Antibacterial and Antioxidant Activity of Metabolites From Bioconverted Docosahexaenoic 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]. Antimicrobial 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 have been reported on various enveloped viruses, parasites and pathogenic bacteria such as Pseudomonas aeruginosa, Bacillus subtilis, Listeria monocytogenes, Helicobacter pylori, Staphylococcus aureus and Neisseria gonorrhoea [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 (NH4)2SO4, 3 g KH2PO4, 0.5 g MgSO4.7H2O, 15 mg EDTA, 4.5 mg ZnSO4.7H2O, 4.5 mg CaCl2.2H2O, 0.3 mg FeSO4.7H2O, 1 mg MnCl2.4H2O, 1 mg H2BO3, 0.4 mg Na2MoO4.2H2O, 0.3 mg CuSO4.5H2O, 0.3 mg CoCl2.6H2O and 0.1 mg KI; The pH was adjusted to 5.5 using HCL, and the media was sterilized through a 0.22µm 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 carbonsources present in the original inoculum, samples were transferred (2.5µL) 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 3 mL 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 µL trimethylsilyl trifluoroacetamide (BSTFA) was added, and the mixture was incubated at 70°C for 30 min and dried under a stream of nitrogen. The fatty acids were dissolved in 100 µL hexane for GC/MS analysis [15].

**GC-MS Analysis**

Fatty acid composition analysis was performed on the Shimadzu GCMS OP 2020 that employed a fused silica column, packed with SH-Rxi-%Sil MS (30 m × 0.25 mm ID × 250µm df) 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 spectra 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 were selected 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 the gut 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 Docosahexaenoic acid.

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

After 24 h, bioconvertedDocosahexaenoic 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 convertend Docosahexaenoic 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 various 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., Escherichia coli,* Citrobacter spp. Bioconverted DHA extractagains*t B. cereus with the diameter of inhibition zone about 25mm, 26mm, 24mm. *Bifidobacterium spp., Lactobacillus spp., Escherichia coli Citrobacter spp., 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 invivostudy 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 persons[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 enteridis* 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. enteridis* 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                | Enterococcus spp.    | +             | Cocci | -        | -     | -        | -       | +  | -  | +      | +                 | +                        |
| H2                | Lactobacillus spp.   | +             | Rod   | -        | -     | -        | -       | -  | +  | -      | +                 | +                        |
| H3                | Clostridium spp.     | +             | Bacilli | +    | -     | -        | -       | -  | -  | -      | +                 | -                        |
| H4                | Eschericia coli      | -             | Bacilli | +    | -     | +        | -       | +  | -  | -      | +                 | -                        |
| H5                | Citrobacter spp.     | -             | Bacilli | +    | -     | +        | -       | +  | -  | -      | -                 | -                        |
| H6                | Enterobacter spp.    | -             | Rod   | +        | -     | -        | -       | +  | -  | -      | +                 | -                        |
| H7                | Proteus spp.         | -             | Rod   | +        | -     | -        | -       | +  | -  | +      | +                 | -                        |
| H8                | Streptococci spp.    | +             | cocci | -       | -     | +        | -       | +  | -  | -      | -                 | -                        |
| H9                | Bacillus spp.        | +             | Bacilli | +    | +     | +        | -       | +  | -  | -      | +                 | -                        |
| H10               | Staphylococcus spp.  | +             | cocci | +       | -     | +        | -       | -  | +  | -      | -                 | -                        |
| H11               | Bifidobacterium spp. | +             | Rod   | -       | -     | -        | -       | -  | -  | -      | +                 | -                        |
| H12               | Clostridium spp.     | +             | Coccobacilli | +    | -     | -        | +       | -  | -  | -      | +                 | -                        |
| H13               | Leuconostoc spp.     | +             | Cocci | -       | -     | -        | -       | -  | +  | -      | -                 | -                        |
| H14               | Enterococcus spp.    | +             | Cocci | -       | -     | -        | -       | -  | +  | -      | -                 | -                        |
| H15               | Fusobacterium spp.   | -             | Rod   | -       | -     | -        | -       | -  | +  | -      | +                 | -                        |
