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Research Article

Changes in the immune functions and susceptibility to *Listeria monocytogenes* infection in mice fed dietary lipids

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Summary The direct examination of the effects that fish oil diets (composed of long-chain *n*-3 polyunsaturated fatty acids) exert on immune system function indicates a reduction of host natural resistance to infectious diseases mainly because of a suppression of immune function generated by the fatty acids contained in this diet. Here, we evaluated the concentration of IL-12, IL-4, prostaglandin E_2 and leukotriene B_4 in the serum from BALB/c mice receiving four different diets. Each group was fed a diet that differed only in the source of fat: a low-fat diet (2.5% by weight), an olive oil diet (20% by weight), a fish oil diet (20% by weight) or a hydrogenated coconut oil diet (20% by weight). Mice were fed for 4 weeks and then infected with the intracellular pathogen *Listeria monocytogenes*. An initial reduction in the Th1-type response as a result of a decrease in IL-12p70 secretion, an inefficient action of IL-4 (Th2-type response) and no modification of pro-inflammatory lipid-mediator production could be, at least in part, the key events responsible for the inadequate elimination of *L. monocytogenes* from the spleens of mice fed a fish oil diet. Furthermore, our results suggest that the type of dietary lipids may affect the circulating concentration of IL-12p70 and IL-4, leading to a modulation in the protective cellular immune response to *L. monocytogenes* infection.

Key words: dietary lipids, fish oil, host natural resistance, hydrogenated coconut oil, immunomodulation, interleukin, leukotrienes, *Listeria monocytogenes*, olive oil, prostaglandins.

Introduction

Our current knowledge about the interactions of certain dietary lipids and immunity opens new clinical perspectives for the intervention of these substances as preventive agents capable of modulating immune function. For many years, different studies have revealed that dietary fish oil (FO) or vegetable oils are able to exert immunosuppressive effects.[1–3] As a result, some dietary oils have led to a reduction in the severity of different inflammatory diseases[4] and their incidence,[5] as well as a modulation of colon carcinogenesis.[3] Thus, long-chain *n*-3 polyunsaturated fatty acids (contained in FO) or monounsaturated fatty acids (contained in olive oil [OO]) affect a wide range of immune functions that include lymphocyte proliferation, cytokine production, NK-cell activity, adhesion molecules and antigen presentation in both humans and animals.[1] Nevertheless, numerous studies have determined that the anti-inflammatory effects exerted by certain dietary lipids could cause an impairment of host natural resistance and, therefore, the elimination of the infectious agents is less effective.[3,6–9] On the basis of these arguments, several investigations have demonstrated a reduction of natural resistance to infection induced by *Listeria monocytogenes*,[6–8] *Mycobacterium tuberculosis*[10] or *Salmonella typhimurium* serovar Typhimurium[11] in animals fed an FO diet. Numerous factors appear to be involved in the severe reduction of host resistance. Thus, modulation of cytokine production by certain fatty acids is a possible factor leading to the decreased ability to eliminate infectious agents.[12,13] IL-12 is an inflammatory cytokine that plays a critical role in the development of Th1-type immune responses, and offers an important protective cellular immune response against a variety of pathogenic agents.[14] It is a heterodimer that consists of 35 and 40 kDa subunits.[15] Bioactive cytokine is produced as a 70 kDa protein (IL-12p70), but the homodimer IL-12p40 antagonizes the activity of IL-12p70.[16]

Recent studies have determined that *n*-3 polyunsaturated fatty-acid diets are involved in the reduction of this pro-inflammatory cytokine in an early stage of *L. monocytogenes* infection.[12,13] In contrast, fatty-acid-rich diets are not related to the modulation of IL-4 production,[17] although it is important to emphasize that this cytokine is rapidly produced after *L. monocytogenes* infection, but it decreases soon after.[18] Hence, this event contributes to a reduction of host resistance against *L. monocytogenes*.[19] Similarly, the generation of lipid mediators (such as prostaglandins and leukotrienes), which are crucially important as potent regulators of immune responses, is altered by dietary lipids.[20,21]

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In the present study, we investigated the effect of several dietary lipids, such as FO, OO and hydrogenated coconut oil (HCO), on the circulating concentrations of IL-12p70, IL-4, prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) in the serum from mice experimentally infected with a virulent strain of *L. monocytogenes*, a ubiquitous Gram-positive facultative intracellular bacterial pathogen. Here, an initial reduction of IL-12p70 production by the administration of FO or HCO diets might be a critical factor involved in increased host susceptibility to *L. monocytogenes* infection.

