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Original article

## Polyunsaturated fatty acids affect intestinal anaphylactic response in BALB/c mice sensitized with $\beta$ -lactoglobulin

*Les acides gras polyinsaturés affectent la réponse anaphylactique intestinale chez la souris BALB/c sensibilisée à la  $\beta$ -lactoglobuline*

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### Abstract

**Objective.** – Polyunsaturated fatty acids (PUFA) have immunomodulatory proprieties and their use in the prevention and treatment of allergy symptoms is proposed as a therapeutic option. The present study investigates the effect of n-3 and n-6 PUFA on intestinal anaphylactic response in mice allergic to  $\beta$ -lactoglobulin ( $\beta$ -lg), a major allergen in cow milk proteins.

**Material and methods.** – Female BALB/c mice were fed by gavage for 15 days with either fish oil (FO) (n-3 PUFA) or corn oil (CO) (n-6 PUFA) at different concentrations (0.6%, 1%, 1.5% V/W) and were then sensitized with  $\beta$ -lg. To study the local allergic manifestations in the intestine, electrophysiological parameters (short-current circuit,  $I_{sc}$ , in  $\mu A/cm^2$  and tissue conductance,  $G$ , in  $mS/cm^2$ ) were measured in jejunum segments in an Ussing chamber, while morphological changes were assessed by histological analysis.

**Results.** – FO at 0.6% V/W significantly decreased  $I_{sc}$  ( $\mu A/cm^2$ ) values ( $P < 0.05$ ). All other doses of both oils proved ineffective. The three doses of FO (but not CO) significantly reduced tissue conductance ( $mS/cm^2$ ) values ( $P < 0.05$ ,  $P < 0.001$ ). Histological analysis showed morphological improvement in all groups with increased villus height ( $P < 0.01$ ,  $P < 0.001$ ).

**Conclusion.** – Low-dose PUFA supplementation, especially with n-3 PUFA, considerably reduced part of the intestinal damage resulting from sensitization with  $\beta$ -lg in BALB/c mice.

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**Keywords:** n-3 PUFA; n-6 PUFA;  $\beta$ -lactoglobulin; BALB/c; Ussing chamber

### Résumé

**Objectif.** – Les acides gras polyinsaturés (AGPI) ont des propriétés immunomodulatrices, leur utilisation pour la prévention et le traitement des symptômes d'allergie est proposée comme une stratégie thérapeutique. Le présent travail étudie l'effet des AGPI n-3 et n-6 sur la réponse anaphylactique intestinale chez des souris rendues allergiques à la  $\beta$ -lactoglobuline ( $\beta$ -lg), un allergène majeur des protéines du lait de vache.

**Matériels et méthodes.** – Des souris BALB/c femelles gavées pendant 15 jours avec de l'huile de poisson (FO) (AGPI n-3) ou de l'huile de maïs (CO) (AGPI n-6) à différentes doses : 0,6 %, 1 %, 1,5 % V/Pds sont ensuite sensibilisées avec la  $\beta$ -lg. Pour étudier les manifestations allergiques locales au niveau intestinal, les paramètres électrophysiologiques ; le courant de court circuit  $I_{sc}$  ( $\mu A/cm^2$ ) et la conductance tissulaire  $G$  ( $mS/cm^2$ ) ont été mesurés en chambre de Ussing, une étude histologique a été aussi réalisée.

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**Résultats.** – FO à 0,6 % V/Pds a significativement diminué les valeurs de l'Isc ( $\mu\text{A}/\text{cm}^2$ ) ( $p < 0,05$ ). Les autres doses des deux huiles se sont révélées inefficaces. Les trois doses de FO (mais pas celles de CO) ont significativement diminué la conductance ( $\text{mS}/\text{cm}^2$ ) ( $p < 0,05$ ,  $p < 0,001$ ). L'analyse histologique a montré une amélioration morphologique pour tous les groupes avec une augmentation de la hauteur villositaire ( $p < 0,01$ ,  $p < 0,001$ ).  
**Conclusion.** – Les AGPI n-3 à faible dose réduisent considérablement certains des dommages intestinaux induits par la sensibilisation à la  $\beta$ -lg.  
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**Mots clés :** AGPI n-3 ; AGPI n-6 ;  $\beta$ -lactoglobuline ; BALB/c ; Chambre de Ussing

