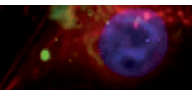
molecular oral microbiology



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A novel bioactivity of omega-3 polyunsaturated fatty acids and their ester derivatives

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SUMMARY

Fish oil, enriched in omega-3 polyunsaturated fatty acids (n-3 PUFA), is widely used as a dietary or nutritional supplement with numerous benefits, including as an anti-inflammatory particularly linked to atherosclerosis. While n-3 PUFA have been suggested to be able to improve oral health through a reduction in inflammation through elevations in these fatty acids in serum and cellular membranes, information is lacking for the possibility that these fatty acids could directly impact the survival and growth of the oral bacteria that trigger the chronic inflammatory responses. The n-3 fatty acids, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and α-linolenic acid (ALA), and their fatty acid ethyl esters, ALAEE, EPAEE, DHAEE were analysed for antibacterial activity against oral pathogens. This study demonstrated a novel bioactivity of the three major n-3 PUFA, EPA, DHA, and ALA, and their ester derivatives. Our experimental data indicated that n-3 PUFA and their ester derivatives exhibited strong antibacterial activity against various oral pathogens, including *Streptococcus mutans*, *Candida albicans*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis*. This study suggested that n-3 PUFA could have a positive therapeutic effect for improving oral health via their antibacterial activities, besides their anti-inflammatory effects.

INTRODUCTION

Fish oil is widely used as a supplement to improve diets because it contains the n-3 polyunsaturated fatty acids (PUFA), which are essential nutrients for normal physiological functions. In addition, fish oil has been suggested to help regulate cholesterol, reduce the risk of depression, and have a neuroprotective action in Parkinson's disease (Bousquet *et al.*, 2008; Kamphuis *et al.*, 2006; Pérez-Echarri *et al.*, 2009). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the major n-3 PUFA in fish oil (Kris-Etherton *et al.*, 2009; Moghadasian, 2008). The n-3 fatty acids refer to a family of unsaturated fatty acids that have a final carbon–carbon double bond in the n-3 position, from the methyl end of the fatty acid. The most widely available source of EPA and DHA for humans comes from cold-water oily fish such as salmon, herring, and mackerel. The human body cannot synthesize n-3 PUFA *de novo*, but it can form 20- and 22-carbon unsaturated n-3 fatty acids from the 18-carbon n-3 fatty acid, α-linolenic acid (ALA) (Doughman *et al.*, 2007). A similar fatty acid, linoleic acid, is an n-6 fatty acid, which has a different bioactivity from ALA. Linoleic acid is used in the celllular biosynthesis of arachidonic acid. As consumer products, n-3 PUFA are usually converted into n-3 fatty acid ethyl esters, which are incorporated in the diets because of the stability of n-3 fatty acid ethyl esters (Logan, 2003; Reis *et al.*, 1990).

It is reported that EPA and DHA can serve as precursors to eicosanoids, which can reduce inflamma-

tion. Recent studies have shown that n-3 PUFA can reduce inflammation in the oral cavity of rats challenged with specific oral bacteria (Campan *et al.*, 1997; Hasturk *et al.*, 2006). Recent studies also reported that fish oil dietary supplementation may have potential benefits as a host modulatory agent in the prevention and/or adjunctive management of periodontitis (Bendyk *et al.*, 2009; Campan *et al.*, 1997; Eberhard *et al.*, 2002). *Porphyromonas gingivalis*-infected rats treated with n-3 PUFA had significantly less alveolar bone resorption. These results demonstrated the effectiveness of an n-3 PUFA-supplemented diet in modulating alveolar bone resorption following *P. gingivalis* infection, and supported the idea that n-3 fatty acids may be a useful adjunct in the treatment of periodontal disease. In addition, n-3 PUFA reduce the inflammation in the oral cavity by modulating gene expression; rats on an n-3 PUFA diet exhibited decreased proinflammatory cytokine gene expression (interleukin-1β, tumor necrosis factor-α) and enhanced interferon-γ, catalase and superoxide dismutase messenger RNA expression compared with rats fed a corn oil diet, supporting a diet-induced modulation of host inflammatory reactions (Kesavalu *et al.*, 2007).