Materials and Methods

Animals and diets

BALB/c mice 8–10 weeks old were purchased from the University of Jaén (breeding colony of Servicios Técnicos de Investigación, University of Jaén). They were housed in cages in an environmentally controlled room at a temperature of 24°C with a 12 h light–dark cycle. Mice were randomly allocated to receive one of four diets for 4 weeks and each group was allowed access ad libitum to water as well as to their respective diets. Experimental diets contained either OO (20%), FO (20%) or HCO (20%). In addition, another group was fed a low-fat (LF) diet (2.5% of fats), which was used as control. The composition of the experimental diets is shown in Table 1.

Bacterial cell preparation, infection and blood collection

Listeria monocytogenes was grown in blood tryptic soy agar (TSA) medium (Scharlau Chemie, Barcelona, Spain) for 24 h at 37°C. At the end of the experimental feeding period, each mouse was infected with a virulent strain of *L. monocytogenes* (10^5 CFU/mL) injected through the tail vein, and peripheral blood was isolated at 0, 24 or 48 h after experimental infection for the determination of interleukin secretion. For the quantification of viable bacteria from the spleen, mice were infected with 10^4 CFU/mL in the same conditions, and measurements were carried out at 24, 48, 72 and 96 h after experimental infections. Mice were anaesthetized with diethyl ether and the blood was drawn from the retro-orbital plexus into tubes containing heparin (20 U/mL blood). Serum was obtained after centrifugation of the tubes at 1500 *g* for 30 min. Finally, serum samples were stored at –80°C for subsequent analysis.

ELISA for cytokines

ELISA kits (R&D Systems, Minneapolis, MN, USA) were used for determination of IL-12p70 and IL-4 concentration in the sera samples. Results were calculated against standard curves generated using known amounts of recombinant cytokines in accordance with the manufacturer's instructions in a microplate reader (Whittaker 2001, Salzburg, Austria) at a wavelength of 450 nm. Limits of detection for these assays were <2.5 pg/mL (IL-12p70) and <2 pg/mL (IL-4). Samples were assayed in duplicate.

Determination of prostaglandin E_2 and leukotriene B_4

ELISA kits (R&D Systems) were used for determination of eicosanoid concentration (PGE_2 and LTB_4). Samples were diluted 1:2 in assay buffer. The measurement of PGE_2 and LTB_4 production was performed in accordance with the manufacturer's protocols in a microplate reader (Whittaker 2001) at a wavelength of 405 nm. Limits of detection for these assays were <15.9 pg/mL (PGE_2) and <19.4 pg/mL (LTB_4). Samples were assayed in duplicate.

Table 1 Composition of the experimental diets†

Components	Diet (g/kg)
Casein	200
D,L-methionine	3
Corn starch	315
Sucrose	155
Fibre	80
Fats‡	200
Mineral mix	35
Vitamin mix	10
Choline	2

†BALB/c mice were fed their respective diets for 4 weeks; ‡oils incorporated in the diets were olive oil, fish oil or hydrogenated coconut oil (the control group was fed a diet containing 2.5% lipid by weight [low-fat diet]).

Quantification of bacterial load in spleens of Listeria-challenged mice

Mice were infected with 10^4 CFU/mL in order to determine the capacity of host immune response to eliminate *L. monocytogenes*. Briefly, spleens were quickly removed and weighed under sterile conditions. Then, spleen cells were prepared by homogenizing the spleen between frosted-glass slides in distilled water under sterile conditions. Cells were disrupted by treatment with distilled water in order to release intracellular bacteria at 24, 48, 72 and 96 h after experimental infection with *L. monocytogenes*. Then, serial 10-fold dilutions of each sample were made and an aliquot of 10 μL of each dilution was transferred onto blood TSA medium to determine the number of live *L. monocytogenes* in the spleen. Plates were incubated at 37°C for 24 h, the number of CFU was counted and the values were expressed as $\log_{10}$ viable bacteria.