## 1. Introduction

n-3 PUFA alpha-linolenic acid (ALA; 18:3n-3) and n-6 PUFA linoleic acid (LA; 18:2, n-6) are essential FA and must be provided by the diet. Via an enzymatic pathway that includes desaturases and elongases, n-6 and n-3 PUFA are converted to biologically active long-chain polyunsaturated fatty acid (LCP-PUFA), LA is converted into arachidonic acid (AA; C20:4 n-6) via gamma-linoleic acid (GLA; C18:3 n-6) and di-homo-gamma-linoleic acid (DGLA; C20:3 n-6), while ALA is transformed to eicosapentaenoic acid (EPA; C20:5 n-3) and docosa-hexaenoic acid (DHA; C22:6 n-3).

The literature reports the immunomodulatory proprieties of PUFA [1,2]. These substances can exert potent, sustaining, but opposing effects on immune activity [3]. n-3 PUFA are generally considered anti-inflammatory, while n-6 fatty acids are known to be proinflammatory at high intake levels [4].

Several studies have been conducted to investigate the effects of PUFA on Th2-mediated hypersensitivity, IgE-mediated food allergies and their symptoms [5–8], but to our knowledge their effects on intestinal anaphylactic response secondary to food allergy has not been studied.

Intestinal anaphylaxis can be defined as a dysfunction in fluid and electrolyte regulation with increased vascular permeability, resulting in massive fluid shifts from the intravascular to the extravascular space [9].

To study the local allergic manifestations in the intestine in response to food allergens, an in vitro approach could be adopted starting with systemic sensitization of an animal model with antigen plus adjuvant. This approach uses intestinal segments removed from sensitized animals, mounted in Ussing chambers, and challenged in vitro with antigen delivered to the luminal or the serosal surface of the tissue. The tissue is voltage clamped, allowing the measurement of net active ion transport across it [10].

The typical responses of the intestinal epithelium to allergens are an increase in watery diarrhea due to the stimulation of chloride secretion, and increased inward protein permeability from lumen to blood [11]; indeed, several studies have related increased intestinal Cl<sup>-</sup> secretion to local allergic reaction [12,13].

Since cow milk protein allergy (CMPA) is one of the most common food allergies among young children [14], we thought it would be of value to investigate a possible preventive effect of n-3 and n-6 PUFA against intestinal anaphylactic response secondary to sensitization with a major bovine serum allergen,  $\beta$ -lactoglobulin ( $\beta$ -lg), in a CMA animal model.

## 2. Materials and methods

### 2.1. Reagents

$\beta$ -lactoglobulin, fish oil (F8020) rich in n-3 PUFA, corn oil (C8627) rich in n-6 PUFA, and all other reagents used in this experiment were purchased from Sigma (France), except for alum [Al (OH)<sub>3</sub>], and calcium chloride, which were obtained from Merck (France). All materials and instruments for the Ussing chamber were purchased from physiologic instruments (San Diego, CA, USA).

### 2.2. Animals

BALB/c mice were housed and had mated in the animal facility of nutrition physiology and food safety laboratory-university of Oran1, originally purchased from the Pasteur Institute in Algiers. Animals were kept in a controlled environment at 22 °C with 12-hour light and dark cycles, and they had ad libitum access to a standard diet and water.

All animals were handled in accordance with current Algerian legislation covering animal welfare.

### 2.3. Experimental design

5-week-old offspring of BALB/c females weighing  $20 \pm 2$  g were divided into 7 groups: 6 experimental groups were given either fish oil (FO) or corn oil (CO) by gavage at different doses, namely 0.6%, 1% or 1.5% volume/weight (v/w), for 15 consecutive days prior to immunization with  $\beta$ -lg. A control group (CL) also received a solution of PBS 1/10 by gavage in a single dose of 0.6% V/W for 15 days and were then immunized with  $\beta$ -lg. Details of the experimental and immunization protocols are shown in (Fig. 1). At the end of the study, the mice were sacrificed and jejunum segments were removed for histological analysis and local anaphylactic study in an Ussing chamber by the measurement of electrophysiological parameters, i.e. short current circuit Isc ( $\mu\text{A}/\text{cm}^2$ ) and tissue conductance G ( $\text{mS}/\text{cm}^2$ ).

