fielding and Fielding, 2002). The liver is also a significant organ for the synthesis of HDL particles. However, unlike VLDL particles that are assembled intracellularly, HDL particles are formed within the sinusoidal capillaries near the surface of hepatocytes (Lewis and Rader, 2005). The first step in synthesizing the HDL particle is the production of APOA-I protein in the hepatocyte ER, which is phospholipidated intracellularly and secreted into the sinusoidal capillaries (Maric et al., 2005). The liver can secrete cholesterol and phospholipids for the lipidation of APOA-I aided by the ABCA1 transporter (Tang and Oram, 2009). This lipidation of APOA-I facilitates the formation of a pre-beta HDL particle, which travels to the peripheral tissues to gain more cholesterol. In circulation, lecithin-cholesterol acyltransferase (LCAT) protein (synthesized in the liver and secreted into the blood) converts the free cholesterol of the pre-beta HDL (from the sinusoidal capillaries) to cholesterol esters (Deng et al., 2022), increasing the ability of the pre-beta HDL to take up more lipids leading to the formation of a mature HDL particle. Maturated HDL incorporates cholesterol from cells and tissues through its interaction with the scavenger receptor class B type 1 (SR-B1) (to a lesser extent), ATP-binding cassette sub-family G member 1 (ABCG1) and ABCA1 and passive diffusion. These HDL particles after reaching the liver transfer cholesterol via its interaction with

SR-B1. However, the protein SR-BI performs only a selective cholesterol uptake without internalizing the APOA-I, which enters circulation to form new HDL particles (Yu, 2022). Overall, the lipoprotein metabolism in the liver and intestine tissue is a complex but precisely regulated process that orchestrates the delivery of lipids to muscle and adipose tissue by chylomicrons and VLDLs and the removal of excess cholesterol from cells by HDL particles. However, the consumption of high-calorie or lipid-rich diets disturbs the delicate balance of the lipoprotein metabolism in the body resulting in a dyslipidemic lipoprotein profile.

1.5 Characteristics of dyslipidemia

A diet rich in unfavorable fats can cause dyslipidemia by altering the lipoprotein levels in circulation. Saturated fats (butter, margarine, palm oil and sunflower oil) can elevate blood cholesterol and LDL cholesterol levels (Siri-Tarino et al., 2010). On the other hand, trans fat (microwave popcorn, frozen pizza, French fries, fried chicken, coffee creamer, etc.) can increase the LDL and triacylglycerols and decrease HDL (Dhaka et al., 2011). Dietary cholesterol (red meat, dairy, fried foods, butter, etc.) can increase the plasma cholesterol levels (Griffin and Lichtenstein, 2013). The lipoprotein imbalance triggered by an unhealthy diet may have far-reaching effects on, among others, plasma lipoprotein levels. Cellular membrane dynamics, organellar functions, and signaling pathways.

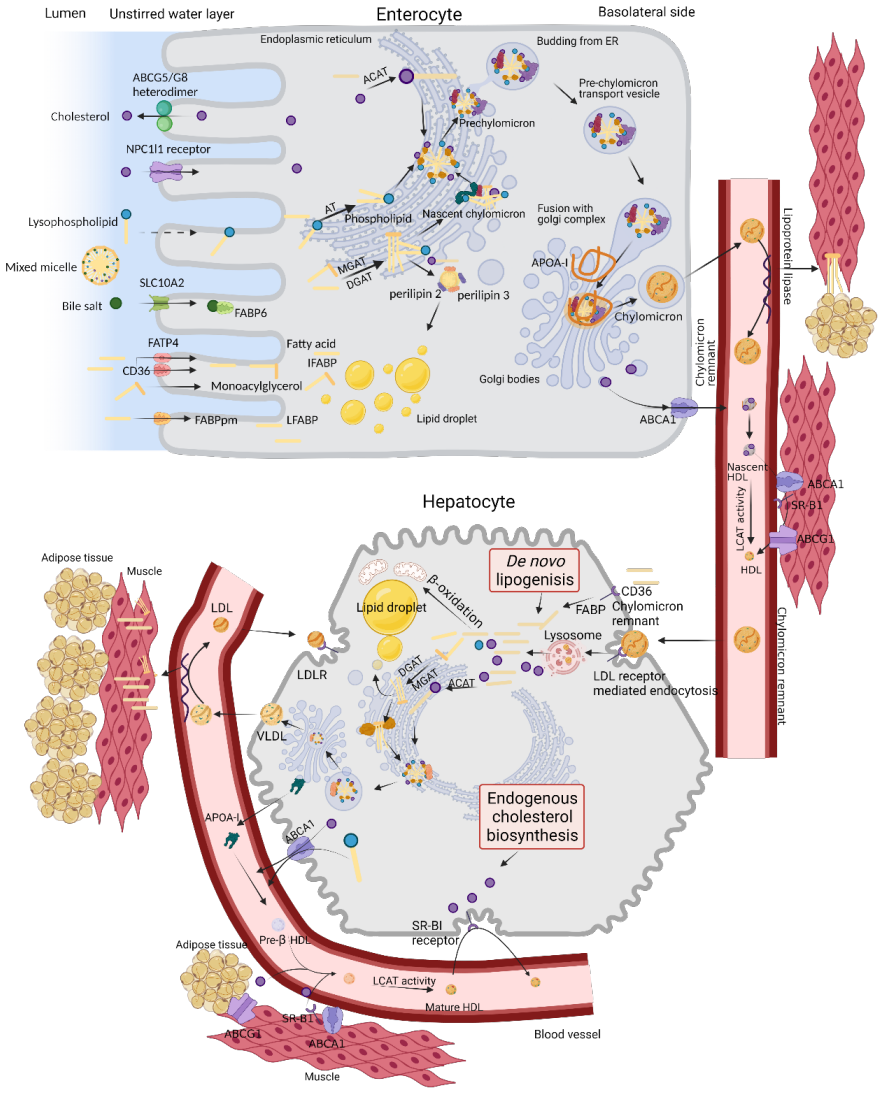


Figure 5. Overview of intestinal and hepatic lipoprotein metabolism. Lipid digestion starts with the hydrolysis of dietary fat in the intestine lumen. The hydrolyzed lipids along with bile acids form mixed micelles, which carry lipids to the enterocytes through the mucus layer and the unstirred water layer. Mixed micelles are broken down near the apical membrane of the enterocytes to release the constituent lipids and bile salts.

Most of the lipid species are transported into the enterocyte with the help of transporter proteins except the lysophospholipids which can cross the membrane by passive diffusion. Intestinal fatty acid binding protein, liver fatty acid binding protein, cluster of differentiation 36 and fatty acid transporter protein 4 facilitate the transport of fatty acids. Niemann-Pick C1-like 1 transporter carries cholesterol

across the enterocyte membrane and fatty acid binding protein 6 and solute carrier family 10 member 2 are responsible for the absorption of bile salts. Inside the enterocytes re-esterification of the lipids by the enzymes monoacylglycerol acyltransferases, diacylglycerol acyltransferases, acyl-CoA:cholesterol acyltransferases takes place, resulting in the formation of triacylglycerol and cholesterol esters.

In the rough ER, MTTP induces a structural change in the APOB-48 protein enabling its lipidation with cholesterol and phospholipids, resulting in the formation of nascent chylomicron particles. These nascent chylomicrons mature in the Golgi complex by acquiring APOA-I and are finally exported by the basolateral transporters of the enterocyte. Cholesterol is also exported by ABCA1 transporter and taken up by the APOA-I secreted by the intestine. This initiates the formation of a nascent HDL particle.

The action of lipoprotein lipase in the blood capillaries converts chylomicrons to chylomicron remnants particles that are taken up by the liver following the interaction between hepatocytic low-density lipoprotein receptor with the APOE protein of the chylomicron remnant. The endocytosed remnants are subjected to lysosomal degradation. The nascent VLDL particle is formed by the lipidation of the apolipoprotein B-100 protein by microsomal triglyceride transfer protein. After it enters the blood capillaries of the muscle and adipose tissue, triacylglycerols are released by the action of lipoprotein lipase, thereby forming VLDL remnants and LDL. Pre-beta HDL particles are formed within the sinusoidal capillaries near the surface of hepatocytes. APOA-I is phospholipidated in the hepatocytes and later secreted into the blood, where it acquires cholesterol and phospholipids, resulting in the formation of a pre-beta HDL particle.

In circulation, lecithin-cholesterol acyltransferase protein converts the free cholesterol of the pre-beta HDL to cholesterol esters, elevating its ability to take up more lipids to form a mature HDL particle. Finally, the HDL transfers cholesterol to the liver via its interaction with hepatic scavenger receptor class B type 1 receptor. The hepatic cholesterol, delivered by HDL/diet or synthesized endogenously, is used to produce bile salts. The cholesterol from the HDL will be part of the reverse cholesterol transport and will be partly excreted. Note that the features in the illustration are not drawn to scale. (Created with BioRender.com)

1.5.1 Excess lipid-induced fate of lipoproteins

The circulating LDL cholesterol has APOB-100 in its membrane for binding to cell surface receptors. APOB-100 facilitates the uptake of LDL cholesterol by the liver cells by binding to LDLR. The expression of APOB-100 and LDLR are essential for the liver to perform its functions to maintain the LDL homeostasis in circulation (Dietschy, 1998; Goldstein & Brown, 2009). Lipid levels above the recommended daily intake can alter the expression of the aforementioned proteins. Excessive lipid intake will challenge its uptake by available LDL receptors, as it will not be sufficient to bind to and endocytose APOB-containing particles. The protein, proprotein convertase subtilisin/kexin type 9 (PCSK9) binds to LDL receptors in the hepatocytes to promote their lysosomal

degradation and reduce the number of LDL receptors on the cell. The consequent relative deficiency of LDLR in the hepatocytes leads to a rise in blood LDL level. The excess LDL particles in circulation can cross the endothelial membrane of the blood vessels. The penetration of LDL particles activates the endothelial cells to express monocyte adherence proteins (like vascular cell adhesion molecule 1 and intracellular cell adhesion molecule 1) (Galkina and Ley, 2007) and proinflammatory cytokines (monocyte chemoattractant protein-1 and interleukin-8) (Wu et al., 2017) leading to the attachment of monocytes to the endothelial cells, their conversion to macrophages and their trans-endothelial migration that mark the beginning of the development of atherosclerotic plaques (Daniels & Couch, 2014). Conversely, when the consumption of lipids is reduced, the level of LDL in the blood reduces gradually in response to the increase in the expression of LDLR.

Lipid-rich diets, in particular saturated fat rich diets increase the levels of VLDLs and chylomicrons, in the circulation (Adiels et al., 2008, DiNicolantonio and O'Keefe, 2018). A considerable proportion of the lipids in these lipoproteins are triacylglycerols and cholesterols. Therefore, a sustained high consumption of lipid-rich diets can increase the triacylglycerol content and the total cholesterol content in the circulation. The compensatory mechanism to reduce the cholesterol is to increase the circulating HDL cholesterol during the consumption of cholesterol-rich diets. This strategy will increase the biosynthesis of HDL and reduce its breakdown rate, as reported in rat (Hayek et al., 1993) and human studies (Greene et al., 2005, Herron et al., 2003).

1.5.2 Effect of diet-induced dyslipidemia on cell membranes and organellar functions

Cholesterol is important to maintain organellar integrity, and membrane fluidity and structure. It also acts as a precursor of hormones, vitamin D and bile salts. However, high amounts of dietary cholesterol can suppress cholesterol biosynthesis by reducing the expression and activity of the key enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) that is present in the ER (DeBose-Boyd, 2008). Furthermore, excess consumption of triacylglycerols and cholesterol can stimulate hepatocytes to store

these lipids in the form of neutral lipid droplets (Rudel et al., 2005, Lindeboom et al., 2015), and this is a characteristic of dyslipidemia. Such accumulation of neutral lipids can be benign and normally will not cause inflammation, but excess storage is toxic to liver tissues (Heeren and Scheja, 2021).

Excess lipids can also affect the functions and organization of the plasma membrane. The lipid bilayer of the plasma membrane stores 90% of total cellular cholesterol (Marquardt et al., 2016, Krause and Regen, 2014), and cholesterol accounts for about 30% of the total plasma membrane lipids (Ikonen, 2008). In the plasma membrane, cholesterol exists as lipid rafts along with sphingolipids and proteins (Kraft, 2013). These rafts perform signaling functions (Cherezov et al., 2007) besides regulating the membrane order and fluidity (Simons and Vaz, 2004). Alterations in the cellular cholesterol levels can affect the relative abundance of proteins involved in several pathways like regulation of actin cytoskeleton, focal adhesion, and cell-cell junction proteins (Suica et al., 2015).

Cholesterol also interferes with the functions of intracellular organelles, especially those of ER and mitochondria, that are highly sensitive to dietary cholesterol. It is estimated that the cholesterol content in the ER membrane is only 1% of the total cellular cholesterol (Lange, 1991). The chaperone proteins in the ER, like heat shock protein 70 family member and lectin chaperones calnexin and calreticulin, facilitate folding and post-translational modifications of the newly synthesized proteins in the ER (Totani et al., 2020). Certain chaperone proteins require calcium for their proper functioning (Michalak et al., 2002) and the Ca^{2+} pumps, sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in the ER membrane ensure that there is sufficient Ca^{2+} in the ER lumen. However, higher amounts of membrane cholesterol reduce the Ca^{2+} reserve in the ER lumen (Li et al., 2004). This, in turn, hampers the folding of the newly synthesized proteins in the ER lumen and activates the unfolded protein response (UPR). The UPR suppresses protein translation machinery to maintain homeostasis. Furthermore, the condition elevates the mRNA levels of ER chaperone proteins such as binding protein (BiP) and glucose-regulated protein 94 (GRP94) (Westerheide and

Morimoto, 2005) and stromal derived factor proteins (Tiwari et al., 2013) that are connected to ER-associated degradation component (Iwayanagi et al., 2011) which is essential to clear the unfolded proteins (Ren et al., 2021). Thus, the activation of UPR counters the adverse effects of high cholesterol levels on the ER and helps to maintain normal cell functions under hyperlipidemic stress. However, a sustained UPR can eventually stimulate apoptosis to ensure minimum damage to the tissues (Sano and Reed, 2013).

Reactive oxygen species (ROS) are produced in different compartments of cells in an organism. Among them, mitochondria stand out from the rest by generating 90% of ROS. Although ROS are continuously produced via mitochondrial bioenergetics and oxidative metabolism (Hernansanz-Agustín and Enríquez, 2021), high-fat diets can increase the fatty acid oxidation and the associated ROS (Sikder et al., 2018, Jiang et al., 2021). The non-vesicular movement of cholesterol from the plasma membrane to the organelles is regulated to a large extent by steroidogenic acute regulatory related lipid transfer domain (STARD) proteins (Garbarino et al., 2012). The mRNA expression of STARD proteins is downregulated by a cholesterol-rich diet probably as a compensatory mechanism to avoid cholesterol exposure to mitochondrial membrane (Soccio et al., 2002). On the other hand, cholesterol accumulation in the mitochondria affects their physical properties and fluidity (van Meer et al., 2008). Consequently, excess cholesterol reduces the glutathione transport into the mitochondrial matrix by interfering with the functioning of the glutathione transporter, namely solute carrier family 25 member 11 (SLC25A11) (Mei et al., 2012). The antioxidant enzymes, glutathione-S-transferases (GST) also protect mitochondria against endogenous oxidative stress and exogenous toxins. GSTs are found in cytosol and microsomes also and these enzymes are known as the second phase enzyme detoxification system as they catalyze conjugation of electrophilic substrates to glutathione. It has been reported that microsomal glutathione-S-transferases can also alleviate the effects of ROS generated in the cells (Johansson et al., 2010). Thus, the studies reported here suggest that induced dyslipidemia not only leads to atherosclerosis but also affects cells

and organelles by inducing ER stress, ROS production and lipid accumulation. Sustained ER stress and ROS production can activate apoptosis (Szegezdi et al., 2006) and damage DNA (Martins et al., 2021), respectively. Lipid accumulation in the hepatocytes can progress to fatty liver disease. Therefore, maintaining the lipoprotein balance is key to preventing the development of several diseases.

1.6 Mitigating dyslipidemia

Dyslipidemia can often lead to CVDs and hence, there are many drugs to prevent and treat dyslipidemia. The primary emphasis in these drug-based treatments is to lower the circulating LDL cholesterol and triacylglycerol content. These drugs have different modes of action through which they lower the blood lipid and lipoproteins. For example, there are drugs that reduce the blood LDL levels or prevent the formation of dyslipidemia-causing lipoproteins. Orlistat is a pancreatic and gastric lipase inhibitor that hampers the absorption of dietary lipids (Seo et al., 2019), probably helping in reducing total cholesterol and LDL cholesterol (Kwon et al., 2022). The protein PCSK9 regulates the degradation of LDL receptors, and inhibitors of PCSK9 like Alirocumab can increase the LDL receptors in the liver to reduce the blood LDL levels. Lomitapide, which is a systemic MTTP inhibitor, prevents the formation of chylomicrons and VLDL, thereby diminishing the LDL cholesterol levels (Cuchel and Rader, 2013). APOB antisense oligonucleotides help in reducing blood LDL and VLDL levels, by inhibiting the synthesis of APOB-100 in the hepatocytes, which are key proteins of LDL and VLDL particles (Mullick et al., 2011). There are drugs that affect the cholesterol absorption and biosynthesis. For example, Ezetimibe hampers cholesterol absorption in the intestine by interfering with the activity of the cholesterol absorption facilitator, namely NPC1L1 receptor. Another class of drug, namely statins (hydroxy-methyl-glutaryl-coenzyme A reductase inhibitors), can suppress the endogenous cholesterol biosynthesis of the body, by inhibiting the activity of the enzyme HMGCR. There are drugs that can promote the formation of HDL particles. For example, APOA1 mimetic peptides promote the formation and function of HDL particles. Though these drugs are efficient in mitigating dyslipidemia either through reducing cholesterol absorption and

biosynthesis or by reducing the levels of LDL or increasing the HDL particles, they have side-effects such as steatorrhea and vitamin deficiency (Tak and Lee, 2021), myalgia and hepatotoxicity (Zhou et al., 2017), elevated hepatic transaminase activity and liver toxicity (Florentin et al., 2008), injection-site erythema and muscle pain (Stoekenbroek et al., 2018). These mild to severe side effects of antihyperlipidemic drugs have necessitated the discovery of alternative approaches which can help alleviate dyslipidemia.

Dietary and lifestyle interventions are regarded as the first-line therapy for dyslipidemia; managed-nutrition can help alleviate several non-communicable diseases including CVDs. In recognition of the importance of dietary habits, some regional (Mediterranean and Nordic diets) and specialized (Paleo and Portfolio) diets are recommended for their health benefits (Cena and Calder, 2020, De la et al., 2021, Glenn et al., 2021). Mediterranean and Nordic diets are rich in n-3 polyunsaturated fatty acids (PUFAs) and Paleo and portfolio diets contain β-glucans (Pastore et al., 2015, Marcason, 2015).

1.7 Mitigating dyslipidemia using bioactive compounds from novel microbial sources

Consumption of β-glucans and n-3 PUFAs effectively mitigate dyslipidemia as they influence signaling pathways differently to regulate tissue metabolism. β-glucans are structural or storage polysaccharides consisting of glucose units that are linked by β-glycosidic bonds. These bioactive compounds are found in the cell wall of grains, mushrooms, and microorganisms like bacteria, yeast, and microalgae. β-glucans have been shown to have antihyperlipidemic effect through varied modes of action. These polysaccharides are non-digestible and form viscous gels in the digestive tract, thereby hampering the diffusion of bile acids to the brush-border membrane to prevent their reabsorption in the intestine (Naumann et al., 2019) and stimulate the subsequent increase in bile acid biosynthesis (Ellegård and Andersson, 2007). Therefore, consumption of β-glucans can prevent dyslipidemia by reducing the total and LDL

cholesterol (Whitehead et al., 2014). Another important mechanism by which β-glucans mitigate dyslipidemia is by stimulating the β-oxidation of fatty acids in the peroxisome (Tahri-Joutey et al., 2021) and mitochondria (Bastin, 2014). Along with the retinoid X receptor, peroxisome proliferator-activated receptors (PPARs) activated by β-glucans bind as obligate heterodimers to specific genomic regions known as PPAR-responsive regulatory elements and control the expression of several networks of genes involved in lipid metabolism (Ahmadian et al., 2013). Several lines of evidence indicate that dietary β-glucans can also suppress the PPAR-γ signaling pathway which regulates the development of adipocytes (Kanagasabapathy et al., 2013, Li et al., 2019, Tang et al., 2020), but upregulate PPAR-α which regulates β-oxidation of fatty acids (Huang et al., 2014). β-glucans can also increase the DNA binding of PPARβ/δ transcription factor which can regulate β-oxidation in the muscle and adipose tissues (Dushkin et al., 2009, Tanaka et al., 2003). The physical properties of β-glucans govern the responses evoked by them (Du et al., 2019). However, there are source-specific differences in molecular weight, among others. Two novel sources of β-glucans are the microalgae *Phaeodactylum tricornutum* and *Euglena gracilis*. The β-glucans from these microalgae differ markedly from the β-glucan from oats (a well-known antihyperlipidemic β-glucan) in terms of molecular weight (Table 1). It has been observed that the low molecular weight (1.56×10^5 g/mol) β-glucan extracted from oats bound to substantial amount of bile acid compared to the high molecular weight (6.87×10^5 g/mol) β-glucan (Kim and White, 2010). However, viscosity of the β-glucan increased with the molecular weight (9.04×10^5 g/mol), thus hampering the absorption of dietary and biliary lipids (Kim and White, 2013). Moreover, studies have reported that β-glucans of medium (2.9×10^5 g/mol) to high (1.3×10^6 g/mol) molecular weight are more effective in reducing serum cholesterol than β-glucan of low molecular weight (Wang et al., 2016). These studies indicate that the difference in molecular weight is an important factor that can determine the antihyperlipidemic activity of β-glucans. A few studies have also explored the ability of β-glucans derived from *Euglena* and *Phaeodactylum* to alter the lipid metabolism in vertebrates. β-glucans from *E.*

gracilis significantly downregulated the expression of genes connected to digestion, fatty acid metabolic process, and lipid catabolic process in the intestine and upregulated genes related to lipid catabolic process in the liver, and consequently reduced the serum total and LDL cholesterol levels in obese mice (Aoe et al., 2021). A randomized controlled trial in humans reported that consuming an extract of *P. tricornutum* significantly lowered the total cholesterol, LDL cholesterol, and triacylglycerol contents in the serum of humans (Stiefvatter et al., 2022).

Table 1. Molecular weight of β-glucans from different sources

Source	Molecular weight (g/mol)	Branching pattern	Reference
Avena sativa (oats)	$>20\times10^5$	β-1,3 and/or β-1,4 glycosidic bonds	(Åman et al., 2004)
P. tricornutum	1×10^4	Linear β-1,3-glucan	(Yang et al., 2022)
E. gracilis	5×10^5	β -1,3 glucan	(Sonck et al., 2010)

Similar to β-glucans, n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA) also have several bioactive properties that can accrue health benefits. Fatty fish like salmon contains n-3 LCPUFA, with high content of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which have antihyperlipidemic properties (Kontostathi et al., 2021). These n-3 PUFAs downregulate the gene and protein expression of NPC1L1 transporter (Alvaro et al., 2010) and can increase β-oxidation through their effect on PPARα (Tahri-Joutey et al., 2021). Dietary fish oil also increases HDL cholesterol (Gao et al., 2020), which is a key particle that provides protection against dyslipidemia by facilitating reverse cholesterol transport. Although randomized control trials have not shown any benefits of increasing HDL against CVD incidences (Kaur et al., 2014), several observational studies have revealed that low HDL content is associated with CVD-related incidences (Jung et al., 2022). Thus n-3 PUFA consumption can mitigate

dyslipidemia by increasing HDL and lowering the triacylglycerol content in circulation (Gao et al., 2020).

Schizochytrium sp. is a heterotrophic fungus belonging to the order Thraustochytriales within the phylum Heterokonta. The n-3 PUFA content in this microbe accounts for 40% (w/w) of its total fatty acids. The fatty acid composition of *Schizochytrium* oil is different from the conventional fish oils. Like fish oil (EPA:14.9%, DHA 13% of total fatty acids), *Schizochytrium* oil is also rich in n-3 PUFAs (EPA 15%, DHA 39% of total fatty acids) but in contrast to fish oil it has a higher content of saturated fatty acids (Sarker et al., 2016, Santigosa et al., 2020). Recent studies have investigated the antihyperlipidemic effects of these novel microbial oils. Dietary *Schizochytrium* oil elevated the expression of *Insulin-induced gene 1* (*Insig-1*) and *Ldlr* genes in the liver of rats and also reduced the plasma total cholesterol, triacylglycerol and LDL cholesterol contents (Komprda et al., 2015). Consumption of dietary *Schizochytrium* oil for three weeks reduced the expression of intestinal *Npc1l1* and hepatic *Hmgcra* and *Ldlr* genes in hamsters and reduced the plasma total cholesterol and triacylglycerol levels (Chen et al., 2011).

These studies indicate that even though the beneficial effects of these bioactive compounds from novel microbial sources have been established, most studies have focused on changes in the blood lipid profile and expression of genes connected to lipid metabolism. There is a paucity of information regarding the holistic effects of novel microbial β-glucans and n-3 PUFAs. As mentioned above, an array of pathological conditions such as oxidative stress, hepatic lipid accumulation, organellar dysfunction, and alterations in cellular cytoskeletal architecture mark dyslipidemia. The effects of novel microbial bioactive factors on these aspects of dyslipidemia have not yet been fully uncovered.

1.8 Transcriptomic and lipidomic approaches to understand dyslipidemia

Lipidomics allows high-throughput screening of lipid molecules. The quantified lipid species will be either novel molecules or already known species that can be associated

with biological systems. Lipidomics has helped to identify novel biomarkers of CVDs that are not among the traditional risk indicators but can be used to predict the onset of the disease. For instance, from among 184 lipids, eight lipids in human plasma were reported as biomarkers of CVD. These eight lipid species included four species of phosphatidylcholines (PC) and one species each of sphingomyelin (SM), diacylglycerol, phosphatidylinositol (PI) and cholesterol ester (CE) (Ottosson et al., 2021). Analysis of plasma lipidome of rats fed a high-fat diet revealed significant alterations of seven fatty acid species–palmitic acid, hexadecenoic acid, hexanoylcarnitine, tetracosahexaenoic acid, cervonoyl ethanolamide, 3-hydroxytetradecanoic acid, and 5,6-dihydroxy eicosatrienoic acid; and five sterols, namely cholesterol ester (CE 18:2), cholesterol, hydroxytestosterone, 19-hydroxydeoxycorticosterone, and cholic acid. However, natural bioactive compounds can reduce the adverse effects of high-fat diets. For example, 95% ethanolic extract of the mushroom *Poria cocos* can restore the levels of several of the aforementioned lipids (Miao et al., 2016). Another natural product *Chrysanthemum morifolium* and a flavonoid from the flower (luteolin) can reduce the imbalance in certain lipid classes caused by a high-fat diet; the mix could restore the concentration of lysophosphatidylcholines (LPC 18:1, LPC 22:6), phosphatidylcholine (PC 18:1/22:6), cholesterol ester (CE 22:6), ceramide (CER 34:1 (d18:1/16:0)) and sphingomyelin (SM 34:1 (d18:1/16:0)) altered by a high-fat diet (Shon et al., 2020). These studies indicate the potential of lipidome analysis to delineate the mechanisms of actions of natural bioactive compounds.

While the lipidome analysis helps to get a glimpse of all the lipid species in a tissue, a transcriptome is a complete set of RNAs transcribed by cells in a tissue at a particular time. Transcriptomes can reveal clues on specific stages of a physiological condition because cells adjust their transcriptomic landscape in response to different external factors. Quantification of the transcripts gives information regarding the adaptation power or susceptibility of organisms to counter specific events like high-fat uptake or efficacy of novel bioactive compounds. Advances in next-generation sequencing

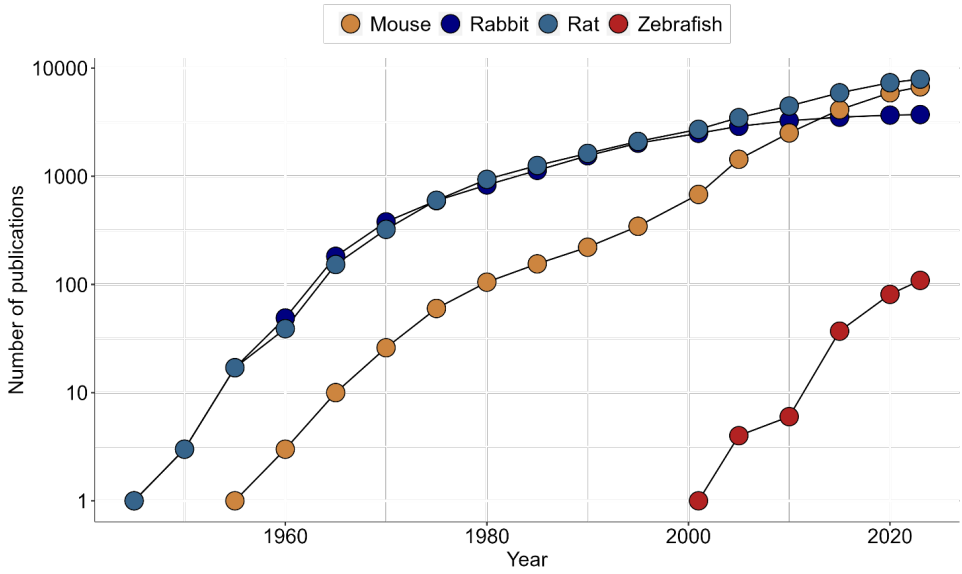
platforms have made it possible to conduct in-depth transcriptomic studies to understand disease mechanisms (Wang et al., 2009).