Statistical analysis

Results were expressed as mean ± SEM. Statistical differences were performed by ANOVA to compare the effects of the experimental diets with the control group (mice fed a LF diet). When significant differences occurred, the treatment means were compared using Fisher's least significant difference test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Body and spleen weights

Measurement of bodyweight of mice fed dietary lipids and experimentally infected with *L. monocytogenes* revealed no significant change after infection (Table 2), with the exception being mice fed a diet containing FO in which a reduction in bodyweight was observed at 72 and 96 h after infection with *L. monocytogenes*. In contrast, no significant increase was detected in the group fed diets containing LF or OO, whereas the bodyweight of mice fed an HCO diet remained unchanged. In general, spleen weights were significantly increased 72 h after experimental infection with *L. monocytogenes*, except in the group fed a diet containing FO, in which a substantial reduction was detected at 96 h after infection ($P < 0.05$).

Table 2 Body and spleen weights of BALB/c mice fed dietary lipids and time since experimental infection with *Listeria monocytogenes* (mean ± SEM of three mice in each period of time)†

	Time since experimental infection (h)				
	0	24	48	72	96
Bodyweight (g)					
Low fat	27.4 ± 1.8	25.7 ± 1.7	25.2 ± 2.0	27.7 ± 2.3	27.5 ± 0.4
Olive oil	27.2 ± 2.0	27.7 ± 2.0	26.3 ± 1.6	29.6 ± 1.2	29.0 ± 1.6
Fish oil	25.6 ± 1.0	27.3 ± 0.9	23.5 ± 1.2	20.1 ± 1.5*	19.7 ± 1.6*
Hydrogenated coconut oil	29.1 ± 1.6	29.3 ± 0.5	28.1 ± 3.3	28.4 ± 0.5	29.0 ± 1.6
Spleen weight (mg)					
Low fat	148.6 ± 14.4	134.6 ± 2.3	137.0 ± 3.6	182.5 ± 17.0*	220.0 ± 30.0*
Olive oil	187.6 ± 20.0	171.3 ± 4.0	184.4 ± 14.3	205.0 ± 15.0*	270.1 ± 12.0*
Fish oil	153.5 ± 20.3	172.3 ± 27.1	177.0 ± 12.2	159.0 ± 25.0	113.5 ± 3.5*
Hydrogenated coconut oil	180.1 ± 11.0	180.4 ± 27.8	162.4 ± 12.5	220.0 ± 20.0*	286.3 ± 15.0*

*$P < 0.05$ compared with values at 0 h within the same group; †BALB/c mice were fed one of four diets that differed only in type of fat.