| H16               | Bacteroides spp.     | -             | 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 | Giulitti 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-OI, 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-fekaiki 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                    | Devi et al., 2021           |
|      |        |      |                                       | Anti-oxidant                   | Idih et al., 2021           |
| 3    | 6.517  | 4.75  | Oxime-, methoxy-phenyl-               | Pancreatic lipase inhibitory   | Anand et al., 2021          |
|      |        |      |                                       | activity.                      |                             |
| 4    | 18.019 | 6.41  | Eicosane                              | Anti-microbial                 | Octarya et al., 2021        |
| 5    | 9.401  | 4.43  | Cyclotrisiloxane, hexamethyl-         | Anti-oxidant                   | Kanimozhi et al., 2021      |
|      |        |      |                                       | Anti-biofilm                   |                             |
| 6    | 16.862 | 4.11  | Hexadecane                            | -                             | -                           |
| 7    | 12.619 | 3.28  | Dodecane, 2,6,11-trimethyl-           | Anti-microbial                 | Octarya et al., 2021        |
|      |        |      |                                       | Anti-oxidant                   | 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[1,2-A]Triazolo[1,2-A]| -                             | -                           |
| 14   | 24.162 | 2.31  | Hexatriacontane                       | Anti-oxidant                   | Jamil et al., 2021          |
|      |        |      |                                       | Anti-microbial                 |                             |
| 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                 | Kerem et al., 2019          |
|      |        |      |                                       | Anti-Oxidant                   | 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         | Tetrupentacontane | 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 | 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 | - | - |
### 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                | Ansari et.al., 2021 |
|      |         |              |                                             | Anti-fungal                   |                     |
|      |         |              |                                             | Anti-biofilm                  |                     |
|      |         |              |                                             | Anti-cancer                   |                     |
| 2    | 22.709  | 10.97        | Octadecanoic acid                           | Anti-bacterial                | Daniels et.al., 2021|
|      |         |              |                                             | Anti-oxidant                  |                     |
| 3    | 23.462  | 10.31        | Palmitoyl chloride                          | -                             |                     |
| 4    | 24.962  | 8.77         | 9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester | Anti-microbial                | Kumari et.al., 2021 |
|      |         |              |                                             | Anti-fungal                   | 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                    | Giulitt et.al, 2021 |
|      |         |              |                                             | Anti-microbial                | Ghavam et.al., 2021 |
| 10   | 25.468  | 2.07         | Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethy | Anti-fungal                   | Khan et.al., 2021   |
|      |         |              |                                             | Anti-microbial                | 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                  | Ji et.al., 2021     |
|      |         |              |                                             | Anti-bacterial                |                     |
| 14   | 25.163  | 1.59         | Octadecanoic acid, 2,3-dihydroxypropyl ester | Anti-microbial                | Kumari et.al., 2021 |
|      |         |              |                                             | Anti-oxidant                  | Vural et.al., 2021  |
| 15   | 15.771  | 1.50         | Phenol, 2,4-Bis(1,1-Dimethylethyl)-          | Anti-fungal                   | Devi et.al., 2021   |
|      |         |              |                                             | Anti-oxidant                  | 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                | -                             |                     |
| 18   | 18.023  | 0.80         | Eicosane                                    | Anti-microbial                | Octarya et.al., 2021|
| 19   | 16.866  | 0.75         | Hexadecane                                  | -                             |                     |
