Journal of Aquatic Food Product Technology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/wafp20

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To cite this article: Hong-Deok Kim , Soo-Bin Lee , Sung-Hwan Eom , Daeung Yu , Suengmok Cho , Young-Mog Kim & Seon-Bong Kim (2021) *In vitro* Synergistic Antibacterial Effect of Ozonized Antarctic Krill Oil in Combination with Antibiotics against Bacterial Skin Pathogens, Journal of Aquatic Food Product Technology, 30:1, 133-137, DOI: 10.1080/10498850.2020.1854408

To link to this article: https://doi.org/10.1080/10498850.2020.1854408

Published online: 01 Dec 2020.

JOURNAL OF AQUATIC FOOD PRODUCT TECHNOLOGY
2021, VOL. 30, NO. 1, 133–137
https://doi.org/10.1080/10498850.2020.1854408

In vitro Synergistic Antibacterial Effect of Ozonized Antarctic Krill Oil in Combination with Antibiotics against Bacterial Skin Pathogens

Hong-Deok Kim[a], Soo-Bin Lee[a], Sung-Hwan Eom[b], Daeung Yu[c], Suengmok Cho[a], Young-Mog Kim[a]*, and Seon-Bong Kim[a]*

[a]Department of Food Science and Technology, Pukyong National University, Busan, Republic of Korea; [b]Department of Food Science and Technology, Dongeui University, Busan, Republic of Korea; [c]Department of Food and Nutrition, Changwon National University, Changwon, Republic of Korea

ABSTRACT

Ozonized Antarctic krill oil (OAKO) exhibited a significant antibacterial activity against bacterial skin pathogens that cause various skin infectious diseases such as *Propionibacterium acnes, Staphylococcus epidermidis, Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Minimum inhibitory concentration (MIC) values of OAKO against these bacteria ranged from 128 to 256 μg/ml. Furthermore, MIC values of antibiotics such as erythromycin and lincomycin were dramatically reduced in combination with OAKO against antibiotic-resistant bacteria. The combination of OAKO with erythromycin exhibited significant synergistic effect with fractional inhibitory concentration (ΣFIC) 0.375 and 0.503 against erythromycin-resistant *P. acnes* and *P. aeruginosa*, respectively. Hence, these results indicate synergistic antibacterial effects of restoration traditional antibiotics with OAKO.

KEYWORDS

Antibacterial agent; antibiotic resistant; skin pathogens; synergistic effect; ozonized krill oil

Introduction

Krill oil (KO) extracted from Antarctic small crustaceans (*Euphausia superba*) has gained increasing attention, because of its healthy functional properties such as anti-oxidant, anti-inflammation, anti-obesity, and prevention of cardiovascular diseases (Berge et al. 2014; Fasano et al. 2014; Kim et al. 2018a). These effects are mainly due to components present in KO, such as a high amount of unsaturated omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Interestingly, KO possesses a high content of phospholipids, whereas other fish oils are mainly composed of triglycerides. In addition, KO contains a natural pigment called astaxanthin (Tou et al. 2007; Xie et al. 2017). These are reasons that KO has gained increasing attention as an attractive resource for health and medicinal applications (Kim et al. 2018b).

Bacterial skin pathogens such as *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa*, and *Propionibacterium acnes* are often involved in the development of abnormal follicular keratinization and inflammation known as acne vulgaris (Kim et al. 2018a; Lee et al. 2014). Antibiotic applications used to treat bacterial infections often result in antibiotic-resistant bacteria. The field of synergistic therapy of traditional antibiotics with naturally derived agents has increased the production of effective antibacterial agents (Eom et al. 2016; Kim et al. 2018a, 2017; Lee et al. 2014). Ozone-treated oils have been applied as a therapeutic agent for diverse skin problems, including burns, wound healing, inflammation, and

CONTACT Young-Mog Kim ymkim@pknu.ac.kr; Seon-Bong Kim owlkim@pknu.ac.kr Pukyong National University, Busan 48513, Republic of Korea.

*Author contributed equally to this work.

periodontitis (Valacchi et al. 2005). In our previous study, it was revealed that ozone-treated KO (OAKO) showed an anti-inflammatory effect in lipopolysaccharide-stimulated RAW 264.7 macrophages (Kim et al. 2018b). However, there are no published materials on antibacterial activity of KO and OAKO. Therefore, the aim of this study was to evaluate the antibacterial effects of KO and OAKO, and the synergistic effects of the combination of oils with known antibiotics against bacterial skin pathogens.

