Antibacterial and Antioxidant Activity of Metabolites From Bioconverted Docosaheanoic Acid Using Gut Bacteria

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Abstract

Docosahexaenoic acid (DHA) is an essential fatty acid necessary for brain development in both infants and adults. However, the role of gut microbiome and their metabolites produced from DHA remain unclear. In present study, the bacterial isolates *Lactobacillus spp.*, *Clostridium spp.*, *Escherichia coli*; *Staphylococcus spp.*, *Enterococcus spp.* were used to convert the metabolites from DHA with SM medium supplemented with 200mg of DHA as substrate. The metabolites were extracted after 24 hours of incubation at 37°C and analyzed by GC/MS. The antimicrobial activity of these metabolites confirmed their effectiveness against clinical pathogens such as *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis* and *Staphylococcus aureus*.

Introduction

DHA (docosahexaenoic acid) is an important nutrient needed during critical life stages (such as lactation) and for immunity [1]. It is vital during pregnancy and infancy because it plays a critical role in the brain development [2]. DHA accounts for over 90% of the omega-3 fatty acids in human brain, as well as up to 25% of its overall fat content. It is primarily found in cell membranes, where it improves the fluidity of the membranes and gaps between cells. This allows nerve cells to transmit and receive electrical signals more easily [3]. Therefore, adequate levels of DHA appear to make nerve cell communication simpler, faster, and more effective. Low levels in the brain or eyes may cause communication between cells to slow down, resulting in blurry vision or impaired cognitive function. DHA is essential for brain tissue growth and function, especially during infancy and development [4]. For the eyes and brain to grow normally, it accumulates in the central nervous system. The baby's DHA levels are determined by its ingestion during the third trimester of pregnancy, with the largest accumulation occurring in the brain during the first few months of life [5]. Antibacterial activity is exhibited by DHA derivatives through a variety of mechanisms, all of which mainly involve bacterial cell membrane perturbation. DHA has anti-inflammatory properties and prevents tumorigenesis, in addition to its antimicrobial and antiviral properties [6, 7, 8, 9]. Following cerebral ischemia, the nerve cell membrane releases DHA, which induces oxidative stress and neurotrophin activation, and is metabolized to NPD1-like DHA, which prevents ischemic nerve cell death. These findings suggested that DHA combined with NPD1 could prevent ischemic neuronal damage [10].

Later, DHA having a higher unsaturation level than EPA, was demonstrated to have an inhibitory effect on gram negative bacteria that surpasses that of EPA [11]. Long chain fatty acids are well known to be inhibitory on gram positive bacteria even at low concentrations. However, gram negative bacteria are known for their complex lipopolysaccharide layer as compared to the gram positive bacteria. But, PUFA are known to have inhibitory effect on these strains as compared to saturated fatty acids as they are readily incorporated into the outer cell membranes of these organisms, where they significantly increase membrane fluidity. It is possible that by opening up permeability channels, the concentration gradients necessary between the organism and its environment may be dissipated resulting in fatality of the organism [12]. Among these, DHA is one of the most effective fatty acid compounds. In addition to its documented antimicrobial and antiviral properties, DHA possesses anti-inflammatory activity and inhibit tumorigenesis [6, 7, 8, 9]. Although microbial bioconversion of EPA and DHA was reported but the antimicrobial activity of bioconversion products have not been investigated so far [11]. The microbicidal activity of selected LCUFAs and their derivatives has been reported on various enveloped viruses, parasites and pathogenic bacteria such as *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Listeria monocytogenes*, *Helicobacter pylori*, *Staphylococcus aureus* and *Neisseria gonorrhea* [6, 11]. Fish oil decreases the proliferation of tumor cells, whereas arachidonic acid, a long chain n-6 fatty acid, increases their proliferation [13]. Therefore, in this study, we investigated whether the antimicrobial properties of DHA derivatives against clinical isolates, thereby supporting their use in the treatment of chronic infection in patients.

Materials And Methods

Growth Media

The following ingredients were present in the Sole Carbon Source (SCS) media used to grow normal gut flora (in grams per liter): 5 g $(\text{NH}_4)_2\text{SO}_4$, 3 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 mg EDTA, 4.5 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 4.5 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1 mg H_3BO_3 , 0.4 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.3 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.3 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.1 mg KI; The pH was adjusted to 5.5 using HCL, and the media was sterilized through a 0.22 μm filter. Solid medium was prepared by adding 15 g agar per liter of liquid SCS media followed by autoclaving at 121°C [14].

Fecal Sample Collection

The stool samples were taken from four healthy volunteers ranging in age from 20 to 75 years' old who lived in the same neighborhood. Within 15 h of collection, samples were sent to the researchers, packed with parafilm, then transported and stored in ice.

Isolation and Characterization of Gut Microbial Flora from Human Fecal Sample

Approximately 125mg of fecal material was dissolved in 5mL of SCS media. To avoid the transfer of residual alternative carbon sources present in the original inoculum, samples were transferred (2.5 μL) into fresh SCS media (5mL) and incubated at 37°C for 24–48 h. The isolates from the liquid cultures were obtained by plating cultures on an SCS agar medium plate and incubated at 37°C for 24–48 h [14]. The single colonies were picked and re-streaked on SCS plates and further isolates were identified by morphological and biochemical characteristics.