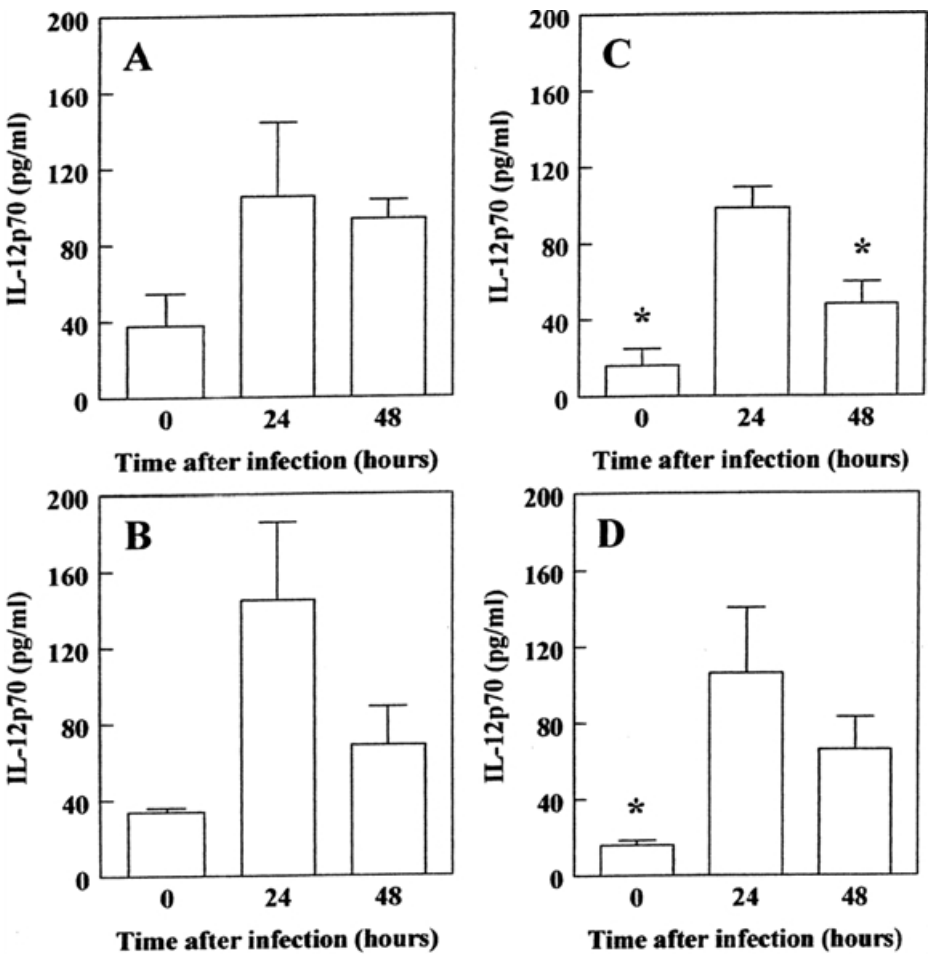

Figure 1 Determination of IL-12p70 secretion from the serum of peripheral blood of mice fed different dietary lipids for 4 weeks and infected with *Listeria monocytogenes*. BALB/c mice ($n = 5$ in each dietary group) were fed (A) a low-fat (LF) diet, (B) an olive oil diet, (C) a fish oil diet or (D) a hydrogenated coconut oil diet and infected with 10^5 viable *L. monocytogenes*. IL-12p70 was measured at 0, 24 and 48 h after bacterial infection. Quantification of IL-12p70 secretion in experimental samples was made by extrapolation from ELISA results using various concentrations of rIL-12 as a standard. Results are means ± SEM of two independent determinations in duplicate. *$P < 0.05$ compared with the LF group.

Measurement of IL-12p70 and IL-4 secretion

Analysis of IL-12p70 cytokine secretion, a cytokine which plays a crucial role in host defence against *L. monocytogenes*, revealed a significant reduction after dietary lipid administration in the groups fed diets containing FO or HCO (at 0 h of experimental infection) (Fig. 1C,D). In general, Fig. 1 shows an increase in IL-12p70 secretion 24 h following experimental infection with *L. monocytogenes* in all the groups, whereas a significant reduction in IL-12p70 secretion was observed after 48 h of infection particularly in the group fed a diet containing FO when compared with the control LF group ($P < 0.05$; Fig. 1C). In contrast, a substantial increase in IL-4 production was evident at 24 and 48 h after infection in the OO and FO groups (Fig. 2B,C), whereas a significant reduction in IL-4 production was observed in the HCO group 48 h after experimental infection (Fig. 2D), when compared with mice receiving the LF diet (control group; Fig. 2A). Finally, it is also important to note the initial increase in IL-4 production, which was observed after administration of the OO diet ($P < 0.05$).