Materials and methods

All chemicals including antibiotics were supplied by Sigma Chemical Co. (St. Louis, MO, USA). The Antarctic krill used in this study was captured in the Antarctic Ocean and provided in frozen state by Dongwon Inc. (Busan, Korea); it was then stored at −70°C. KO was extracted using hexane and treated with ozone gas at 3.006 g/h using an ozone generator, as described in Kim et al. (2018b). The four types of bacterial strains used in this study were obtained from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea) and the American Type Culture Collection (ATCC; Manassas, VA, USA). Bacterial cells were cultured as described by Kim et al. (2018a). The antibacterial efficacy of KO and OAKO was qualitatively evaluated by disk diffusion assay (Lee et al. 2014). In brief, 0.1 ml of bacterial culture, containing approximately 10^5–10^6 CFU/ml, was spread on Mueller Hinton agar (MHA; Difco Inc., Detroid, MI, USA). Sterile paper disks, 6 mm in diameter, loaded with 1 mg and 5 m of extracted oil sample were placed onto MHA plates. Then, the MHA plates were incubated at 37°C for 24 h, and the diameter of inhibition zone was measured. All experiments were conducted in triplicate, and mean values were calculated. In addition, minimum inhibitory concentration (MIC) assay was performed to evaluate the quantitative antibacterial efficacy of KO and OAKO, as described by Kim et al. (2018a). To estimate the synergistic effect, an integrated approach of the fractional inhibitory concentration (FIC) index was calculated by the combination of oils (KO and OAKO) with three selected antibiotics (Charway et al. 2019; Kim et al. 2018c). The synergic effect was defined as follows: ΣFIC value of <0.5 indicates synergistic effect; a value of 0.5 to <1.0 indicates weak synergistic effect; a value of 1.0 to <2.0 indicates indifferent effect; and the value of >2.0 indicates antagonistic effect.

Results and discussion

As shown in Table 1, OAKO at 1 and 5 mg/disc exhibited a zone of inhibition in range of 9.0 to 18.3 mm against bacterial skin pathogens tested in this study and also showed stronger antibacterial efficacy than that of KO. These results are in agreement with the previous findings that ozonized oil exhibited strong antibacterial activity (Montevecchi et al. 2013; Sechi et al. 2001). In this study, there was no significant difference in antibacterial efficacy in relation to the

Table 1. Disk diffusion assay of ozonized Antarctic krill oil (OAKO) against bacterial skin pathogens.

		Clear zone on plate (mm)			
Strains	Concn.	KO	OAKO (48 h)	OAKO (96 h)	OAKO (144 h)
Propionibacterium acnes KCTC 3314	1 mg/disk	–[1)]	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0
	5 mg/disk	-	13.0 ± 0.0	12.0 ± 1.7	11.3 ± 1.2
Staphylococcus epidermidis ATCC 14990	1 mg/disk	-	9.0 ± 0.0	9.0 ± 0.0	9.7 ± 0.6
	5 mg/disk	8.0 ± 0.0	13.0 ± 0.0	12.0 ± 1.7	11.3 ± 1.2
Staphylococcus aureus KCTC1927	1 mg/disk	-	12.7 ± 0.6	16.0 ± 0.0	12.0 ± 1.7
	5 mg/disk	9.0 ± 0.0	19.3 ± 1.2	18.3 ± 0.6	20.7 ± 2.3
Pseudomonas aeruginosa KCTC 1637	1 mg/disk	-	13.0 ± 0.0	13.0 ± 0.0	10.7 ± 0.6
	5 mg/disk	9.0 ± 0.0	17.7 ± 0.6	16.7 ± 0.6	15.7 ± 0.6

KO: Non-treated Antarctic krill oil. OAKO, Krill oil was treated with ozone gas for 48 h, 96 h and 144 h, respectively.[1)]-, not detected.

Table 2. Minimum inhibitory concentrations (MIC) of ozonized Antarctic krill oil (OAKO) and antibiotics against bacterial skin pathogens.

	MIC (μg/mL)				
Strains	KO	OAKO	Erythromycin	*Lincomycine*	Tetracycline
Propionibacterium acnes KCTC 3314	>2,048	256	1,024	1,024	32
Pseudomonas aeruginosa KCTC 1637	>2,048	256	16	128	0.5
Staphylococcus aureus KCTC 1927	>2,048	128	2	8	0.5
Staphylococcus epidermidis ATCC 14990	>2,048	256	0.5	8	0.5
Soussy's MIC breakpoints[1)]			1–4	2–8	4–8

KO, Non-treated Antarctic krill oil. OAKO, Krill oil was treated with ozone gas for 48 h.[1)]Soussy et al. (1994).

treatment time of ozone (48 h, 96 h, and 144 h); therefore, the 48 h ozonized OAKO was used in following experiments.