### 2.4. Measurement of intestinal anaphylactic response

The Ussing chamber provides a physiological system for measuring the transportation of ions, nutrients and drugs across various epithelial tissues [15]. A jejunum fragment was mounted in the Ussing chamber according to the protocol described by Grar et al. (2015) [13]. After a stabilization phase, the tissue was challenged with 60  $\mu\text{g}/\text{ml}$  of  $\beta$ -lg deposited on the serosal

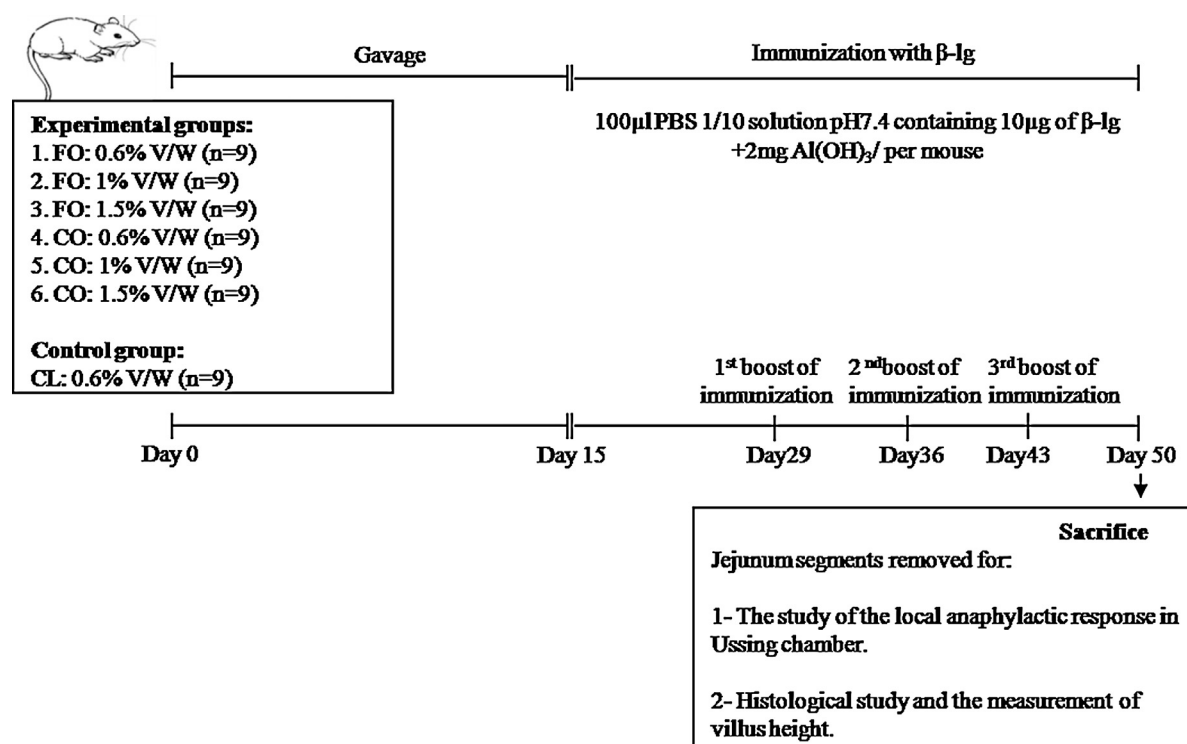


Fig. 1. Protocol diagram.

side. The electrophysiological parameters of short current circuit  $I_{sc}$  ( $\mu A/cm^2$ ) and tissue conductance  $G$  ( $mS/cm^2$ ) were then measured for a period of 10 min per experiment.

The  $I_{sc}$  ( $\mu A/cm^2$ ) represents the sum of net ion flux across the epithelium in the absence of an electrochemical gradient (mainly  $Na^+$ ,  $Cl^-$ , and  $HCO_3^-$ ). Transepithelial electrical conductance ( $G$  ( $mS/cm^2$ )) is an index of the epithelial integrity calculated according to Ohm's law. The increase in  $I_{sc}$  ( $\Delta I_{sc}$ ) and  $G$  ( $\Delta G$ ) is the difference between the peak value after  $\beta$ -Ig challenge and the base-line value.

## 2.5. Histological analysis

Jejunum segments were processed for histological analysis using the Hould method (1984), with tissue being fixed in 10% buffered formalin solution at pH 7.2 and stained with hematoxylin-eosin [16]. An optical microscope was used to investigate the effect of PUFA intake on mucosal damage after  $\beta$ -1 g sensitization. Villus height was measured at 6 points for each sample using an optical microscope equipped with a micrometer.