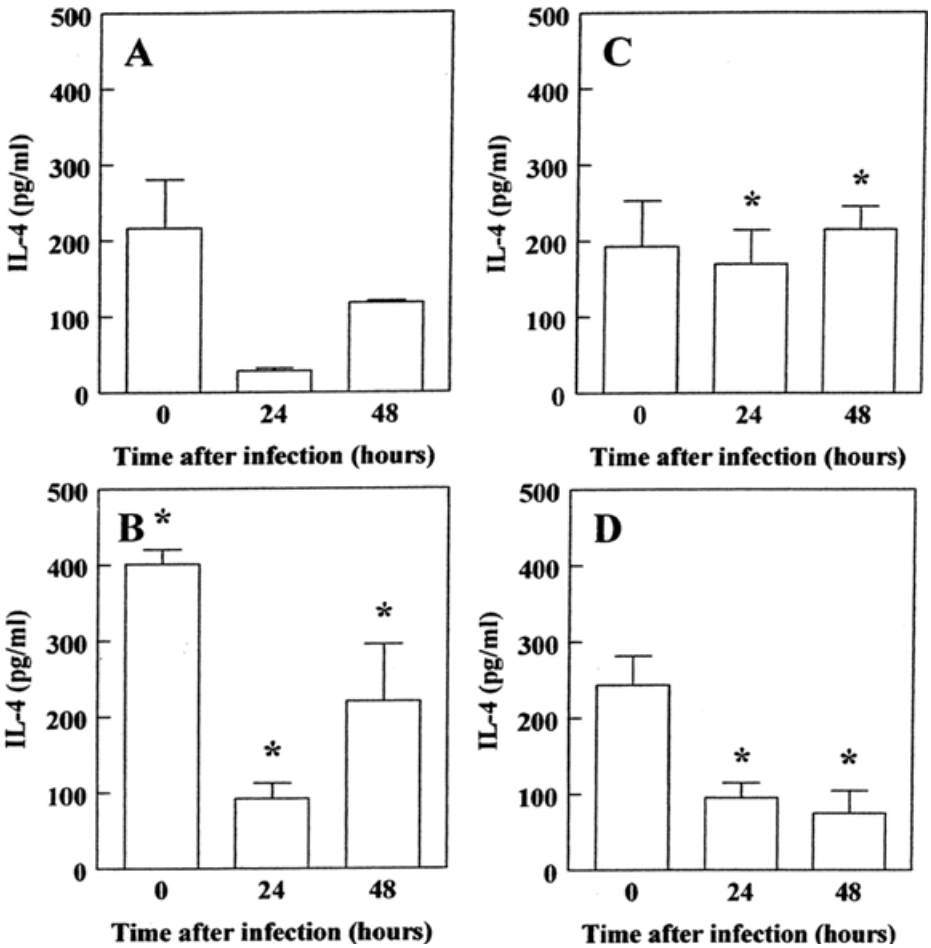

Figure 2 Determination of IL-4 secretion from the serum of peripheral blood of mice fed different dietary lipids for 4 weeks and infected with *Listeria monocytogenes*. BALB/c mice ($n = 5$ in each dietary group) were fed (A) a low-fat (LF) diet, (B) an olive oil diet, (C) a fish oil diet or (D) a hydrogenated coconut oil diet and infected with 10^5 viable *L. monocytogenes*. IL-4 was measured at 0, 24 and 48 h after bacterial infection. Quantification of IL-4 secretion in experimental samples was made by extrapolation from ELISA results using various concentrations of rIL-4 as a standard. Results are means ± SEM of two independent determinations in duplicate. $*P < 0.05$ compared with the LF group.

Determination of prostaglandin E_2 and leukotriene B_4

In the present study, we did not find any alteration in PGE_2 levels in the serum of mice fed dietary lipids after experimental infection with *L. monocytogenes* when compared with PGE_2 production in mice fed a LF diet (data not shown), whereas the levels of LTB_4 decreased significantly at 0 and 24 h after *L. monocytogenes* infection in mice fed a diet containing FO ($P < 0.05$; Fig. 3).

No statistically significant increase in LTB_4 levels was observed after 24 or 48 h of infection in mice fed a diet containing HCO, compared with the control LF group.

Quantification of Listeria monocytogenes in spleens of mice

Data presented in Table 3 show a significant increase in the number of *L. monocytogenes* recovered from the spleens of mice fed a diet containing FO at 24, 48, 72 and 96 h after experimental infection with the bacterium ($P < 0.05$). It should be noted, however, that there was a significant reduction in the number of viable bacteria recovered from the spleens of mice fed diets containing OO or HCO 72 h after experimental infection compared with the control LF group ($P < 0.05$). In general, the numbers of viable bacteria recorded from the spleens of animals peaked at 72 h after infection in all four experimental groups, but this increase was most marked in the group fed an FO diet ($P < 0.05$). Despite this increase in the number of viable bacteria at 72 h, a general reduction was observed 96 h after experimental infection with *L. monocytogenes*.

Discussion

Numerous studies have examined the importance of certain fatty acids as modulators of immune-system functions.[1,2] This immune status can be altered by different factors. Thus, modulation of cytokine production appears to constitute one of the multiple factors associated with these changes.[20] Therefore, an increase in the susceptibility of the host to infectious agents has been reported as a direct consequence of the alteration of immune functions by dietary fatty acids.[6–9,22] In fact, early investigations reported that mice fed a diet rich in lard exhibited an impaired immune response, because *L. monocytogenes* persisted in the livers of infected mice.[23]