Transcriptomic approaches have been employed to understand biomarkers of dyslipidemia. A recent blood transcriptomic study revealed 40 differentially expressed genes (DEGs) associated with dyslipidemia, which were mainly related to metabolism, and functions of mitochondria and immune system (Plaza-Florido et al., 2021). Among the DEGs, many were identified as hub genes that are key drivers of, among others, type 2 diabetes, macrophage phospholipid metabolism and inflammation. Transcriptomic analysis of the blood cells has also provided insights into the association of dyslipidemia with other diseases like type 2 diabetes and periodontitis (Corbi et al., 2020). These human studies indicate the potential of transcriptomic approaches in unraveling unique insights into dyslipidemia, though most of them are observational studies. There are only a few randomized controlled trials that have explored the effect of dyslipidemia on the tissue transcriptome of organisms. To understand dyslipidemia in humans, researchers have been primarily relying on blood cells because of the difficulty in obtaining tissues. The development of animal models has significantly increased our understanding of the tissue-specific molecular effects of dyslipidemia and its mitigation through dietary components.

1.9 Zebrafish as an emerging model of dyslipidemia

Since the latter half of the 20th century, animal models have been used to study dyslipidemia–to understand the associated mechanisms and the efficacy of different therapeutic agents. The blood lipid profile in animals can be skewed towards a dyslipidemic condition through genetic manipulation, selective breeding, or dietary interventions (Andreadou et al., 2020). In 1909, Ignatowski developed the first dyslipidemic animal model by inducing the formation of atherosclerotic plaques in rabbits by feeding the animals a high-fat diet (Ignatowski, 1909). Although rabbit,

mouse, and rat models are extensively used to understand dyslipidemia, zebrafish is a promising model to study dyslipidemia (Figure 6).



The first study on the effects of diet-induced hyperlipidemia in zebrafish was published

Figure 6. Increase in the number of publications on hyperlipidemia. Results of hyperlipidemia studies using rat, mouse and rabbit models started emerging during the period 1945-1960, while such reports based on zebrafish studies started appearing only in early 2000s. The list is obtained from the MeSH database of PubMed using the terms "hyperlipidemia" with "rabbit", "rat", "mouse" or "zebrafish".

in 2009 (Stoletov et al., 2009). In this study, adult zebrafish were fed 4% cholesterol for 8 to 12 weeks to increase the total cholesterol and triacylglycerols in the blood. The suitability of zebrafish to study dyslipidemia was confirmed by assessing the development of the hallmarks of atherosclerosis in mammals–accumulation of lipids in the vascular system and uptake of cholesterol by myeloid cells. This pioneering study has spurred research using diet-induced hyperlipidemia models. Researchers started investigating the molecular basis of dyslipidemia and screening effective therapeutic agents that stall the progression of dyslipidemia using similar zebrafish models (Tainaka et al., 2011, Baek et al., 2012, Jin and Cho, 2011). There is clear resemblance between the intestinal anatomy of humans and zebrafish. Although zebrafish lacks a

functional stomach, its gut has different segments, namely foregut, midgut, and hindgut similar to those of mammals (Wallace et al., 2005). Regulation of transcripts of the epithelial cells in different segments of zebrafish gut is highly conserved with that of mammals (Lickwar et al., 2017). Furthermore, the small size, optical transparency, fully sequenced genome, large clutch size, and short generation time of zebrafish have made it a promising animal model to study several immune and metabolic disorders. Several mutant zebrafish models like the *apoc2*$^{-/-}$ (Liu et al., 2015), *ldlr*$^{-/-}$ (Liu et al., 2018) and *apoeb*$^{-/-}$ (Hu et al., 2022) have been developed recently to study dyslipidemia. However, one of the foremost advantages of using zebrafish for studying dyslipidemia is the feasibility of developing a model by feeding high-fat diets to the fish. This advantage can be partly attributed to APOB biology in zebrafish. Mammals have two major types of APOB proteins: APOB-100 and APOB-48. Both these forms are encoded by a single gene, *APOB* in humans and *Apob* in mice. APOB-100 results from the translation of the full APOB mRNA resulting in the formation of a 512 kDa protein. However, in the intestine, splicing of the APOB pre-mRNA results in formation of a smaller 246 kDa APOB-48 protein, consisting of 48% of amino acids at the N-terminal of APOB-100. In zebrafish, three orthologues, namely *apoba*, *apobb.1* and *apobb.2* of *APOB* have been discovered, and the sequence similarities of these genes with the human *APOB* are 54.2, 43.0, and 29%, respectively (Otis et al., 2015). However, there is no report of APOB-48 protein in zebrafish, possibly due to lack of an RNA editing enzyme or other factors that are essential to generate a premature stop codon in the *apob* pre-mRNA (Conticello et al., 2005, Chester et al., 2003). Lack of APOB-48 in zebrafish is likely the reason for rapid development of dyslipidemia in zebrafish as APOB coated (which are operationally equivalent to APOB-100) zebrafish chylomicrons are, most likely, not cleared rapidly from the circulation. Moreover, in zebrafish, the LDL particles can be formed from chylomicrons (Schlegel, 2016). Besides the three APOB orthologues mentioned above, *apoa1a* and *apoa1b* are orthologues of *APOA-I* gene in humans, *apoea* and *apoeb* are orthologous of the *APOE* gene and *apoa4a*, *apoa4b.1*, *apoa4b.2* and *apoa4b.3* are orthologous of human *APOA4*. These genes in

zebrafish are also transcriptionally regulated by high-fat diets (Otis et al., 2015). The orthologs of other genes which code for proteins such as MTTP, LDLR, SRB1 and LCAT that are involved in lipoprotein metabolism have also been identified in zebrafish (O'Hare et al., 2014, Marza et al., 2005) (Figure 7).

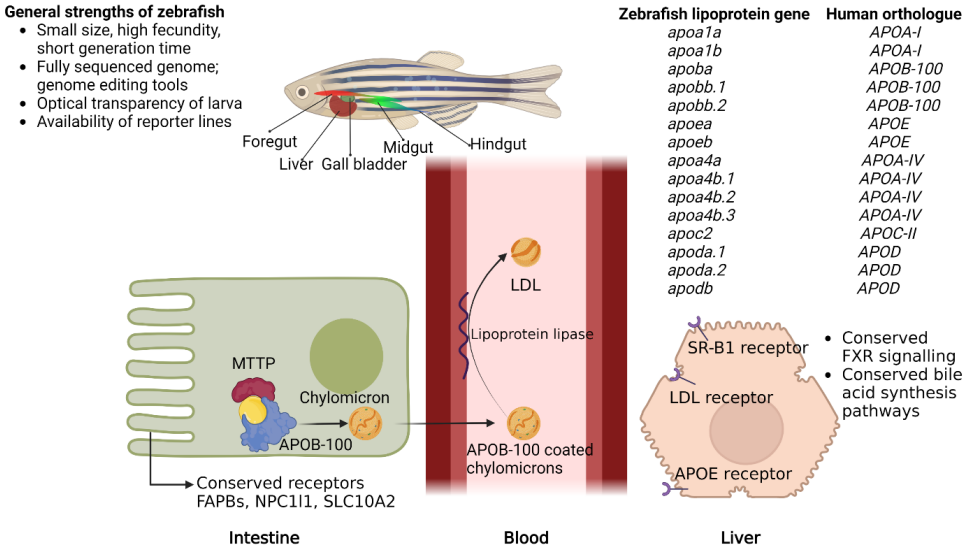


Figure 7. Overview of the characteristics in zebrafish that facilitates the development of a dyslipidemia model. Zebrafish possesses a gastrointestinal system that is largely similar to that of humans. This system contains the major organs required for lipid digestion, absorption, and metabolism, except the stomach. Zebrafish genome has orthologs of human genes important for lipid metabolism, including microsomal triglyceride transfer protein, fatty acid binding proteins, cholesterol transporter Niemann-Pick C1-Like 1 and bile transporter solute carrier family 10 member 2. An important distinction between zebrafish and humans is that zebrafish only produces full-length APOB protein (APOB-100) and lacks the truncated form (APOB-48). Unlike mammalian chylomicrons, the APOB-100 coated chylomicrons in zebrafish are not cleared rapidly and these lipoproteins can be converted to LDL after interacting with lipoprotein lipase in the blood capillaries of the peripheral tissues. Zebrafish genome has several paralogues of other mammalian apolipoprotein genes, that are also transcriptionally regulated by the lipid content in the diet. Furthermore, key components that are required for hepatic bile salt synthesis and transport pathways are also conserved in zebrafish. (Created with BioRender.com)

2. OBJECTIVES

Zebrafish is an emerging model species that can be used to understand diet-based prevention of dyslipidemia. Although several studies have confirmed the suitability of this model to investigate the physiology behind this disorder, the specific changes in zebrafish intestine during dyslipidemia have not been deciphered in detail. *One of the aims of this PhD project was to generate in-depth information about the changes in the intestine of the zebrafish model of diet-induced dyslipidemia.* The intestine was chosen because of its ability to regulate the absorption of dietary lipids and assemble them into chylomicrons, which eventually determine the complete lipoprotein profile of the blood. Digestion, absorption, and repackaging of dietary lipids into lipoproteins are accompanied by substantial changes in the transcriptional landscape of the intestine. Therefore, I focused on the alterations in the intestinal transcriptome of the zebrafish fed high cholesterol diet.

Some studies have reported the effect of β-glucans and n-3 PUFAs to prevent dyslipidemia. However, source-based differences in these nutraceuticals can govern their bioactive potential and generate differential responses in the tissues. There is a paucity of knowledge regarding the impact of β-glucans and n-3 PUFAs from novel microbial sources. Hence, *this PhD project also focused on the effects of β-glucans and n-3 PUFAs from novel microbial sources on the intestinal and hepatic transcriptomic landscapes as well as the plasma lipidomic profile in the zebrafish model of dyslipidemia* (Figure 8).

Accordingly, the objectives were framed as:

i. To understand the impact of dietary cholesterol, β-glucans, and simvastatin on the intestinal transcriptome of zebrafish.
ii. To elucidate the effect of dietary n-3 PUFA-rich microbial oil on the intestinal transcriptome of the zebrafish model of dyslipidemia.

iii. To explore the outcomes of a combination of dietary β-glucans and n-3 PUFA-rich microbial oil on hepatic transcriptome and plasma metabolome of a zebrafish model of dyslipidemia.

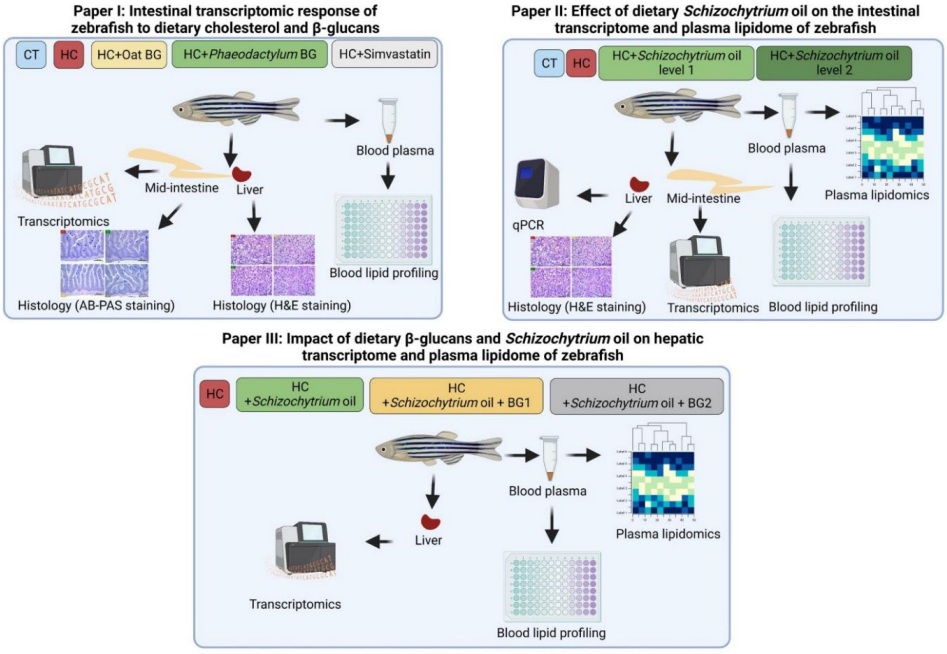


Figure 8. Overview of the studies performed in this PhD project. CT: control group, HC: High cholesterol group; BG: β-glucan; BG1: *Phaeodactylum tricornutum* β-glucan; BG2: *Euglena gracilis* β-glucan. (Created with BioRender.com)

3. GENERAL DISCUSSION

Dyslipidemia, characterized by an altered blood lipid and lipoprotein profile, is a risk factor that triggers the development of cardiovascular diseases. In this PhD project, three studies were conducted to generate knowledge about the tissue-specific molecular changes associated with diet-induced dyslipidemia and the ability of β-glucans and n-3 PUFAs to counter the health condition in zebrafish.

The first study explored the impact of dietary cholesterol, microalga-derived β-glucan, and simvastatin on the blood lipoprotein levels, histological architecture of the liver and intestine, and the intestinal transcriptome of zebrafish (**Paper I**). The second study investigated the effect of dietary n-3 PUFA-rich microbial oil from *Schizochytrium* sp. on the blood lipoprotein and total triacylglycerol levels, liver histology and gene expression profiles, intestinal transcriptome, and plasma lipidomic landscape of the zebrafish model of dyslipidemia (**Paper II**). In the third study (**Paper III**), the effect of combining dietary microbial oil with β-glucans from different sources on the blood lipoprotein and total triacylglycerol levels, hepatic transcriptome, and plasma lipidome of the zebrafish model of dyslipidemia was elucidated.

3.1. Zebrafish model of dyslipidemia

3.1.1. A dyslipidemia model by feeding excess cholesterol

A high-cholesterol diet was employed to dysregulate the cholesterol metabolism and develop a dyslipidemic blood profile in zebrafish (**Papers I, II and III**). In humans, genetic defects or unhealthy diet can be the causes of altered cholesterol metabolism that lead to dyslipidemia, characterized by abnormal lipoprotein patterns (Weissglas-Volkov and Pajukanta, 2010, Sharifi et al., 2019). Likewise, in zebrafish, dyslipidemia can be induced through genetic or dietary manipulation. To understand the effects of dyslipidemia from genetic defects or unhealthy diets, several models have been developed that are classified according to their translational resemblance to human dyslipidemia (Andreadou et al., 2020). Although, gene knockout models in rodents (Oppi et al., 2019) and zebrafish (Liu et al., 2015, Liu et al., 2018) are appropriate for

understanding the genetic defects-caused dyslipidemia, diet-induced dyslipidemia models should be used to understand the exact effects of lipid-rich diets in humans (Ka and Jin, 2021). However due to difficulties in inducing dyslipidemia and related disorders through dietary intervention alone, most rodent studies that target dyslipidemia use gene-knockout models (Andreadou et al., 2020). In contrast, by overfeeding or by administering high cholesterol diets to zebrafish dyslipidemia models that replicate the lipid-caused changes in lipoprotein levels and cholesterol metabolism in humans can be developed to study the disorder.

In humans, dyslipidemia may not be often associated with obesity and several studies have established the distinct molecular basis of both obesity and diet-induced dyslipidemia (Højland et al., 2016, Badoud et al., 2015). Previous studies have exploited overfeeding and lipid-rich diets to induce dyslipidemic blood profile in zebrafish (Oka et al., 2010, Landgraf et al., 2017). These dietary approaches can induce obesity and the responses cannot be solely associated with dyslipidemia. Therefore, it is difficult to separate the molecular changes associated with dyslipidemia from those of obesity. To avoid this, only purified cholesterol was employed in the experiments conducted for this PhD project (**Papers I, II and III**). This strategy was adopted because cholesterol does not provide energy to the cells. Purified cholesterol at 5% inclusion in the diets elevated the circulating cholesterol and lipoprotein levels (**Papers I and II**) without affecting the condition factor of the experimental fish (Figure 9). This indicates the reliability of the cholesterol-induced dyslipidemia model to study specific aspects of the disorder without inducing obesity. Similar dyslipidemia models were generated by feeding moderate amounts of dietary lipids to *Ldlr* knock-out mice; this approach developed a dyslipidemic blood profile while avoiding an obese condition (Hartvigsen et al., 2007). In mammals, lipid-rich diets can increase the HDL particles and other lipoproteins (Greene et al., 2005, Herron et al., 2003). However, we did not find any significant increase in the plasma HDL concentration of zebrafish fed a high cholesterol diet (**Papers I and II**). Like other teleosts, zebrafish plasma has an HDL-dominating lipoprotein profile. The HDL cholesterol was higher than the LDL cholesterol (**Papers I**

and II). However, after providing a high-cholesterol diet, the lipoprotein profile tends to become β-dominant by a substantial increase in the LDL cholesterol and a slight increase in HDL fraction (Stoletov et al., 2009). We also found that a cholesterol-rich diet can increase the LDL levels in zebrafish (**Papers I and II**). Thus, zebrafish fed high-cholesterol diet is unable to reduce the cholesterol load by increasing the HDL particles in circulation. Therefore, feeding excess cholesterol to zebrafish is an ideal strategy to generate a diet-induced dyslipidemia model without a concomitant increase in HDL or development of obesity (Figure 9). Dietary cholesterol can increase the circulating total triacylglycerol level, which is a biomarker of CVDs, by increasing the biosynthesis of triacylglycerols and decreasing the β-oxidation of fatty acids in the liver (Fungwe et al., 1993, Wang et al., 2010).

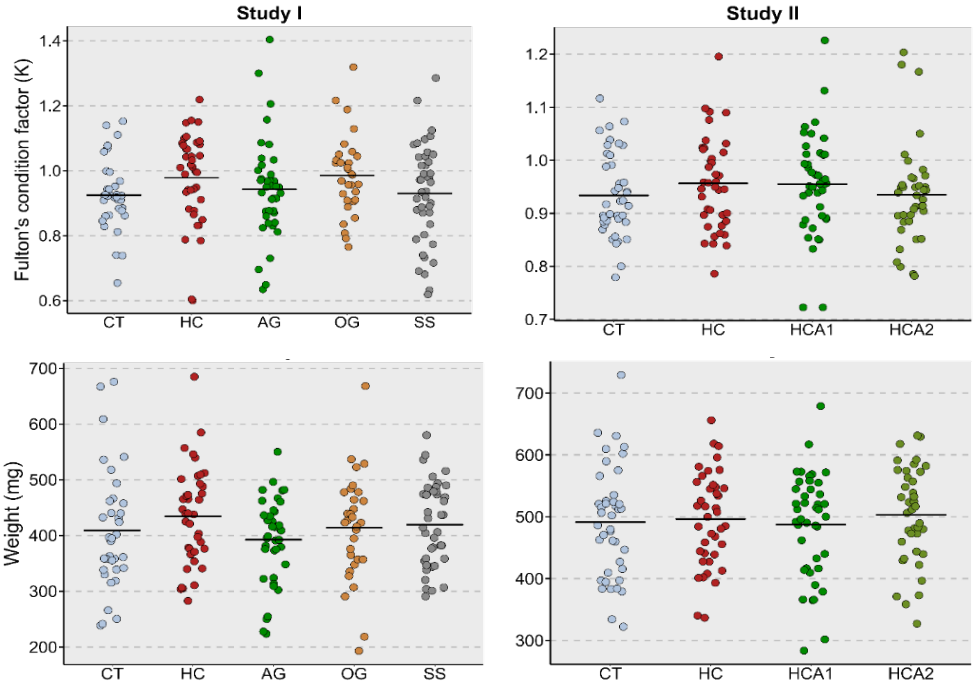


Figure 9. Condition factor and final weight of the zebrafish fed different experimental diets. There were no changes in the condition factor and weight of the experimental fish after feeding cholesterol at an inclusion of 5% in the diet for 12 weeks. CT: control group; HC: high-cholesterol group; AG: *Phaeodactylum tricornutum* β-glucan fed group; OG: oat β-glucan fed group; SS: simvastatin fed group; HCA1: microbial oil (3.1% dietary inclusion) fed group; HCA2: microbial oil (6.6% dietary inclusion) fed group.

The plasma total triacylglycerol was not elevated by dietary cholesterol, but the expression of genes that control lipid β-oxidation–*acyl-CoA thioesterase 22 (acot22), apolipoprotein Db (apodb*) was decreased in zebrafish intestine (**Paper II**). APOD is involved in LPL activity and improved catabolism of VLDL particles and elevated APOD production in mice resulted in significant reduction in plasma triacylglycerol levels in mice (Perdomo et al., 2010). The lower expression of *apodb* is likely indicating higher levels of triacylglycerols, the content of which is higher in LDL particles (**Paper II**). The expression of human orthologs of the zebrafish gene *acot22* was higher in oxidative tissues, indicating their role in mitochondrial β-oxidation (Tillander et al., 2019). Transcriptomic studies also revealed downregulated expression of *low-density lipoprotein receptor b* (*ldlrb*)*, low density lipoprotein receptor a* (*ldlra*) (**Paper I**) and *low density lipoprotein receptor-related protein 2b (lrp2b*) in the intestine of zebrafish model of dyslipidemia (**Paper II**). Proteins encoded by these genes serve critical functions in lipoprotein metabolism. LDLR proteins control the uptake of circulating LDLs into different tissues (Xia et al., 2021). Likewise, the LRP2 protein is also a receptor that recognizes APOB-100, and deficiency of the LRP2 protein is implicated in hyperlipidemia (Mii et al., 2007). The downregulated expression of *ldlra*, *ldlrb,* and *lrp2b* genes indicates a possible subdued clearance of LDL from circulation, as indicated by the increased LDL levels in zebrafish plasma (**Papers I and II**). Thus, excess dietary cholesterol can induce hyperlipidemia in zebrafish, as evidenced by the elevated total cholesterol and LDL particles, without increasing the HDL particles or developing obesity.

3.1.2. Cholesterol uptake and biosynthesis in the intestine of zebrafish fed excess cholesterol

In the intestine, absorption of cholesterol is regulated by controlling the expression of NPC1L1 protein which is the principal transporter of cholesterol in the enterocytes. The expression of the gene, *Npc1l1* was downregulated in mice fed a high cholesterol diet (Davis et al., 2004). The transcriptome sequencing (**Papers I and II**) revealed that expression of the gene *npc1l1* in the intestine of zebrafish did not respond to excess

cholesterol, indicating that zebrafish is not utilizing this transporter to regulate cholesterol absorption in the intestine.

Although cholesterol performs several critical functions in vertebrates, it is not an essential dietary component as it can be synthesized endogenously. However, since cholesterol biosynthesis is an energy and oxygen-consuming process, the biosynthetic pathway is activated only when there is cholesterol deficiency. Thus, dietary cholesterol largely determines the rate of endogenous biosynthesis. The principal rate-limiting enzyme in the cholesterol biosynthetic pathway is HMGCR (Duan et al., 2022). Transcription of the gene encoding HMGCR is downregulated directly by high intracellular cholesterol levels. In the intestine of zebrafish, the expression of several genes that code for cholesterol biosynthesis enzymes, including HMGCR, was downregulated by feeding excess cholesterol (**Papers I and II**). However, a high cholesterol diet did not significantly alter the expression of *Hmgcr* in the intestine of mice (Desmarchelier et al., 2012). Overall, it seems that intestinal expression of mice *Npc1l1* gene changes with dietary cholesterol, but not that of zebrafish orthologue whereas the expression of *hmgcra* gene changes with dietary cholesterol in zebrafish intestine, but not in mice.

The contribution of *de novo* synthesis of cholesterol to the total cholesterol pool in mice is 160 mg per day per kg weight, whereas it is only about 5 mg per kg per day in humans. About 40% of the total endogenously produced cholesterol is synthesized in the liver of mice compared to 7% produced in the liver of humans. The intestine also contributes significantly to the cholesterol pool through endogenous biosynthesis in humans (Dietschy and Turley, 2002). Although the total contribution of the intestine to endogenous biosynthesis in zebrafish has not been explored, the results in **Papers I and II** indicate the presence of a strict transcriptional (*3-hydroxy-3-methylglutaryl-CoA reductase a, squalene epoxidase a*, *lanosterol synthase*, *3-hydroxy-3-methylglutaryl-CoA synthase 1* and *cytochrome P450,* and *family 26, subfamily A, polypeptide 1*) regulatory mechanism that controls intestinal cholesterol biosynthesis in response to dietary cholesterol levels. This cholesterol-sensing mechanism in the zebrafish

intestine was exploited in the studies described in this PhD thesis to understand the development of dyslipidemia and its mitigation through diet-based strategies.

3.1.3. Hepatic vacuolization in zebrafish fed excess cholesterol

The organization of hepatocytes in the liver of zebrafish is similar to that of mammals (Pham et al., 2017). Consumption of high-fat diets can be the cause of accumulation of lipids in the liver of humans and mice. Hepatic steatosis can be developed in zebrafish by feeding the fish a diet containing high levels of fructose and fat (Katoch and Patial, 2021). Lipid droplets in the hepatocytes consist of a neutral lipid (triacylglycerols and/or cholesterol esters) core that is surrounded by a phospholipid and protein (which include perilipins 2, 3 and 5) monolayer. Humans have five *PLIN* genes (*PLIN* 1-5), whereas, in zebrafish, there are *plin1-3* and a unique *plin6* gene (Granneman et al., 2017). The expression of *plin2* was not altered in the liver of zebrafish model of dyslipidemia (**Paper II**). Although diet-induced hepatocyte vacuolation was reported in zebrafish larvae (Ding et al., 2022), *plin2* is not expressed in the liver of zebrafish larvae (Wilson et al., 2021).

Cholesterol-rich diets can cause the infiltration of lipids including cholesterol and triacylglycerols into the liver. This could be due to insufficient cholesterol export (reduced conversion to VLDL, HDL or bile acids) from the hepatocytes (Ioannou, 2016) or reduced β-oxidation. We did not observe any significant differences in the expression of *scarb1* (involved in the uptake of cholesterol from circulating HDL) and *abca1a* (involved in the export of nascent HDL particles from the hepatocyte to the circulation) genes (**Paper II**). This indicates that the accumulation of lipid droplets in hepatocytes might not occur through increased uptake of HDL cholesterol or reduced production of HDL in the hepatocyte. However, high-cholesterol diets caused a significant increase in the circulating LDL cholesterol (**Papers I and II**), indicating the mobilization of cholesterol/triacylglycerols into the APOB containing lipoproteins. A previous study has also revealed that a high-fat diet stimulates the expression of *apob* genes (involved in VLDL synthesis) in zebrafish (Otis et al., 2015). This indicates that the

increase in LDL cholesterol content caused by a high-cholesterol diet (**Papers I and II**) is likely an attempt by the fish to reduce the cholesterol load in the liver.

3.1.4. Cytoskeletal disorganization and organellar dysfunction in zebrafish fed excess cholesterol

Cholesterol is an integral part of the plasma membrane, and its vesicular transport requires an intact cytoskeleton with microtubules. Changes in cholesterol metabolism may affect membrane-cytoskeleton interactions (Klausen et al., 2006). Transcriptome sequencing revealed the downregulation of several genes connected to cytoskeletal organization in zebrafish fed a high-cholesterol diet (**Papers I and II**). In line with the present findings, recent studies have also documented cholesterol-induced alteration of the expression of cytoskeletal proteins (Sarkar et al., 2022, Maerz et al., 2019). Cholesterol-induced downregulation of the expression of genes related to cytoskeleton organization may alter numerous functions like intracellular cytoskeletal tethering to the membranes and signal transduction in the cells.

Cholesterol transport (non-vesicular) inside the cells is regulated mainly by STARD proteins. STARD4 proteins are involved in the transport of cholesterol from the plasma membrane to the ER (Garbarino et al., 2012). The mRNA expression of STARD4 is downregulated in mice fed a cholesterol-rich diet (Soccio et al., 2002). The expression of *stard4* (**Paper I**) and *stard5* (**Paper II**) in the intestine was downregulated by the excess cholesterol in the diet. Not much is known about the specific function of *stard5* in zebrafish, but its orthologue in humans is involved in the binding of cholesterol and 25-hydroxycholesterol, and increased expression of *STARD5* leads to a marked increase in microsomal free cholesterol levels (Rodriguez-Agudo et al., 2005). Excess cholesterol also impairs the functioning of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA, calcium pump) in the ER membrane, thereby reducing the ER Ca^{2+} reserve (Li et al., 2004) and inducing ER stress. Endoplasmic reticulum stress by a high cholesterol diet was indicated by the enrichment of gene ontology terms like endoplasmic reticulum membrane, cellular response to topologically incorrect protein, and cellular response to unfolded protein (**Paper I**). High cholesterol-induced misfolding of proteins may

have severe consequences on cellular lipid metabolism. A recent study in zebrafish has reported that feeding emulsion of chicken egg yolk, rich in cholesterol, can also induce ER stress in the intestinal enteroendocrine cells that regulate lipid metabolism through the production of hormones (Ye et al., 2019). Thus, ER stress in different tissues may lead to dyslipidemia (Basseri and Austin, 2012) and plays a critical role in the pathogenesis of various cardiovascular diseases (Choy et al., 2018).