Bioconversion of Docosahexaenoic Acid

Bioconversion was described previously [11] and carried out in five set 50 mL of SM broth with supplement of DHA tablet which contains 200mg of DHA were added to 24 h old cultures of five different gut bacteria to the five set of SM broth individually and followed by continued incubation for an 24 h at 37°C and bioconversion was allowed to proceed.

Extraction of Fatty Acids from Bio-converted Broth

Bio-converted broth were suspended in 3mL of 4 molL⁻¹ sodium hydroxide, and incubated at 90°C for 90 min. After cooling, the pH of the sample was adjusted to 2 with Hydrochloric acid. Fatty acids were then extracted by adding 2 mL anhydrous diethyl ether and separated by centrifugation at 5500 × g for 10 min. The upper phase was removed and dehydrated by adding anhydrous sodium sulfate. The dehydrated fatty acids were collected and dried under a stream of nitrogen. Next, 50 µLbistrimethylsilyltrifluoroacetamide (BSTFA) was added, and the mixture was incubated at 70°C for 30 min and dried under a stream of nitrogen. The fattyacids were dissolved in 100 µL hexane for GC/MS analysis [15].

GC-MS Analysis

Fatty acid composition analysis was performed on the Shimadzu GCMS QP 2020 that employed a fused silica column, packed with SH-Rxi-%Sil MS (30 m × 0.25 mm ID × 250µmdf) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 280°C during the chromatographic run.1µL of extract sample injected into the instrument the oven temperature was as follows: 40°C (2 min); followed by 280°C at the rate of 10°C min⁻¹ and 280°C, where it was held for 3 min. The mass detector conditions were: transfer line temperature 280°C; ion source temperature 230°C; and ionization mode electron impact at 70 eV, a scan time 0.2 s and scan interval of 0.1 s. The fragments from 40 to 550 Da. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2017) library.

Antibacterial activity of bio-converted DHA extract

The antimicrobial activity of the compound was determined using agar-well diffusion method. The microorganisms *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis* and *Staphylococcus aureus* were obtained from the SKS Clinical Laboratory, Salem, Tamil Nadu, India. The Gentamycin containing discs (Nam KhoaBioTek Company, Vietnam) (10µg/mL) were used as positive controls and negative controls were sterilized distilled water containing paper discs. 900µg of the bio-converted DHA extract was weighed and dissolved in 10mL Dimethyl sulfoxide (DMSO) to obtain a concentration of 90µg/mL of the extract; this was the initial concentration of the extract used to determine the antimicrobial activity of the extract. Mueller Hinton agar was prepared by following the medium sterilized at 121°C for 15 min; the sterilized medium was then poured into sterile petri dishes. The plates were allowed to cool and solidify. The sterilized medium was seeded with 0.1mL of the standard inoculum was spread evenly over the surface of medium with a sterile swab. Wells were bored into the solidified inoculated using a standard broth borer of 6 mm in diameter. 0.1mL of the solution of the extract of concentration 90µg/mL was then introduced into each well on the medium. The inoculated medium was then incubated at 37°C for 24 h after which each plate was observed for the zone of inhibition of growth which was measured with a transparent ruler and the result recorded in millimeters.

Results

Isolation and Characterization of Gut Microbial Flora from Human Fecal sample

There were 65 colonies from the human fecal sample. Based on colony morphology, 16 bacteria wereselected for further biochemical characteristics [Table 1] which was defined by Bergey's Manual. Among 16 bacterial isolate screened from colony morphological characteristics, *Lactobacillus spp.*, *Clostridium spp.*, *Escherichia coli*, *Staphylococcus spp.*, *Enterococcus spp.* are thegut bacteria which was predominantly present in both normal human intestine and the disease human intestine[16]. These five gut bacteria were further used for bioconversion of Docosaehxaenoic acid.

Bioconversion of DHA using Gut bacteria and GC-MS Analysis of Metabolites from Bio-converted DHA extract

After 24 h, bioconvertedDocosahexanoic acid broth was subjected to fatty acid extraction. The crude extract was further analyzed for GC-MS analysis and antibacterial activity.The Normal Gut flora which was isolated from human fecal converted Docosaehxaenoic acid into various therapeutic metabolites analyzed by GC-MS analysis and it has various medicinal properties are tabulated [Fig. 1, Table 2, Table 3, Table 4, Table 5, and Table 6].