## 2.6. Statistical analysis

Data are expressed as means  $\pm$  SEM. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) with Tukey's post-hoc test using GraphPad Prism 5 software. A value of  $P < 0.05$  was considered the threshold of statistical significance.

## 3. Results

In ussing chamber, stimulation with  $\beta$ -Ig of jejunal fragments of the CL group induced an increase in the  $I_{sc}$  and  $G$  values ( $\Delta I_{sc} = 17.40 \pm 2.39 \mu A/cm^2$ ,  $\Delta G = 6.41 \pm 1.67 mS/cm^2$ ). In comparison to this group, all experimental groups showed decreased  $I_{sc}$  and  $G$  values after stimulation with  $\beta$ -Ig (Fig. 2, Fig. 3). However, the decrease in  $I_{sc}$  values was only statistically significant in the group supplemented with FO at 0.6% V/W (Fig. 2A) ( $P < 0.05$ ) and the  $G$  values were statistically significant in all FO-supplemented groups regardless of the dose used (Fig. 3A).

Histological analysis showed that the impaired intestinal architecture observed in the CL group was improved in all groups supplemented with both n-3 and n-6 PUFA at different doses (Fig. 4). The impairments consisted mainly of villus atrophy and crypt hyperplasia, although it is important to note that some morphological damage still remained in different experimental groups. The beneficial effect exerted by n-3 and n-6 PUFA was evidenced by a very significant increase in villus height ( $P < 0.01$ ,  $P < 0.001$ ) compared to the CL group (Fig. 5).

## 4. Discussion

In *in vitro* studies, it is well established that antigen challenge of tissue taken from sensitized animals induces a rapid increase in current across such tissue due to active chloride secretion by intestinal epithelial cells [17] and a decrease in epithelial barrier function associated with permeation of macromolecules across the epithelial tight junctions [18]. Indeed, many studies have found that intestinal permeability increases after food allergy

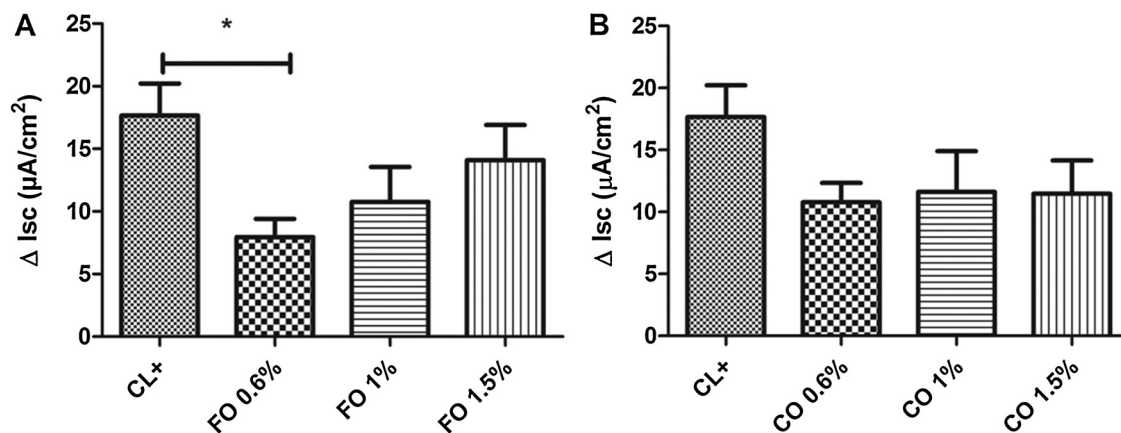


Fig. 2. Effect of FO (A) and CO (B) at different doses on  $\Delta I_{sc}$  values after  $\beta$ -Ig challenge. Data are presented as means  $\pm$  SEM,  $n = 9$ /group. \*  $P < 0.05$ , one-way ANOVA with Tukey's post-hoc test.