The MIC assay quantitatively demonstrated the antibacterial activities of the OAKO in the range of 128–256 μg/ml, while no antibacterial effect of the KO was observed against bacterial strains (Table 2). It was also observed that the antibacterial efficacy of the OAKO against *P. acnes* was higher than commercial antibiotics (erythromycin and lincomycin) used in the treatment of bacterial infection (Lee et al. 2014). Thus, these results suggest a potent antibacterial activity of the OAKO against bacterial skin pathogens tested in this study (Jang et al. 2006; Lezcano et al. 2000). It was supposed that the antibacterial effect of ozonized oils was mainly originated by generating reactive oxygen species (ROS) from double bonds of polyunsaturated fatty acids (Guerra-Blanco et al. 2017; Valacchi et al. 2005).

Results seen in Table 2 reveal that *P. acnes* KCTC 3314 and *P. aeruginosa* KCTC 1637 strains are resistant to antibiotics according to the criteria of Soussy's MIC breakpoints (Soussy et al. 1994). One effective way to control antibiotic-resistant pathogens is to restore the antibacterial activity of traditional antibiotics with combined usage of a natural antibacterial agent (Eom et al. 2016; Kim et al. 2018a, 2017; Lee et al. 2014). MIC values of antibiotics in combination with the OAKO against *P. acnes* and *P. aeruginosa* were in range of 2–16 μg/ml, while MICs of antibiotics alone were in 32–1,024 μg/ml (Table 3), representing a synergistic effect of the OAKO and antibiotics. Furthermore, FIC values revealed that the synergistic antibacterial effect in the combination of OAKO-erythromycin and OAKO-tetracycline against *P. acnes* was median ΣFIC 0.375 and 0.343, respectively. Additionally, a weak synergy effect was observed in the combination of OAKO-erythromycin and OAKO-lincomycin against *P. aeruginosa*, with median

Table 3. Fractional inhibitory concentration (FIC) indices of ozonized Antarctic krill oil (OAKO) in combination with antibiotics against bacterial skin pathogens.

Strain	Test compound	MIC (μg/mL)	Median ΣFIC	ΣFIC_{max}	ΣFIC_{min}	Minimum concentration for observing synergy
Propionibacterium acnes KCTC 3314	OAKO	256	0.375	1.031	0.625	0.5
	Erythromycin	1,024				16
	OAKO	256	1.002	1.25	0.313	0.5
	Lincomycin	1,024				16
	OAKO	256	0.343	1.125	0.251	0.5
	Tetracycline	32				8
Pseudomonas aeruginosa KCTC 1637	OAKO	256	0.503	1.016	0.156	0.5
	Erythromycin	16				8
	OAKO	256	0.625	1.5	0.516	256
	Lincomycin	128				2

OAKO, Krill oil was treated with ozone gas for 48 h; ΣFIC, the sum of FICs; ΣFIC_{min}, minimum ΣFIC; ΣFIC_{max}, the maximum ΣFIC.The FIC index indicated synergistic; <0.5, weak synergy; 0.5 to <1.0, indifferent; 1.0 to <2.0, antagonistic; ≥2.0. ΣFIC was calculated for each well with the equation: $\Sigma FIC = FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$, where MIC_A and MIC_B are the MICs of drugs A and B alone, respectively, and C

ΣFIC 0.503 and 0.625, respectively. Lower synergistic effect was recorded in the combination of OAKO-lincomycin against *P. acnes*, with median ΣFIC 1.002. Previous studies stated that there was a synergistic antibacterial activity in traditional antibiotics with different natural antibacterial agents against bacterial skin pathogens (Charway et al. 2019; Eom et al. 2016; Kim et al. 2018c, 2018a, 2017; Lee et al. 2014). There are no published materials relevant to the antibacterial activity of OAKO and its synergistic antibacterial effect. In conclusion, findings obtained from this study suggest that the OAKO could be a novel natural antibacterial agent to restore the antibacterial effects of traditional antibiotics in combined therapy against antibiotic-resistant bacterial skin pathogens.

Acknowledgments

Authors are thankful to Dongwon Inc. Busan, Korea for supplying antarctic krill.

Disclosure statement

No potential conflict of interest was reported by the author(s).

ORCID

Daeung Yu http://orcid.org/0000-0002-2185-4959
Suengmok Cho http://orcid.org/0000-0001-5951-5832
Young-Mog Kim http://orcid.org/0000-0002-2465-8013

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