Anti-bacterial activity of crude extract from bioconverted DHA

From the GC-MS analysis, compounds from bioconverted DHA have varies medicinal properties and most of the compounds have similar property of antimicrobial activity. To ensure that we evaluate the antibacterial activity for their extract. The antibacterial activity of bioconverted DHA extract was presented in [Table 7]. Among 5 tested microorganisms, the extract featured the strongest antimicrobial effect of *Bifidobacterium spp.*, *Eschericia coli*, *Citrobacter spp.* Bioconverted DHA extractagainst*B. cereus* with the diameter of inhibition zone about 25mm, 26mm, 24mm. *Bifidobacterium spp.*, *Lactobacillus spp.*,*Eschericiacoli*,*Citrobacterspp.*,*Enterobacter spp.*bioconverted DHA extract showed activity against*Staphylococcus aureus* (20mm, 19mm, 18mm, 20mm), *Escherichia coli*(15mm, 16mm, 14mm, 15mm, 17mm), *Pseudomonas aeruginosa* (19mm, 18mm, 20mm, 19mm, 20mm), and *Salmonella enteritidis* (16mm, 15mm, 14mm, 15mm, 13mm). There was no activity found against the DMSO and distilled water. The findings suggest the potential application of bioconverted DHA extract as the antimicrobial agent, because the diameter of inhibition zone of extract were near to that of positive controls.

Discussion

Lactobacillus spp., *Clostridium spp.*, *Escherichia coli*, *Staphylococcus spp.*,and*Enterococcus spp.* have multiple roles in the human body which stabilize the gastric acid, hepatic bile and digestive enzymes of gastrointestinal tract and it can modulate gut microbiota and microbiota associated metabolic pathways [17].These gut bacteria are predominantly present in both the healthy and diseased person [16]. Therefore, these microbes were used in *invivo*study to evaluate the relationship between the gut bacteria and DHA. These gut bacteria converted DHA into 82 metabolites [Table 8] which have various therapeutic properties like anti-oxidant, anti-bacterial, anti-depressant, anti-tumor, anti-biofilm, anti-melanogenic, anxiolytic effects, anti-obesity, anti-diarrheal, anti-fungal, anti-cancer, anti-inflammatory, alpha-amylase inhibitory, anti-staphylococcal activity, pancreatic lipase inhibitory activity, and immunotherapeutic agent.

Commonly, *Lactobacillus spp.*, *Clostridium spp.*, *Escherichia coli*, *Staphylococcus spp.*, *Enterococcus spp.* are present in healthy person[18] but decrease if the person is said to be in a diseased state[19]. This could lead to many disorders like depression and other hormonal changes due to the lack of the therapeutic metabolites. We suggest that to improve the role of these therapeutic metabolites in the diseased person, it should be given to them as a combination of DHA and these microbes as probiotics which can increase the normal flora of intestine.

The most common activity among all metabolites obtained from bioconverted DHA extract is the anti-bacterial activity. Hence, in this study the crude extract was evaluated against clinical pathogens and it showed effective and strong antibacterial activity against them. In an earlier study, DHA was bioconverted using *P. aeruginosa* PR3 and the crude extract showed effective antibacterial activity against four gram-positive bacteria, *Bacillus subtilis*, *Listeria monocytogenes*, *Staphylococcus aureus* (ATCC 6538) and *S. aureus* (KCTC 1916) and seven gram-negative bacteria, *Enterobacter aerogenes*, *Escherichia coli*, *E. coli* O157:H7, *Pseudomonas aeruginosa*, *Salmonella enteritidis* and *S. typhimurium*[11]. Whereas, we analyzed the bioconverted DHA with *Lactobacillus spp.*, *Clostridium spp.*, *Escherichia coli*, *Staphylococcus spp.*, *Enterococcus spp.* crude extract which showed an effective antibacterial activity against the clinical pathogens *B. cereus*, *E. coli*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *S. enteritidis* when compared to the standard antibiotic Gentamycin.

Conclusion

From this work, it can be concluded that metabolites from extract of bioconverted DHA with *Lactobacillus spp.*, *Clostridium spp.*, *Escherichia coli*, *Staphylococcus spp.*, *Enterococcus spp.* has many medicinal properties such as antibacterial, antioxidant, anxiolytic, phytotoxicity and antiviral activities. This work reported for the first time the metabolite from this bioconversion to possess an anti-depressant effect. In conclusion, Normal flora of gut can convert the Docosahexaenoic acid into the various therapeutic metabolites.

Declarations

Ethics Approval: Not Applicable.

Consent to Participate: Authors agreed to participate in this research.

Consent for Publication: All the authors have approved the last version of the manuscript for its submission/publication.

Author Contribution: V.K and M.A contributed to the study conception and design; V.K conducted experiments; and V.K, R.A, D.K.S, S.S and M.A contributed to the writing of this manuscript and approved the final version.

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Tables

Table-1: Morphological and Biochemical Characteristics of Gut Bacteria from fecal sample