In contrast to the single continuous membrane of the ER, mitochondria have inner and outer membranes. The cytosolic soluble STARD1 is the principal protein that facilitates the non-vesicular transport of cholesterol across the outer mitochondrial membrane and then into the inner mitochondrial membrane (Elustondo et al., 2017). The gene, *stard1* was downregulated in the intestine of zebrafish fed a high-cholesterol diet (**Paper I**). Alteration of the expression of the mRNA or protein associated with *stard1* leads to lipid accumulation (Larsen et al., 2020). Excess cholesterol feeding to zebrafish also led to altered *stard1* expression (**Papers I and II**), but the lipid accumulation in the intestine of the fish fed excess cholesterol was not different from those fed the control diet (**Paper I**). Cellular cholesterol accumulation is associated with suppressed oxidative phosphorylation in the cells (Chiu et al., 2021). Among other genes, *transmembrane protein 70* (*tmem70*) and *solute carrier family 25 member 25b* (*slc25a25b*) were upregulated in the intestine of the fish fed a high cholesterol diet (**Paper I**). The mammalian orthologues of *tmem70* and *slc25a25b* genes code for proteins which facilitate the assembly of mitochondrial complex V (ATP synthase) and the transport of ATP from the mitochondria to the cytosol, respectively (Satrústegui et al., 2007, Kovalčíkova et al., 2019). Alteration of the expression of the proteins that are encoded by these genes can diminish the metabolic efficiency of mitochondria (Anunciado-Koza et al., 2011, Braczynski et al., 2015). Considering the effect of cholesterol-rich diet on the organization of cytoskeleton, genes which regulate ER and mitochondrial functions, it can be speculated that the reduced expression of *stard1, stard4* and *stard5* may be a homeostatic mechanism to diminish the transport of cholesterol to ER and mitochondria. In contrast, elevated expression of *tmem70,*

slc25a25b and genes connected to ER stress and the downregulation of genes connected to cytoskeletal organization seem to be the effect of excess cholesterol uptake.

Overall, the effects of high cholesterol diet on the intestinal transcriptome, histological parameters, and blood lipid profile of zebrafish were evaluated in **Papers I and II**. The results revealed distinct impacts of dietary cholesterol on the blood lipid profile and liver vacuole formation, which are hallmarks of dyslipidemia. Furthermore, excess cholesterol can adversely affect cholesterol biosynthesis in the intestine. In addition, the zebrafish dyslipidemia model can be used to study cellular events like cytoskeletal disorganization and organellar dysfunction.

3.2. Mode of action of microbe-derived bioactive compounds against dyslipidemia

A departure of physiological indicators from their normal values suggests an increased disease risk. For example, elevated LDL cholesterol and triacylglycerols are critical risk factors for the development of CVD. Conventional drug-based treatments can return these markers to normal levels to reduce the disease risk (Lazarte and Hegele, 2020). High-throughput quantification of gene expression and lipid species have proven indispensable to study the molecular basis of dyslipidemia, to establish novel risk biomarkers of CVDs, and examine the efficacy of different therapeutic agents (Schmidt et al., 2012, Garcia-Jaramillo et al., 2019). The effectiveness of a treatment strategy should be based not only on its ability to normalize the physiological biomarkers but also on its ability to restore the underlying cellular, organellar, and molecular level phenotypes. The efficacy of microbial bioactive compounds in managing diet-induced dyslipidemia in zebrafish was investigated in **Papers I, II and III**. Besides estimating traditional blood biomarkers of dyslipidemia, the ability of selected bioactive agents to alter the transcriptomic and lipidomic landscapes in a zebrafish model of dyslipidemia was also explored in the three studies. The objective of adopting the omics approach was to understand the efficacy of the bioactive agents to normalize the gene

expression levels and lipid species abundance, which were altered by a high-cholesterol diet.

3.2.1. n-3 PUFA-rich microbial oil mitigates dyslipidemia in zebrafish

The American Heart Association dietary guidelines indicate that including at least two servings of fatty fish per week is a healthy habit (Chaddha and Eagle, 2015). Fish oil-based strategies to improve human cardiovascular health require rethinking (Jenkins et al., 2009) because reliance on marine fish as the only source of n-3 fatty acids could be unsustainable. The uncertainty in fish oil supply for human consumption has necessitated the search for novel sources of n-3 LCPUFAs. To this end, certain marine microbes have been identified as rich sources of n-3 LCPUFAs. For the studies described in **Papers II and III**, a microbial (*Schizochytrium* sp.) oil rich in n-3 LCPUFAs was incorporated at two inclusion levels (3.1% and 6.6%) in the diet of the zebrafish model of dyslipidemia. The inclusion levels had a clear effect on the gene expression profile and blood lipid profiles (**Paper II**). Both the inclusion levels significantly increased the HDL cholesterol content of the plasma (**Papers II and III**). However, only the higher dietary level (6.6%) of microbial oil significantly decreased the total cholesterol, LDL cholesterol, and total triacylglycerol levels in the plasma of the zebrafish model. The gene expression in the liver corroborated with the results of the plasma lipid profile, i.e., only the higher level of microbial oil induced a significant difference in the expression levels of the genes associated with HDL metabolism (*scarb1*, *lcat*), and β-oxidation (*cpt1aa*) (**Paper II**). The dose-dependent effect of the dietary microbial oil was more pronounced on the expression of intestinal genes that are involved in cholesterol biosynthesis (**Paper II**). Including 3.1 and 6.6% microbial oil restored the expression of 2 and 7 genes linked to cholesterol biosynthesis, respectively (**Paper II**). The plasma lipidome of zebrafish fed 6.6% microbial oil had higher levels of triacylglycerols rich n-3 PUFAs (**Paper II**). The plasma lipidome of zebrafish fed 3.1% microbial oil had higher levels of phosphatidylcholines rich n-3 PUFAs (**Paper III**). The dose-dependent effects of n-3 PUFA are also reported in animal and human studies (Ding et al., 2022, Conquer and Holub, 1998, Kim et al., 2015). It should be noted that

both the inclusion levels of *Schizochytrium* oil were able to reduce hepatic vacuolization (**Paper II**).

A strong effect of *Schizochytrium* oil on the expression of genes connected to cholesterol metabolism was noted in **Papers II and III**. In the intestine, 6.6% microbial oil normalized the expression of cholesterol biosynthesis genes (**Paper II**). However, in the liver, 3.1% microbial oil downregulated the expression of cholesterol synthesis genes (**Paper III**). Such a downregulation effect of 6.6% inclusion of microbial oil on the liver was not confirmed in **Paper II** because the liver transcriptome was not evaluated in this study. This indicates a tissue-specific and inclusion level-based response to n-3 PUFA-rich microbial oil in the zebrafish model. In vertebrates, the contribution of different tissues to endogenous cholesterol biosynthesis vary widely and the liver is considered the main organ which can regulate the total cholesterol pool in the body (Cohen, 2008). Suppression of cholesterol biosynthesis in the liver by microbial oil indicates an antihyperlipidemic effect which is also observed in other studies using different antihyperlipidemic agents (Shin et al., 2011, Chung et al., 2008). The antihyperlipidemic effect of the microbial oil was reflected in the plasma cholesterol and LDL levels as well (**Paper II**). The reduction in the LDL cholesterol and increase in the expression of the gene *cpt1aa* connected to β-oxidation (**Paper II**) may have contributed to the lowering of plasma total triacylglycerol content by dietary microbial oil (6.6%) in zebrafish. On the other hand, 3.1% microbial oil did not lower the plasma total triacylglycerol content (**Papers II and III**). However, when it was combined with *E. gracilis* β-glucan, 3.1% microbial could reduce the total triacylglycerol in zebrafish plasma. In the intestine, the upregulation of cholesterol biosynthesis by the microbial oil may be due to the lower cholesterol levels induced by the microbial oil.

Dyslipidemia can lead to the accumulation of lipids in metabolic organs, like the liver (Arroyave-Ospina et al., 2021). On the other hand, cholesterol-induced alterations in the expression of genes that code for proteins involved in mitochondrial functioning or glutathione transporter in the mitochondria can lead to oxidative stress. This redox imbalance can accelerate the advancement of dyslipidemia to cardiovascular events as

the LDL cholesterol is prone to oxidative stress. Oxidation of the LDL in the endothelial layer of the blood vessels can expedite the development of atherosclerotic lesions (Poznyak et al., 2021). Therefore cardiovascular events are frequently accompanied by a high ROS load (Dubois-Deruy et al., 2020). Several diet-based treatment therapies focus on reducing ROS production during dyslipidemia (Jiang et al., 2021). Transcriptome analysis revealed the enrichment of glutathione metabolism in the intestine (**Paper II**) and liver (**Paper III**) of zebrafish fed the microbial oil. Several studies have confirmed that dietary *Schizochytrium* oil can improve the antioxidant capacity in various tissues by increasing the expression of glutathione peroxidases and glutathione transferases (Xu et al., 2022, Katerina et al., 2020). n-3 PUFAs can activate the glutathione antioxidant system (Valenzuela et al., 2012) by triggering the expression of several cytoplasmic and microsomal glutathione transferases via PPAR transcription factors (de Vogel et al., 2008). Dietary microbial oil increased the expression of *microsomal glutathione S-transferase 1.1* and *microsomal glutathione S-transferase 1.2* (**Papers II and III**) genes that contain PPAR responsive elements (de Vogel et al., 2008). Therefore, we speculate that activation of the glutathione metabolism pathway might be through PPAR activation by n-3 PUFAs in the liver and intestine of zebrafish. Although, glutathione metabolism was activated by dietary microbial oil both in the intestine and liver of zebrafish, the effect on these tissues was dependent on the inclusion levels of the microbial oil. In the liver, lower level of microbial oil stimulated glutathione metabolism (**Paper III**, based on integrative omics analysis), but in the intestine, only the higher dietary level of *Schizochytrium* oil significantly enriched the glutathione metabolism pathway (**Paper II**, based on the transcriptome). The liver and intestine handle excessive lipids differently. While the intestine is predominantly involved in digestion and absorption, the liver acts as a storage organ for lipids. Total triacylglycerol and total cholesterol content in the liver of zebrafish were correlated with *mgst1* expression (Yang et al., 2018). A high-cholesterol diet triggered lipid accumulation in the liver of zebrafish (**Papers I** and **II**) but not in the enterocytes (**Paper I**).

Overall, the results indicate that dietary microbial oil can mitigate dyslipidemia by downregulating the expression of genes involved in cholesterol biosynthesis, increasing the HDL biosynthesis (**Papers II and III**), reducing the total triacylglycerol content of the blood (**Paper II**), reducing the triacylglycerol species rich in monounsaturated and saturated fatty acids (**Paper III**) and enriching the ROS controlling processes through the activation of PPAR transcription factors.

3.2.2. β-glucans mitigate dyslipidemia in zebrafish through different mechanisms

β-glucans are well-known antihyperlipidemic compounds that can prevent dyslipidemia. β-glucans from oats (*A. sativa*) and microalgae (*P. tricornutum* and *E. gracilis*) were investigated in this PhD project. These glucans differ in their molecular weight (Table 1). It has been proposed that β-glucans with specific differences in molecular weights may interact with the cells in different ways to impart distinct effects on lipid metabolism. Also, differences in the molecular weight of β-glucans may affect key physical properties like viscosity and gel-forming ability and bile acid sequestration (Islam et al., 2022). To understand the possible source-specific effects of β-glucans on lipid and lipoprotein metabolism, the intestinal transcriptome of the fish fed oat and *P. tricornutum* β-glucans was examined in **Paper I**. In **Paper III**, the investigation was to understand the liver transcriptomic and plasma lipidomic changes induced by *P. tricornutum* β-glucan or *E. gracilis* β-glucan. Though the β-glucans from the three sources induced similar effects, i.e., they lowered the lipoprotein and total triacylglycerol levels in the plasma (**Papers I** and **III**), the transcriptomic and lipidomic analysis allowed to differentiate the source-specific effects of the three β-glucans. Although it has been reported that β-glucans of high and medium molecular weight can lower cholesterol, both oat β-glucan and *P. tricornutum* β-glucan restored the total cholesterol and LDL cholesterol levels in the zebrafish model of dyslipidemia but generated unique transcriptomic responses in the intestine (**Paper I**). Oat β-glucan induced an upregulation of the genes connected to β-oxidation and PPAR signaling pathway (**Paper I**). Several studies have revealed the effects of oat β-glucans on lipid

catabolic pathways (Nie and Luo, 2021, Gao et al., 2021). The genes connected to steroid hydroxylase activity and primary bile acid biosynthesis were upregulated by dietary oat β-glucans. These pathways indicate the increased production of bile from cholesterol. β-glucans of high molecular weight increase the viscosity of the luminal content (Mudgil, 2017), and the bile acid binding capacity of the β-glucans (Kim and White, 2011). Therefore, the comparatively higher molecular weight and bile acid binding ability of oat β-glucan may have diminished the absorption of bile, thus stimulating steroid hydroxylase activity and primary bile acid biosynthesis (**Paper I**). Although *P. tricornutum* β-glucan restored the blood lipid profile and mitigated the lipid infiltration into the liver of the zebrafish dyslipidemia model (**Paper I**), it did not substantially affect the transcriptomic landscape of the intestine and liver (both in terms of the total number of DEGs and the number of DEGs connected to lipid metabolism, **Papers I and III**). However, lipidomic analysis revealed that triacylglycerols rich in monounsaturated and saturated fatty acids had lower abundance in zebrafish fed *P. tricornutum* β-glucan (**Paper III**). Triacylglycerols rich in monounsaturated and saturated fatty acids are important markers of dyslipidemia (Siri-Tarino et al., 2010). β-glucans from *P. tricornutum* and *E. gracilis* had distinct effects on the liver transcriptome and plasma lipidome (**Paper III**). Comparison of the liver transcriptome of the fish fed the two microalgae-derived β-glucans revealed their differential effect on genes related to ER stress, spliceosome, MAPK signaling pathway and arginine and proline metabolism (**Paper III**). Comparison of the plasma lipidomes of zebrafish fed the two β-glucans did not reveal any differentially abundant triacylglycerols or cholesterol esters. However, compared to *E. gracilis* β-glucan and microbial oil, dietary *P. tricornutum* β-glucan and microbial oil significantly increased the abundance of several phosphatidylcholines in the plasma, an effect that was also observed when the fish were fed *Schizochytrium* oil and not β-glucan (**Paper III**). In vertebrates, phosphatidylcholines are synthesized via two main pathways– phosphatidylethanolamine N-methyltransferase (PEMT) pathway in which a sequential methylation of phosphatidylethanolamine produces phosphatidylcholines and the

cytidine diphosphate (CDP)-choline pathway which uses dietary choline and 1,2-diacylgycerol molecules to produce phosphatidylcholines (Jackowski and Fagone, 2005, Walkey et al., 1999). Since no significant enrichment of the genes associated with these pathways was noted in zebrafish fed *E. gracilis* or *P. tricornutum* β-glucans, it is plausible that the increase in the PUFA-rich phosphatidylcholines in the plasma of the fish may be a direct effect of *Schizochytrium* oil along with β-glucans. Phosphatidylcholines account for about 5% of the total lipids in *Schizochytrium* (Wang and Wang, 2012), and it has been reported that the dietary microbial oil can increase phosphatidylcholines in the skeletal muscles of pigs (Dannenberger et al., 2022). Combination of *Schizochytrium* oil and *E. gracilis* β-glucan also suppressed the total triacylglycerol level in the plasma and downregulated the genes connected to the PPAR signaling pathway in the liver (**Paper III**). These effects were not observed when the same amounts of microbial oil were provided without β-glucan in the diet (**Papers II and III**). The combination of microbial oil with *E. gracilis* β-glucan impacted the plasma lipidome in a different way compared to the impact of *Schizochytrium* oil alone. This combination increased the abundance of alkyl lysophosphatidylcholine species, but decreased the abundance of phosphatidylcholines in the dyslipidemic model (**Paper III**). This is in contrast to the lipidomic changes that were noted in zebrafish fed *Schizochytrium* oil without β-glucan supplementation– reduction in abundance of triacylglycerol species and cholesterol esters (**Papers II and III**). Although the microbial oil increased the HDL cholesterol, combining dietary *Schizochytrium* oil with β-glucans (from both microalgae) diminished the effect of *Schizochytrium* oil on the plasma HDL (**Papers II and III**). Dietary β-glucans can lower the total cholesterol and LDL cholesterol levels without affecting the circulating HDL cholesterol (**Paper I**) (Whitehead et al., 2014). Therefore, the reduction in the HDL cholesterol levels could be due to the ability of dietary β-glucans to hamper HDL production by *Schizochytrium* oil. One of the mechanisms could be that β-glucans inhibit the absorption of n-3 PUFA-rich *Schizochytrium* oil. β-glucans can restrict the absorption of long-chain fatty acids by the cells (Drozdowski et al., 2010) and reduce lipid digestibility (Aoe et al., 2019). Some

anti-dyslipidemic drugs also hamper the absorption of lipids (Alanazi et al., 2022) which can in turn reduce the circulating total triacylglycerol levels in circulation (Sahebkar et al., 2017). In **Paper III**, the circulating total triacylglycerol content was lower in zebrafish fed *E. gracilis* β-glucan and n-3 PUFA rich microbial oil (**Paper III**) but not in fish fed the same amount (3.1%) of n-3 PUFA rich microbial oil alone (**Papers II and III**). Therefore, it can be speculated that β-glucans likely hampered the ability of the microbial oil to increase the circulating HDL cholesterol. There were also similarities in the effects of dietary *Schizochytrium* oil alone and *Schizochytrium* oil along with *P. tricornutum* β-glucan on plasma lipidome i.e. triacylglycerols rich in monounsaturated and saturated fatty acids and cholesterol esters were reduced and phosphatidylcholines rich in long-chain fatty acids were elevated in both the groups (**Paper III**).

Overall, these results indicate a differential mode of action of the β-glucans, based on the intestine and liver transcriptome and plasma lipidome of zebrafish. This necessitates the need to discriminate the β-glucans from sources to develop diet-based therapeutic strategies to manage dyslipidemia.

4. CONCLUSION

This PhD thesis provides novel information regarding the effect of a high-cholesterol diet on the blood lipid profile, hepatic lipid accumulation, and the intestinal transcriptome of zebrafish. Excess cholesterol altered the expression of genes involved in cholesterol biosynthesis, cytoskeletal organization, ER and mitochondrial function in the intestine of a zebrafish dyslipidemia model. Expression of cholesterol biosynthesis genes in the intestine reflected the dyslipidemic blood profile in the zebrafish model.

The thesis also describes the efficacy of dietary n-3 PUFA-rich microbial oil and β-glucans from novel microbial sources to prevent dyslipidemia in this zebrafish model. Supplementation of dietary microbial oil mitigated diet-induced dyslipidemia in zebrafish by restoring the expression of intestinal genes involved in cholesterol biosynthesis, increasing the HDL cholesterol levels, reducing the total triacylglycerol content of the blood, and triacylglycerols rich in saturated and monounsaturated fatty acids and increasing the triacylglycerols rich in long-chain polyunsaturated fatty acids.

Effects of β-glucans on the intestinal and hepatic transcriptomes and plasma lipidomic profile are also provided in this thesis. β-glucans obtained from novel microbial sources restored the total cholesterol and LDL cholesterol levels and reduced hepatic lipid accumulation in the zebrafish dyslipidemia model. Combining microbial oil with *E. gracilis* β-glucan significantly reduced the plasma total triacylglycerol levels, downregulated the genes connected to the PPAR signaling pathway in the liver, and increased the abundance of lysophosphatidylcholine species in the plasma of the zebrafish dyslipidemia model. On the other hand, combining microbial oil with *P. tricornutum* β-glucan increased the abundance of phosphatidylcholines rich in long-chain polyunsaturated fatty acids and reduced the abundance of triacylglycerols with monounsaturated and saturated fatty acids in the plasma of the zebrafish model.

Thus, this thesis provides insights into the molecular changes in the intestine, liver and plasma associated with dyslipidemia in the zebrafish model and the efficacy of the

bioactive compounds that can be exploited to devise dietary intervention strategies to manage dyslipidemia (Figure 10).

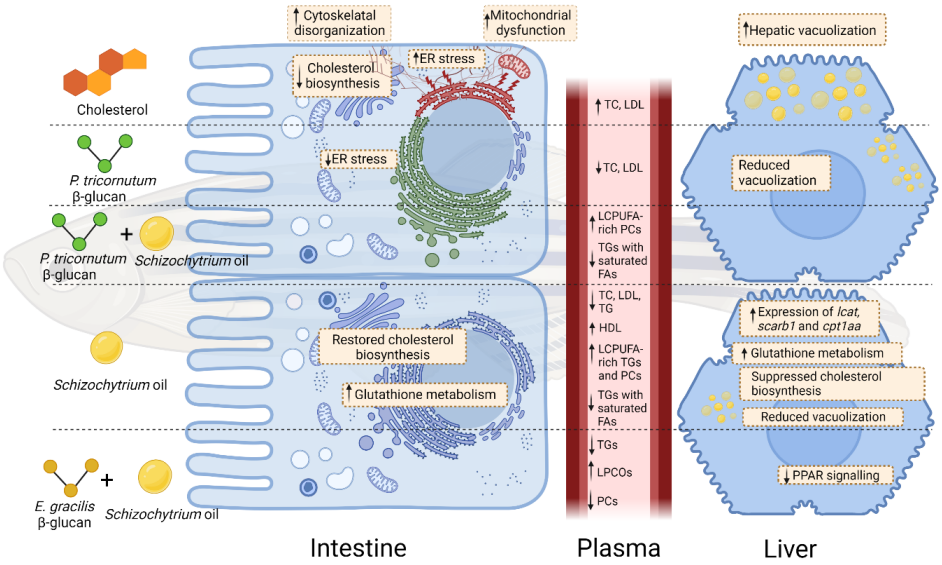


Figure 10. Graphical abstract of the key findings in this thesis. TG: total triacylglycerol, LPCO: alkyl lysophosphatidylcholines, PC: phosphatidylcholines, LCPUFA: Long-chain polyunsaturated fatty acid, HDL: high density lipoprotein, LDL: low density lipoprotein, FA: fatty acid, TC: total cholesterol*, lcat: lecithin-cholesterol acyltransferase, scarb1: scavenger receptor class B, member 1, cpt1aa: carnitine palmitoyltransferase 1Aa,* ER: endoplasmic reticulum *(Created with BioRender.com).*

5. CONTRIBUTION TO THE FIELD

The studies performed in this PhD thesis have generated knowledge about the molecular events that are associated with the development of dyslipidemia in a zebrafish model. Dyslipidemia is often associated with ER stress and mitochondrial dysfunction, but such events in zebrafish have not been deciphered in detail. Hence, the intestine transcriptome of zebrafish fed a high cholesterol diet was analyzed to generate knowledge about organellar dysfunction and cytoskeletal disorganization. Modelling of such dysfunctions that are associated with lipid rich diets is critical to establish zebrafish as a model for dyslipidemia studies. This information is intended to promote the use of the zebrafish as an alternative model of dyslipidemia to understand the mechanism of action of existing treatment strategies that target the intestine.

Plasma lipidome was targeted to understand the efficacy of novel bioactive compounds to prevent dyslipidemia. The study represents the first-ever attempt to explore the plasma lipidomic landscape of zebrafish. The lipidome analysis revealed crucial insights into the effects of *Schizochytrium* oil on the zebrafish model of dyslipidemia. Lipidomics screening also helped elucidate the effects of *Phaeodactylum* β-glucan. This knowledge could be useful for future studies that target the plasma lipidomics.

This thesis also highlighted the mechanism of action of n-3 PUFA rich lipids in managing dyslipidemia. Although human and rodent studies have indicated that n-3 PUFAs can reinforce reverse cholesterol transport, the present thesis has extended this observation to the zebrafish model. This thesis also revealed the importance of discriminating the mechanisms of action of β-glucans from different sources. β-glucans from three different sources were used in this thesis. Though the effects of molecular weight on the bioactivity of β-glucans have been revealed in previous publications, few *in vivo* studies have employed omics approach to delineate the effects of molecular weight on the bioactivity of β-glucans.

Therefore, based on the results generated in the thesis, it can be proposed that zebrafish is a reliable *in vivo* model to investigate dyslipidemia and the relationship between physical properties and bioactivity of nutraceuticals.

6. FUTURE PERSPECTIVES

Though the studies revealed crucial insights into the perturbation of lipoprotein levels and suppression of intestinal cholesterol biosynthesis by excess cholesterol, the contribution of cholesterol biosynthesis in the intestine to the total cholesterol pool in the zebrafish is yet to be revealed in detail. It will be helpful to elucidate the relative contribution of different tissues to the *de novo* biosynthesis of cholesterol in zebrafish intestine using stable isotopes. This information is critical in order to decipher the contribution of the intestine towards cholesterol biosynthesis.

Molecular changes in the plasma lipidome of the zebrafish dyslipidemia model were investigated after feeding the fish either microbial oil alone or in combination with β-glucans from different sources. However, the focus was not on exploring the plasma lipidomic landscape in fish fed with and without a high-cholesterol diet. Future studies should investigate the effect of a high-fat diet on the lipidome of both plasma and the liver. Such studies will help to set a benchmark of alterations in the lipidomic landscape against which the restorative effects of treatment therapies could be compared.

Excessive dietary cholesterol alters the expression of genes involved in lipid metabolism linked to the intestine. Single cell RNA sequencing can provide information about the transcriptomic changes in different cell populations in response to lipid rich diets. Imaging flow cytometry and fluorescent dyes/cell markers can be employed to understand lipid accumulation/quantify lipid-associated markers in the intestinal cells of the zebrafish. In addition, imaging flow cytometry can be used to reveal the morphological adaptations of specific intestinal cells in response to high-fat diet. This will open new frontiers in dyslipidemia research and lead to development of novel therapeutic approaches that will specifically target cell populations involved in lipid uptake and processing.

The thesis indicated that excessive cholesterol interferes with the expression of genes which code for proteins that deliver cholesterol to mitochondria, ATP synthase assembly and export of ATP from mitochondria. Recent studies have revealed changes

in the mitochondrial morphology in response to high fat diets. The altered morphology and expression of genes involved in mitochondrial function translates to diminished energy output and metabolic efficiency of the cells. Employing advanced respirometry mitochondrial energy metabolism should be studied to understand the effect of dietary cholesterol on mitochondrial function. Cholesterol-induced alterations in mitochondrial functions can be related to behavioral aberrations using high throughput platforms like Ethovision.

Another important aspect that regulates cholesterol metabolism in vertebrates is the gut microbiota. Bacteria that possess the gene *intestinal sterol metabolism A* can convert dietary cholesterol to coprostanol (Kenny et al., 2020). Individuals who harbor these microbes in the gut have lower serum total cholesterol, indicating the critical role of gut microbiota in cholesterol metabolism. Zebrafish can be employed as a model species to understand the role of gut microbiota in lipid metabolism. Metatranscriptome sequencing of the zebrafish gut microbiota may reveal cholesterol and bile metabolizing genes. This information can be exploited to select probiotics for human use (O'Toole et al., 2017).

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Paper I

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Management of Hypercholesterolemia Through Dietary ß-glucans–Insights From a Zebrafish Model

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Consumption of lipid-rich foods can increase the blood cholesterol content. β-glucans have hypocholesterolemic effect. However, subtle changes in their molecular branching can influence bioactivity. Therefore, a comparative investigation of the cholesterol-lowering potential of two β-glucans with different branching patterns and a cholesterol-lowering drug, namely simvastatin was undertaken employing the zebrafish (*Danio rerio*) model of diet-induced hypercholesterolemia. Fish were allocated to 5 dietary treatments; a control group, a high cholesterol group, two β-glucan groups, and a simvastatin group. We investigated plasma total cholesterol, LDL and HDL cholesterol levels, histological changes in the tissues, and explored intestinal transcriptomic changes induced by the experimental diets. Dietary cholesterol likely caused the suppression of endogenous cholesterol biosynthesis, induced dysfunction of endoplasmic reticulum and mitochondria, and altered the histomorphology of the intestine. The two β-glucans and simvastatin significantly abated the rise in plasma cholesterol levels and restored the expression of specific genes to alleviate the endoplasmic reticulum-related effects induced by the dietary cholesterol. Furthermore, the distinct patterns of transcriptomic changes in the intestine elicited by the oat and microalga β-glucans impacted processes such as fatty acid metabolism, protein catabolic processes, and nuclear division. Oat and microalgal β-glucans also altered the pattern of lipid deposition in the liver. Our study provides insights into the effectiveness of different β-glucans to alleviate dysfunctions in lipid metabolism caused by dietary cholesterol.

Keywords: β-glucans, cholesterol, lipids, microalgae, RNA-Seq, zebrafish

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INTRODUCTION

The drastic shift in the global food system has driven a new trend in consuming calorie-rich and highly processed foods (1). Increased consumption of lipid-rich diets is directly correlated with the risk of cardiovascular diseases (CVDs) (2, 3). Cholesterol is one of the main components in the western diet, but it is not an essential nutrient for vertebrates as this form of lipid can be synthesized

de novo (4). However, cholesterol obtained from diets is known to have a significant impact on lipoproteins and their levels in the blood (5). The two classes of lipoproteins, namely high density lipoprotein (HDL) and low density lipoprotein (LDL) have distinct properties. LDL is considered as the main proatherogenic lipoprotein. Accumulation and subsequent oxidation of LDL in the arterial wall triggering an inflammatory response is a critical step toward the development of atherosclerosis (6). On the other hand, HDL performs cholesterol efflux from peripheral tissues to the liver, and the lipoprotein is known to possess anti-inflammatory and antioxidative effects as well as LDL oxidation lowering ability (7). The liver and intestine are the two principal organs that regulate the circulating lipoproteins and cholesterol homeostasis in the body, and specific receptors are known to facilitate metabolism linked to bile and dietary cholesterol (8, 9). The dietary cholesterol, along with other lipids and the synthesized apolipoproteins forms chylomicrons (10, 11), which are finally released into the blood. The endogenous pathway of cholesterol metabolism generates LDLs and the amounts of LDL cholesterol present in the circulation is largely determined by the rate of uptake by specific receptors in the liver (12). If the cholesterol content of the hepatocytes is high, LDL receptor activity is decreased (13), causing a reduction in the uptake of LDL by the hepatocytes. This in turn increases the amounts of LDL in circulation. Higher LDL cholesterol content in the blood is directly associated with lifestyle diseases like ischemic heart disease and stroke (3, 14).