While it is known that n-3 PUFA have anti-inflammatory activity, little is known about the effect of n-3 PUFA on the actual growth and survival of oral bacteria. This study tested three major n-3 PUFA: EPA, DHA, ALA, and their methyl esters: EPAME, DHAME, ALAME and ethyl esters: EPAEE, DHAEE, ALAEE for their antibacterial activities. Our experimental data demonstrated that n-3 PUFA and their ester derivatives exhibited antibacterial activity against oral pathogens, such as *Streptococcus mutans*, *Candida albicans*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis*.

MATERIALS AND METHODS

Bioassay screening of fatty acids and ester derivatives

The three n-3 fatty acids, EPA, DHA, ALA, and the fatty acid ethyl esters, ALAEE, EPAEE, DHAEE were purchased from Cayman Chemicals (Ann Arbor, MI, USA) and the methyl esters, ALAME, EPAME, DHAME, were purchased from Sigma (St. Louis, MO, USA). Oral microbial species *S. mutans* ATCC 25175, *P. gingivalis* ATCC 33277, *C. albicans* ATCC 2091, *A. actinomycetemcomitans* JP2, and *F. nucleatum* ATCC 25586 were purchased from the American Type Culture Collection (Manassas, VA, USA). Tryptic soy broth-yeast extract (TSBYE) medium and anaerobe broth were purchased from Oxoid Ltd. (Cambridge, UK). Growth conditions were at 37°C in a Plas-Labs anaerobic chamber with 85% N_2, 10% H_2, and 5% CO_2 (Lansing, MI, USA). Various concentrations of EPA, DHA, ALA, and their methyl and ethyl esters were prepared in 99.5% ethanol solution and were tested against *S. mutans* as follows: 5 μl n-3 fatty acids, and methyl and ethyl ester solutions were prepared and tested for bioactivity by adding the solutions to each well of 96-well plate containing 200 μl TSBYE medium and 10% bacteria from the overnight culture, with 5 μl of 99.5% ethanol solution as negative control. The plate was then incubated in an anaerobic chamber for 16 h. After overnight incubation, 3 μl of the culture solution was diluted 10^5 times and plated onto blood agar plates (Remel®, Lenexa, KS). The plates were incubated anaerobically at 37°C for 48 h. Then, the colony-forming units were determined for each plate. Similar procedures were performed for analysis of *C. albicans*, *A. actinomycetemcomitans*, *F. nucleatum*, and *P. gingivalis*.

Preparation and analysis of glucosyltransferase

Crude glucosyltransferase (GTF) was prepared from *S. mutans* (Liu & Osawa, 2009). A 500-ml culture of *S. mutans* was grown in TSBYE medium in an anaerobic chamber for 3 days. The culture was harvested and centrifuged at 9000 $\times$ *g* for 15 min. The bacterial pellet was resuspended in 20 ml of 0.01 M (pH 5.5) sodium acetate buffer. The bacterial suspension was then sonicated five times for 30 s each time at 4°C. The bacterial lysate was centrifuged at 800 $\times$ *g* for 20 min. The supernatant was brought to 55% saturation with ammonium sulfate and allowed to sit for 3 days at 4°C. The precipitate was collected by centrifugation at 800 $\times$ *g* for 20 min. The precipitates were redissolved in 10 ml of 0.01 M (pH 5.5) sodium acetate buffer.

The ability of the fatty acids to inhibit GTF was assessed using a modification of the phenol–sulfuric acid method (Al-Hershi *et al.*, 2005). The reaction

mixture was made by adding 75 μl of 0.1 M (pH 6.2) sodium acetate containing 5% sucrose, followed by 25 μl crude GTF solution (150 mg/ml protein), and in the presence or absence of the n-3 fatty acids or ethyl esters (a final concentration of 50 μg/ml of n-3 fatty acids or ethyl esters). The reaction mixture was incubated for 1 h at 37°C. It was then centrifuged for 5 min at 9000 × *g*. The pellet was saved and 150 μl of 1 M HCl was added and boiled in water for 30 min, followed by addition of 150 μl 1 M NaOH to neutralize the solution. An aliquot (200 μl) of the reaction solution was placed in a microcentrifuge tube to which 5 μl of 99% phenol was added, followed by the rapid addition of 500 μl sulfuric acid. The reaction was incubated for 10 min, shaken, and then placed into a 30°C water-bath for 10–20 min. Color was stabilized for a few hours at room temperature and the optical density at 450 nm was determined to estimate formation of glucan.