Bacterial Isolate	Bacterial Name	Gram Staining	Shape	Motility	Spore	Catalase	Oxidase	MR	VP	Indole	Nitrate Reduction	Fermentation of Fructose
H1	<i>Enterococcus spp.</i>	+	Cocci	-	-	-	-	-	+	-	+	+
H2	<i>Lactobacillus spp.</i>	+	Rod	-	-	-	-	-	-	-	+	+
H3	<i>Clostridium spp.</i>	+	Bacilli	+	+	-	-	-	-	-	-	+
H4	<i>Eschericia coli</i>	-	Bacilli	+	-	+	-	+	-	+	+	-
H5	<i>Citrobacter spp.</i>	-	Bacilli	+	-	+	-	+	-	-	+	-
H6	<i>Enterobacter spp.</i>	-	Rod	+		+	-	-	+	-	+	-
H7	<i>Proteus spp.</i>	-	Rod	+		+	-	+	-	+	+	-
H8	<i>Streptococci spp.</i>	+	cocci	-		-		+	+	-	-	-
H9	<i>Bacillus spp.</i>	+	Bacilli	+	+	+	-	+	+	-	-	+
H10	<i>Staphylococcus spp.</i>	+	cocci			+	-	+	-	-		-
H11	<i>Bifidobacterium spp.</i>	+	Rod	-	-	-	-			-	-	+
H12	<i>Clostridium spp.</i>	+	Coccobacilli	-	+	-	-	+	-	-	-	+
H13	<i>Leuconostocspp</i>	+	Cocci	-		-	-					-
H14	<i>Enterococcus spp.</i>	+	Cocci	-	-	-	-					-
H15	<i>Fusobacterium spp.</i>	-	Rod	-	-	-	-	-	-	+	-	+
H16	<i>Bacteroides spp.</i>	-	Rod	-	-	+	-			-		-

Table-2: Metabolites from bioconverted DHA using *Lactobacillus spp.*

S.No	R.Time	Percentage %	Compound Name	Medicinal Properties	References
1	22.176	13.27	2-Nonadecanone	Anti-depression Anti-bacterial Anti-tumor	Lee et.al., 2018 Mihigo et.al., 2015 Figueiredo et.al., 2014
2	21.941	12.91	Z,Z-6,27-Hexatriactontadien-2-one	-	-
3	21.164	8.47	cis-9-Hexadecenal	Anti-biofilm Anti-melanogenic Anti-fungal	Hoda et.al., 2019 Hoda et.al., 2020
4	21.395	7.89	Eicosanal-	Anti-depressant Anxiolytic effects and Anti-oxidant	Uddin et.al., 2021
5	26.623	7.54	Stigmast-5-En-3-Ol, Oleat	Anti-obesity	Ghinmy et.al., 2019
6	26.922	5.07	Tetrapentacontane	Anti-microbial Anti-Oxidant	Kerem et.al., 2019 Xuanji et.al., 2016
7	22.489	3.26	Oleic Acid	Anti-tumor Anti-Microbial	Giulitt et.al, 2021 Ghavam et.al., 2021
8	22.698	3.20	Octadecanoic acid	Anti-bacterial Anti-oxidant	Daniels et.al., 2021
9	21.275	2.15	(Z)-3-(Heptadec-10-en-1-yl)phenol	Anti-bacterial Anti-diarrheal Anti-oxidant	Van et.al., 2020 Nemkul et.al., 2021
10	20.193	2.69	2-Heptadecanone		
11	20.774	2.05	n-Hexadecanoic acid	Anti-bacterial Anti-fungal Anti-biofilm Anti-cancer	Ansari et.al., 2021
12	24.730	1.95	Heneicosane	Anti-inflammatory	Barda et.al., 2021
13	15.782	1.65	Phenol, 2,4-Bis(1,1-Dimethylethyl)-	Anti-fungal Anti-oxidant	Devi et.al., 2021 Idih et.al., 2021
14	19.326	1.64	Hexadecanal	-	-
15	21.880	1.34	8,11-Heptadecadienal, (8Z,11Z)-	-	-
16	21.102	1.28	9,17-Octadecadienal, (Z)-	Antimicrobial agent	Insanu et.al., 2021
17	23.985	0.99	2-Pentacosanone	-	-
18	16.871	0.99	Hexadecane	-	-
19	27.063	0.97	8-Octadecanone	Antimicrobial metabolite	Tayung et.al., 2011
20	25.397	0.89	Stigmasta-5,22-Dien-3-Ol, Acetat, (3-Beta,2	Antimicrobial	Richa Bhardwaj 2018
21	28.040	0.72	9-Heptadecanone	-	-
22	27.786	0.70	Z,Z-6,28-Heptatriactontadien-2-one	Alpha-amylase Inhibition And Antioxidant Activity	Unnikrishnan et.al., 2015

Table-3: Metabolites from bioconverted DHA using Clostridium spp.