A previous study from our laboratory revealed a reduction in mouse survival as well as an increase in the number of viable bacteria from the spleens of mice fed diets containing FO or OO and infected with *L. monocytogenes*.[6] Here, we confirm these findings and we examine the modulation of IL-12p70 and IL-4 secretion by dietary lipids and their effects on immune resistance to infection. The present study reflects the data obtained from a murine model experimentally infected with the facultative intracellular bacterium *L. monocytogenes*. Our results suggest an important reduction of both

body and spleen weights in mice fed an FO diet. These findings appear to indicate a progressive damage in murine immune status as a consequence of an inefficient ability to eliminate this pathogen. We have measured IL-12p70 secretion after experimental infection with *L. monocytogenes* because this cytokine has been shown to be critically important for the optimal development of Th1-type responses against this bacterium. However, recent investigations have determined that the administration of dietary lipids does not affect Th2-type cytokines (anti-inflammatory cytokines that suppress Th1 responses) and, in particular, IL-4 production is not altered.[24] Nevertheless, in the present study, the changes of IL-4 levels in mice fed unsaturated fatty acids and experimentally infected with *L. monocytogenes* do not appear to constitute a crucial event in the elimination of this bacterium, because a significant increase in IL-4 production was observed at 24 and 48 h after infection in the OO and FO groups, whereas a reduction in IL-4 production was observed in the HCO group at 48 h after infection. In spite of these differences, a similar recovery of bacteria from spleens of mice fed OO and HCO diets was apparent 48 h after infection.

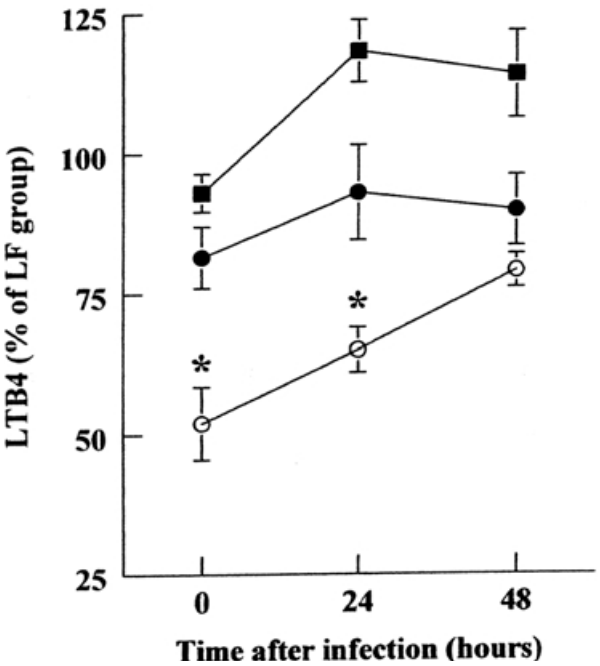

Figure 3 Measurement of leukotriene B_4 (LTB_4) production from the serum of peripheral blood of mice fed dietary lipids for 4 weeks and experimentally infected with *Listeria monocytogenes*. BALB/c mice ($n = 5$ in each dietary group) were fed a low-fat (LF) diet, (●) an olive oil diet, (○) a fish oil diet or (■) a hydrogenated coconut oil diet and experimentally infected with *L. monocytogenes* for 0, 24 and 48 h after bacterial infection. The LF group was considered as control. Values from the LF group were taken as 100%. Results were expressed as percentage of LTB_4 production. Data are means ± SEM of two independent determinations in duplicate. *$P < 0.05$ compared with the LF group.

Fritsche *et al.* reported that the change of fat type in animal diet alters the *in vivo* IL-12 response to *L. monocytogenes* infection.[12] Nevertheless, it is important to determine whether this effect persists during the infectious process with this bacterium. In the present study, the main changes were observed in the group fed an FO diet, in which different alterations were observed in the concentration of IL-12p70. In the group fed an FO diet, we detected a rapid increase in IL-12p70 at 24 h as well as a significant reduction in IL-12 production at 48 h after experimental infection as compared with the LF group, whereas a significant reduction in IL-12p70 levels was found initially (0 h). In fact, this last result is in agreement with Fritsche *et al.* who reported that *n*-3 polyunsaturated fatty acids contained in an FO diet are responsible for the reduction of IL-12 levels in animals experimentally infected with *L. monocytogenes*.[12,13] In the present study however, a significant reduction in IL-12p70 secretion was detected in the group fed an HCO diet at 0 h of experimental infection, which indicates that saturated fatty acids might also be involved in the regulation of IL-12 secretion. Evidence from a recent report has indicated that these effects could be related to an increase in cell death promoted by *n*-3 polyunsaturated fatty acids, which modulate T-cell functions by a selective deletion of the Th1 subset while maintaining or enhancing Th2-mediated humoral immune responses.[25]