Dietary and endogenous factors are known to adversely affect cholesterol uptake and biosynthesis, but some diet components, paradoxically, can stall the progression of lifestyle diseases by regulating the total circulating cholesterol and LDL-cholesterol levels. In fact, encouraging outcomes through dietary and therapeutic interventions have spurred an interest in developing strategies to manage LDL-cholesterol through diet. Targeting LDL-cholesterol reduction through diets is a better approach because medicines are associated with many side effects. Statins are a class of drugs commonly used to manage hypercholesterolemia. These drugs though effective, exhibit myotoxic side effects which include myalgia, myositis and rhabdomyolysis (15, 16). As an alternative, dietary intervention can be adopted to maintain cholesterol homeostasis. The benefits of using natural bioactive compounds are multifaceted if they are employed to arrest hypercholesterolemia (17, 18). β-glucans found in plants, microalgae and fungi have several unexploited properties, and subtle changes in their structural organization can elevate their efficacy. For example, fungal β-glucans, with β-(1, 3) backbone and (1, 6) linkages, have immunostimulatory and anti-tumor properties (19). In contrast, β-glucan from cereals, which have β-(1, 4) linkages, help lower cholesterol and blood glucose (20). The marine microalga *Phaeodactylum tricornutum,* is a rich source of β-glucan (21), and this polysaccharide has a linear chain of β-(1, 3) linkage and branching at the C-6 position (22). Studies have reported that the functionality and biopotency of β-glucans are largely determined by their molecular structure (23). Although branching differences between microalgal β-glucans and oat glucans are well known (24), it is unclear how these structural differences affect bioactivity *in vivo*. It is plausible that β-glucans from different sources are recognized by specific receptors, the affinity of which varies based on the molecular structure of the β-glucans (25). In addition, each β-glucan type may have its distinct gel-forming ability (26), prebiotic property (27) and influence on microbiota to affect the blood cholesterol levels (28). Moreover, we cannot discount the effect of interaction of these polysaccharides with other food components. Consumption of oat β-glucan with food and juices lead to different outcomes in humans. For example, beverages enriched with oat is effective in decreasing the LDL or total cholesterol in humans (29), whereas bread with oat β-glucan can decrease LDL cholesterol, total/HDL cholesterol ratio and LDL/HDL cholesterol ratio (30) indicating the impact of the food matrix on the efficacy of β-glucans.

A comparative investigation of dietary β-glucans from two sources with different molecular structures was undertaken employing a zebrafish model of hypercholesterolemia. Zebrafish is considered a valuable model species to understand abnormalities in lipid metabolism that result in disease progression. Fundamental lipoprotein pathways are conserved in humans and zebrafish (31) and adult zebrafish fed a cholesterol-rich diet are highly susceptible to hypercholesterolemia (32, 33). This model also allowed us to test the therapeutic potential of two dietary components which can eventually be considered as hypercholesterolemia-controlling agents in humans (34). We targeted the intestine tissue because of its importance in dietary lipid uptake and regulation of cholesterol metabolism (35).

We hypothesized that dietary β-glucans with different molecular structures may influence the circulating cholesterol levels and their associated responses in the intestine. RNA sequencing was employed to understand the underlying impact of dietary cholesterol, β-glucans and simvastatin on the intestinal cholesterol metabolism of zebrafish at a molecular level.

MATERIALS AND METHODS

Experimental Fish

Three hundred male (14-month-old) zebrafish, *Danio rerio*, were used for the experiment. To obtain this experimental fish stock, the adult fish were bred in-house in the zebrafish facility of Nord University, Norway, following standard protocols (36). The eggs were maintained in E3 medium and incubated at 28 °C in an incubator until hatching i.e., at around 50 h post-fertilization. From 4 to 14 days post-fertilization, the larvae were fed the commercial micro diet Zebrafeed® (SPAROS Lda, Olhão, Portugal) of $< 100\,\mu m$ particle size and *Artemia nauplii*, *ad libitum*. From 15 days post-fertilization (advanced larval stage) onwards, they were fed only micro diets of 100-200 μm particle size (Zebrafeed®). On month 14, the fish were randomly distributed into 30 tanks (6 tanks per treatment group) of a freshwater flow-through system (Zebtec Toxicological Rack, Tecniplast, Varese, Italy) with 3.5 L tank capacity. The stocking density was ten fish per tank. The fish were acclimatized in the flow-through system for 2 weeks and were fed control diet during this period. The water temperature in the tanks was 28 ± 0.5°C, and the water flow rate was 2.5 L/h. The dissolved oxygen in the tanks ranged between 7 and 8 ppm (oxygen saturation >

85%). A 14L:10D photoperiod was maintained throughout the experimental period.

β-Glucans, Diets and Feeding Experiment

The alga β-glucans employed in the present study originated from the microalga *Phaeodactylum tricornutum*, strain SAG 1090-1b (culture collection from the University of Goettingen) also designated as CCAP strain 1052/1B or UTEX 640. *P. tricornutum* (SAG 1090 1b) grown under nitrogen-depleted conditions in flat panel airlift reactors was harvested, concentrated via centrifugation to 250–270 g L^{-1} (Clara 20, Alfa Laval) and frozen at −20°C. For further processing, the biomass was thawed and diluted to 100 g L^{-1} with deionized water. After disrupting the algal cells with a ball mill (PML-2, Bühler), the biomass was centrifuged and the supernatant, containing the enriched fraction of β-glucan, was freeze-dried (Cat. Number: J326XP-IM-4, Avanti J-26 XP, Beckman Coulter). Besides β-glucan, the alga product contained a low level of protein (3.7%) and fat (<0.5%), and the remaining fraction could be presumed as other soluble carbohydrates. The oat β-glucans used in the study was a commercial product PromOat® (Lantmännen Oats AB, Sweden). According to the manufacturer, the oat β-glucan product also contains high levels of other carbohydrates including starch and low amounts of protein (4%) and fat (0.5%). SPAROS Lda. prepared the five experimental diets (**Supplementary Table 1**): 4 high-cholesterol diets and 1 control diet. The high cholesterol diet (HC) had 5.1% inclusion of purified cholesterol. We selected this level based on previous studies on zebrafish (32, 33). A standard low cholesterol diet without supplementation of purified cholesterol served as the control diet (CT). The fatty acids in the two basal diets, CT and HC (**Supplementary Table 2**) were profiled by Eurofins Food Testing Lisboa (Alcochete, Portugal).

The algal (AG) and oat glucan (OG) diets had 2.5% (with 30% purity, final content 0.8%) each of alga-derived and oat-derived β-glucans, respectively incorporated into the HC diet. The SS diet contained 50 mg kg^{-1} of simvastatin (Cat. Number: S6196, SigmaAldrich, St. Louis, MO, USA) in the HC diet; the inclusion level was based on a previous study (37). Thus AG, OG and SS diets had all the ingredients in the HC diet in addition to the respective test compound. The daily feeding rate was 4% of total biomass per day. The fish were fed thrice a day during the 12-week feeding trial; at 08:00, 13:00, and 18:00.

Sampling

At the end of the experimental period, fish were sacrificed by immersing in 200 mg L^{-1} of tricaine methane sulfate (Cat. Number: E10521, SigmaAldrich), which was buffered with 200 mg L^{-1} of sodium bicarbonate (Cat. Number: S5761, SigmaAldrich). Blood drawn by tail ablation method (38) was collected in a heparinized tube, and was centrifuged at 5,000 *g* for 10 min at 4 °C to collect the plasma (n = 5 per group; 5 fish from each tank pooled). The middle intestine (n = 5 to 6 per group) was carefully dissected and snap-frozen in liquid nitrogen followed by storage at −80°C until use.

Plasma Total Cholesterol, LDL Cholesterol and HDL Cholesterol Estimation

The total, LDL, and HDL cholesterol levels in the plasma were estimated using the HDL and LDL/VLDL Cholesterol Assay Kit (Cat. Number: ab65390, Abcam, Cambridge, UK). Plasma from five fish per tank was pooled and considered as one replicate sample. Two microliters of this pooled plasma were used for quantifying the total, LDL- and HDL-cholesterol content, following the manufacturer's instructions.

Transcriptomic Analysis of Intestine

To extract total RNA, the frozen intestine samples were briefly homogenized in QIAzol lysis reagent (Cat. Number: 79306, Qiagen, Hilden, Germany) at 6,500 rpm for 2 × 20 s in a Precellys 24 homogenizer (Cat. Number: P000669-PR240-A, Bertin Instruments, Montigny-le-Bretonneux, France). RNA was extracted from the tissue homogenate using Direct-zol™ RNA MiniPrep (Cat. Number: R2052, Zymoresearch, CA, USA) following the manufacturer's instructions. The RNA concentration and integrity were determined using Qubit 4 Fluorometer (Cat. Number: Q33238, Thermo Fisher Scientific, Waltham MA, USA) and Tape Station 2200 (Cat. Number: G2964AA, Agilent Technologies, Santa Clara, CA, USA). Only RNA samples exhibiting RIN value >7 were used to construct the RNA-Seq libraries. Libraries were prepared as described in our previous publication (39) using the NEBNext Ultra™ RNA Library Prep Kit (Cat. Number: E7760S, NE Biolabs, Ipswich, MA, USA) with the poly (A) mRNA magnetic isolation module (Cat. Number: E7490S, NE Biolabs). Briefly, one μg of total RNA was used for library preparation and after Poly(A) enrichment, mRNA was fragmented to 100–200 nt length. Next, the first and second strands of cDNA was synthesized, and then the cDNA was purified, end-repaired and used for adaptor ligation followed by barcoding using NEBNext Multiplex Oligos (Cat. Numbers: E7600S and E7780S, NE Biolabs). PCR enrichment was done for nine cycles, and the amplified libraries were purified using AMPure XP beads (Cat. Number: A63881, Beckman Coulter, Inc., Brea, USA). The barcoded libraries were then pooled and loaded at 1.4pM on the Illumina NextSeq 500 sequencer (Cat. Number: SY-415-1001,Illumina, San Diego, CA, USA) using the NextSeq 500/550 High Output Kit (Cat. Number: FC-404-2005, Illumina) for 75 bp single-end sequencing at the genomics platform of Nord University (Bodø, Norway). The average mapping percentage for the whole data set was 91.4% (**Supplementary Table 3**).

Bioinformatic Analysis

The quality of the reads was assessed using the *fastQC* command. Adapter sequences and low quality reads (Phred quality score, Q<30) were trimmed from the raw reads using the fastp software (40). The reads were then aligned to the reference zebrafish genome downloaded from NCBI (release 100) using HISAT2, version 2.2.1, which uses an indexed reference genome for alignment (41). The reads were annotated using *featureCounts*

to obtain the read counts that belong to each gene (42). Differential expression analyses of the genes across the treatment groups was performed using the R package *DESeq2* (version 1.30.0). Transcripts with an absolute Log_2 fold change of 1 and an adjusted *p*-value (q-value) of < 0.05 (Benjamini-Hochberg multiple test correction method) were considered significantly differentially expressed and used for gene ontology (GO) and KEGG pathway analysis. The gene ontology enrichment was performed with Database for Annotation, Visualization and Integrated Discovery (*DAVID*) version 6.8 and the *clusterProfiler* package (version 3.18.0) in R. The packages *ggplot2* (version 3.3.3), *pheatmap* (version 1.0.12) and *GOplot* (version 1.0.2) were employed to visualize the data. Gene ontology networks were generated using Cytoscape (43) (version 3.8.2). In addition, long lists of gene ontology terms were summarized into non-redundant terms using the REViGO online tool (44).

Histological Analysis of the Liver and the Intestine

The liver and the mid-intestine samples ($n = 8$ per group) were fixed in 3.7 % (v/v) phosphate-buffered formaldehyde solution (pH 7.2) at 4 °C for 24 h. Standard histological procedures were followed for dehydration, processing, and paraffin embedding as described by Bancroft and Gamble (45). The paraffin blocks thus prepared were sectioned using a microtome (Microm HM355S, MICROM International GmbH, Walldorf, Germany). Four micrometer thick longitudinal sections were cut and mounted on SuperFrost® slides (Menzel, Braunschweig, Germany). A robot slide stainer Microm HMS 760X (MICROM International GmbH) was used to stain the intestine sections with Alcian Blue (Cat. Number: A3157, SigmaAldrich) and Periodic Acid Schiff's Reagent (Cat. Numbers: 375810 and 1.09033, SigmaAldrich)(AB-PAS, pH 1) and the liver sections with hematoxylin (Cat. Number: H9627, SigmaAldrich) and eosin (Cat. Number: 861006, SigmaAldrich). Light microscopy photomicrographs were taken with the Leica DM3000 LED microscope (Leica Camera AG, Wetzlar, Germany) fitted with Leica MC 190HD camera (Leica Camera AG). The software ImageJ (46) was used for quantifying the histological indices. The histological indices that were evaluated were: length of the villi, width of lamina propria, submucosa thickness and tunica muscularis thickness, as described in our previous publication (47) (**Supplementary Figure 1**). Liver vacuolation was assessed by evaluating two parameters-average lipid vacuole area and average lipid vacuole number in a selected area of the liver (**Supplementary Figure 2**). Shapiro-Wilk test and Bartlett's test were employed to confirm the data normality and homoscedasticity, respectively. Parametric *t*-test and one-way ANOVA were performed where the normality assumptions were met. In the case of non-parametric data, statistical differences were identified using the Wilcoxon-Mann-Whitney test and Kruskal-Wallis test. Tukey's test (parametric data) and Dunn's test (non-parametric data) were employed to understand the differences between groups of interest.

qPCR Verification of the RNA Seq Results

Differential expression of selected genes from the transcriptome data was verified by qPCR analysis. The same samples used for RNA-Seq were employed for qPCR-based verification, and reactions were run with a sample size of 5 per group. One µg of total RNA from each sample was reverse transcribed using the QuantiTect reverse transcription kit (Cat. Number: 205311, Qiagen), according to the manufacturer's instructions. The cDNA was further diluted ten times with nuclease-free water and used as a PCR template. The PCR reactions were conducted using the SYBR green (Cat. Number: 04707516001, Roche Holding AG, Basel, Switzerland) in LightCycler® 96 Real-Time PCR System (Cat. Number: 05815916001, Roche Holding). Relative expression of selected genes was determined based on the geometric mean of reference genes (*actb1*, *eef1a* and *rpl13α*), for which we employed the primers that are reported previously (48). We designed the primers for the selected genes using the Primer-BLAST tool in NCBI. The primers were then checked for secondary structures such as hairpin, repeats, self and cross dimer by NetPrimer (Premier Biosoft, Palo Alto, USA). The primers for the target genes are listed in **Supplementary Table 4**. The data were checked for normality (Shapiro-Wilk test) and homoscedasticity (Bartlett's test), based on which, the statistical difference was determined by Student *t*-test or Welch two-sample *t*-test.

RESULTS

In the present study, we fed adult zebrafish with a control low cholesterol diet (CT) or high cholesterol diets (HC, AG, OG, SS) that contained 5.1% cholesterol.

Purified Dietary Cholesterol Altered the Plasma Cholesterol Profile

We examined the effect of purified dietary cholesterol on different cholesterol species that are present in the plasma of adult zebrafish. Total, LDL- and HDL-cholesterol in fish fed the HC diet was compared with those of the CT group. We found an apparent ($p < 0.1$) increase in the total cholesterol content and a significant ($p < 0.05$) increase in the LDL-cholesterol content in the plasma of the HC group (**Figures 1A,B**). On the other hand, the HDL-cholesterol levels of the two study groups were not significantly different (**Supplementary Figure 3A**).

β-Glucans and Simvastatin Reduced the Plasma Cholesterol Levels

The effect of supplementation of different forms of β-glucans and simvastatin on the plasma cholesterol level in zebrafish was assessed to evaluate their effectiveness in keeping the proatherogenic lipoprotein levels under control. We found a significant reduction of total cholesterol in the AG and OG groups compared to the HC group (**Figure 2A**). The LDL-cholesterol in the AG, OG and SS were lower compared to the HC group (**Figure 2B**). Again, we did not observe significant differences in the plasma HDL-cholesterol of the treatment groups (**Supplementary Figure 3B**).

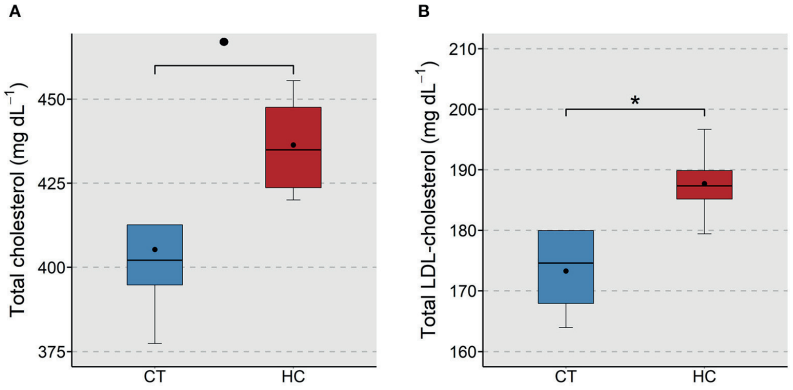

FIGURE 1 | Total and LDL-cholesterol levels in the plasma of zebrafish fed a low or a high cholesterol diet. Boxplots show the total cholesterol **(A)** and LDL-cholesterol **(B)** levels (mg dL^{-1}) in the plasma of zebrafish fed either a control (CT) diet or a high cholesterol (HC) diet for a period of 12 weeks. Black dots inside each box indicate the mean values of the corresponding groups. * indicates $p < 0.05$ and • indicates $p < 0.1$.

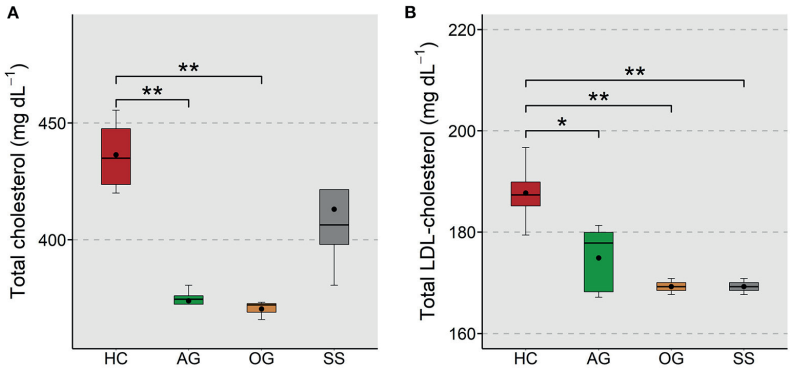

FIGURE 2 | Total and LDL-cholesterol levels in the plasma of zebrafish fed glucans or simvastatin. Boxplots show total cholesterol **(A)** and LDL-cholesterol **(B)** levels (mg dL^{-1}) in the plasma of zebrafish fed high cholesterol (HC) diet or HC diet supplemented with either algal glucan (AG) or oat glucan (OG) or simvastatin (SS), for a period of 12 weeks. Black dots inside each box indicate the mean values of the corresponding groups. **indicates $p < 0.01$ and * indicates $p < 0.05$.

Dietary Cholesterol Affected Steroid and Bile Biosynthesis and Endoplasmic Reticulum-Linked Genes in the Intestine

To assess whether the cholesterol content in the HC group impacted the cholesterol metabolism in the intestine, we compared the transcriptomes of the HC group and the CT group. The analysis revealed 71 downregulated and 109 upregulated genes ($|\text{Log}_2$ fold-change$| \geq 1$, q-value < 0.05) in the HC group compared to the CT group (**Supplementary Table 5**). The KEGG pathway enrichment analysis of the downregulated genes revealed significant suppression of steroid biosynthesis, terpenoid backbone biosynthesis and primary bile acid biosynthesis pathways. We found significant enrichment of pathways such as protein export, phagosome, and protein processing in the endoplasmic reticulum (ER) by the upregulated genes (**Figure 3A**).

Furthermore, the GO analysis revealed significant enrichment of several associated biochemical processes like cholesterol and sterol metabolic processes, sterol and steroid biosynthetic processes because of the downregulated genes in the HC group (**Figure 3B**). On the other hand, the GO enrichment analysis with the upregulated genes led to the enrichment of ER membrane, ER lumen and nuclear outer membrane reticulum membrane network (**Figure 4**).

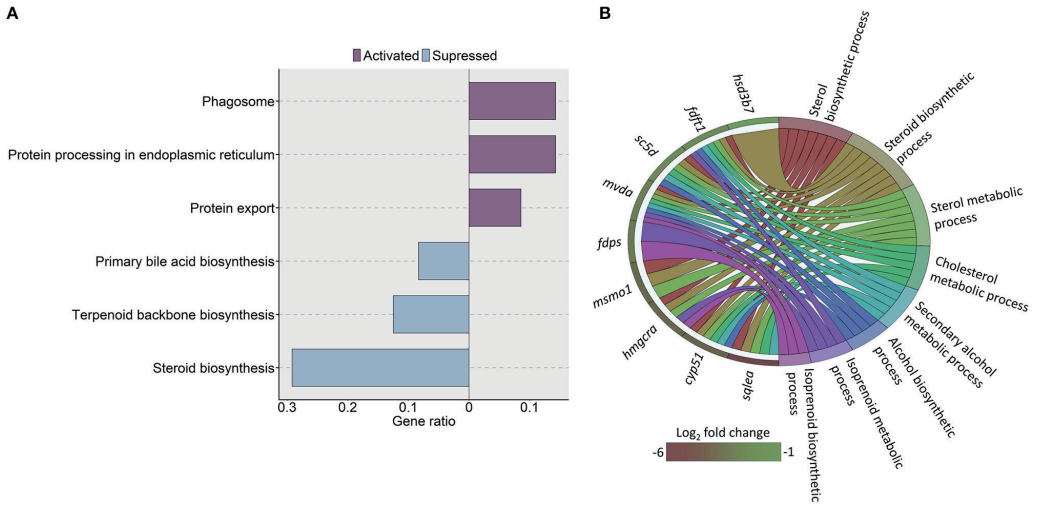

FIGURE 3 | KEGG pathways and GO terms that were enriched in zebrafish fed a high cholesterol diet. **(A)** Significantly altered KEGG pathways in the high cholesterol diet (HC) group, based on the altered genes in the HC group compared to the low cholesterol (CT) diet group. Each treatment group consisted of at least five biological replicates. **(B)** Chord diagram showing the link between the enriched GO terms and the associated genes that were detected as downregulated in the HC vs. CT group comparison. Genes that were considered for the enrichment analyses are shown on the left half of the circle. The gradient color bar intensity varies with the Log_2 fold change.

Algal Glucan Regulated the Expression of Genes Involved in the Intestinal Lipid Metabolism and Vacuole Formation

Since the plasma cholesterol levels were affected by algal glucan supplementation in the zebrafish diet, we hypothesized that the algal glucans could alter the cholesterol metabolism in the intestine. To assess this, we compared the intestinal transcriptome of the AG group with that of the HC group. This analysis revealed 19 downregulated and 43 upregulated genes ($|Log_2$ fold-change$| \geq 1$, q-value < 0.05, **Figure 5A**, **Supplementary Table 6**) in the AG group. Several important genes that regulate the lipid metabolism - *cytochrome P450, family 4, subfamily V, polypeptide 8* (*cyp4v8*), *ATP-binding cassette, sub-family F (GCN20), member 2a* (*abcf2a*), vacuole formation-*cathepsin L.1* (*ctsl.1*), *cathepsin Bb* (*ctsbb*), *IFI30 lysosomal thiol reductase* (*ifi30*) and ER stress-*calumenin a* (*calua*) were differentially regulated by the algal β-glucan. GO analysis based on the upregulated genes revealed significant enrichment of vacuole, lytic vacuole and lysosome (**Figure 5B**). Our analysis did not detect any significant enrichment of KEGG pathways based on the differentially expressed genes from the AG vs. HC group comparison.

Oat Glucan Altered Genes Associated With Lipid Metabolism and Endoplasmic Reticulum in the Intestine

Transcriptomic analysis of the oat glucan group revealed 177 upregulated and 223 downregulated genes ($|Log_2$ fold-change$| \geq 1$, q-value < 0.05, **Supplementary Table 7**). The gene ontology analysis of the upregulated genes revealed processes like autophagy, steroid hydroxylase activity, lipid catabolic process and lipid oxidation (**Figure 6A**). The GO analysis of the downregulated genes revealed the suppression of several processes like response to ER stress, ER associated protein degradation (ERAD) pathway and *de novo* protein folding (**Figure 6B**). KEGG pathway enrichment analysis using the differentially expressed genes revealed the activation (based on the upregulated genes) of PPAR signaling pathway, fatty acid degradation, mitophagy-animal, primary bile acid biosynthesis and the suppression (based on the downregulated genes) of protein export, ribosome biogenesis in eukaryotes, and protein processing in ER (**Figure 7A**).

Simvastatin Impacted Genes Connected to Protein Synthesis and Endoplasmic Reticulum in the Intestine

Transcriptomic analysis of the simvastatin fed (SS) group vs. high cholesterol-fed (HC) group revealed 242 upregulated and 224 downregulated genes ($|Log_2$ fold-change$| \geq 1$, q-value < 0.05, **Supplementary Table 8**). Gene ontology analysis of the downregulated genes revealed the enrichment of several terms like translational elongation, rRNA modification, rRNA metabolic process and peptide biosynthetic process (**Figure 7B**). KEGG pathway enrichment analysis of the upregulated genes in the SS group revealed activation of lysosome, mitophagy-animal, and other glycan degradation pathways. In contrast, protein export, ribosome biogenesis in eukaryotes and protein processing in ER

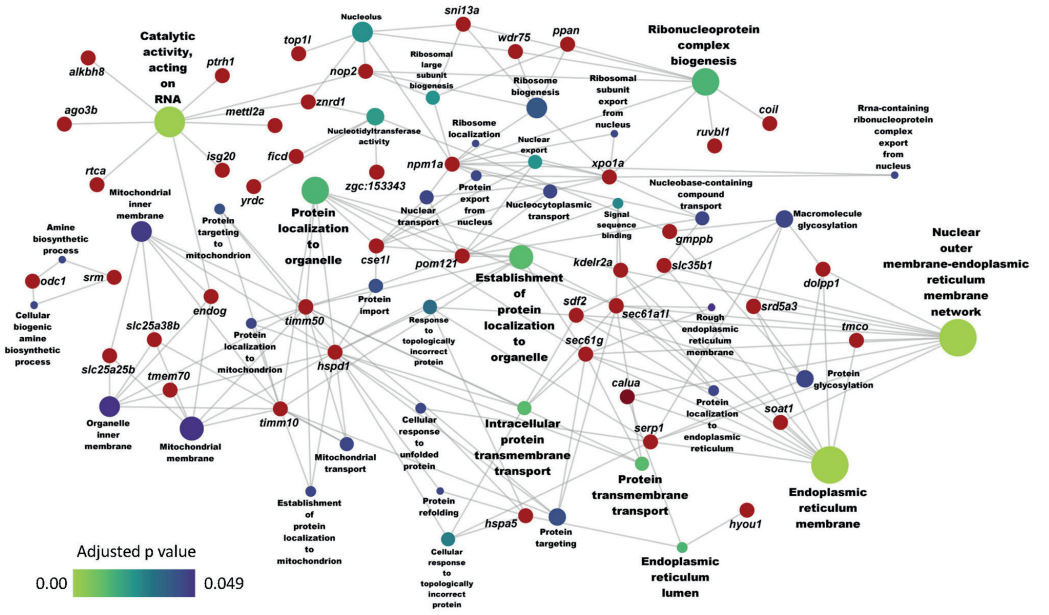

FIGURE 4 | Network showing the link between enriched GO terms and the associated genes that were upregulated in zebrafish fed the high cholesterol (HC) diet compared to the control (CT) diet. Genes that were considered for the enrichment are written near the red circles. The gradient color bar intensity varies with the adjusted *p* value (Benjamini-Hochberg method) for each GO term. Node size of each GO term increases with the number of mapped genes.