S.No	R.Time	Percentage%	Compound Name	Medicinal Properties	References
1	22.996	15.86	Octadecanoic acid	Anti-bacterial Anti-oxidant	Daniels et.al., 2021
2	22.743	13.93	6-Octadecenoic acid	Anti-bacterial	Al-fekaiki1 et.al., 2017
3	20.948	10.92	n-Hexadecanoic acid	Anti-bacterial Anti-fungal Anti-biofilm Anti-cancer	Ansari et.al., 2021
4	27.000	8.34	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	Anti-microbial Anti-Fungal	Kumari et.al., 2021 Antonio et.al., 2021
5	27.214	7.11	Octadecanoic acid, 2,3-dihydroxypropyl ester	Anti-microbial Anti-oxidant	Kumari et.al., 2021 Vural et.al., 2021
6	25.389	4.40	9-Octadecenoic acid (Z)-, oxiranylmethyl ester	-	-
7	26.680	4.30	n-Propyl 9-octadecenoate	-	-
8	25.582	3.50	Glycidyl palmitate	Anti-staphylococcal activity	Houdkova et.al., 2021
9	21.398	11.29	Eicosanal-	Anti-depressant Anxiolytic effects and Anti-oxidant	Uddin et.al., 2021
10	21.168	3.01	cis-9-Hexadecenal	Anti-biofilm Anti-melanogenic Anti-fungal	Hoda et.al., 2019 Hoda et.al., 2020
11	25.490	2.83	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethy	Anti-fungal Anti-microbial	Khan et.al., 2021 Kumari et.al., 2021
12	26.786	2.31	1-Cyclohexyldimethylsilyloxybutane	-	-
13	26.880	2.26	Octadecanoic acid, propyl ester	-	-
14	24.491	1.75	Eicosanoic acid	Anti-bacterial	Pavani et.al., 2021
15	6.560	1.43	Oxime-, methoxy-phenyl-	Pancreatic lipase Inhibitory activity.	Anand et.al., 2021
16	28.188	1.41	Dotriacontane	Anti-fungal	Prakash et.al., 2021
17	4.716	1.18	Hexanal	Anti-fungal	Shuaibing Zhang et.al., 2021
18	21.940	1.15	Z,Z-6,27-Hexatriactontadien-2-one	-	-

Table-4: Metabolites from bioconverted DHA using Escherichia coli.

S.No	R.Time	%	Compound Name	Medicinal Properties	References
1	4.165	17.03	Benzene, Methyl-	-	-
2	15.766	10.84	Phenol, 2,4-Bis(1,1-Dimethylethyl)	Anti-fungal Anti-oxidant	Devi et.al., 2021 Idih et.al., 2021
3	6.517	4.75	Oxime-, methoxy-phenyl-	Pancreatic lipase Inhibitory activity.	Anand et.al., 2021
4	18.019	4.61	Eicosane	Anti-microbial	Octarya et.al., 2021
5	9.401	4.43	Cyclotrisiloxane, hexamethyl-	Anti-oxidant Anti-biofilm	Kanimozhi et.al., 2021
6	16.862	4.11	Hexadecane	-	-
7	12.619	3.28	Dodecane, 2,6,11-trimethyl-	Anti-microbial Anti-oxidant	Octarya et.al., 2021 Khalaf et.al., 2021
8	14.351	3.12	Tetradecane	-	-
9	19.115	3.03	Heneicosane	Anti-inflammatory	Barda et.al., 2021
10	4.460	2.65	3-Hexanone	-	-
11	22.308	2.62	Octadecane	-	-
12	4.538	2.39	2-Hexanone	-	-
13	24.218	2.32	1H,5H-Cyclopropa[G][1, 2, 4]Triazolo[1,2-A]	-	-
14	24.162	2.31	Hexatriacontane	Anti-oxidant Anti-microbial	Jamil et.al., 2021
15	20.757	1.90	1-Butyl 2-(8-Methylnonyl) Phthalate #	-	-
16	26.220	1.90	Silikonfett Se30 (Grevels)	-	-
17	9.209	1.86	Octane, 6-Ethyl-2-Methyl-	-	-
18	3.365	1.82	Furan, tetrahydro-2,5-dimethyl-	-	-
19	24.112	1.50	Cyclodecasiloxane, eicosamethyl-	Immunotherapeutic agent	Gullilat et.al., 2021
20	25.846	1.48	Tetrapentacontane	Anti-microbial Anti-Oxidant	Kerem et.al., 2019 Xuanji et.al., 2016
21	12.282	1.41	Benzene, 1,3-Bis(1,1-Dimethylethyl)-	Anti-cancer	Jenifer et.al., 2021
22	11.521	1.38	Dodecane	-	-
23	20.574	1.29	4-Pentyl-Cyclohexanecarboxylic Acid	-	-

Table-5: Metabolites from bioconverted DHA using *Staphylococcus* spp.