RESULTS

The effect of n-3 fatty acids on the growth of *S. mutans* was examined. Significant antibacterial activity was found for EPA, DHA, and ALA. This antibacterial activity demonstrated a dose–response killing for each of the fatty acids (Fig. 1). EPA showed the greatest activity (93% of inhibition at concentration 2.5 μg/ml), with ALA showing 66.5% inhibition, and DHA the least effective (48.5% inhibition) at a concentration of 2.5 μg ml^{-1}.

Significant antibacterial activities were also found in n-3 fatty acid methyl esters, EPAME, DHAME, and ALAME. These modified compounds also demonstrated a dose-dependent inhibition (Fig. 2). ALAME showed the strongest antibacterial activity (80% of inhibition at a concentration of 2.5 μg ml^{-1}), with both EPAME (80% of inhibition at a concentration of 2.5 μg ml^{-1}) and DHAME (80% of inhibition at a concentration of 2.5 μg ml^{-1}) being considerably less effective.

Significant antibacterial activities were observed with the n-3 fatty acid ethyl esters, EPAEE, DHAEE, and ALAEE. The antibacterial activity of the n-3 fatty acid ethyl esters exhibited a dose–response killing (Fig. 3), with DHAEE and ALAEE appearing somewhat more effective than the native fatty acids at lower doses, and with ALAEE being the most inhibitory (85.6% inhibition at 2.5 μg ml^{-1}). Both EPAEE

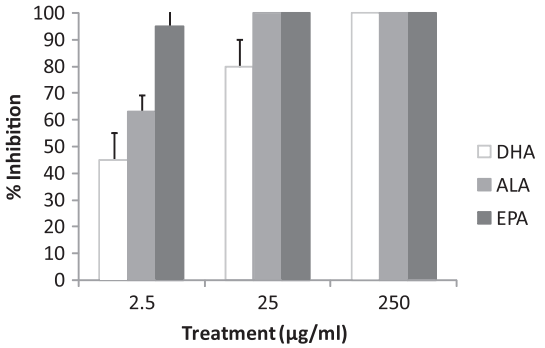

Figure 1 Effects of the n-3 fatty acids on *Streptococcus mutans* growth. Dose–response inhibition by n-3 fatty acids, docosahexaenoic acid (DHA), α-linolenic acid (ALA), and eicosapentaenoic acid (EPA) is shown. Three different concentrations (250, 25, and 2.5 μg ml^{-1}) of the n-3 fatty acids were used. Percentage of inhibition is based upon the ratio of colony-forming units at 24 h in the n-3 polyunsaturated fatty acid cultures to the colony-forming units in the cultures at 0 h time point × 100.

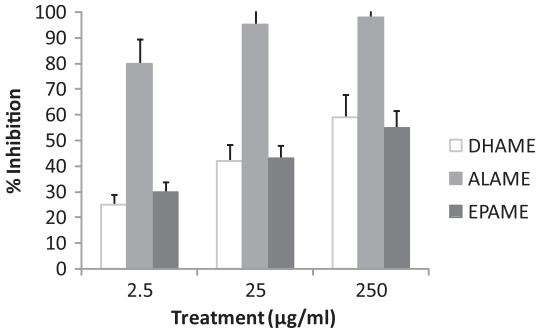

Figure 2 Effects of the n-3 fatty acid methyl esters on *Streptococcus mutans* growth. The percentage of inhibition and dose–response of n-3 fatty acid methyl esters of docosahexaenoic acid (DHAME), α-linolenic acid (ALAME), and eicosapentaenoic acid (EPAME) are shown. Three different concentrations (250, 25, and 2.5 μg ml^{-1}) of the n-3 fatty acid methyl esters were used.

(72% inhibition) and DHAEE (61% inhibition) were less effective at 2.5 μg ml^{-1}.