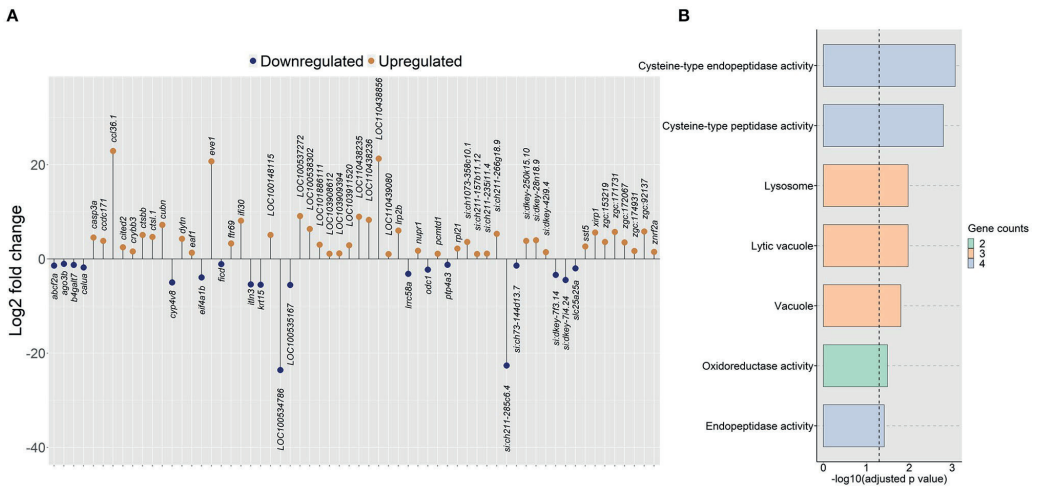

FIGURE 5 | Genes and GO terms that were regulated in zebrafish fed the algal glucan diet. **(A)** Differentially expressed genes obtained by comparing the algal glucan group (AG) with the high cholesterol (HC) group ($|\text{Log2 fold-change}| \geq 1$, $q\text{-value} < 0.05$). **(B)** Bar plot showing enriched GO terms in the AG group, based on genes that were upregulated in the AG group compared to the HC group. Bar colors represent the gene count. For both analyses, each treatment group consisted of at least five biological replicates.

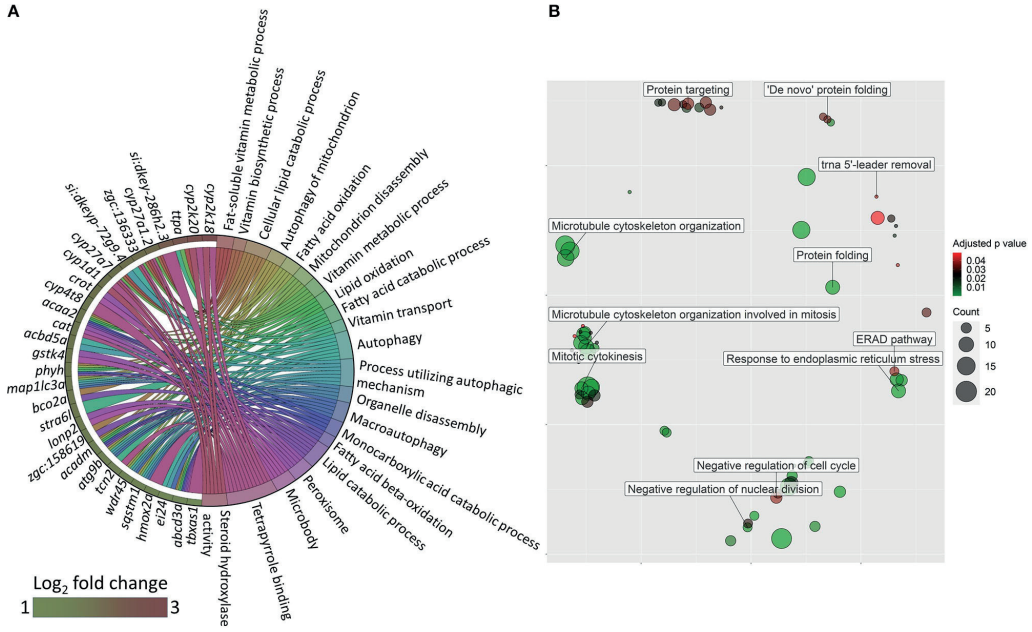

FIGURE 6 | GO terms that were altered in zebrafish fed the oat glucan diet. **(A)** Chord diagram showing the link between enriched GO terms and the associated upregulated genes in the oat glucan (OG) group compared to the high cholesterol diet (HC) group. Genes that were considered for the enrichment are shown on the left half of the circle. The gradient color bar intensity varies with the Log_2 fold change. **(B)** Enriched GO terms in the OG group. Only the non-redundant GO terms are marked in the bubble plot, and the analysis was performed using the genes that were downregulated in the OG group compared to the HC group. The color of the circles ranging from green to red indicates the order of adjusted *p* value (0.01–0.049). Circle sizes are proportional to the gene count.

pathways were likely suppressed by simvastatin feeding (**Figure 7C**).

The Intestinal Transcriptome Responds Differentially to Diet-Induced Hypercholesterolemia and Cholesterol-Lowering Agents

We examined the genes that were downregulated by dietary cholesterol (HC vs. CT) and upregulated by dietary algal glucan (AG vs. HC), oat glucan (OG vs. HC) and simvastatin (SS vs. HC). Algal glucan, oat glucan or simvastatin were able to restore the expression of the genes that were downregulated by cholesterol feeding (**Figure 8**, **Supplementary Figure 4**). We found 13 genes downregulated in the intestine when the fish were fed high cholesterol diet, but their expression was restored by algal glucan feeding. Similarly, we found that the expression of 30 genes was restored by oat glucan feeding. Likewise, simvastatin feeding also helped in bringing back the expression of 38 genes. To understand the differential effects of the oat and algal β-glucans, we explored the disparate alteration of gene expression. Among the 124 highly ($|Log_2$ fold-change$| \geq 2.5$, q-value < 0.05) differentially expressed genes obtained from the AG vs. HC and OG vs. HC comparisons, 9 genes were shared and 105 were detected as unique (30 genes in AG vs. HC and 85 genes in OG vs. HC) (**Supplementary Figure 5A**). Among these 105 uniquely expressed genes, the expression of 95 genes differed in the algal and oat β-glucan fed groups (**Supplementary Figure 5B**). Twenty-five genes exhibited higher normalized counts in the OG group compared to the AG group. On the other hand, 70 genes exhibited higher normalized counts in the AG group compared to the OG group. Furthermore, the GO analysis of these 95 genes (**Supplementary Figure 6**) revealed the processes like nuclear division, cellular protein catabolic process, cellular macromolecular catabolic process that were predominantly enriched in group fed algal glucans. On the other hand, GO terms like fatty acid metabolic process, long-chain fatty acid metabolic process and oxoacid metabolic process were predominantly enriched based on the upregulated genes in the OG group compared to the AG group.

Diets Altered the Vacuolization in the Liver and Micromorphology of the Intestine

We assessed the changes in hepatocyte vacuoles to understand the probable consequence of plasma hyperlipidemia; by measuring the average size and number of vacuoles in a selected area of the liver. We did not observe a significant

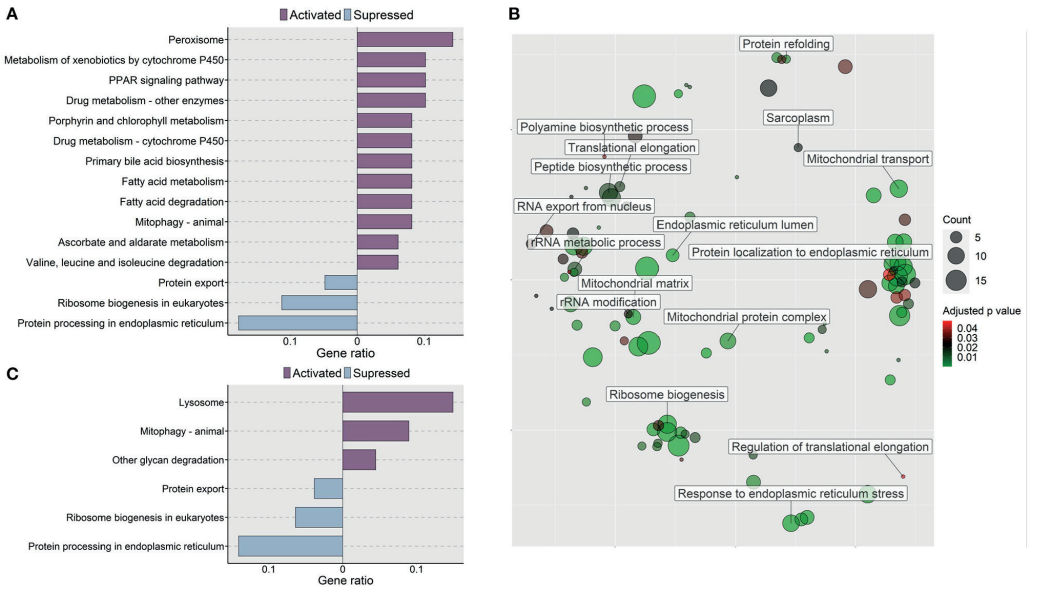

FIGURE 7 | Molecular effects of the oat glucan and simvastatin supplemented diets. **(A)** KEGG pathways that were altered in zebrafish fed the oat glucan (OG) diet. The enrichment analyses were performed by considering the differentially expressed genes in the OG fed zebrafish compared to the fish fed the high cholesterol (HC) diet. **(B)** Enriched GO terms in simvastatin fed (SS) group. Only the non-redundant GO terms are marked in the bubble plot, and the analysis was performed using the genes that were downregulated in the SS group compared to the HC group. The color of the circles ranging from green to red indicates the order of adjusted *p* value. Circle sizes are proportional to the gene number. **(C)** KEGG pathways that were altered in zebrafish fed simvastatin. The enrichment analyses were performed by considering the differentially expressed genes in the SS group compared to the HC group. Each treatment group consisted of at least five biological replicates.

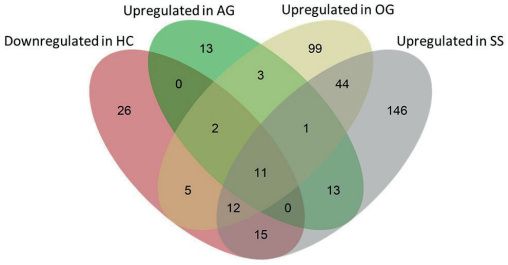

FIGURE 8 | Venn diagram showing the total number of genes that were affected by the experimental diets. The pink ellipse includes the genes that were downregulated in the HC group compared to the CT group. Green, yellow and gray ellipses include genes that were upregulated (compared to the HC group) in the AG, OG and SS groups, respectively. Thirteen (2 + 11), 30 (2 + 5 + 11 + 12) and 38 genes (12 + 15 + 11) that were downregulated in the HC group were upregulated in the AG, OG and SS groups, respectively.

increase in either the size or number of vacuoles in the fish fed high cholesterol diet (HC) compared to the CT diet (**Supplementary Figure 7**). On the other hand, the HC diet group had significantly larger vacuole area in their liver compared to the OG, AG and SS groups. Also, a trend for a lower number of vacuoles was observed in the OG ($p = 0.07$) and SS ($p = 0.08$) groups compared to the HC group (**Figure 9**). Fish fed the HC diet had longer intestinal villi compared to the CT group (**Supplementary Figure 8**). Furthermore, the algal glucan fed group had shorter villi compared to the HC and OG groups. The HC diet also significantly reduced the lamina propria thickness compared to the other groups and tunica muscularis thickness compared to glucan fed groups (**Figure 10**).

Verification of the Expression of Selected Genes From the Transcriptomic Data

From our RNA-Seq data, we selected 7 differentially expressed genes from different group comparisons for verification by qPCR (**Figure 11A**). These genes have critical functions in maintaining cholesterol homeostasis through regulation of cholesterol and bile acid biosynthesis (*hmgcra, abcf2a, cyp27a7*), vacuole formation (*ctsl.1*), protein synthesis (*xpo1a*), molecular chaperoning (*endog*) and lipoprotein (*ldlra*) receptor expression. Overall, the alterations in the expression of the selected genes agreed with the changes in the transcriptomic data (**Figure 11B**).

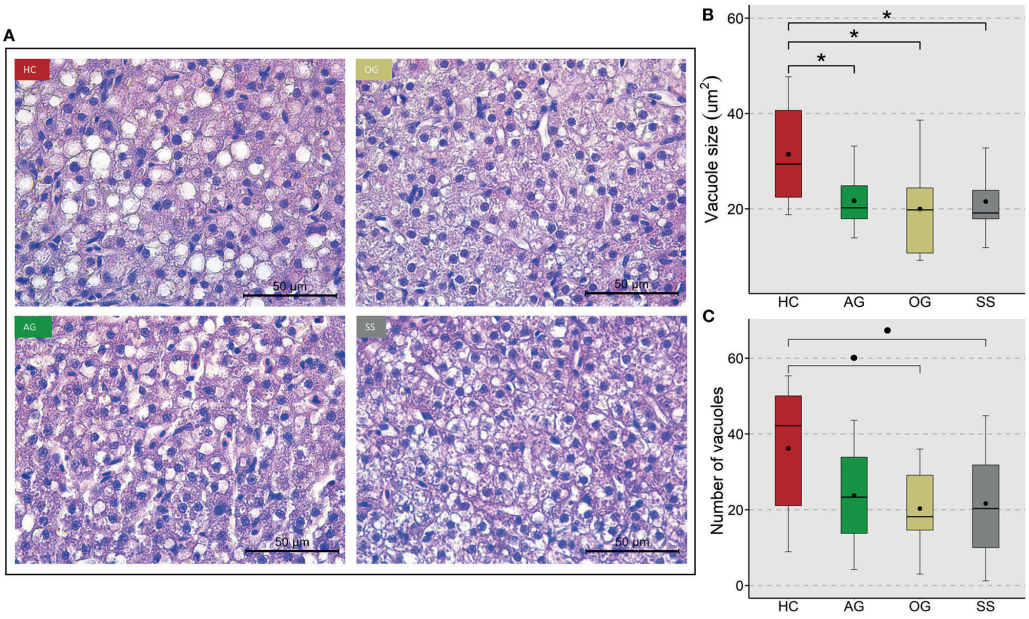

FIGURE 9 | Lipid accumulation in the liver of zebrafish fed four experimental diets. **(A)** Representative images of the liver of zebrafish. Scale bar = 50 µm. Boxplots show average size **(B)** and average number **(C)** of vacuoles in the liver of fish fed high cholesterol (HC) diet or HC diet supplemented with either algal glucan (AG) or oat glucan (OG) or simvastatin (SS), for a period of 12 weeks. Black dots inside each box indicate the mean values of the corresponding groups. * indicates $p < 0.05$ and • indicates $p < 0.1$. Each treatment group consisted of eight biological replicates.

DISCUSSION

Consumption of a fiber-rich diet can be considered a healthy approach to prevent many diseases, including cancer, obesity, and hypercholesterolemia (49–51). In contrast to cholesterol-lowering drugs, β-glucans, the dietary fibers with bioactive properties, can positively impact lipid metabolism without exerting any side effects (52). Hence, in the present study, we employed two types of β-glucans; one derived from oats and the other from the alga, *P. tricornutum*. The rationale behind using β-glucans from different sources was to understand their distinct ability to lower cholesterol levels; due to their differential molecular structure, solubility, varying mode of action such as their gel-forming ability, prebiotic nature, effect on bile acids and microbiota. The synergy of β-glucans with their residues, as well as with the components in the food matrix also determines their bioactivity. In fact, for many decades β-glucans were thought to mitigate hypercholesterolemia through their ability to both increase the viscosity of the luminal contents and bind to bile acids (53), a property dependent on the branching pattern of β-glucans. Studies have revealed that consumption of 3 grams of β-glucans per day can mitigate hypercholesterolemia (28). There are certain products with proven cholesterol-lowering potential, e.g., PromOat® (Lantmännen Oats AB, Kimstad, Sweden) and OatWell™ (CreaNutrition, Lutterworth, UK) with European Food Safety Authority (EFSA) certificates; they are marketed in Europe, and the companies tout about the gel-forming ability of their products. On the other hand, a number of supplements for example EcoGard® (EderaGen Helse, Bergen, Norway) composed of fungal β-1,3 / 1,6-glucan and BioGlena™ (Algatechnologies Inc. Eilot, Israel) composed of *Euglena gracilis* β-1,3-glucan, are marketed as immunomodulators. We have used β-glucans from the microalga *P. tricornutum* and PromOat® in the present study. It is now well known that the effects of dietary β-glucans go beyond the lumen of the intestine i.e., they can alter the expression of lipid metabolism related genes of the intestinal cells (54). The cellular and molecular effects of β-glucans depend on their interaction with the cells via their specific receptors and signaling pathways. This interaction is dependent on the structural features making the source of β-glucans a vital factor that defines the hypocholesterolemic potential. In this context, the differential efficacy of β-glucans from different natural sources in alleviating hypercholesterolemia should be investigated because the polysaccharides may impact the host organs in multiple ways and with varying efficacies. In the marine diatom *P. tricornutum*, the main β-glucan form is chrysolaminarin, which consists of a linear β-1,3-glucan chain with limited β-1,6-glucan branching. β-glucans from oat

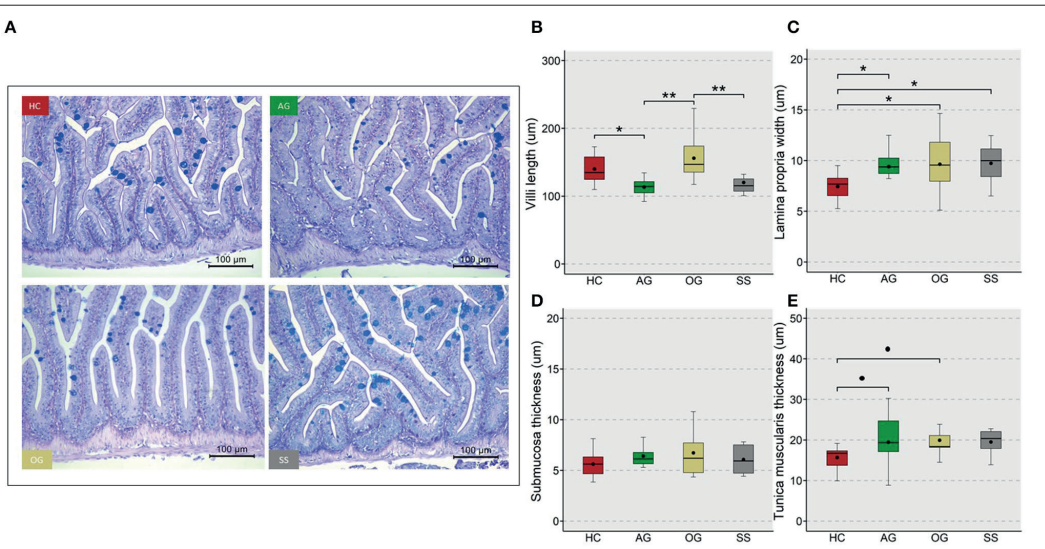

FIGURE 10 | Micromorphology and feature indices of the intestine of the zebrafish. Representative images **(A)** from the different study groups. Scale bar = 100 µm. Boxplots show average villi length **(B)**, lamina propria width **(C)**, submucosa thickness **(D)** and tunica muscularis thickness **(E)** in the mid intestine of the fish fed experimental diets for a period of 12 weeks. Black dots inside each box indicate the mean values of the corresponding groups. ** indicates $p < 0.01$, * indicates $p < 0.05$, and • indicates $p < 0.1$. Each treatment group consisted of eight biological replicates.

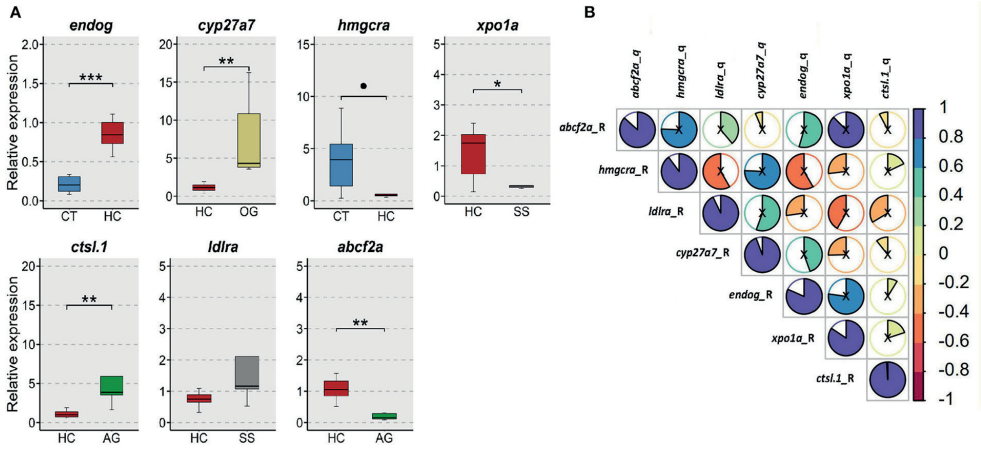

FIGURE 11 | Verification of the expression of selected genes from the transcriptomic data. **(A)** Relative expression of 7 selected genes in the intestine of the zebrafish. CT, fish fed low cholesterol; HC, fish fed high cholesterol; AG, fish fed algal glucan; OG, fish fed oat glucan; SS, fish fed simvastatin. $n = 5$. *** indicate $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$, and • indicates $p < 0.1$. Each treatment group consisted of five biological replicates. **(B)** Correlation between the normalized counts from the RNA-Seq data and relative gene expression from the qPCR data.

comprise mainly β-D-glucopyranosyl monomers connected by either β-1,3 glucosidic bonds and/or β-1,4 glucosidic bonds. Although not measured in the present study, other researchers have indicated that chrysolaminarin has a low molecular weight, ranging between 1 and 20 kDa (55, 56), while oat beta-glucan has a high molecular weight, around 2,000 kDa (57). We compared these two sources of β-glucans–terrestrial plant (oat) and aquatic plant (microalga)–to enhance our understanding of

the source-specific responses in the intestine, a crucial organ that regulates cholesterol metabolism. The plasma profile of zebrafish fed the two sources of glucans revealed that they are equally effective in mitigating hypercholesterolemia. The transcriptome comparisons revealed the plausible differential mode of action exerted by oat and microalga β-glucans to nullify the effects of hypercholesterolemia.

Cholesterol in Diet Altered the Plasma Cholesterol Profile and Cholesterol Synthesis and Affected Important Organelle Functions

In our study, plasma total cholesterol and LDL-cholesterol were higher in adult zebrafish fed the HC diet. Previous studies have also reported that a high cholesterol diet can increase plasma cholesterol levels in adult zebrafish (32, 33). The rise in plasma cholesterol level induced by the high dietary cholesterol could be indicative of increased cholesterol absorption in the intestine or reduced clearance by the liver of the fish. The latter is less likely to be the case because there was no significant change in vacuolization in the liver of fish from the HC group compared to the CT group. *Niemann–Pick type C1-like 1* (*NPC1L1*) receptor, present on the apical surfaces of the intestinal epithelial cells, is an important transporter that facilitates the absorption of diet-derived free cholesterol via enterocytes. The expression of the *Npc1l1* gene in the intestine of mice was reduced by cholesterol feeding to maintain homeostasis of cholesterol metabolism (8). However, in our study *npc1l1* gene did not respond to 5.1% dietary cholesterol treatment. Another study that employed 3% purified cholesterol also did not report any change in the expression of *npc1l1* in the zebrafish intestine (58). This indicates that a homeostasis maintenance to regulate the absorption of cholesterol through the downregulation of *npc1l1* gene expression is probably missing in zebrafish intestine. This lack of homeostasis-maintenance mechanism directed toward cholesterol metabolism (59) makes zebrafish a unique model to recapitulate dyslipidemia without any genetic interventions.

Pathway analysis of the differentially expressed genes revealed that high dietary cholesterol levels can suppress *de novo* synthesis of cholesterol and bile (biosynthesis of steroid/terpenoid/bile acids) in the intestine, probably due to its increased absorption. Under normal circumstances, suppression of endogenous biosynthesis occurs because cholesterol biosynthesis requires significant inputs such as acetyl-CoA, ATP, oxygen and the reducing factors NADPH and NADH (60). Seven genes that are involved in cholesterol biosynthesis were downregulated in the HC diet fed fish, and this included *3-hydroxy-3-methylglutaryl-CoA reductase a* gene (*hmgcra*) that is involved in the formation of mevalonate, a rate-limiting step in cholesterol synthesis. These results probably indicate that zebrafish intestinal cholesterol metabolism is responsive to dietary cholesterol. The high intracellular cholesterol levels are likely to alter the functions of organelles such as ER and mitochondria because the upregulated genes from the HC vs. CT comparison were associated with the GO terms linked to ER and mitochondrial inner membrane and mitochondrial transport. It is known that cholesterol alters the inner mitochondrial membrane, thereby affecting its microviscosity (61, 62). Moreover, high intracellular cholesterol levels also impact the glutathione influx into the mitochondria, thus leading to a higher accumulation of the reactive oxygen species (ROS). The GO terms mitochondrial inner membrane and mitochondrial transport were enriched because of the upregulation of the genes *endonuclease G* (*endog*), *translocase of inner mitochondrial membrane 50 homolog (S. cerevisiae)* (*timm50*), *transmembrane protein 70* (*tmem70*), *solute carrier family 25 member 25b* (*slc25a25b*), *solute carrier family 25 member 38b* (*slc25a38b*) and *translocase of inner mitochondrial membrane 10 homolog (yeast)* (*timm10*). The gene *endog* is an important regulator of oxidative stress-mediated apoptosis. Exposure to ROS-producing agents is also known to induce the expression of *Endog* in rats (63). The *slc25a38b* gene codes for a protein that interacts with mitochondrial outer-membrane fusion proteins and maintains mitochondrial morphology (64). On the other hand the protein coded by *slc25a25b* is a calcium-binding molecule that transports nucleotides and cofactors across the mitochondria (65) and is involved in mitochondrial homeostasis. Altered expression of *Slc25a25* in mice was implicated in the resistance to diet-induced dysregulation of lipid metabolism (66). Cholesterol may also disrupt the assembly of the respiratory supracomplexes in the mitochondria (67). The genes *tmem70*, *timm50* and *timm10* are involved in the transport of different proteins and biogenesis of supracomplexes in the mitochondria (68, 69). We also observed dietary cholesterol-induced upregulation of the phagosome pathway in the HC group. The protein coded by the *SEC61 translocon gamma subunit* (*sec61*) gene normally transports proteins from the cytosol to the ER, but it can also reverse transport proteins from the ER to the cytosol for degradation (70). It is known that at least a subset of ER proteins contributes to the phagosome pathway (71). Furthermore, *sec61* is involved in transferring membrane protein from the ER to proteasome for destruction (72). These results indicate the impact of dietary cholesterol on ER, mitochondria, lipid metabolism and protein misfolding.

Algal β-Glucan Impacted Certain Genes Connected to Lipid Metabolism and Cholesterol Efflux in the Intestine

Developing diet-based mitigation strategies against CVDs requires an understanding of the impact of therapeutic agents on circulating LDL-cholesterol levels. The reduction of the circulating total and LDL-cholesterol by dietary algal glucan was comparable to the protective effect provided by oat glucan and simvastatin. Although the comparison of the intestine transcriptome of the AG group with that of the HC group did not reveal precise pathways that explain the hypocholesterolemic effect of the algal glucan, the downregulation of key genes linked to lipid metabolism and vacuole formation could be informing the involvement of the molecules in lipid processing in the intestine tissue. Algal glucan possibly activated the processes linked to the GO term lysosome, as inferred from the upregulation of the genes *ifi30*, *ctsbb* and *ctsl.1*. Lysosomes

are intracellular membrane-bound organelles characterized by an acidic pH and they contain a variety of hydrolytic enzymes. Cathepsin L1 (Ctsl1) and Cathepsin B (Ctsbb) are the most abundant lysosomal proteases and they participate in autophagy (73). Recognition of β-glucan by the membrane-associated receptors leads to lysosome activation through the unconventional vesicle-mediated secretion (74).

We observed downregulation of *keratin 15* (*krt15*) in the intestine of the algal glucan fed fish. The protein encoded by *krt15* is responsible for the structural integrity of mucosa, and it is expressed in the intestinal crypts and villi (75). The human keratin 14 gene is responsive to cellular levels of cholesterol the depletion of which can downregulate the gene (76). The expression of the gene *krt15* in our study likely indicates a response to reduced intracellular cholesterol. We also found a marked downregulation of the *cyp4v8* gene in the intestine of the alga glucan fed fish. Zebrafish have 94 *cyp* genes, which perform diverse functions; the *cyp4* clan contains four genes, including *cyp4v8*. Its ortholog in humans, *CYP4V2*, has been associated with the formation of omega-hydroxylated products (77). Activation of the lysosome pathway and the downregulation of *cyp4v8* in zebrafish fed algal glucan diet could be indicating an alteration in cholesterol transport because disturbances in the human ortholog is linked to lysosomal cholesterol transport (78). Another important downregulated gene in the AG group was *abcf2a*. ATP-binding cassette proteins are involved in the efflux of cholesterol from the intestine back to the lumen (79, 80). Western high energy diets are known to alter this gene, the expression of which is highly cell specific; while the *Abcf2* gene was upregulated in the endothelium, its expression in the parenchymal and Kupffer cells of the liver was not changed by the diet (81). Although the precise function of zebrafish *abcf2a* is not clearly described, its reduced expression indicates that this paralogue may be involved in the basolateral efflux of cholesterol in the intestine (82). Overall, the downregulation of *abcf2a*, *cyp4v8* and *krt15* genes indicate a possible effect of the microalgal β-glucan on the intestinal cholesterol metabolism and the efflux of cholesterol in the zebrafish model.

Oat Beta Glucan Enhanced Lipid Catabolism in the Intestine

Dietary oat glucan specifically impacted the lipid metabolism of the intestinal tissue, and most of the GO terms indicated a link to lipid catabolism. The activation of the primary bile acid synthesis pathway in the intestine points towards the conversion of cholesterol to bile acids. Oat β-glucan is known to increase bile excretion, necessitating the *de novo* synthesis of bile (28). We found an indication of peroxisome activation, probably because of the need for certain enzymes present in the peroxisome (83). The upregulation of *cytochrome P450, family 27, subfamily A, polypeptide 1, gene 2* (*cyp27a1.2*) and *cytochrome P450, family 27, subfamily A, polypeptide 7* (*cyp27a7*) genes are linked to the conversion of cholesterol to bile. We detected the enrichment of PPAR signaling pathway because of the upregulation of, among others, the *acyl-CoA dehydrogenase medium chain* (*acadm*) gene that is involved in the breakdown of medium-chain fatty acids in the mitochondria.