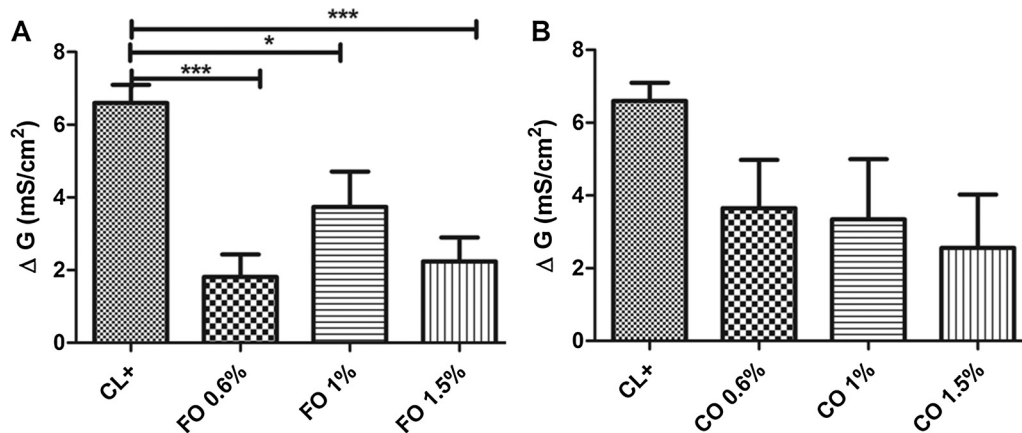


Fig. 3. Effect of FO (A) and CO (B) at different doses on  $\Delta G$  values after  $\beta$ -Ig challenge. Data are presented as means  $\pm$  SEM,  $n = 9$ /group. \*  $P < 0.05$ , \*\*\*  $P < 0.001$  one-way ANOVA with Tukey's post-hoc test.

[19] and that a constitutive abnormality in intestinal antigen handling is not the primary cause for the development of food allergy, but rather a secondary phenomenon [20]. In the study by Saidi et al. (1995), placing bovine  $\beta$ -Ig (the sensitizing antigen) on the luminal surface of jejunal biopsies from infants with active CMA induced an increase in CI-secretion and an increase in ionic conductance, which constitutes an index of epithelial integrity [21].

The beneficial effect of n-3 PUFA at 0.6% V/W demonstrated in this experiment could be explained by their ability to reduce food allergic response in general and therefore its symptoms. Indeed, several studies showed that diet supplementation with n-3 PUFA from fish oil led to a reduction of IgE, IgG1 antibodies and gut inflammatory response against food antigen (ovalbumin) in sensitized BALB/c mice [6]. Fish oil also reduced the generation of a whey-specific humoral response by B cells, leading to attenuated production of Th2-type immunoglobulins (IgE and IgG1) and reduced acute skin response to whey sensitization in C3H/He-OuJ mice [7]. Further, according to studies, n-3 PUFA from fish oil possibly target the mast cell directly, which may affect mast cell phenotype or degranulation [22]. Once activated mast cells release histamine, among other mediators, via a

cascade of intracellular reactions, histamine promotes CI-secretion and inhibits NaCl absorption [23].

The non-significant decrease in  $I_{sc}$  values observed in mice treated with higher doses of n-3 PUFA (1%, 1.5% V/W) compared to the CL group suggests that increasing the dose of ingested n-3 PUFA does not necessarily correlate with decreased intestinal anaphylactic response. Such results may be explained by the variability of the physiological responses observed in mice of the same group. The study by Broughton et al. (1997) compared the effects of 'low'-dose and 'high'-dose fish oil in adult atopic asthmatics; high n-3 PUFA ingestion resulted in improved lung function in more than 40% of subjects, however some patients did not respond to high n-3 PUFA intake, which in some cases worsened respiratory function. This study suggests that there are subjects who respond positively to fish-oil intervention and subjects whose response may be worsened by such intervention [24].

All three doses of FO showed an improvement in epithelial integrity represented by a significant decrease in  $G$  values compared to the CL group. The study by Shin et al. (2017) showed that reducing the dietary n-6 to n-3 PUFA ratio attenuated inflammatory indices and sustained epithelial tight junction integrity

