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Alimentary Tract

Krill oil reduces intestinal inflammation by improving epithelial integrity and impairing adherent-invasive *Escherichia coli* pathogenicity

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ABSTRACT

Background: Krill oil is a marine derived oil rich in phospholipids, astaxanthin and omega-3 fatty acids. Several studies have found benefits of krill oil against oxidative and inflammatory damage.
Aims: We aimed at assessing the ability of krill oil to reduce intestinal inflammation by improving epithelial barrier integrity, increasing cell survival and reducing pathogenicity of adherent-invasive *Escherichia coli*.
Methods: CACO2 and HT29 cells were exposed to cytomix (TNFα and IFNγ) to induce inflammation and co-exposed to cytomix and krill oil. E-cadherin, ZO-1 and F-actin levels were analyzed by immunofluorescence to assess barrier integrity. Scratch test was performed to measure wound healing. Cell survival was analyzed by flow cytometry. Adherent-invasive *Escherichia coli* LF82 was used for adhesion/invasion assay.
Results: In inflamed cells E-cadherin and ZO-1 decreased, with loss of cell–cell adhesion, and F-actin polymerization increased stress fibres; krill oil restored initial conditions and improved wound healing, reduced bacterial adhesion/invasion in epithelial cells and survival within macrophages; krill oil reduced LF82-induced mRNA expression of pro-inflammatory cytokines.
Conclusions: Krill oil improves intestinal barrier integrity and epithelial restitution during inflammation and controls bacterial adhesion and invasion to epithelial cells. Thus, krill oil may represent an innovative tool to reduce intestinal inflammation.

1. Introduction

Intestinal homeostasis is highly regulated through a complex interaction between mucosal immunity, epithelium integrity, gut microbiota and nutrients. The latter are increasingly known as a variable playing a critical role in this process either by direct interaction with the epithelium or by altering microbiota composition or acting as allergens. Both inflammation and cancer can ensue upon dysregulation of this homeostasis.

Omega-3, n-3 polyunsaturated fatty acids (PUFAs), are termed "essential fatty acids" and usually obtained from the diet, because they cannot be synthesized by human cells. In particular, α-linolenic acid (ALA) is a n-3 PUFA that is endogenously converted into eicosapentaenoic acid (EPA) and subsequently into docosahexanoic acid (DHA). The anti-inflammatory properties of omega-3 PUFAs have been extensively studied for their role in preventing and treating many human inflammatory disorders [1–4].

The consumption of seafood provides a number of health benefits, many of which come from the presence of an adequate amount of PUFAs, including EPA and DHA, and antioxidants [5,6]. Most studies have supported the value of fish oil-derived PUFAs in inhibiting inflammation and improving inflammatory diseases, such as asthma, allergic diseases, cardiovascular disease, and diabetes [7–10]. In the gut, fish oil has proven to be strongly protective in animal models of bowel inflammation [11–15], although controversial results have been reported in human intestinal inflammation, especially in inflammatory bowel disease (IBD) [2,7,16–18]. Intriguingly, an important role of fish oil in modulating the gut microbiota has also arisen [19–22].

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More recently, krill oil (KO), an extract prepared from a species of antartic krill, *Euphasia superba*, containing omega-3 fatty acids, phospholipid-derived fatty acids and the natural pigment, astaxantin, has emerged for its purported health benefits, including the management and treatment of inflammatory and metabolic disorders [5,23–28].

KO is comparable to fish oil as dietary source of n-3 PUFAs, although KO comprises phospholipids and triglycerides, while fish oil only contains triglycerides. Pre-clinical studies have shown that fatty acids attached to phospholipids are absorbed into target organs, such as heart, brain and liver of animals, better than when attached to glycerol as triglycerides [29]. Besides, when quantitatively similar doses of n-3 PUFAs are administered, KO seems to have a greater effectiveness in promoting lipid catabolism [30], down-regulating the activity of pathways involved in hepatic glucose production as well as lipid and cholesterol synthesis [31]. Recently, Ramprasath et al. observed increased plasma concentrations of EPA and DHA with consumption of KO compared with fish oil and suggested that this was due to differences in absorption and bioavailability based on the structural difference of the two oils [32,33]. KO characteristically contains astaxantin, the orange-pinkish carotenoid that is currently emerging as a potent antioxidant and anti-inflammatory element with proven beneficial effects on cancer, diabetes and immune system [5,34–39].

Studying host–microbiota interactions are fundamental to understanding the mechanisms involved in intestinal homeostasis and inflammation. Perturbations to the structure of complex commensal communities (referred to as dysbiosis) can trigger a series of immune-mediated diseases, including IBD [40,41].

Recent studies have identified specific bacterial species with increased prevalence in patients with Crohn's disease (CD), one of the two forms of IBD, amongst which is the adherent-invasive *Escherichia coli* (AIEC) [42]. AIEC are characterized by enhanced epithelial adhesion and invasion, survival within macrophages and biofilm formation [43]. For these reasons, they are thought to play a role in the pathogenesis of IBD [44].