Simvastatin Altered Protein Metabolism in the Intestine

Simvastatin is a widely accepted cholesterol-lowering drug. Downregulation of genes and suppression of GO terms linked to ER evoked by both simvastatin and β-glucans validate the ability of glucans to mitigate the effects of high dietary cholesterol in the zebrafish model. Although statins are considered safe hypocholesterolemic drugs for humans, they are often associated with statin-associated skeletal muscle problems (84, 85). The protein synthesis machinery (86) can be impaired, resulting in statin-induced myopathy. Such effects have been reported in zebrafish as well (87). However, we are the first to report simvastatin-caused transcriptomic responses in the intestine of a vertebrate model organism. Based on our results, dietary simvastatin suppressed translation, rRNA modification, rRNA metabolic process and peptide biosynthetic process indicating an impact of the product on the protein synthesis machinery of the intestine. Statin-induced depletion of cholesterol in the striated muscle cells destabilizes membrane potential and alters ion balance in the cells thus affecting protein synthesis (88). Suppression of cholesterol biosynthesis by statins also leads to the deficiency of intermediates like farnesyl pyrophosphate and ubiquinone which are generated during cholesterol biosynthesis. These intermediates are important for sarcoplasm and mitochondrial functions and their unavailability may trigger myopathy (89). Prior studies have also revealed the suppressive effect of cholesterol-lowering drugs on protein synthesis (90). Although simvastatin at 50 mg kg^{-1} of diet revealed an effect on the protein synthesis in the intestine, the impact on the tunica muscularis thickness in the zebrafish intestine was not evident in the present study. Akin to the observations in the fish fed with algal glucan, simvastatin also led to lysosomal activation. Likewise, ER-related processes were suppressed by both oat glucan and simvastatin.

Adverse Effects of Dietary Cholesterol Were Alleviated by β-Glucans and Simvastatin

The ER membrane holds many enzymes associated with cholesterol metabolism. High cholesterol-induced misfolding of proteins influences ER homeostasis. Several GO terms like endoplasmic reticulum membrane, cellular response to topologically incorrect protein, and cellular response to unfolded protein were enriched in the HC diet group. It has been proposed that cholesterol-induced dysfunction of the ER calcium pumps affects the calcium-dependent chaperones and consequently ER protein folding (91). The subsequent ER stress causes the progression of cardiovascular diseases (92). Our study revealed that all three treatments–algal glucan, oat glucan, and simvastatin–can alleviate the adverse effects on ER in the intestine of the zebrafish. Although the AG vs. HC comparison did not reveal any suppression of GO terms or pathways related to ER stress, we observed the downregulation of the gene *calua* in fish fed the algal glucan. This gene codes for an ER chaperone protein, a potent suppressor of ER stress mediated signaling cascade and is considered a marker of ER stress. The downregulation of *calua* expression has also been associated

with the attenuation of ER stress (93). Therefore, we speculate that algal glucan can also relieve the intestinal tissue from cholesterol-induced ER stress. Oat glucan and simvastatin, on the other hand, reduced the expression of several heat shock proteins (HSPs) in the intestine. HSPs comprise a group of highly conserved, ubiquitous molecules, promoting proper folding and assembling of polypeptides. An important aspect of unfolded protein response is the upregulation of HSP genes (94, 95). Expression of HSP genes like *heat shock protein 5* (*hspa5*), *heat shock protein 90, beta (grp94), member 1* (*hsp90b1*), *hypoxia up-regulated 1* (*hyou1*) and *heat shock cognate 70-kd protein, tandem duplicate 3* (*hsp70.3*) was downregulated in the intestine of zebrafish fed oat glucan and simvastatin. We speculate that the observed downregulation of molecular chaperone genes that code for HSPs and Calua is an indication of reduced ER stress.

The distinct alterations in the intestinal transcripts evoked by algal and oat glucans have indicated the possible synergistic actions that could be exploited to counter hypercholesterolemia (**Supplementary Figure 9**). Our results indicate that algal glucans are effective against hypercholesterolemia, possibly through the downregulation of the cholesterol transporter gene *abcf2a* and the cytochrome P450 family gene, *cyp4v8*. Reduced expression of the *cyp4v8* ortholog in humans is known to increase the free cholesterol content in cells (78), whereas alterations in the expression of *abcf2a* gene could suppress basolateral transport of free cholesterol in enterocytes (82). Overall, the alterations in the expression *abcf2a* and *cyp4v8* genes indicate a possible accumulation of free cholesterol in the enterocytes of fish fed the AG diet. On the other hand, in the OG group, we found an increased steroid hydroxylase activity and primary bile acid biosynthesis activity. This indicates a compensatory mechanism of *de novo* bile synthesis possibly activated in response to reduced bile absorption in the intestine stimulated by oat glucans (28, 96). A synergy between the two glucans could activate their specific mechanisms, i.e., reduced cholesterol transport and reduced bile absorption to eventually reduce the circulating cholesterol levels. It could be hypothesized that the synergistic cholesterol-lowering effect of the two glucans can further reduce the dependence on the statins.

Cholesterol and β-Glucans Affected Vacuolization in the Liver and Micromorphology of the Intestine

Zebrafish is an excellent model for studying liver diseases because of the homology of the organ system compared to humans. Although zebrafish has a unique hepatic anatomy, ongoing research has revealed the conserved cell populations, transcriptional profile and signaling pathways associated with zebrafish and the human liver (97). Moreover, the cells in the zebrafish liver are involved in cholesterol metabolism and lipid storage (98). Therefore, zebrafish has been used to study fatty liver disease and is considered a model species to investigate liver metabolic dysfunctions. As for the extent of vacuolization in the liver of fish from different groups, although the mean number of vacuoles was higher in the HC compared to the CT group, we did not observe any significant differences. We speculate that the non-significant difference in vacuolization is linked to the age of the experimental fish and the dietary lipid levels. One-year-old adult fish were chosen for two purposes: to retrieve enough plasma for biochemical analyses and because they can be employed to mimic human diet-induced dyslipidemia. A high vacuolization tendency has been observed in the liver of 16 to 22-month-old zebrafish fed 5% dietary lipid (99). Furthermore, lipid and glycogen vacuolization has also been observed in previous studies wherein zebrafish were fed 11 to 13% lipids (100, 101). The low cholesterol diet in our study with 12% lipid also led to lipid accumulation in the liver of zebrafish. Such lipid vacuolization is likely due to the perturbed energy homeostasis that was induced by formulated feeds (102). Unfortunately, the optimum dietary lipid requirement of zebrafish has not been investigated in detail (103, 104).

β-glucans and simvastatin were able to reduce the vacuolization in the liver of zebrafish. The ability of β-glucan and simvastatin to mitigate fat deposition in the liver has been reported earlier (105, 106). Our findings indicate that β-glucans have a hypolipidemic effect that extends beyond the intestinal tissue. We also found significant changes in the histology of the mid intestine. A general increase in villi length and thinning of the lamina propria was observed in the HC group compared to the CT group. Like in mammals, the zebrafish villi length is regulated by stem cell proliferation at the base and apoptosis at the tip of the villi. Dysregulated lipid metabolism leading to obesity increases the villi length in humans (107). Suppression of endogenous cholesterol biosynthesis (108) and higher intracellular levels of cholesterol (109) induce hypertrophy in different types of cells. Cholesterol acts as a mitogen for intestinal stem cells, and an increase in cellular cholesterol content activates stem cell proliferation both *in vivo* and *ex vivo* (110). Therefore, dietary cholesterol may have increased the stem cell proliferation in the mid intestine leading to the observed increase in the villi length and an associated lamina propria thinning.

Other Factors That May Impact the Lipid Metabolism and Efficacy of β-Glucans

In the present study, we did not investigate the changes in the intestinal microbiota of the fish. Nevertheless, it is known that gut microbiota regulates the total cholesterol, circulating LDL-cholesterol and triglyceride levels that are considered as risk factors of cardiovascular disease (111). β-glucans lower the LDL, total cholesterol and serum triglyceride levels through the proliferation of intestinal microbes that possess bile salt hydrolase (BSH) activity (112, 113). BSH activity can deconjugate bile acids, that are ligands for farnesoid X receptor (FXR), which is the intestinal bile acid sensor and the controller of liver bile acid production and lipid metabolism (114); this function of FXR is conserved in mammals and zebrafish (115). The positive effects of the β-glucan-driven increase in the BSH activity on gut physiology and lipid metabolism are not yet fully clarified.

The purity of β-glucans and feed manufacturing processes can also govern the efficacy of β-glucans to counter hypercholesterolemia. Several compounds other than β-glucans that are present in oats can modulate the metabolic activity in humans (116, 117). As for the extract from the microalga, *P. tricornutum*, functional carbohydrates other than glucans might have influenced the responses in zebrafish.

In our study, the β-glucans from the two sources were 30% pure and therefore, we cannot rule out the contribution of other molecules in the test products. They might have acted synergistically to evoke the responses that we report here. Studies are being conducted to reduce the residues in β-glucans supplements (118). Furthermore, processing can modify the physicochemical characteristics such as molecular weight, extractability and the resulting viscosity (119). The low-shear extrusion process that was adopted to produce the zebrafish feeds employed a moderate processing temperature (50–60 °C) and fluidised air drying at 80 °C for approximately 10–12 min. Extrusion can alter the branching structure and hence reduce the molecular weight. Although β-glucans of high molecular weight are regarded as efficient in lowering the serum cholesterol and shaping the microbiota to reduce the risk of developing cardiovascular disease (120), the efficacy of β-glucans is dependent on many other factors e.g., residues in the product, molecular structure, viscosity, solubility and the surrounding food matrix (23, 26, 116). Future studies regarding the application of β-glucans should assess the effects of food processing variables on the structural integrity and impact on the biological activity of β-glucans.

In conclusion, our study showed that the intestine transcriptome of the zebrafish was highly sensitive to dietary cholesterol. Furthermore, endogenous cholesterol biosynthesis was suppressed, and ER and mitochondrial functions were affected by the dietary cholesterol. Our transcriptome analyses revealed the efficacy of β-glucans as well as simvastatin to counter hypercholesterolemia, by restoring the expression of several genes that were altered by dietary cholesterol. It seems that the three compounds work to reduce the stress on the ER. While the action of algal glucan is likely via lysosome and cholesterol efflux from the intestine, the oat glucan might have employed the cholesterol catabolism path to move to a normocholesterolemic condition. We also observed a significant adverse effect of simvastatin on the protein metabolism in the intestinal tissue. Future studies should explore the combinatorial effect of the two glucans or one of the glucans and the statin because the investigations will disclose new clues to their synergistic effect to tackle hypercholesterolemia.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The approval for the conduct of this study was obtained from the Norwegian Animal Research Authority (FDU ID: 22992).

AUTHOR CONTRIBUTIONS

VK, JD, AG, and MC: conceptualization and study design. AG, SR, and PS: feeding experiment and tissue sampling. AG and SR: library preparation and bioinformatic analysis. AG and IV: histological analysis. SR: qPCR analysis. AG: biochemical tests. AG and VK: manuscript writing. All authors: manuscript revisions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.797452/full#supplementary-material

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Protective mechanisms of a microbial oil against hypercholesterolemia: evidence from a zebrafish model

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A Western diet elevates the circulating lipoprotein and triglyceride levels which are the major risk factors in cardiovascular disease (CVD) development. Consumption of long-chain omega-3 fatty acids can stall the disease progression. Although these fatty acids can significantly impact the intestine under a hypercholesterolemic condition, the associated changes have not been studied in detail. Therefore, we investigated the alterations in the intestinal transcriptome along with the deviations in the plasma lipids and liver histomorphology of zebrafish offered DHA- and EPA-rich oil. Fish were allocated to 4 dietary treatments: a control group, a high cholesterol group and microbial oil groups with low (3.3%) and high (6.6%) inclusion levels. We quantified the total cholesterol, lipoprotein and triglyceride levels in the plasma. In addition, we assessed the liver histology, intestinal transcriptome and plasma lipidomic profiles of the study groups. The results suggested that higher levels of dietary microbial oil could control the CVD risk factor indices in zebrafish plasma. Furthermore, microbial oil-fed fish had fewer liver vacuoles and higher mRNA levels of genes involved in β-oxidation and HDL maturation. Analyses of the intestine transcriptome revealed that microbial oil supplementation could influence the expression of genes altered by a hypercholesterolemic diet. The plasma lipidomic profiles revealed that the higher level of microbial oil tested could elevate the long-chain poly-unsaturated fatty acid content of triglyceride species and lower the concentration of several lysophosphatidylcholine and diacylglycerol molecules. Our study provides insights into the effectiveness of microbial oil against dyslipidemia in zebrafish.

KEYWORDS

DHA, EPA, cardiovascular disease, plasma lipidomics, RNA seq, bioactive compounds

1. Introduction

Disorders of the heart and blood vessels are grouped under the term cardiovascular diseases (CVDs). According to the World Health Organization, in 2019, about 18 million global deaths were due to CVDs, and most of such mortalities were witnessed in Asia and Europe (1, 2). The primary risk factor that instigates the development of CVDs is unhealthy food, the consumption of which can cause an imbalance in blood lipoprotein species (3). Lipoproteins are the primary carriers of cholesterol and altered lipoprotein levels are consequences of, among other factors, excess cholesterol consumption (4). Circulating cholesterol and lipoprotein levels can be restored

with medication that is effective in hampering the intestinal absorption of excess dietary lipids (5, 6). However, the use of drugs, though effective, may induce severe side effects (7, 8). For instance, orlistat, a well-known pancreatic and gastric lipase inhibitor that prevents the absorption of lipids can, in some patients, induce steatorrhea and vitamin deficiency (9). On the other hand, statins which are used to inhibit cholesterol biosynthesis can cause muscle pain (10). Ezetimibe obstructs the absorption of cholesterol by interfering with the Niemann-Pick C1-Like 1 transporter for cholesterol absorption in the intestine. However, clinical observation of hepatitis has been associated with ezetimibe consumption (7). The side effects of the currently employed drugs indicate the need to identify alternate approaches to stall the progression of CVDs. Dietary bioactive compounds can be used to manage hypercholesterolemia because they act directly on the intestine (11). Furthermore, certain fatty acids can lower the risk of CVDs by increasing the proportion of small high density lipoprotein (HDL) particles, reducing the overload of cholesterol in them and elevating the cholesterol efflux capacity (efficiently performed by small HDL) of the particles (12, 13). Among these fatty acids, the long-chain omega-3 fatty acids, especially eicosapentaenoic acid (EPA, 20:5 $n-3$) and docosahexaenoic acid (DHA, 22:6 $n-3$), are believed to be effective in controlling CVDs (14).

Eicosapentaenoic acid and DHA can exert anti-atherogenic effects by lowering the circulating triacylglycerol (TAG) content (15), which is an independent risk factor that triggers the development of CVDs (16). EPA and DHA-rich fish oil is known to reduce the total cholesterol (17) and low-density lipoprotein (LDL) cholesterol content and increase the circulating high-density lipoprotein (HDL) cholesterol content (18) in the blood without affecting the size of HDL particles (19). However, HDL is a heterogenous population of particles and comprise two major subclasses, namely HDL2 which are larger and less dense compared to HDL3 particles which are smaller in size (20). The quantity of the total HDL cholesterol is dominated by HDL2 compared to HDL3 which is more efficient at reverse cholesterol transport (21). It must be noted that n-3 PUFA supplementation induces an increase in the cholesterol content of the HDL2 and a reduction in the cholesterol of HDL3 (19, 22). Interestingly, there exists an inverse relationship between high-density lipoprotein cholesterol (HDL-C) and the incidence of CVDs (23). HDL particles have the ability to acquire cholesterol from peripheral tissues and transfer it to the liver where it is converted to bile, a process known as reverse cholesterol transport (RCT) (24). One of the key players in RCT activity is hepatic scavenger receptor class B member 1 (SR-B1), but the conformation of apolipoprotein A-I (APOA1) influences HDL binding to SR-B1 (25). Another player in RCT activity is lecithin-cholesterol acyltransferase (LCAT). LCAT catalyzes the conversion of free cholesterol to esterified cholesterol, leading to the maturation of HDL particle. Mature HDL incorporates cholesterol from cells and tissues through its interaction with the SR-BI (to a lesser extent), ATP-binding cassette sub-family G member 1 (ABCG1) and ABCA1 and passive diffusion and transfers cholesterol to the liver *via* its interaction with SR-B1 (26–28). Dietary DHA and EPA impact the circulating HDL and SR-BI and LCAT activities, as observed in different animal models (29, 30). Dietary n-3 PUFAs are known to have a positive correlation with liver SR-B1 expression and the associated RCT (12) and lipids rich in EPA and DHA can interact better with SR-B1 (31).

Furthermore, these omega-3 fatty acids can alter CVD predictors, like Castelli I, Castelli II, atherogenic coefficient and atherogenic index (28, 32) which are considered better markers of CVD progression than absolute lipoprotein or lipid values (33–35). Another important mechanism by which these LC-PUFAs can exert their beneficial effect is by altering the circulating lipid species, which can be identified by studying plasma lipidome. Lipidomic studies have indicated that LC-PUFA consumption can selectively reduce the content of C12, C14 and C16 fatty acids (36) and increase LC-PUFA content of TAGs (37, 38).

The impact of omega-3 fatty acids on lipid metabolism has been revealed by studies that focused on alteration in plasma and liver lipids. Understanding the effect of DHA- and EPA-rich diet on the intestine is essential because it is the primary site where lipid absorption and packaging into chylomicrons and other lipoproteins take place (39). Nevertheless, the ability of EPA and DHA to manage dyslipidemia *via* their influence on the intestine has seldom been studied using appropriate models. To address this need, we investigated the effects of a microbial oil rich in EPA and DHA on the intestine transcriptome. *Schizochytrium* has been investigated in previous studies for its ability to reduce total cholesterol and LDL cholesterol levels (40, 41). Like fish oil (EPA:14.9%, DHA 13% of total fatty acids), *Schizochytrium* oil is also rich in n-3 PUFAs (EPA 15%, DHA 39% of total fatty acids). However, in contrast to fish oil it has a higher content of saturated fatty acids (42, 43). Zebrafish model was used as the experimental species (44) in this study because we wanted to model diet-induced dyslipidemia. Zebrafish is an emerging model species for studying diet-induced dyslipidemia. In terms of morphology as well as the features that aid in digestion and absorption, zebrafish intestine resembles that of mammals (45, 46). A recent study has identified the orthologues of mammalian lipoproteins, and dietary lipids can modulate their expression (47). Therefore, we employed a diet-induced hypercholesterolemia model of zebrafish that simulates the dysregulated lipid metabolism in vertebrates to explore the effects of microbial oil rich in EPA and DHA on the intestine transcriptome. We also studied the plasma lipidomic profiles to understand the changes in the plasma of zebrafish that were fed EPA- and DHA-rich microbial oils. Additionally, we studied the plasma lipidomic profiles to understand the changes in the plasma of zebrafish that were fed EPA- and DHA-rich microbial oils.

2. Materials and methods

2.1. Experimental fish

The approval for the conduct of this study was obtained from the Norwegian Animal Research Authority (FDU ID: 22992). All the experimental procedures involving animals were in accordance with the EU Directive 2010/63 on the use of animals for scientific purposes. To obtain the 240 male zebrafish (6-month-old) required for the study, adult zebrafish (AB line) were bred in-house at the zebrafish facility of Nord University, Norway, following standard protocols (48). Zebrafish eggs were obtained by breeding sexually mature males and females. Fish in five tanks were used for breeding, and in each of these tanks there were 15 males and 30 females. They were community bred and

300–400 eggs were obtained from each tank. The eggs were maintained in E3 medium and incubated at 28°C in an incubator until hatching, i.e., around 50 h post-fertilization. From 4 to 14 days post-fertilization, the larvae were fed (*ad libitum*) the commercial micro diet Zebrafeed® of SPAROS Lda, Olhão, Portugal (< 100 µm particle size) and *Artemia nauplii*. From 15 days post-fertilization (advanced larval stage) onwards, the fish were fed micro diets of 100–200 µm particle size (Zebrafeed®). When the fish were 5-month-old, they were randomly distributed into 24 tanks (6 tanks per treatment group) on a freshwater flow-through system (Zebtec Toxicological Rack, Tecniplast, Varese, Italy) with 3.5 l tank capacity. We selected only male zebrafish for our study as previous reports have indicated sex-based differences in lipid metabolism (49, 50). The stocking density was ten fish per tank. The experimental fish were acclimatized in the flow-through system for 4 weeks during which period they were fed the experimental control diet. The water temperature in the tanks was 28 ± 0.5°C, and the water flow rate was 2.5 l/h. The dissolved oxygen in the tanks ranged between 7 and 8 ppm (oxygen saturation > 85%). A 14 l:10D photoperiod was maintained throughout the experiment.

2.2. Diets and feeding regimen

The four experimental diets were prepared by SPAROS Lda. (Supplementary Table 1): one control diet and three high-cholesterol diets. A standard zebrafish diet without the purified cholesterol served as the control diet, CT. The high cholesterol (HC) diet had 5.1% (*w*/*w*) inclusion of purified cholesterol and 6.6% inclusion of soybean oil. We selected the cholesterol level based on previous studies with zebrafish (51, 52). Two diets were prepared by incorporating the microbial oil derived from *Schizochytrium* sp. (Veramaris, Delft, Netherlands): HCA1 and HCA2 in which the inclusion level of the microbial oil was 3.1 and 6.6%, respectively; replacing 3.5 and 6.6% of soybean oil. The EPA:DHA ratio in the CT and HC diets was 1.45, whereas the ratio in the HCA1 and HCA2 diets were 0.49 and 0.45, respectively (Supplementary Table 1). The daily feeding rate was 4% of the total biomass in the tanks. This ration was split into three feeding events per day—at 08:00, 13:00, and 18:00—to allow the fish to consume the feed instantly so that no leftover feed remained in the tanks during the experimental period of 12 weeks.

2.3. Sampling

At the end of the feeding trial, fish were euthanized by immersion (for 3 min) in 200 mg L^{-1} of tricaine methanesulfonate (Cat. Number: E10521, SigmaAldrich, Saint Louis, U.S.), which was buffered with 200 mg L^{-1} of sodium bicarbonate (Cat. Number: S5761, SigmaAldrich). The blood collected by tail ablation (53) was centrifuged at 5000 *g* for 10 min at 4°C to obtain the plasma. There are seven distinct regions in the gut of zebrafish based on their gene expression profiles. The mid-region of the gut has high expression of genes like *fatty acid binding protein 2, intestinal* (*fabp2*), *apolipoprotein A-Ia* (*apoa1a*) and apolipoprotein A-IV a (*apoa4a*) that are involved in lipid metabolism (54). It has also been reported that the main fatty acid transporter gene *thrombospondin receptor* (*cd36*) and cholesterol homeostasis genes like *apolipoprotein Bb, tandem duplicate 1* (*apobb.1*), *cytochrome P450, family 7, subfamily A, polypeptide* 1 (*cyp7a1*), *apolipoprotein Ba* (*apoba*), *cholesteryl ester transfer protein* (*cetp*) and *apolipoprotein a-Ib* (*apoa1b*) have significantly higher expression in the mid-intestine region of zebrafish compared to the anterior and posterior regions (55). Therefore, mid-intestine was considered for RNA Seq. The whole liver was also dissected and snap-frozen in liquid nitrogen and stored at −80°C for qPCR study.

2.4. Plasma cholesterol and triglyceride estimation

The total, LDL, and HDL cholesterol levels in the plasma were estimated using the HDL and LDL/VLDL Cholesterol Assay Kit (Cat. Number: ab65390, Abcam, Cambridge, United Kingdom). Total triacylglycerides in the plasma were estimated using the Triglyceride Assay Kit (Cat. Number: ab65336, Abcam), according to the manufacturer's instructions. Each treatment consisted of six replicates and each replicate was a pool of plasma from 6 fish per tank. The CVD risk indices were calculated using the following four equations (56):

$$\text{Castelli I index} = \text{Total cholesterol} / \text{HDL cholesterol}. \quad (1)$$

$$\text{Castelli II index} = \text{LDL cholesterol} / \text{HDL cholesterol}. \quad (2)$$

$$\text{Atherogenic index} = \log\left(\text{TAG} / \text{HDL}\right). \quad (3)$$

$$\text{Atherogenic coefficient} = \begin{pmatrix}\text{Total cholesterol}\\ -\text{HDL cholesterol}\end{pmatrix} / \text{HDL cholesterol}. \quad (4)$$

2.5. Intestine RNA-sequencing and bioinformatic analyses

To extract total RNA, the frozen intestine samples were briefly homogenized in QIAzol lysis reagent (Cat. Number: 79306, Qiagen, Hilden, Germany) at 6500 rpm for 2 × 20 s in a Precellys 24 homogenizer (Cat. Number: P000669-PR240-A, Bertin Instruments, Montigny-le-Bretonneux, France). RNA was extracted from the tissue homogenate using Direct-zol™ RNA MiniPrep kit (Cat. Number: R2052, Zymoresearch, CA, United States) following the manufacturer's instructions. The RNA concentration and integrity were checked using Qubit™ RNA Broad Range (BR) Assay Kit (Cat. Number: Q10210, Thermo Fisher Scientific, Waltham MA, United States) with a Qubit 4 Fluorometer (Cat. Number: Q33238) and Tape Station 2,200 (Cat. Number: G2964AA, Agilent Technologies, Santa Clara, CA, United States). Only the RNA samples that had RIN value > 7 were used to construct RNA-Seq libraries. Library preparation and sequencing was performed by Novogene Europe (Cambridge, United Kingdom). The mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamers followed by the second strand

cDNA synthesis. The libraries were end repaired, A-tailed, adapter ligated, size selected, amplified, and finally purified. The libraries were quantified by Qubit and real-time PCR. Furthermore, a Bioanalyzer (Agilent technologies) detected the size distribution. The barcoded libraries were then pooled and loaded on the Illumina NovaSeq 6,000 Sequencing system (Illumina, San Diego, CA, United States) to obtain 150 bp paired end reads. For each sample, a minimum of 20 million paired raw reads were obtained, with an average of 22.3 million reads per sample (Supplementary Table 2). The quality of the raw reads was assessed using the *fastQC* command. Low quality reads (Phred quality score, $Q < 30$) were filtered from the raw reads using the *fastp* software (57). The filtered reads were then aligned to the reference zebrafish genome downloaded from NCBI (release 106) after indexing using HISAT2, version 2.2.1 (58). The average mapping percentage for the whole dataset was 87.5%. The reads were annotated using *featureCounts* to obtain the read counts of each gene (59). Differential expression analyses of the genes in the treatment groups were performed using the R package *DESeq2* (version 1.30.0). Transcripts with an absolute Log_2 fold change of ≥ 1 and an adjusted p value of < 0.05 (Benjamini-Hochberg multiple test correction method) were considered significantly differentially expressed and were used for gene ontology (GO) and KEGG pathway analyses. The GO enrichment was performed with Database for Annotation, Visualization and Integrated Discovery (*DAVID*) version 6.8 (60) and *clusterProfiler* package (version 3.18.0) in R. The R packages *ggplot2* (version 3.3.3), *pheatmap* (version 1.0.12) and *GOplot* (version 1.0.2) were employed to visualize the data. GO term-gene networks were generated using *Cytoscape* 3.8.2 (61).

2.6. Liver histomorphometry

The liver from the experimental fish ($n = 9$–12 per group) was dissected and immediately fixed in 3.7% (*v*/*v*) phosphate-buffered formaldehyde solution (pH 7.2) at 4°C for 24 h. Standard histological procedures were employed for dehydration, processing, and paraffin embedding, as described by Bancroft and Gamble (62). The paraffin blocks thus prepared were sectioned using a microtome (Microm HM355S, MICROM International GmbH, Walldorf, Germany). Four micrometer thick sections were cut and mounted on SuperFrost® slides (Menzel, Braunschweig, Germany). A robot slide stainer Microm HMS 760 × (MICROM International GmbH) was used to stain the liver sections with hematoxylin (Cat. Number: H9627, SigmaAldrich) and eosin (Cat. Number: 861006, SigmaAldrich). Light microscopy photomicrographs were taken with Leica DM3000 LED microscope (Leica Camera AG, Wetzlar, Germany) fitted with Leica MC 190HD camera (Leica Camera AG). The software *ImageJ* (63) was used to analyze the images. Liver vacuolation was assessed by evaluating two parameters—average vacuole area and average vacuole number in randomly selected areas of the liver (64). Shapiro–Wilk and Bartlett's tests were employed to confirm normality and homoscedasticity of the data, respectively. One-way ANOVA was employed where the assumptions were met. In the case of non-parametric data, statistical differences were evaluated using the Kruskal–Wallis test.