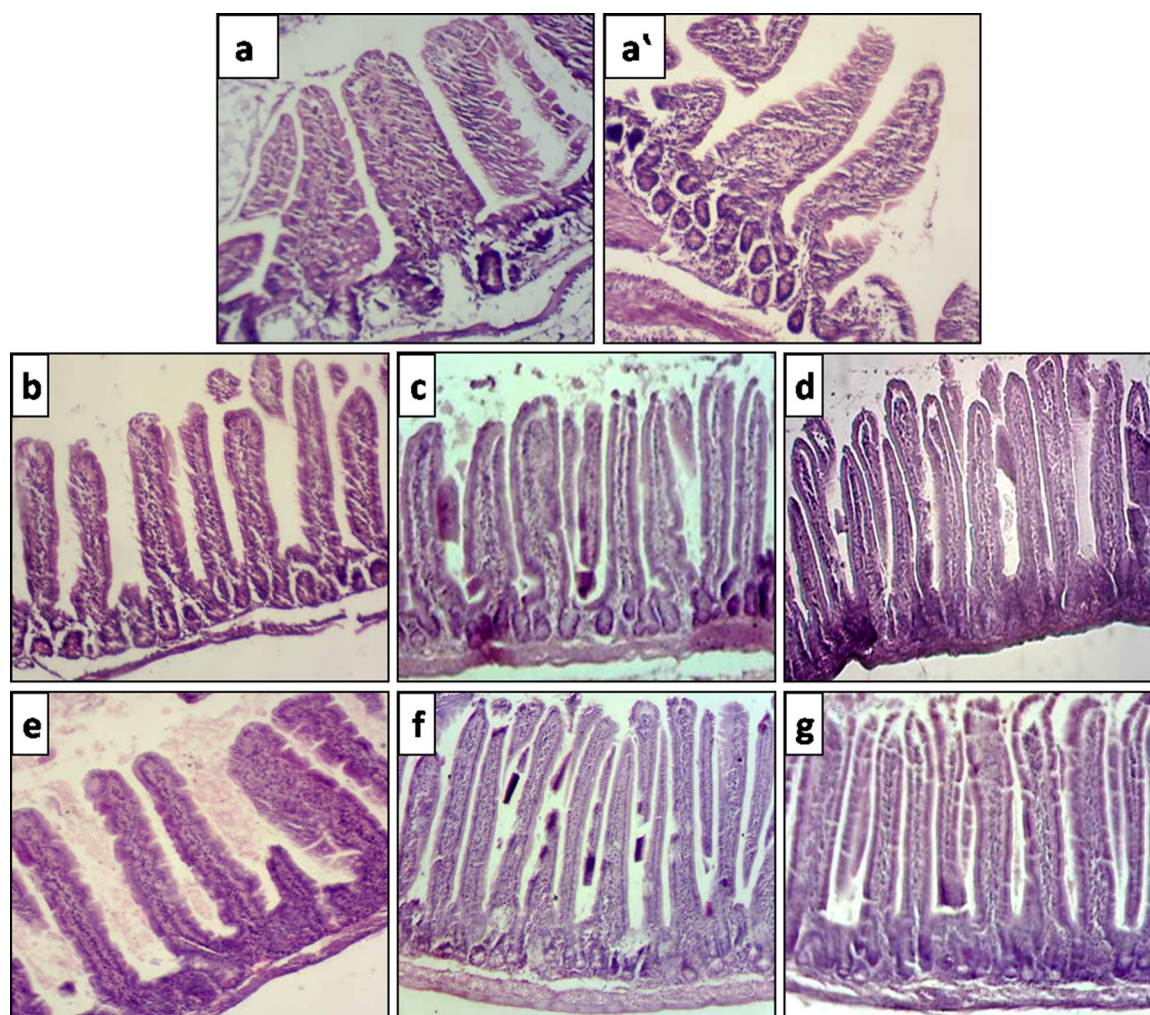


Fig. 4. Light microscopy ( $G \times 100$ ) showing histological sections of jejunal tissues stained with hematoxylin and eosin showing the effects of different doses of FO and CO in  $\beta$ -Ig sensitized mice: (a, a') CL; (b) FO 0.6%; (c) FO 1%; (d) FO 1.5%; (e) CO 0.6%; (f) CO 1%; (g) CO 1.5%.