Hence, in the present study, we aimed at assessing the capacity of KO to down-regulate intestinal inflammation by inducing epithelial functional and morphological restitution, improving cell survival and reducing the adhesiveness and invasiveness of AIEC bacteria.

2. Materials and methods

2.1. KO

KO (Giellepi SPA, Milan, Italy), whose specific composition is phospholipids (51%, w/w), DHA (7%, w/w), eicosapentaenoic acid EPA (12%, w/w), omega-3 (ω-3, or PUFA-3) (21%, w/w), astaxanthin (0.04%, w/w), was used at a concentration of 250 mg/l in all experiments (dose of 750 μg/well). The manufacturer supplied a Certificate of Analysis for the use in nutritional supplements and functional food, which includes: total of active ingredients, physical and chemical tests and microbiological tests.

2.2. Corn oil

Corn oil (CO) (Sigma, St. Louis, MO, USA), whose specific composition consists for the most part of fatty acid (C18:1 20.0–42.2%; C18:2 39.4–62.0%), was used at a concentration of 250 mg/l in our experiments (dose of 750 μg/well).

The manufacturer supplied a Certificate of Analysis for the use in nutritional supplements and functional food, including active ingredients, physical and chemical tests and microbiological tests.

2.3. Bacterial strain LF82

The adherent, invasive *E. coli* strain, LF82 (ileal Crohn's strain, kindly provided by Prof. Arlette Darfeuille-Michaud, Clermont Université, Université d'Auvergne, Clermont-Ferrand, France) was cultured in MacConkey agar plates for 24 h at 37 °C and then subcultured in Luria Bertani Broth (LB, Oxoid, Basingstoke, U.K.) with overnight incubation at 150 rpm, 37 °C.

2.4. Cell lines

CACO2, HT29 (human colorectal adenocarcinoma cell lines) and RAW 264.7 (mouse leukemic monocyte macrophage) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MA, USA). CACO2 and HT29 were grown at confluence at 37 °C in Dulbecco's minimum essential medium (DMEM, Gibco, Life Technologies, Carlsbad, CA, USA) and McCoy's 5A medium (Gibco), respectively, supplemented with 10% inactivated foetal calf serum (FCS, Euroclone, Milan, Italy) and 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Biochrom, Berlin, Germany). RAW 264.7 were grown at confluence at 37 °C in RPMI 1640 medium (Gibco), supplemented with 10% FCS and 2 mM L-glutamine.

2.5. Scratch test

Wound healing was assessed by the scratch test as previously described [45]. Briefly, confluent HT29 cells were seeded on to a 6-multi-well plate at a density of 2×10^5 cells/ml. The linear scratch was made with a 10 μl sterile pipette tip and the gap widths (1 mm at day 0) were measured after 24 h. Then, cells were exposed to a cytomix of pro-inflammatory cytokines tumour necrosis factor α (TNFα 10 ng/ml) and interferon γ (IFNγ 250 ng/ml) to induce inflammation or co-exposed to cytomix and KO for 24 h. Scratch wound healing was observed under a Nikon inverted microscope (Nikon, Tokyo, Japan) at 10× magnification.

2.6. Flow cytometry

5×10^5 cells were seeded in multi-well plates with McCoy's 5A medium without serum. The day after cells were exposed to the cytomix or co-exposed to cytomix and KO for 18 h. Then, cells were fixed in cold 70% ethanol and kept overnight at +4 °C. Centrifuged cells were resuspended in 1 ml of propidium iodite/RNase staining buffer (BD Biosciences, San José, CA, USA). Samples were incubated for 15 min at +4 °C and analyzed by flow cytometry (FACSCalibur flow cytometer BD Biosciences). For each sample, 2×10^4 cells were analyzed. Cell cycle distribution and hypodiploid DNA content were calculated by Cell Quest software (BD Biosciences).

2.7. Immunofluorescence

Cells exposed to the cytomix or co-exposed to cytomix and KO were grown at confluence on a microscope glass slide for 24 h. Then, cells were fixed for 10 min in PBS with 4% paraformaldehyde. Cells were washed in PBS, permeabilized by incubation for 10 min in PBS-0.1% Triton X-100 (Sigma), and then blocked for 30 min in PBS-1% BSA. For E-cadherin and ZO-1 (Tight Junction Protein 1) staining, samples were incubated with anti-E-cadherin (1:100 dilution, BD Transduction Laboratories) and anti-ZO-1 (1:100 dilution, BD Transduction Laboratories) for 1 h and then with the secondary anti-mouse antibody Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) for 30 min. For F-actin staining, samples were incubated with Alexa Fluor 488-conjugated Phalloidin (1:50 dilution, Molecular Probes) according to manufacturer's instructions. In both cases