S.No	R.Time	Percentage %	Compound Name	Medicinal Properties	References
1	28.162	7.18	Decanedioic acid, bis(2-ethylhexyl) ester	Anti-fungal	Rafiq et.al., 2021
2	20.769	6.67	n-Hexadecanoic acid	Anti-bacterial Anti-fungal Anti-biofilm Anti-cancer	Ansari et.al., 2021
3	22.688	5.70	Octadecanoic acid	Anti-bacterial Anti-oxidant	Daniels et.al., 2021
4	4.164	5.56	Benzene, Methyl-	-	-
5	8.781	5.49	1-Hexanol, 2-Ethyl-	-	-
6	15.772	5.11	Phenol, 2,4-Bis(1,1-Dimethylethyl)-	Anti-fungal Anti-oxidant	Devi et.al., 2021 Idih et.al., 2021
7	23.653	4.90	Ergosta-5,7,9(11),22-tetraen-3-ol, (3.beta.,22E)-	-	-
8	24.174	3.72	Tetrapentacontane	Anti-microbial Anti-Oxidant	Kerem et.al., 2019 Xuanji et.al., 2016
9	18.024	2.88	Eicosane	Anti-Microbial	Octarya et.al., 2021
10	16.866	2.68	Hexadecane	-	-
11	28.368	2.56	Squalene	-	-
12	15.499	2.17	Hexadecane, 2,6,10,14-Tetramethyl-	-	-
13	19.120	2.06	Heneicosane	Anti-inflammatory	Barda et.al., 2021
14	22.473	1.96	Oleic Acid	Anti-tumor Anti-microbial	Giulitt et.al, 2021 Ghavam et.al., 2021
15	12.623	1.90	Dodecane, 2,6,11-trimethyl-	Anti-microbial Anti-oxidant	Octarya et.al., 2021 Khalaf et.al., 2021
16	14.356	1.88	Tetradecane	-	-
17	24.115	1.67	Cyclodecasiloxane, eicosamethyl-	Immunotherapeutic agent	Gullilat et.al., 2021
18	6.999	1.48	1-Propene, 3,3-Dichloro-	-	-
19	25.824	1.48	Bis(2-ethylhexyl) phthalate	-	-
20	22.314	1.44	Hexatriacontane	Anti-microbial	Jamil et.al., 2021
21	22.421	1.44	10(E),12(Z)-Conjugated linoleic acid	Anti-carcinogen Anti-Inflammatory	Hornung et.al., 2005
22	4.450	1.36	3-Hexanone	Anti-microbial Anti-oxidant	Khalaf et.al., 2021
23	4.525	1.20	2-Hexanone	-	-
24	20.411	1.15	Hexadecanoic acid, methyl ester	Anti-oxidant	Ahamefula Sunday et.al., 2021
25	24.814	1.13	1,16-Dibromohexadecane	-	-
26	18.671	0.99	Tetradecanoic acid	Anti-oxidant Anti-bacterial	Ji et.al., 2021
27	20.709	0.98	2,6,10,15,19,23-Hexamethyltetracosane	-	-
28	9.212	0.95	Octane, 6-Ethyl-2-Methyl-	Anti-cancer	Jenifer et.al., 2021
29	20.581	0.94	4-Pentyl-Cyclohexanecarboxylic Acid	-	-
30	4.766	0.90	2-Hexanol	Antimicrobial Anti-inflammatory	Javaid et.al., 2021
31	16.065	0.88	Hexadecane, 2,6,10,14-Tetramethyl	-	-

S.No	R.Time	Percentage %	Compound Name	Medicinal Properties	References
32	12.730	0.85	4-Propylbenzaldehyde	-	-
33	11.525	0.81	Dodecane	-	-
34	16.778	0.81	1-Hexadecanol	-	-
35	4.691	0.76	3-Hexanol	Anti-bacterial	Rojas et.al., 2021
36	13.282	0.75	Dodecane, 4,6-dimethyl-	-	-

Table-6: Metabolites from bioconverted DHA using *Enterococcus* spp

S.No	R.Time	Percentage%	Compound Name	Medicinal Properties	References
1	20.803	15.04	n-Hexadecanoic acid	Anti-bacterial Anti-fungal Anti-biofilm Anti-cancer	Ansari et.al., 2021
2	22.709	10.97	Octadecanoic acid	Anti-bacterial Anti-oxidant	Daniels et.al., 2021
3	23.462	10.31	Palmitoyl chloride	-	-
4	24.962	8.77	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	Anti-microbial Anti-fungal	Kumari et.al., 2021 Antonio et.al., 2021
5	27.952	10.69	Butyl 4,7,10,13,16,19-docosahexaenoate	-	-
6	23.874	7.70	Glycidyl palmitate	-	-
7	25.364	5.43	9-Octadecenoic acid (Z)-, oxiranylmethyl ester	-	-
8	3.172	5.02	Heptane	-	-
9	22.488	8.71	Oleic Acid	Anti-tumor Anti-microbial	Giulitt et.al, 2021 Ghavam et.al., 2021
10	25.468	2.07	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethy	Anti-fungal Anti-microbial	Khan et.al., 2021 Kumari et.al., 2021
11	27.861	2.73	Methyl 4,7,10,13,16-docosapentaenoate	-	-
12	26.959	1.87	cis-10-Pentadecenoic acid, isobutyl ester	-	-
13	18.677	1.65	Tetradecanoic acid	Anti-oxidant Anti-bacterial	Ji et.al., 2021
14	25.163	1.59	Octadecanoic acid, 2,3-dihydroxypropyl ester	Anti-microbial Anti-oxidant	Kumari et.al., 2021 Vural et.al., 2021
15	15.771	1.50	Phenol, 2,4-Bis(1,1-Dimethylethyl)-	Anti-fungal Anti-oxidant	Devi et.al., 2021 Idih et.al., 2021
16	22.429	1.46	9,12-Octadecadienoic Acid (Z,Z)-	Anti-bacterial	Jabbar et.al., 2021
17	25.561	1.28	Myristic acid glycidyl ester	-	-
19	18.023	0.80	Eicosane	Anti-microbial	Octarya et.al., 2021
20	16.866	0.75	Hexadecane	-	-
21	24.915	0.61	3-({[2-(4-Fluorophenyl)Ethyl]Amino}Methyl	-	-
22	19.118	0.59	Heneicosane	Anti-inflammatory	Barda et.al., 2021
23	12.623	0.48	Dodecane, 4,6-dimethyl-	-	-