2.7. Hepatic gene expression analysis

We performed qPCR to understand the effect of the supplementation of microbial oil on hepatic gene expression, focusing on genes linked to (i) fatty acid β-oxidation in mitochondria and peroxisomes (*cpt1aa*, *acaa*), (ii) intracellular lipid droplets (*plin2*), and (iii) HDL metabolism (*lcat*, *scarb1* and *abca1a*). One μg of total RNA from each sample was reverse transcribed using the QuantiTect reverse transcription kit (Cat. Number: 205311, Qiagen), according to the manufacturer's instructions. The cDNA was further diluted ten times with nuclease-free water and used as a qPCR template. The qPCR reactions were carried out using SYBR green (Cat. Number: 04707516001, Roche Holding AG, Basel, Switzerland) in a LightCycler® 96 Real-Time PCR System (Cat. Number: 05815916001, Roche Holding). Relative expression of selected genes was determined based on the geometric mean of previously reported reference genes (*actb1*, *eef1a1l1* and *rpl13α*) (65) following the protocol described by Livak and Schmittgen (66). We designed the primers for the selected genes using the Primer-BLAST tool in NCBI. The primers were then checked for secondary structures such as hairpin, repeats, self and cross dimers by *NetPrimer* (Premier Biosoft, Palo Alto, United States). The primers for the target genes are listed in Supplementary Table 3. The efficiency of all primers was confirmed by the method described by Pfaffl (67). The data were checked for normality (Shapiro–Wilk test) and homoscedasticity (Bartlett's test), based on which, the statistical difference was determined by one-way ANOVA or Kruskal-Wallis test.

2.8. Plasma lipidome profiling

Lipidome profiling was carried out by MS-Omics (Vedbæk, Denmark). Plasma samples were mixed 1:9 with isopropanol containing 0.1 M benzothiazolone hydrazone and internal standards before transferring to SpinX filters. The samples were then mixed in a vortex for 60 s and left at room temperature for 10 min before placing in a −20°C freezer overnight. The following day, samples were left at room temperature for 30 min before centrifuging (14,000 rpm/5°C/2 min). Finally, the samples were mixed with eluent. The analysis was carried out using a Thermo Scientific Vanquish LC (Thermo Fisher Scientific) coupled to Thermo Q Exactive HF MS (Thermo Fisher Scientific). The lipids extracted from the samples were ionized in positive and negative ionization mode using an electrospray ionization interface. Then, chromatographic separation of lipids was carried out on a Waters® ACQUITY Charged Surface Hybrid (CSH™) C18 column (2.1 × 100 mm, 1.7 μm; Waters Corporation, Milford, United States) at 55°C. The mobile phases consisted of (A) acetonitrile/water (60:40) and (B) isopropanol/acetonitrile (90:10), both with 10 mM ammonium formate and 0.1% (*v*/*v*) formic acid. Lipids were eluted in a two-step gradient by increasing B in A from 40 to 99% over 18 min, and the flow rate was 0.4 ml/min. The obtained peak areas were extracted using Compound Discoverer 3.2 (Thermo Fisher Scientific). Thereafter, compounds were identified at four levels; Level 1: identification by retention times (compared against in-house authentic standards), accurate mass (with an accepted deviation of 3 ppm), and MS/MS spectra; Level 2a: identification by retention times (compared against in-house authentic standards), accurate mass (with an accepted deviation of 3 ppm); Level 2b: identification by accurate mass (with an accepted deviation of 3 ppm), and MS/MS spectra; Level 3: identification by accurate mass alone (with an accepted deviation of 3 ppm). The obtained lipidome data were analyzed employing *MetaboAnalyst 5.0* (68). The data were log transformed and auto-scaled (mean-centered and divided by the standard deviation of

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each variable; Supplementary Figure 1) before downstream analyses. Principal component analysis was performed using the *mixomics* package in *R 4.2.1* to understand the differential clustering of the study groups. A $|\text{Log}_2$ fold change$| \geq 1$ and a p value of < 0.05 were considered to identify the significantly altered lipid species. The over representation analysis (ORA) based on the significantly altered lipids was performed using a reference of 1,072 sub chemical class of lipid sets. The ORA, which employs the hypergeometric test, was performed using the differentially abundant lipid species. A p value cut-off of < 0.05 and a minimum lipid species count ≥ 2 for each lipid set were considered as significantly enriched lipid classes. *Cytoscape 3.9.0* and *ggplot2* package in *R 4.2.1* were employed to present the data.

3. Results

3.1. Microbial oil lowered the cholesterol and TAG levels in the plasma

We examined the effect of two levels of dietary microbial oil (HCA1-low level and HCA2-high level) on the circulating cholesterol and TAG levels in zebrafish. Total cholesterol (values presented as mg dL^{-1}) of the HC group (1544.6 ± 590.9; a model of hypercholesterolemia) was significantly higher ($p < 0.001$) compared to the CT group (386.9 ± 130.1; Figure 1A). The HCA2 group (912.0 ± 400.4) had significantly ($p < 0.05$) lower total cholesterol compared to the HC group. However, the total cholesterol levels of the HCA1 (988.9 ± 308.4) and HCA2 groups were significantly higher compared to the CT group. While the HC group had a significantly ($p < 0.05$) higher plasma LDL cholesterol (values presented as mg dL^{-1}, 365.3 ± 203.5) compared to the CT group (106.0 ± 72.8), the LDL cholesterol level of HCA2 group (147.7 ± 107.9) was not significantly different ($p > 0.05$) from the CT group (Figure 1B). The HCA1 group also had significantly ($p < 0.05$) higher level of LDL cholesterol (371.6 ± 198.27) compared to CT. A significantly higher ($p < 0.01$) amount of HDL cholesterol (values presented as mg dL^{-1}) was found in HCA2 group (260.5 ± 27.7) compared to the CT group (160.4 ± 32.7; Figure 1C). We also found that the HCA2 group had a higher proportion of esterified cholesterol content (0.85 ± 0.1) compared to the other groups though the increase was not statistically significant (Supplementary Figure 2). We further investigated the hypolipidemic effect of HCA2 diet by estimating the TAG levels (values presented as mg dL^{-1}) in the different diet groups. The HCA2 group (416.2 ± 126.5) had a significantly ($p < 0.05$) lower level of triglycerides compared to the CT (675.0 ± 187.0), HC (618 ± 66.1) and HCA1 (697.0 ± 188.5) groups (Figure 1D).

Using the plasma TC, LDL cholesterol, HDL cholesterol and TAG values of the treatment groups, we estimated the different CVD risk

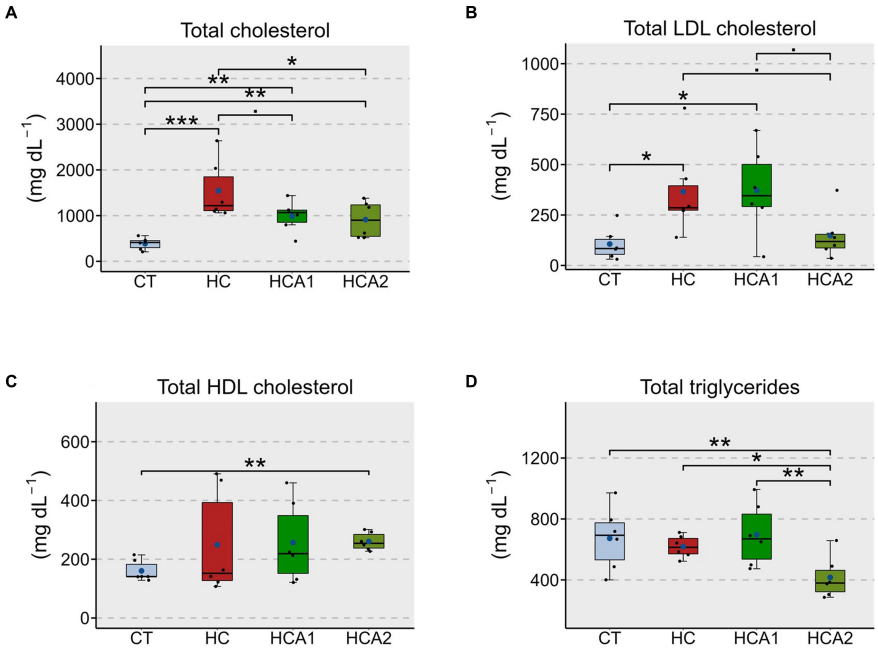

FIGURE 1
Alteration of the plasma lipids in zebrafish fed different experimental diets for a period of 12 weeks. Boxplots show total cholesterol **(A)**, LDL cholesterol **(B)**, HDL cholesterol **(C)** and total triglyceride **(D)** contents in the plasma of the fish. Blue dots inside boxplots indicate the mean values of the corresponding groups. CT-fish fed the control diet; HC-fish fed the high cholesterol diet (5.1% inclusion); HCA1-fish fed microbial oil (3.1% inclusion); HCA2-fish fed microbial oil (6.6% inclusion). ***p<0.001, **p<0.01, *p<0.05, and ·p<0.1. Each treatment group consisted of six biological replicates.

indices: Castelli I index (Equation 1), Castelli II index (Equation 2), Atherogenic index, AI (Equation 3) and Atherogenic coefficient, AC (Equation 4). The HC group had a significantly higher Castelli I index (9.5 ± 7.1) compared to the CT group (2.5 ± 1.0; Figure 2A). The HCA2 group had significantly lower values for all four predictors (Castelli I = 3.52 ± 1.6, Castelli II = 0.57 ± 0.5, AI = 0.43 ± 0.3, AC = 2.52 ± 1.6) of cardiovascular disease (PCVD), compared to the HC group (Castelli II = 2.2 ± 1.7, AI = 1.10 ± 0.7, AC = 8.53 ± 7.1; Figures 2A–D). Furthermore, the HCA2 group had significantly lower atherogenic index compared to CT (1.41 ± 0.4) and HCA1 (1.08 ± 0.7) groups (Figure 2C). Correlation between plasma lipid species and PCVD is presented in Supplementary Figure 3A. HDL was found to have a negative correlation with PCVD. We also observed a significantly positive correlation ($R = 0.69$, $p = 0.00019$) between Castelli I index and LDL cholesterol content (Figure 2E). The correlation between TAG:HDL ratio and LDL cholesterol was also positive ($R = 0.37$) but not significant ($p = 0.086$; Supplementary Figure 3B). The principal component analysis biplot revealed a separation of the HC group from the CT and HCA2 groups along the principal component 1 (PC1) that captured 77% variability (Figure 2F). We also found a separation of the HC1 and HCA2 groups, predominantly driven by differences in the HDL and TAG levels of the two groups. The differences in the CT and HC groups are due to the total cholesterol content.

3.2. HCA2 diet altered the liver histomorphology and gene expression

The relative expression of the *lcat* gene was increased by two-fold ($p < 0.05$) in the HCA2 group compared to the CT, HC and HCA1 groups (Figure 3A). The expression of *scarb1* in the HCA2 group increased 9-fold ($p < 0.05$) compared to the CT, HC and HCA1 groups (Figure 3B). We observed a two-fold increase ($p < 0.05$) in the mRNA level of the gene *cpt1aa* in the HCA2 group compared to the CT and HC groups (Figure 3C). However, we did not detect a statistically significant difference in the relative expression of *plin2*, *acaa* and *abca1a* (Figures 3D–F). We also investigated histological changes in the liver to understand the effect of different diets on vacuolization (Figure 4A). We observed a significantly higher number of vacuoles (Figure 4B) in the HC group compared to CT ($p < 0.001$). Furthermore, both HCA1 and HCA2 groups had significantly lower vacuole number ($p < 0.01$ and $p < 0.05$, respectively) compared to the HC group. Although, the mean vacuole size of the study groups was not

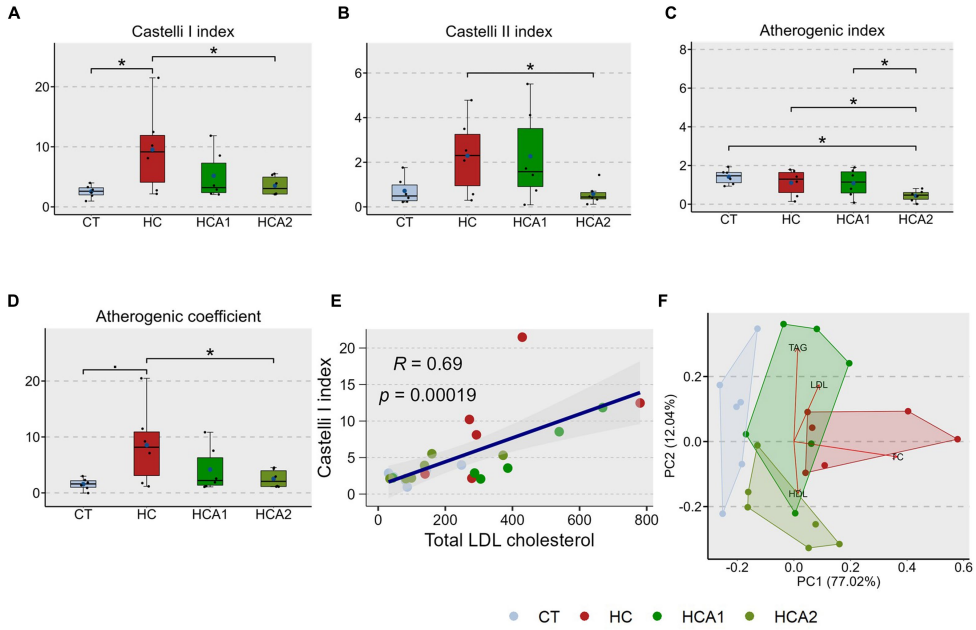

FIGURE 2
Alteration of the cardiovascular disease (CVD) risk indices in zebrafish fed different experimental diets for a period of 12 weeks. Boxplots show Castelli I index **(A)**, Castelli II index **(B)**, Atherogenic index **(C)** and Atherogenic coefficient **(D)** of plasma from the fish. Blue dots inside boxplots indicate the mean values of the corresponding groups. Scatter plot **(E)** shows the correlation between Castelli I index and total LDL cholesterol levels. Biplot **(F)** indicates the separation of the CT and HCA2 groups from the other 2 study groups and variables associated with each group. CT-fish fed the control diet; HC-fish fed the high cholesterol diet (5.1% inclusion); HCA1-fish fed microbial oil (3.1% inclusion); HCA2-fish fed microbial oil (6.6% inclusion). *p<0.05 and *p<0.1. Each treatment group consisted of six biological replicates.

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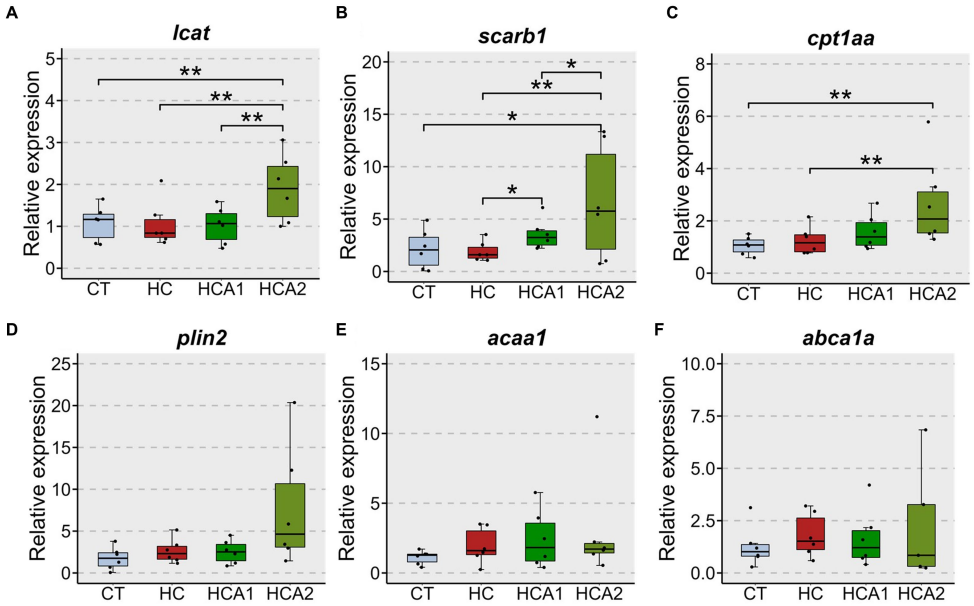

FIGURE 3
Relative expression of selected genes in the liver of zebrafish fed different experimental diets. *lecithin-cholesterol acyltransferase* (*lcat*) **(A)**; *scavenger receptor class B, member 1* (*scarb1*) **(B)**; *carnitine palmitoyltransferase 1Aa* (*cpt1aa*) **(C)**; *perilipin 2* (*plin2*) **(D)**; *acetyl-CoA acyltransferase 1* (*acaa1*) **(E)**; *ATP-binding cassette, sub-family A (ABC1), member 1A* (*abca1a*) **(F)**. Black dots indicate the relative expression of the respective genes in each sample. CT-fish fed the control diet; HC-fish fed the high cholesterol diet (5.1% inclusion); HCA1-fish fed microbial oil (3.1% inclusion); HCA2-fish fed microbial oil (6.6% inclusion). $^{**}p<0.01$ and $^{*}p<0.05$. Each treatment group consisted of six biological replicates.

significantly different ($p > 0.05$; Figure 4C), we detected a significant correlation ($R = 0.57$, $p < 0.001$) between vacuole size and number in the liver of the different diet groups (Figure 4D).

3.3. The intestinal transcriptome reflected diet-induced hypercholesterolemia in zebrafish

We analyzed the intestinal transcriptome of zebrafish from the different treatment groups. The comparison between HC and CT groups revealed 164 differentially expressed genes (DEGs), of which 146 were downregulated and 18 were upregulated in the HC group (Supplementary Table 4). These included *cytochrome P450, family 26, subfamily A, polypeptide 1* (*cyp26a1*), *cubilin* (*cubn*), *3-hydroxy-3-methylglutaryl-CoA reductase a* (*hmgcra*), *steroidogenic acute regulatory protein* and *3-hydroxy-3-methylglutaryl-CoA synthase 1* (*soluble; hmgcs1*). The KEGG pathway enrichment employing the downregulated DEGs revealed a significant suppression of the steroid biosynthesis pathway (Supplementary Table 5). Furthermore, the GO enrichment analysis based on the downregulated DEGs revealed significant enrichment of several GO terms in two separate clusters. One of the clusters was linked to cholesterol metabolism and included terms like cholesterol biosynthetic process, steroid metabolic process and steroid biosynthetic process (Figure 5). The second cluster included GO terms like microtubule cytoskeleton organization, cilium organization, microtubule bundle formation and axoneme assembly. The upregulated genes in the HC group were not found to significantly enrich any KEGG pathways or GO terms.

3.3.1. Microbial oil altered the intestinal transcriptome of the zebrafish model of hypercholesterolemia

Since the microbial oil in the diet was able to lower the plasma total and LDL cholesterol levels in zebrafish, we wanted to understand if these effects can be associated with the intestinal transcriptome. We first compared the intestinal transcriptome of the HCA1 group with that of the CT group. This analysis revealed 177 DEGs, of which 15 were upregulated and 162 were downregulated in the HCA1 group (Supplementary Table 6). GO enrichment analysis employing the downregulated DEGs revealed terms like cholesterol biosynthetic process, steroid metabolic process, and steroid biosynthetic process, microtubule cytoskeleton and microtubule cytoskeleton organization (Figure 6).

Downregulated DEGs-based KEGG pathway enrichment revealed a significant suppression of steroid biosynthesis and terpenoid biosynthesis (adjusted p value < 0.01) in the HCA1 group (Supplementary Table 7). These enriched GO terms and pathways were similar to the results of the comparison between the transcriptomes of the HC and CT groups. Given that the results of the HC vs. CT and HCA1 vs. CT comparisons were similar,

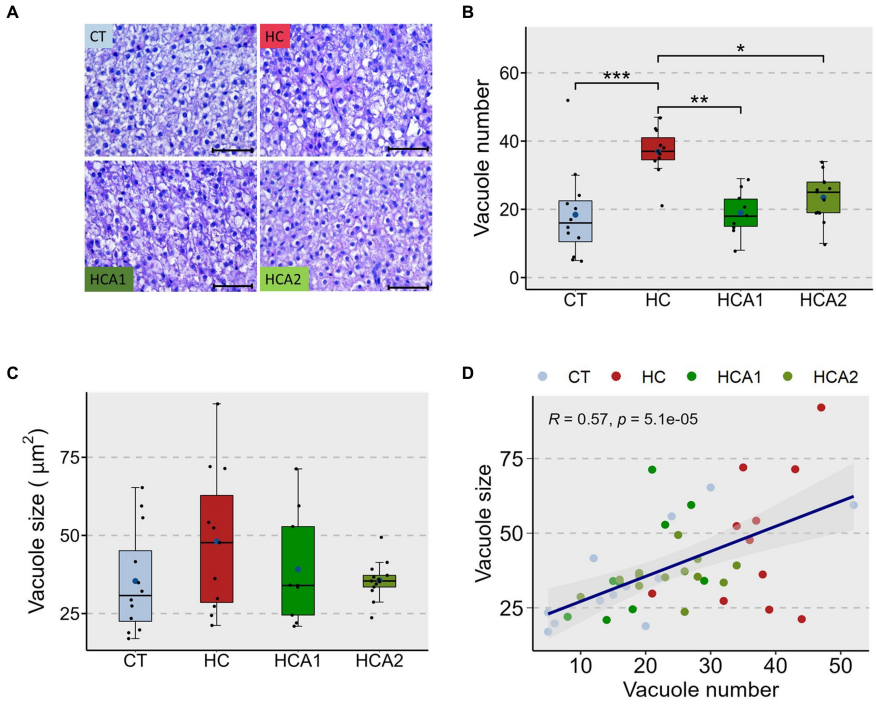

FIGURE 4
Histomorphology of the liver of zebrafish fed different experimental diets for a period of 12 weeks. Representative histological images **(A)** of the liver of zebrafish. Dot-plot shows average number **(B)** and average size **(C)** of vacuoles in the liver of fish fed control (CT) diet, high cholesterol (HC) diet, HC diet supplemented with lower (HCA1) or higher (HCA2) levels of microbial oil. The scatter plot **(D)** shows correlation between average vacuole number and average vacuole size in the liver of zebrafish. ***p<0.001, **p<0.01 and *p<0.05. Each treatment group consisted of 9–12 biological replicates. Scale bar=50µm.

we wanted to know if a higher level of the microbial oil can mitigate the effects of dietary high cholesterol. Hence, we performed the HCA2 vs. CT comparison. This analysis revealed 182 DEGs, of which 162 were downregulated and 20 were upregulated in the HCA2 group (Supplementary Table 8). The GO terms that were enriched by the downregulated DEGs included glucose metabolic process, ADP metabolic process, microtubule cytoskeleton organization, nuclear division and cell cycle process (Figure 7). However, we did not find any enriched GO terms linked to cholesterol metabolism. Steroid biosynthesis and terpenoid biosynthesis pathways were not enriched based on the downregulated DEGs in the HCA2 group. The differentially upregulated DEGs led to the enrichment of KEGG pathways like glutathione metabolism, drug metabolism-cytochrome p450 and metabolism of xenobiotic by cytochrome p450 (Supplementary Figure 4; Supplementary Table 9).

3.3.2. HCA2 diet influenced the expression of genes involved in cholesterol biosynthesis

We first studied the influence of the HCA2 diet on the expression of all the genes that were altered by the high cholesterol diet, i.e., which were differentially expressed in HC vs. CT comparison. Of the 146 differentially downregulated genes in the HC vs. CT comparison, the normalized counts of 13 genes were increased in the HCA2 group (Supplementary Figure 5). Many of these 13 genes are involved in cholesterol biosynthesis. We selected 8 genes that were significantly downregulated in the HC vs. CT comparison (Figure 8A). In the HCA1 group, 6 out of the aforementioned 8 genes were significantly downregulated, compared to the CT group (Figure 8B). However, in the HCA2 group, only one gene was significantly downregulated and 7 were not differentially expressed (Figure 8C) compared to the CT group indicating a possible effect on cholesterol biosynthesis. Hierarchical clustering of the normalized counts of the genes revealed that the gene expression profile of HCA2 group was similar to the CT group (Figure 8D).

3.3.3. HCA2 diet increased the expression of genes involved in lipoprotein metabolism

We also performed a comparison of the transcriptome of the HCA2 and HC groups (Figure 9A). Thirteen DEGs were identified, of which 5 were upregulated and 8 were downregulated in the HCA2 group (Figure 9B; Supplementary Table 10). The GO enrichment analysis of the upregulated DEGs revealed the enrichment of terms like chylomicron, lipoprotein transport and lipid transport (Figure 9C).

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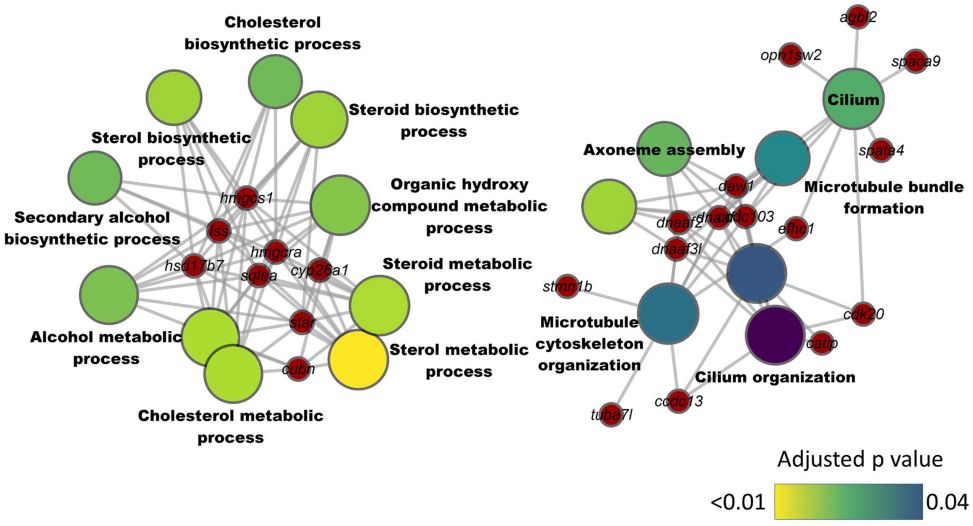

FIGURE 5
Network plot showing the link between enriched GO terms and the associated genes that were downregulated in the intestine of zebrafish fed high cholesterol diet. The significantly downregulated genes from the HC vs. CT comparison are written on the red nodes. Only the non-redundant GO terms are labelled in the network plot. For each GO term, the gradient color varies with the adjusted *p* value (Benjamini–Hochberg method). Adjusted *p* value (Benjamini–Hochberg method)<0.05 and minimum gene count of 2 were set as cut-off parameters for each GO term.

3.4. HCA2 diet altered the plasma lipidome of the zebrafish model

In order to gain deeper insights into the impact of dietary microbial oil supplementation on lipid species in plasma, we compared the global lipidomic profile of the HCA2 group with the HC group. The 331 lipid species annotated at level 1 belonged to 14 different lipid classes (Figure 10A; Supplementary Table 11) and they were used for further analysis. Unsupervised principal component analysis revealed a clear group-based clustering of the samples (Figure 10B). Subsequently, 40 plasma lipid species were identified as significantly altered in the HCA2 group compared to the HC group (Figure 10C; Supplementary Table 11). These 40 significantly altered lipids belonged to 7 classes. Among these were 5 species of TAGs that had significantly higher abundance. On the other hand, 35 species which included cholesterol ester (CE), diacylglycerols (DAG), free fatty acids (FA), alkyl lysophospholipids (LPCO), alkyl phosphatidylcholines (PCO) and sphingomyelines had significantly lower abundance in the HCA2 group compared to HC group (Figure 10D). The HCA2 group had significantly increased TAGs with 22:6 or 20:5 fatty acids. On the other hand, the diacylglycerols which had lower abundance were rich in 18:1, 16:0, 16:1 and 18:2 fatty acids. Furthermore, cholesterol ester 18:3 was also significantly reduced in the HCA2 group. ORA of the significantly altered lipids revealed an enrichment of the TAGs, lysophosphatidylcholines, diradylglycerols and diacylglycerols in the plasma of the HCA2 group (Figure 11A). A correlation network analysis revealed a high correlation between several differentially abundant lipid species. We found that the differentially abundant diacylglycerols, lysophospholipids and phosphatidylcholines were positively correlated. The TAGs were positively correlated among themselves. On the other hand, most of the other differentially abundant lipid species and the differentially abundant TAGs were negatively correlated (Figure 11B).