| 20   | 24.915  | 0.61         | 3-((2-(4-Fluorophenyl)Ethyl)Amino)Methyl     | -                             |                     |
| 21   | 19.118  | 0.59         | Heneicosane                                 | Anti-inflammatory             | Barda et.al., 2021  |
| 22   | 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.   | *Bacillus cereus*          | 25 24 26 25 24 28 0 0                                                                 |
| 2.   | *Escherichia coli*         | 15 16 14 15 17 18 0 0                                                                 |
| 3.   | *Pseudomonas aeruginosa*   | 19 18 20 19 20 21 0 0                                                                  |
| 4.   | *Salmonella enteritidis*   | 16 15 14 15 13 17 0 0                                                                  |
| 5.   | *Staphylococcus aureus*    | 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       | Lee et.al., 2018 |
|      |        |              |               | Anti-bacterial        | Mihigo et.al., 2015 |
|      |        |              |               | Anti-tumor            | 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      | Hoda et.al., 2019 |
|      |        |              |               | Anti-melanogenic      | Hoda et.al., 2020 |
|      |        |              |               | Anti-fungal           |            |
| 4    | 21.395 | 7.89         | Eicosanal-    | Anti-depressant       | Uddin et.al., 2021 |
|      |        |              |               | Anxiolytic effects and Anti-oxidant | |
| 5    | 26.623 | 7.54         | Stigmas-5-En-3-Ol, Oleat | Anti-obesity | Ghinmy et.al., 2019 |
| 6    | 26.922 | 10.27        | Tetrapentacontane | Anti-microbial | Kerem et.al., 2019 |
|      |        |              |               | Anti-oxidant          | Xuanji et.al., 2016 |
| 7    | 22.489 | 13.93        | Oleic Acid    | Anti-tumor            | Giuliani et.al, 2021 |
|      |        |              |               | Anti-microbial        | Ghavam et.al., 2021 |
| 8    | 22.698 | 32.53        | Octadecanoic acid | Anti-bacterial      | Daniels et.al., 2021 |
|      |        |              |               | Anti-oxidant          |            |
| 9    | 21.275 | 2.15         | (Z)-3-(Heptadec-10-en-1-yl)phenol | Anti-bacterial | Van et.al., 2020 |
|      |        |              |               | Anti-diarrheal        | Nemkuli et.al., 2021 |
| 10   | 20.193 | 2.69         | 2-Heptadecanone | Anti-bacterial       | Ansari et.al., 2021 |
|      |        |              |               | Anti-fungal           |            |
|      |        |              |               | Anti-biofilm          |            |
|      |        |              |               | Anti-cancer           |            |
| 11   | 20.774 | 34.68        | n-Hexadecanoic acid | Anti-bacterial | |
|      |        |              |               | Anti-fungal           | |
|      |        |              |               | Anti-biofilm          | |
| 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 | Devi et.al., 2021 |
|      |        |              |               | Anti-oxidant          | 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 | Unnir Krishnan et al., 2015 |
| 23   | 27.000 | 17.11        | 9-Octadecenoic acid (Z); 2,3-dihydroxypropyl ester | Anti-microbial | Kumari et.al., 2021 |
|      |        |              |               | Anti-fungal           | Antonio et.al., 2021 |
| 24   | 27.214 | 8.7          | Octadecanoic acid, 2,3-dihydroxypropyl ester | Anti-microbial | Kumari et.al., 2021 |
|      |        |              |               | Anti-oxidant          | 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-                          | Anti-fungal, Anti-microbial | Khan et. al., 2021, Kumari 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[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-MethylInonyl) 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     | Guilliat 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     | Guilliat 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                    | Hornung et al., 2005             |
|      |        |            |                                        | Anti-Inflammatory                  |                                  |
| 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                       | Ji et al., 2021                  |
| 65   | 20.709 | 0.98       | 2,6,10,15,19,23-Hexamethyltetrasosane  | -                                  |                                  |
| 66   | 9.212  | 0.95       | Octane, 6-Ethyl-2-Methyl              | Anti-cancer                        | Jenifer et al., 2021             |
| 67   | 20.581 | 0.94       | 4-Pentyl-Cyclohexane-carboxylic Acid  | -                                  |                                  |
| 68   | 4.766  | 0.90       | 2-Hexanol                             | Antimicrobial Anti-inflammatory    | 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-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