Table – 7: Antibacterial activity of Bioconverted DHA extract

S.No.	Clinical Pathogens	Zone of Inhibition (mm)							
		Bioconverted DHA extract of Bifidobacterium spp. (90µg/mL)	Bioconverted DHA extract of Lactobacillus spp. (90µg/mL)	Bioconverted DHA extract of Escherichia coli (90µg/mL)	Bioconverted DHA extract of Citrobacter spp. (90µg/mL)	Bioconverted DHA extract of Enterobacter spp. (90µg/mL)	Gentamycin (10µg/mL) (Positive Control)	DMSO	Sterile Distilled water (Negative Control)

1.	<i>Bacillus cereus</i>	25	24	26	25	24	28	0	0
2.	<i>Escherichia coli</i>	15	16	14	15	17	18	0	0
3.	<i>Pseudomonas aeruginosa</i>	19	18	20	19	20	21	0	0
4.	<i>Salmonella enteritidis</i>	16	15	14	15	13	17	0	0
5.	<i>Staphylococcus aureus</i>	20	19	18	20	19	21	0	0

Table – 8: Metabolites from Bioconverted DHA using Gut Bacteria

S.No	R.Time	Percentage %	Compound Name	Medicinal Properties	References
1	22.176	13.27	2-Nonadecanone	Anti-depression Anti-bacterial Anti-tumor	Lee et.al., 2018 Mihigo et.al., 2015 Figueiredo et.al., 2014
2	21.941	12.91	Z,Z-6,27-Hexatriactontadien-2-one	-	-
3	43.881	11.48	cis-9-Hexadecenal	Anti-biofilm Anti-melanogenic Anti-fungal	Hoda et.al., 2019 Hoda et.al., 2020
4	21.395	7.89	Eicosanal-	Anti-depressant Anxiolytic effects and Anti-oxidant	Uddin et.al., 2021
5	26.623	7.54	Stigmast-5-En-3-Ol, Oleat	Anti-obesity	Ghinmy et.al., 2019
6	26.922	10.27	Tetrapentacontane	Anti-microbial Anti-oxidant	Kerem et.al., 2019 Xuanji et.al., 2016
7	22.489	13.93	Oleic Acid	Anti-tumor Anti-microbial	Giulitt et.al, 2021 Ghavam et.al., 2021
8	22.698	32.53	Octadecanoic acid	Anti-bacterial Anti-oxidant	Daniels et.al., 2021
9	21.275	2.15	(Z)-3-(Heptadec-10-en-1-yl)phenol	Anti-bacterial Anti-diarrheal Anti-oxidant	Van et.al., 2020 Nemkul et.al., 2021
10	20.193	2.69	2-Heptadecanone		
11	20.774	34.68	n-Hexadecanoic acid	Anti-bacterial Anti-fungal Anti-biofilm Anti-cancer	Ansari et.al., 2021
12	24.730	7.63	Heneicosane	Anti-inflammatory	Barda et.al., 2021
13	15.782	31.59	Phenol, 2,4-Bis(1,1-Dimethylethyl)-	Anti-fungal Anti-oxidant	Devi et.al., 2021 Idih et.al., 2021
14	19.326	1.64	Hexadecanal	-	-
15	21.880	1.34	8,11-Heptadecadienal, (8Z,11Z)-	-	-
16	21.102	1.28	9,17-Octadecadienal, (Z)-	Antimicrobial agent	Insanu et.al., 2021
17	23.985	0.99	2-Pentacosanone	-	-
18	16.871	8.53	Hexadecane	-	-
19	27.063	0.97	8-Octadecanone	Antimicrobial metabolite	Tayung et.al., 2011
20	25.397	0.89	Stigmasta-5,22-Dien-3-Ol, Acetat, (3-Beta,2	Antimicrobial	Richa Bhardwaj 2018
21	28.040	0.72	9-Heptadecanone	-	-
22	27.786	0.70	Z,Z-6,28-Heptatriactontadien-2-one	Alpha-amylase Inhibition And Antioxidant Activity	Unnikrishnan et.al., 2015
23	27.000	17.11	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	Anti-microbial Anti-fungal	Kumari et.al., 2021 Antonio et.al., 2021
24	27.214	8.7	Octadecanoic acid, 2,3-dihydroxypropyl ester	Anti-microbial Anti-oxidant	Kumari et.al., 2021 Vural et.al., 2021
25	25.389	9.83	9-Octadecenoic acid (Z)-, oxiranylmethyl ester	-	-