4. Discussion

An unhealthy diet is a critical risk factor that contributes to the development of CVDs (69). While a Western diet can increase CVD-related fatality, Mediterranean diets rich in omega-3 fatty acids effectively mitigate hyperlipidemia and CVDs (70). With their differential action on transcription factors, the omega-3 fatty acids, EPA and DHA may have independent positive effects on the cardiovascular system (71). Most studies on human and animal models investigated the effects of omega-3 fatty acids in fish oil on TAGs and lipoproteins (72, 73). *Schizochytrium* sp. are marine heterotrophic microorganisms rich in n-3 PUFAs. *Schizochytrium* oil contains about 15 and 39% of EPA and DHA, respectively compared to 14.9 and 13% in fish oil and the EPA:DHA ratio in *Schizochytrium* oil is 0.4 and that in fish oil is 1.1 (42, 43). There are several studies which have evaluated the effect of *Schizochytrium* against dyslipidemia. A systematic analysis revealed that marine microorganism-derived oils can reduce the circulating TAG and increase HDL cholesterol in humans (74). But these human studies have been limited to blood lipid values and have not explored the deeper mechanisms of action in the intestine and liver. *Schizochytrium* oil and fish oil could reduce the levels of total

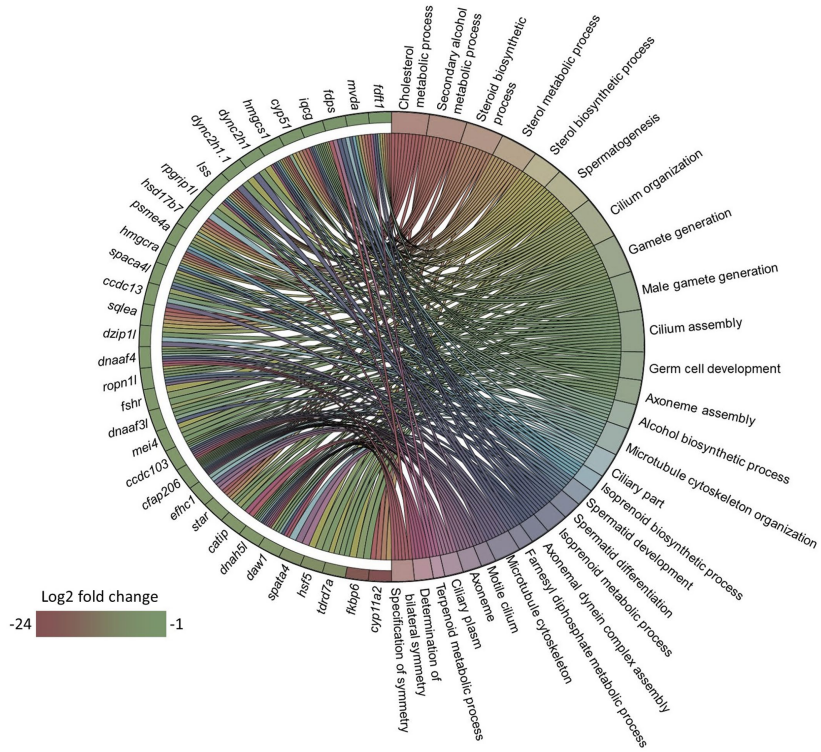

FIGURE 6
Gene ontology (GO) term enrichment based on the downregulated genes in the HCA1 group. Chord diagram showing the link between the enriched GO terms and the associated downregulated genes from the HCA1 vs. CT group comparison. Genes that were considered for the enrichment analyses are shown on the left half of the circle. The gradient color bar intensity varies with the Log_2 fold change.

cholesterol and LDL cholesterol in the plasma of rats, but the triglyceride content was found to reduce only in rats that consumed *Schizochytrium* oil. This microbial oil also increased the expression of genes such as *Insulin-induced gene 1* (*Insig-1*) and *LDL receptor* (*Ldlr*) in the liver of rats, whereas the fish oil could only induce the expression of the latter gene (40). However, *Schizochytrium*-derived oil is also rich in pro-atherogenic palmitic acid which is about 30% of the total fatty acids (42). To understand the effectiveness of the whole oil in preventing CVDs, we used the zebrafish model of hypercholesterolemia, which is characterized by key biomarkers like elevated plasma lipid species and aberrated lipid metabolism (64). We focused on the effect of dietary microbial oil supplementation on the liver, mid-intestine and plasma parameters. The results indicate that the tested high level of the microbial oil can keep the levels of plasma cholesterol and lipoproteins in check. Intestinal transcriptome comparisons also pointed to the effectiveness of microbial oil in alleviating the effects of hypercholesterolemia. Plasma lipidomic analysis revealed a significant increase in the LC-PUFAs of the TAG. This study provides insights into the mechanisms of mitigation of hypercholesterolemia by *Schizochytrium* oil.

4.1. Microbial oil affected the plasma lipid species and hepatic gene expression and vacuolization

We found that a higher level of microbial oil can lower the plasma TC, LDL-C and TAG levels in zebrafish fed a high-cholesterol diet. Plasma TC correlated positively with LDL-C but not with HDL-C levels. Hence, the reduction in TC was likely driven by the decreased plasma LDL-C of the HCA2 group, probably due to the uptake of LDL-C by the peripheral tissues (75). Studies in humans have indicated that elevating the HDL-C concentration without decreasing triglycerides may not prevent CVDs (76). We observed both higher plasma HDL and lower TAG content in the HCA2 group compared to the HC group. There are several mechanisms by which DHA can restore dyslipidemia, including stimulation of β-oxidation (58) and reverse cholesterol transport. We found that the expression of *cpt1aa*, which encodes a mitochondrial transmembrane enzyme required for beta oxidation, was higher in the liver of the HCA2 group. We also found significantly higher amounts of HDL cholesterol in the plasma and increased mRNA levels of *lcat* and *scarb1* in the liver of the HCA2 group. The activity of LCAT and

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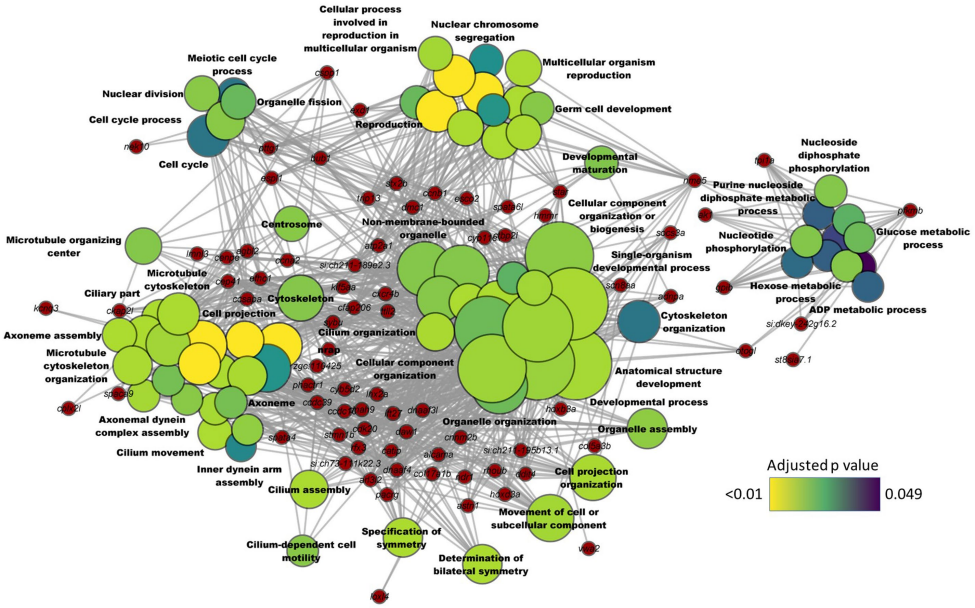

FIGURE 7
Network plot showing the link between enriched GO terms and the associated genes that were downregulated in zebrafish fed microbial oil (HCA2) supplemented diet. The significantly downregulated genes (HCA2 vs. CT) are written on the red nodes. Only the non-redundant GO terms are labelled and for each GO term, the gradient color varies with the adjusted p value (Benjamini-Hochberg method). Adjusted *p* value (Benjamini-Hochberg method)<0.05 and minimum gene count of 2 were set as cut-off parameters for each GO term.

SCARB1 is critical for reverse cholesterol transport, a mechanism by which the body removes excess cholesterol by delivering it to liver (26). Therefore, the increase in plasma HDL and increased expression of *cpt1aa*, *lcat* and *scarb1* in the liver of the HCA2 group may have contributed to reduced circulating TAG. Diets rich in lipids can increase the circulating HDL cholesterol by increasing its biosynthesis and reducing the breakdown rate, as reported in a rat study (77) and human studies (78, 79). However, we did not find any significant increase in HDL concentration in the plasma of zebrafish after feeding a high cholesterol diet. Our previous study also confirmed that plasma HDL concentration in zebrafish remains unaltered after a high cholesterol feeding (64). Like other teleosts, the plasma of zebrafish has an HDL dominant lipoprotein profile (80). Nevertheless we find reports of increase in HDL fraction after feeding with a high cholesterol diet (51).

Although abnormal levels of circulating lipoproteins are considered indicators of CVD development, these parameters are not ideal CVD biomarkers. Ratios of plasma lipoproteins are regarded as better suited in predicting cardiovascular risk in humans (81). In our study, the plasma Castelli risk indices (I and II), atherogenic coefficient and atherogenic indices were significantly improved in the HCA2 group. These ratios are considered reliable parameters in predicting cardiovascular diseases (82, 83). We did not find any significant correlation between TAG:HDL-C ratio and LDL-C content of plasma. However, the Castelli I index was significantly correlated with the LDL-C values. When consumed excessively, lipids will accumulate in the liver (84). Excess cholesterol is accumulated as lipid droplets in hepatocytes, through the action of enzymes located mainly in their endoplasmic reticulum (85), as observed in humans (86) as well as several model species (87, 88). Such excess accumulation of lipids in the liver can lead to lipotoxicity (89). The associated abnormal responses include organellar dysfunction (90), abnormal activation of intracellular signaling pathways (91), chronic inflammation (92) and apoptosis (17, 93). In the present study, 6 month-old zebrafish had significantly more hepatic vacuoles when fed high cholesterol than the control diet. This outcome was not evident in our previous study with 1-year-old zebrafish (64), probably because liver vacuolization increases with age (94, 95). Hence, 6-month-old adult zebrafish can be considered a suitable model for understanding the effects of hypercholesterolemia. The application of two levels of dietary microbial oil resulted in vacuolization to the same extent as noted in the control group. A previous study has indicated that ≥0.5% DHA can increase β-oxidation in the liver of zebrafish (96) which will lead to reduced vacuolization. Increase in the expression of the SR-BI is also associated with a reduction in liver vacuolization (97, 98). In our study, β-oxidation-associated gene *cpt1aa*, and the HDL metabolism-linked gene *scarb1* were upregulated in the HCA2 diet group but not in the HCA1 group, even though vacuolization was reduced in both the groups compared to the HC group. This indicates an alternate vacuole-reduction mechanism in the HCA1 group compared to the abovementioned alteration in the HCA2 group.

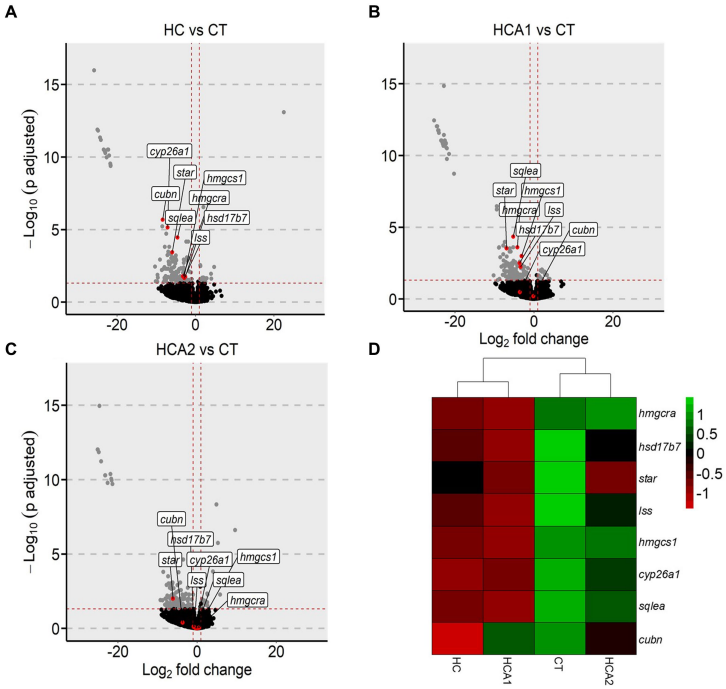

FIGURE 8
Alteration of genes related to cholesterol biosynthesis in zebrafish fed high cholesterol diet with and without microbial oil. Volcano plots highlighting the fold-changes in the intestinal cholesterol biosynthesis-related genes in **(A)** HC vs. CT, **(B)** HCA1 vs. CT and **(C)** HCA2 vs. CT transcriptome comparisons. Heatmap **(D)** showing hierarchical clustering of CT, HC, HCA1 and HCA2 groups. Each treatment group consisted of at least five biological replicates.

4.2. High level of microbial oil can favorably maintain cholesterol metabolism and strengthen antioxidant capacity

In the present study, the HCA1 diet had 3.1% and the HCA2 diet had 6.6% of the microbial oil. We did not study the effect of inclusion of more than 6.6 g of *Schizochytrium* oil/100 g feed. Toxicological studies have not revealed any adverse effects of *Schizochytrium* in rats and pigs, when fed at 3,343 mg/kg/day and 1,121 mg/kg/day, respectively (99, 100). In the present study, *Schizochytrium* oil was fed at a rate of 2,640 mg/kg/day to zebrafish. Furthermore, the oil is considered safe for human consumption (101). Although both diets prevented lipid infiltration into the liver, only the blood lipid profile of the HCA2 group was similar to that in the CT group. As for the genes linked to cholesterol biosynthesis, the intestinal expression of 2 and 7 genes in the HCA1 and HCA2 groups, respectively, was similar to those in the CT group. Compared to the CT group, the HC group had higher plasma TC and LDL-C but caused a suppression of genes linked to cholesterol biosynthesis in the intestine. On the other hand, the lower plasma TC and LDL-C levels along with the unaltered expression of cholesterol biosynthesis genes in the HCA2 group is likely pointing to a greater efficacy of the higher level of dietary microbial oil. This proposition is strengthened by the observation on the upregulation of genes linked to lipoprotein transport, lipid transport and chylomicron in the HCA2 group compared to the HC group, notably due to the upregulation of *apobb.1* and *apoc2* genes in the HCA2 group. The protein coded by the *apobb.1* gene in the intestine is the carrier of absorbed neutral lipids, cholesteryl esters, and TAGs. Templehof et al. (102) revealed that the deletion of *apob* genes in zebrafish can increase lipid infiltration into the liver. The protein coded by *apoc2* is an activator of the lipoprotein lipase enzyme which is in turn needed for the hydrolysis of plasma TAGs, thereby clearing TAGs in circulation. In zebrafish, loss of *apoc2* can lead to hyperlipidemia (103). The upregulation of the expression of *apobb.1* and *apoc2* genes along with the unaltered expression of cholesterol biosynthesis genes in the HCA2 group indicate the need for a high level of microbial oil to counter hyperlipidemia. Microbial oil (HCA2 diet group) caused alteration of genes revealed the enrichment of KEGG pathways like glutathione metabolism, drug metabolism-cytochrome p450 and metabolism of xenobiotic by cytochrome p450. These pathways were enriched because of the upregulation of *microsomal glutathione S-transferase 1.1* and *microsomal glutathione S-transferase 1.2* genes in the intestine of zebrafish. Overexpression of microsomal glutathione S-transferase can provide protection against cytotoxicity and oxidative stress (104). Fish oil supplementation, which is also a rich source of long chain PUFAs, can increase the gene

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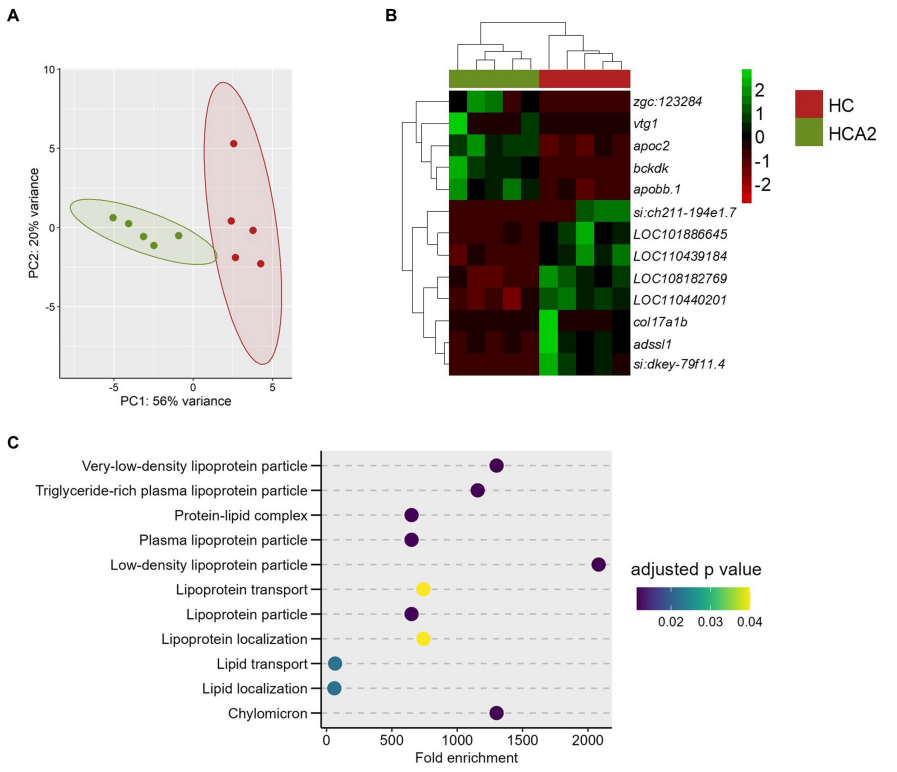

FIGURE 9
Differences in the transcriptome of the HCA2 and HC groups. Principal component analysis **(A)** and Hierarchical clustering **(B)** of the differentially expressed genes (DEGs) in the HCA2 group compared to the HC group. Transcripts with an adjusted *p* value (Benjamini–Hochberg method)<0.05 and $|Log_2$ fold change$|\geq 1$ were considered significantly differentially expressed. Dot-plot **(C)** showing the enriched GO terms, by considering the upregulated genes in the HCA2 vs. HC group comparison. Each treatment group consisted of at least five biological replicates.

expression levels of glutathione transferases to defend against reactive oxygen species production (105). Therefore, our results suggest that the inclusion of microbial oil in the diet activates the antioxidant system in the intestine possibly to combat inflammation and oxidative stress caused by the high cholesterol diet (52, 106).

4.3. Changes in lipidomic profiles mark the protective effect of microbial oil

Lipid-rich diets can cause dyslipidemia that leads to the development of CVDs (107, 108). Recent lipidomic studies on zebrafish have identified many tissue specific lipids; 508 lipids in the liver cells of zebrafish (109), 898 lipids in 7 days old larvae (110) and 2,112 lipids in the right optic nerve of adult zebrafish (111). To our knowledge, this is the first study on the plasma lipidome of zebrafish, reporting 331 lipid species. Most of the 40 differently altered lipid species had lower abundance in the HCA2 group and 5 lipid species of the class TAGs had higher abundance in the HCA2 group compared to the HC group. While reduced serum n-3 PUFA is a CVD risk factor (112), higher circulating DHA levels can lower the risk (113). Dietary omega-3 PUFAs were found to increase n-3 PUFA incorporated plasma and liver phosphatidylcholine, lysophosphatidylcholine, and cholesteryl esters (37). Other studies have identified triglycerides with fewer double bonds and TAGs rich in stearic acid, a saturated fatty acid, as strong predictors of cardiovascular events (114, 115). In our study, the 5 species of triacylglycerols that had higher abundance in the plasma of the HCA2 group were rich in C22:6 and C20:5 fatty acids. Similarly, the proportion of plasma triglycerides containing LC-PUFAs was increased in humans who consumed fish oil (38). Our results indicate a possible dietary microbial oil-induced increase in the LC-PUFA-rich plasma TAGs. Docosapentaenoic acid (C22:5), stearic acid (C18:0) and oleic acid (C18:1) were the three free fatty acids that had significantly lower abundance in the HCA2 group compared to the HC group. Although free stearic acid and oleic acid are generally not considered pro-atherogenic fatty acids (116), EPA + DHA consumption can reduce the content of these fatty acids in plasma of humans (117). We also found a significant reduction in the different species of alkyl lysophosphatidylcholines (LPCO) in

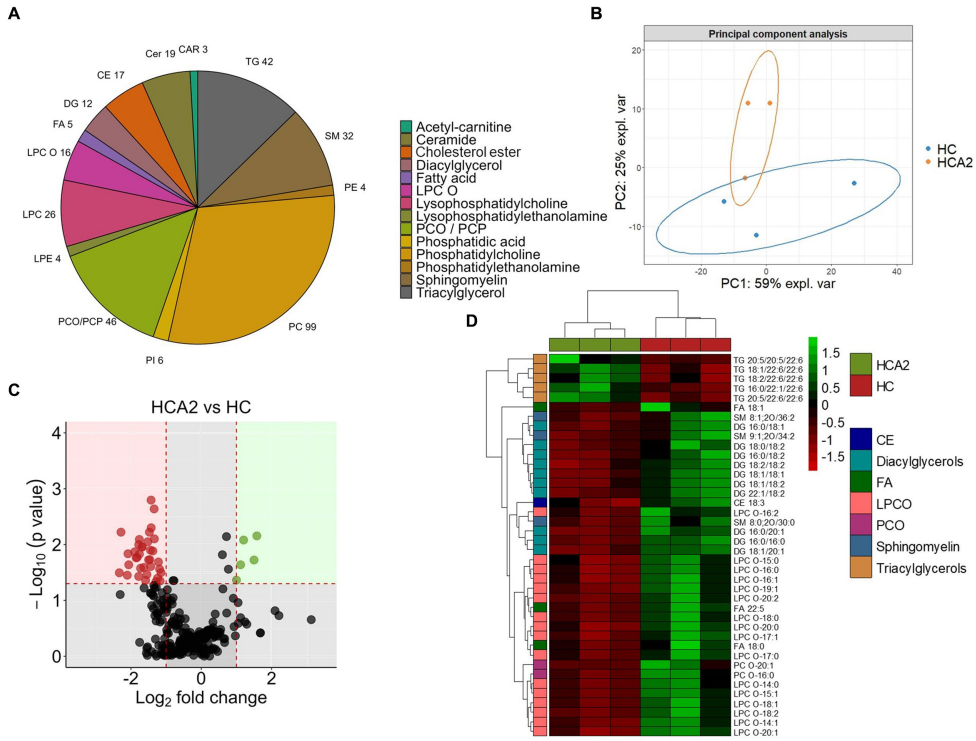

FIGURE 10
Differences in the lipid classes and species in the plasma of zebrafish fed the high cholesterol diet with and without microbial oil for a period of 12 weeks. Pie chart **(A)** of lipid classes in the plasma of zebrafish fed the high cholesterol (HC) diet and microbial oil supplemented (HCA2) diet. PCA **(B)** of the lipidome data from the 6 plasma samples that were used for the analysis (n=3 per group). The first two components captured 59 and 25% of the variation of the data, respectively. **(C)** Volcano plot illustrating the differentially abundant ($|Log_2$ fold-change$|\geq 1$, p value <0.05) lipid species in the HCA2 vs. HC diet group comparison. Red dots indicate downregulated and green dots indicate upregulated lipid species in the HCA2 group compared to the HC group. Heatmap **(D)** showing diet-group based hierarchical clustering of 40 differentially abundant lipid species.

the HCA2 group. These molecules are involved in a broad range of physiological processes and increased LPC levels are biomarkers of dysregulated lipid metabolism (118, 119). Hence, the reduced LPCO levels compared to the corresponding values in the HC group reflects a normal lipid metabolism in the HCA2 group. Several diacylglycerols (DAGs) containing C16 and C18 fatty acids also had lower abundance in the plasma of the HCA2 group. Although, early reports have indicated that dietary DAGs can be beneficial to prevent dyslipidemia (120, 121), circulating levels of DAGs are linked to specific diseases. For instance, liver diseases are associated with increase in plasma C18:1 and C16:1 containing DAGs (122). Furthermore, in humans, metabolic syndrome was correlated with higher plasma C14:0, C16:0 and C18:0 containing DAGs (123). As observed in our study, fish oil supplementation was found to alter the DAG levels in high fat diet fed rats (124). Some lipid species like sphingomyelin, SM 8:1;2O/34:2 and LPC C18:1 have been reported as risk factors of cardiovascular diseases (114). The reduction of these key lipid species in the plasma of zebrafish indicates the effectiveness of microbial oil supplementation against dyslipidemia.

4.4. Microbial oil may not prevent the alteration of cytoskeleton organization

Transcriptomic analyses revealed a consistent enrichment of GO terms linked to microtubule organization based on the downregulated DEGs in the HC, HCA1 and HCA2 groups compared to the CT group. Microtubules, the polarized filament proteins which form the cytoskeleton, are critical for maintaining the polarity of enterocytes (125). Cholesterol is an integral part of the plasma membrane, and it also contributes to the apical polarity of the enterocytes (126). In addition, cholesterol is part of membrane microdomains termed lipid rafts that also contain saturated phospholipids and sphingolipids including glycolipids and sphingomyelin. Cholesterol-enriched rafts are required for cytoskeleton rearrangements (127). Several lines of evidence have indicated that changes in cholesterol metabolism may affect membrane-cytoskeleton interactions (128, 129). In line with our finding, other studies have also documented cholesterol-induced alteration of the abundance of cytoskeletal proteins (130, 131). It seems that both levels of microbial oil were unable to abate the dietary cholesterol-induced suppression of cytoskeletal genes. This indicates that dietary cholesterol

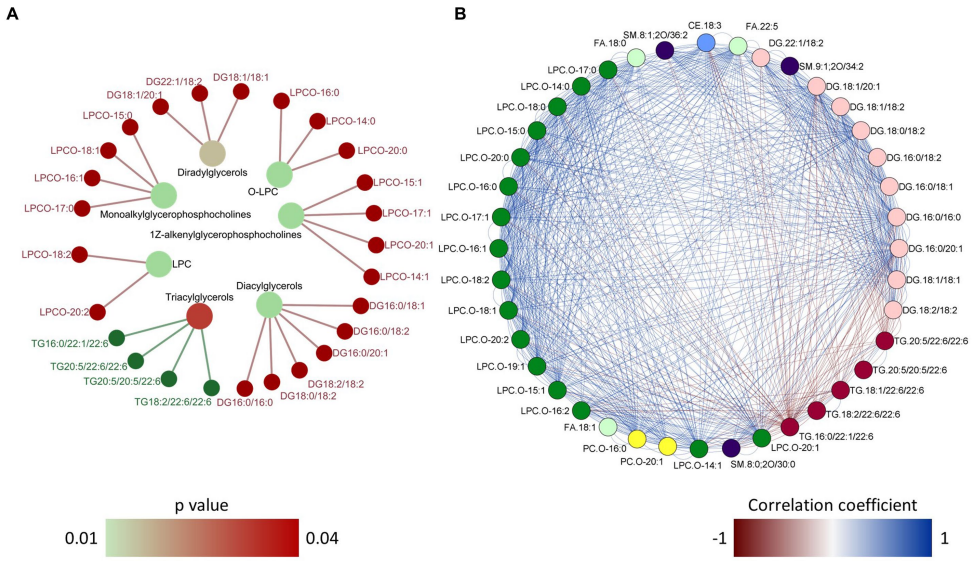

FIGURE 11
Over representation analysis and correlation network of the differentially abundant lipid species. Network plot **(A)** showing the link between the enriched lipid classes and differentially abundant lipid species in the plasma of zebrafish. Over representation analysis was performed using a cut-off of p value < 0.05 after adjusting for multiple testing. The color of the nodes in the outer circle indicate the lipid species that had higher and lower abundance in the HCA2 group compared to the HC group. The gradient color varies with the p value for each enriched lipid class. Network plot **(B)** showing the correlation between the 40 differentially abundant lipid species. The color of the edges indicate positive (blue) or negative correlation. A particular lipid class is color coded and only the links with correlation coefficient greater than 0.75 or less than 0.75 and p value of < 0.05 are shown in the network.

imparts a negative effect on the cytoskeletal elements despite the intervention with the microbial oil. Therefore, a deeper understanding of the impact of cholesterol on the cytoskeletal organization is needed to unravel the tenacious effects of hypercholesterolemia.

5. Conclusion

Taking advantage of a zebrafish hypercholesterolemic model, we demonstrated how a novel EPA and DHA-rich microbial oil could control the negative effects of a high-cholesterol diet. *Schizochytrium*-derived oil impacted the expression of genes involved in lipid metabolism in the liver. Plasma lipidomic profiling revealed the efficacy of the microbial oil in increasing the LC-PUFA content of triacylglycerol species, lowering of the alkyl lysophosphatidylcholine species and several diacylglycerols. The dietary microbial oil-based approach demonstrated through this study, mainly to tackle disrupted cholesterol metabolism, holds promise for a vast majority of the human population afflicted by CVD-associated risks.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, Sequence Read Archive, PRJNA944406.

Ethics statement

The approval for the conduct of this study was obtained from the Norwegian Animal Research Authority (FDU ID: 22992).

Author contributions

VK, JD, and AG: study design. JD: feed preparation. SR and AG: feeding experiment. SR: qPCR analysis. AG: histological analysis. SR and AG: bioinformatic data analysis. AG and VK wrote the manuscript. JF, PO, and MS edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

JD was employed by SPAROS Lda.

The remaining 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.

The handling editor SM declared a past collaboration with the author VK.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1161119/full#supplementary-material

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Every year approximately 18 million people die from heart diseases, making them the most common non-communicable disease. Long-term consumption of fatty foods can alter the normal blood lipid profile by increasing 'bad cholesterol' levels and reducing 'good cholesterol' levels in the blood, a condition known as dyslipidemia. Dyslipidemia is a major risk factor for the development of heart diseases. Although several drugs have been developed to prevent dyslipidemia, they are often associated with side effects. The search for diet-based approaches to controlling dyslipidemia has therefore attracted the interest of researchers. The objective of the research presented in this PhD thesis was to determine whether two dietary compounds, microbial ß-glucans and microbial oil, are effective in preventing the development of dyslipidemia. To explore this, two microbe-derived bioactive compounds were fed to zebrafish, which, like humans, can develop dyslipidemia and fatty liver through the consumption of a cholesterol-rich diet. The results indicated that both microbial ß-glucans and microbial oil can reduce the 'bad cholesterol' level and raise the 'good cholesterol' level in the blood. Furthermore, microbe-derived ß-glucans and oils can reduce the fat droplets in the liver and shape the lipid profile during dyslipidemia.

Thus, this PhD project provides interesting information about the suitability of zebrafish as a research model for studying the efficacy of bioactive compounds in preventing non-communicable diseases. The findings presented in this thesis are quite interesting, so it is expected that the generated knowledge will be used to develop new treatments and preventative measures.

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