To further investigate possible mechanisms of inhibition of *S. mutans*, the capacity of the n-3 fatty acids, fatty acid methyl esters, and fatty acid ethyl esters to inhibit GTF function was assessed. Although the n-3 fatty acids, fatty acid methyl esters, and fatty acid ethyl esters exhibited significant inhibition of the growth of *S. mutans*, they had no effect on isolated GTF activity (data not shown).

The specificities of the n-3 fatty acids, fatty acid methyl esters, and fatty acid ethyl esters against various oral pathogens were also examined and com-

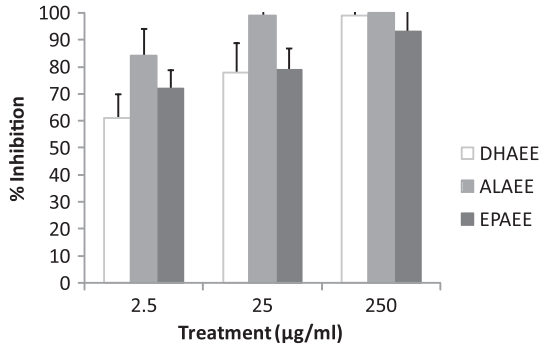

Figure 3 Effects of the n-3 fatty acid ethyl esters on *Streptococcus mutans* growth. The percentage of inhibition and dose–response of n-3 fatty acid ethyl esters of docosahexaenoic acid (DHAEE), α-linolenic acid (ALAEE), and eicosapentaenoic acid (EPAEE) are shown. Three different concentrations (250, 25, and 2.5 μg ml^{-1}) of the n-3 fatty acid ethyl esters were used.

pared (Table 1). The results suggest that modification of the carboxyl group of the n-3 PUFA significantly affects their antimicrobial specificity. In most cases, the native molecules (EPA, ALA, DHA) appeared to be the most active against the oral bacteria, with the methyl esters generally being less effective. However, the range of inhibition was substantially different across the various bacterial species. In contrast, the methyl esters were the most active for inhibiting *C. albicans*, although all forms of the ALA were generally similar. These fatty acids and their esters also exhibited strong antibacterial activity against periodontopathogens *A. actinomycetemcomitans*, *F. nucleatum*, and *P. gingivalis* (Table 1). These fatty acids and their esters have stronger activity against *P. gingivalis* than *A. actinomycetemcomitans* and *F. nucleatum*. As a specificity control, stearic acid was examined. Stearic acid is $C_{18}H_{36}O_2$ and it occurs in many animal and vegetable fats and oils. This fatty acid only showed a 10% inhibition at 250 μg ml^{-1} and 0% inhibition at 25 μg ml^{-1} compared with the n-3 PUFA or their derivatives.

DISCUSSION

Fish oil, often used synonymously with the components of n-3 polyunsaturated fatty acids (PUFA), is widely used as dietary or nutritional supplement. The PUFA have been suggested to contribute varied health benefits, primarily related to activities in minimizing inflammation and as antioxidants (Dyall & Michael-Titus, 2008; Liu & Osawa, 2009; Marik & Varon, 2009). More recently it has been reported that PUFA could improve oral health through these biological activities (Raffaelli *et al.*, 2008). Although oral inflammation is primarily triggered by oral bacteria and fungi, little is known about the effect of PUFA on the survival and growth of the microorganisms that stimulate these responses in the oral cavity. This study demonstrated a novel bioactivity of three major n-3 PUFA and their derivatives on oral microorganisms. The three major n-3 PUFA, EPA, DHA, and ALA, and their methyl and ethyl esters were shown to have antibacterial activities against various oral pathogens, including *S. mutans*, *C. albicans*, and *P. ginigivalis* at 50% inhibitory concentration from 1 to 10 μg ml^{-1}. To date, this is the first study to demonstrate the significant antibacterial activity of n-3 fatty acids and their esters against oral pathogens.