S.No	R.Time	Percentage %	Compound Name	Medicinal Properties	References
26	26.680	4.30	n-Propyl 9-octadecenoate	-	-
27	25.582	3.50	Glycidyl palmitate	Anti-staphylococcal activity	Houdkova et.al., 2021
28	25.490	4.9	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethy	Anti-fungal Anti-microbial	Khan et.al., 2021 Kumari et.al., 2021
29	26.786	2.31	1-Cyclohexyldimethylsilyloxybutane	-	-
30	26.880	2.26	Octadecanoic acid, propyl ester	-	-
31	24.491	1.75	Eicosanoic acid	Anti-bacterial	Pavani et.al., 2021
32	6.560	6.18	Oxime-, methoxy-phenyl-	Pancreatic lipase Inhibitory activity.	Anand et.al., 2021
33	28.188	1.41	Dotriacontane	Anti-fungal	Prakash et.al., 2021
34	4.716	1.18	Hexanal	Anti-fungal	Shuaibing Zhang et.al., 2021
35	4.165	22.59	Benzene, Methyl-	-	-
36	18.019	8.29	Eicosane	Anti-microbial	Octarya et.al., 2021
37	9.401	4.43	Cyclotrisiloxane, hexamethyl-	Anti-oxidant Anti-biofilm	Kanimozhi et.al., 2021
38	12.619	5.18	Dodecane, 2,6,11-trimethyl-	Anti-microbial Anti-oxidant	Octarya et.al., 2021 Khalaf et.al., 2021
39	14.351	5.0	Tetradecane	-	-
40	4.460	4.01	3-Hexanone	Anti-microbial Anti-oxidant	Khalaf et.al., 2021
41	22.308	2.62	Octadecane	-	-
42	4.538	3.59	2-Hexanone	-	-
43	24.218	2.32	1H,5H-Cyclopropa[G][1, 2, 4]Triazolo[1,2-A]	-	-
44	24.162	3.75	Hexatriacontane	Anti-oxidant Anti-microbial	Jamil et.al., 2021
45	20.757	1.90	1-Butyl 2-(8-Methylnonyl) Phthalate #	-	-
46	26.220	1.90	Silikonfett Se30 (Grevels)	-	-
47	9.209	1.86	Octane, 6-Ethyl-2-Methyl-	-	-
48	3.365	1.82	Furan, tetrahydro-2,5-dimethyl-	-	-
49	24.112	1.50	Cyclodecasiloxane, eicosamethyl-	Immunotherapeutic agent	Gullilat et.al., 2021
50	12.282	1.41	Benzene, 1,3-Bis(1,1-Dimethylethyl)-	Anti-cancer	Jenifer et.al., 2021
51	11.521	2.19	Dodecane	-	-
52	20.574	1.29	4-Pentyl-Cyclohexanecarboxylic Acid	-	-
53	28.162	7.18	Decanedioic acid, bis(2-ethylhexyl) ester	Anti-fungal	Rafiq et.al., 2021
54	8.781	5.49	1-Hexanol, 2-Ethyl-	-	-
55	23.653	4.90	Ergosta-5,7,9(11),22-tetraen-3-ol, (3.beta.,22E)-	-	-
56	28.368	2.56	Squalene	-	-
57	15.499	3.05	Hexadecane, 2,6,10,14-Tetramethyl-	-	-
58	24.115	1.67	Cyclodecasiloxane, eicosamethyl-	Immunotherapeutic agent	Gullilat et.al., 2021
59	6.999	1.48	1-Propene, 3,3-Dichloro-	-	-
60	25.824	1.48	Bis(2-ethylhexyl) phthalate	-	-

S.No	R.Time	Percentage %	Compound Name	Medicinal Properties	References
61	22.421	1.44	10(E),12(Z)-Conjugated linoleic acid	Anti-carcinogen Anti-Inflammatory	Hornung et.al., 2005
62	20.411	1.15	Hexadecanoic acid, methyl ester	Anti-oxidant	Ahamefula Sunday et.al., 2021
63	24.814	1.13	1,16-Dibromohexadecane	-	-
64	18.671	5.89	Tetradecanoic acid	Anti-oxidant Anti-bacterial	Ji et.al., 2021
65	20.709	0.98	2,6,10,15,19,23-Hexamethyltetracosane	-	-
66	9.212	0.95	Octane, 6-Ethyl-2-Methyl-	Anti-cancer	Jenifer et.al., 2021
67	20.581	0.94	4-Pentyl-Cyclohexanecarboxylic Acid	-	-
68	4.766	0.90	2-Hexanol	Antimicrobial Antiinflammatory	Javaid et.al., 2021
69	12.730	0.85	4-Propylbenzaldehyde	-	-
70	16.778	0.81	1-Hexadecanol	-	-
71	4.691	0.76	3-Hexanol	Anti-bacterial	Rojas et.al., 2021
72	13.282	0.75	Dodecane, 4,6-dimethyl-	-	-
73	23.462	10.31	Palmitoyl chloride	-	-
74	27.952	10.69	Butyl 4,7,10,13,16,19-docosahexaenoate	-	-
75	23.874	7.70	Glycidyl palmitate	-	-
76	3.172	5.02	Heptane	-	-
77	27.861	2.73	Methyl 4,7,10,13,16-docosapentaenoate	-	-
78	26.959	1.87	cis-10-Pentadecenoic acid, isobutyl ester	-	-
79	22.429	1.46	9,12-Octadecadienoic Acid (Z,Z)-	Anti-bacterial	Jabbar et.al., 2021
80	25.561	1.28	Myristic acid glycidyl ester	-	-
81	24.915	0.61	3-([2-(4-Fluorophenyl)Ethyl]Amino)Methyl	-	-
82	12.623	0.48	Dodecane, 4,6-dimethyl-	-	-

Figures

Figure 1

GC-MS analysis of bioconverted DHA metabolites