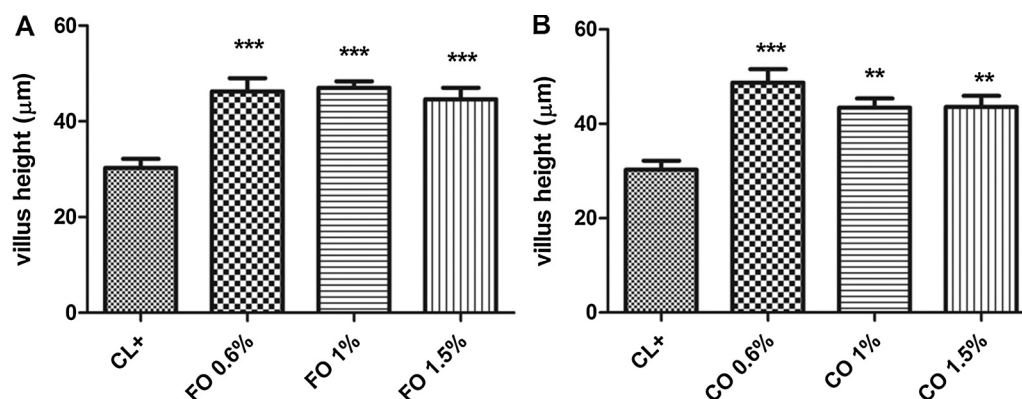


Fig. 5. Effect of FO (A) and CO (B) at different doses on jejunum villus height. Data are presented as means  $\pm$  SEM,  $n = 9/\text{group}$ . \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  one-way ANOVA with Tukey's post-hoc test.

in weaner pigs housed in poor sanitary conditions [25]. The anti-inflammatory properties of n-3 PUFA have been well established and their ability to reduce mucosal damages has been demonstrated in several studies [26,27]. In addition, n-3 PUFA, EPA and DHA changed the lipid environment in tight junction membrane microdomains, and prevented the redistribution of tight junction proteins occludin and ZO-1 [28], which are important

in the regulation of intestinal barrier function [29]. Similar findings were demonstrated by Beguin et al. (2013), using an in vitro intestinal cell translocation model cultivated with n-3 fatty acid and exposed to inflammatory stimuli, which showed that DHA prevented the redistribution of occludin and ZO-1 and that this is induced by inflammatory cytokines [30]. These effects were also observed by Li et al. (2014) [31].

In mice gavaged with different doses of CO (0.6%, 1% and 1.5% V/W), we anticipated increased Isc and G values compared to the CL group.

This assumption was based on the fact that the consumption of n-6 PUFA leads to the formation of specific eicosanoids such as 2-series prostaglandin PGE2 with a proinflammatory profile, which has an essential role in allergic inflammation [6]. However, we found values to be decreased but not statistically significantly so. These results are supported by the study by Willemsen et al. (2008), which demonstrated that specific PUFA of the n-6 (GLA, DGLA and AA) and n-3 series (EPA and DHA) may support natural resistance by enhancing intestinal epithelial barrier integrity in vitro. PUFA ameliorated paracellular permeability after epithelial challenge with allergy-associated cytokine IL-4. LC-PUFA in particular were found to enhance barrier properties, since ALA, LA and GLA were ineffective or less effective. These data support a beneficial role for LC-PUFA in improving gut integrity, thereby contributing to protection against infections and allergic disease [32].

Several papers [5–8] refer to a number of controversial results about the effect of PUFA on Th1 and Th2 responses which could be due according to some authors to the differences in the animal models, the fatty acids content and composition of different oil used, the feeding design or even the duration and timing of intake.

The intestinal architecture improvement observed in all experimental groups compared to the CL group is in concordance with several studies. In the study by Generoso et al. (2015), mice injected with 5-fluorouracil (5-FU) to induce mucositis showed disruption of the ileal mucosa with increased cellular infiltration in the lamina propria and a decreased villus/crypt ratio 72 h after 5-FU injection.

However, mice injected with 5-FU and fed a diet containing n-3 showed decreased mucosal inflammation with maintenance of the villus and crypt length [33]. Based on the study by Willemsen et al. (2008) [32], the effect of corn oil might be due to the effect of n-6 LC-PUFA.

## 5. Conclusions

In this study, we demonstrated the effect of different doses of n-3 PUFA from fish oil and PUFA n-6 from corn oil on the intestinal anaphylactic and inflammatory response in a murine cow's milk allergy model. Our results confirm the beneficial role of n-3 PUFA in the prevention of intestinal mucosal inflammation, and their effect on the intestinal anaphylactic response resulting from food allergy might well be dose dependent. However, at present we cannot draw conclusions concerning the beneficial effects of n-6 PUFA supplementation on the intestinal architecture until more studies are done.

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## Disclosure of interest

The authors declare that they have no competing interest.

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