The structural and functional relationship of n-3 PUFA and their ester derivatives is not certain. Our experimental data demonstrated that both n-3 PUFA and their ethyl ester derivatives EPAEE, DHAEE, and ALAEE exhibited antibacterial activity against the

Table 1 Effects of n-3 fatty acids and their esters on various oral pathogens[1]

	Percentage of inhibition				
Fatty acid	*S. mutans*	*C. albicans*	*A. acinomycet-emcomitans*	*P. gingivalis*	*F. nucleatum*
EPA	100	37	72	100	46
EPAEE	77	59	45	11	43
EPAME	40	76	12	5	3
DHA	80	32	48	71	42
DHAEE	77	64	48	22	31
DHAME	40	78	0	0	0
ALA	100	62	70	100	73
ALAEE	98	72	55	92	31
ALAME	96	60	24	28	32

[1]*Streptococcus mutans*, *Candida albicans*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum*.
The percentage inhibition of n-3 fatty acids, of docosahexaenoic acid (DHA), α-linolenic acid (ALA), and eicosapentaenoic acid (EPA) are shown and their methyl (ME) and ethyl (EE) esters against various oral pathogens at a concentration of 25 μg ml^{-1} is shown. Inhibition was determined by comparison of colony-forming units of treated cultures at 24 h compared with those at 0 h. Final concentration of fatty acids was 25 μg ml^{-1}. Stearic acid was tested as a specificity control and had only 10% inhibition at 250 μg ml^{-1} and 0% inhibition at 25 μg ml^{-1}.

oral bacteria. However, modification of the carboxyl group of fatty acids greatly influenced their antibacterial activities, because the methyl and ethyl esters provided a very different inhibitory profile. In particular, the methyl derivatives were generally poorly antibacterial, but were the most effective for inhibiting the growth of *C. albicans*. Moreover, the modified PUFA demonstrated varied patterns of action against the bacteria, not limited to differences associated with the gram-positive or gram-negative nature of the species. These variations would suggest the potential that the n-3 PUFA and their derivatives have some specificity for interacting with different components of the outer membrane or cell wall of the individual species and altering their growth capacity. From our data in Table 1, it appeared that the free acids, which have hydrophilic head and hydrophobic tail, have better activities than the esters. The free fatty acids resemble the bipolar membrane of the bacterial wall. This could indicate that the target of the n-3 PUFA could be the cellular membrane because fatty acids could possibly penetrate into the cell membrane of the bacteria disrupting normal cell membrane functions and leading to bacterial death. Although our data support the *in vitro* effect, the *in vivo* effects would still need to be empirically determined. However, EPA and ALA had a much stronger antibacterial activity than DHA *in vitro* (Table 1) so it is expected that EPA and ALA will have stronger *in vivo* effects than DHA.

Dental caries is the predominant cause of tooth loss in children and young adults (Kenney *et al.*, 2000). Although caries is a specific bacterial infection associated with a number of species that appear to contribute to the extent and severity of the lesion, a primary etiological agent is *S. mutans* (Wall-Manning *et al.*, 2002). The features of this pathogen include its ability to survive and grow in an acid environment (Belli & Marquis, 1991), as well as it ability to accumulate on teeth via the production of sticky extracellular glucan matrices (Hamada & Slade, 1980). This latter virulence activity is the property of the glucosyltransferase enzymes of the microorganism (Yamashita *et al.*, 1993). Consequently, because we noted the effectiveness of killing of *S. mutans* by the PUFA, we evaluated their ability to inhibit GTF function. None of the PUFA preparations inhibited GTF function so their ability to affect *S. mutans* infections would appear to primarily be via killing the bacteria.

The n-3 PUFA could be readily available adjunctive biomolecules that could be incorporated into various delivery approaches for affecting oral bacteria and their associated diseases, e.g. dental caries, periodontal disease. These compounds, if effective *in vivo*, could be alternatives to the use of the traditional antibiotics that are selectively used to treat oral infections. Importantly, the substantial effect of these agents on *S. mutans* could provide an innovative new approach for controlling these infections in at-risk children and contribute to caries prevention strategies.

Furthermore, our experimental data indicated that n-3 PUFA and their ester derivatives exhibited strong antibacterial activity against various oral pathogens, such as *C. albicans*, and periodontopathogens, *A. actinomycetemcomitans*, *F. nucleatum*, and *P. gingivalis*. This study suggested that n-3 PUFA could have a positive therapeutic effect for improving oral health via their antimicrobial activities, besides their anti-inflammatory effects. Future research will be carried out to study the mechanism of their antimicrobial activity.

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