In contrast, it is also known that *n*-3 polyunsaturated fatty acids are involved in the reduction of eicosanoid production.[26] However, in the present study, we did not find any alteration in the production of PGE_2 from the serum of mice fed dietary lipids after experimental infection with *L. monocytogenes* as compared with PGE_2 production in mice fed a LF diet (data not shown), whereas the levels of LTB_4 decreased at 0 and 24 h after *L. monocytogenes* infection in mice fed an FO diet. In fact, early data indicated that a diet containing long-chain *n*-3 polyunsaturated fatty acids reduced the production of LTB_4 by 50%.[27] Here, we demonstrated a similar reduction of LTB_4 levels in mice fed an FO diet after experimental diet

Table 3 Recovery of viable *Listeria monocytogenes* from spleens of mice fed dietary lipids (means ± SEM of two independent determinations in duplicate after logarithmic [$\log_{10}$] transformation of these variables)†

Time after infection (h):	No. viable bacteria ($\log_{10}$)			
	24	48	72	96
Low fat	2.40 ± 0.70	2.74 ± 0.39	4.01 ± 1.20	2.03 ± 0.90
Olive oil	2.17 ± 0.70	2.60 ± 0.30	3.60 ± 1.90*	2.17 ± 0.80
Fish oil	3.09 ± 0.90*	4.74 ± 1.39*	5.30 ± 2.90*	3.77 ± 1.60*
Hydrogenated coconut oil	2.60 ± 0.30	2.60 ± 0.47	3.69 ± 1.30*	2.83 ± 1.20*

*$P < 0.05$ compared with the low-fat group within the same time period; †BALB/c mice ($n = 5$ in each dietary group) were fed a low-fat diet, an olive oil diet, a fish oil diet or a hydrogenated coconut oil diet.

administration. On the basis of the present results, we suggest that these lipid mediators from the serum of mice fed dietary lipids do not appear to play a crucial role in the regulation of immune functions that lead to the elimination of *L. monocytogenes* in an efficient manner. Nevertheless, these results could depend on the type of animals fed dietary lipids or on the type of microorganisms responsible for the infection, because, for example, the persistence of *M. tuberculosis* infection in guinea pigs fed *n*-3 polyunsaturated fatty acids might be related to a reduction of PGE_2 or LTB_4 synthesis.[28]

With the goal of demonstrating the potential role of certain dietary lipids in the reduction of host natural resistance, we determined the number of viable bacteria in the spleens of infected animals. A significant increase in the number of recovered bacteria was detected in the group fed an FO diet as compared with values from the LF group. This effect could be associated with the significant reduction in IL-12p70 at 0 h. Indeed, an initial reduction of IL-12p70 production was also observed in the mice fed an HCO diet and a significant increase of viable bacteria was quantified at 72 and 96 h after experimental infection in this group. In short, the persistence of bacterial infection could be related to the initial reduction in IL-12 production observed in these groups. Hence, these results confirm previous findings that demonstrated an impairment of host natural resistance against *L. monocytogenes* after the administration of a diet containing FO.[6–9,22] Based on the present results and on previous results, we can speculate that the initial reduction of IL-12p70 production by certain dietary lipids appears to constitute a crucial factor that contributes to reducing the host natural resistance. Similarly, the changes in IL-4 production do not appear to constitute a critical factor responsible for the increase in host susceptibility to *L. monocytogenes* infection. Undoubtedly, it is generally accepted that *n*-3 polyunsaturated fatty acids are more suppressive than monounsaturated fatty acids or saturated fatty acids,[29] and, therefore, this alteration can produce severe effects in the host immune resistance. As a direct consequence, the measurement of mortality after *L. monocytogenes* infection in mice fed these dietary lipids has also shown a similar trend, which is attributed to a reduction in immune resistance to the intracellular pathogen *L. monocytogenes* after dietary lipid administration.[6] Therefore, the effect on IL-4 secretion and particularly IL-12p70 production, which modulate Th2-type and Th1-type response, respectively, could play an important role in the changes in immune function that lead to *L. monocytogenes* elimination.

Further studies will be needed to explain the participation of dietary lipids in the modulation of the immune system, as well as the biological and clinical consequences derived from the changes induced in immune function. Accordingly, the study of certain dietary lipids and their potential effects on immune-system functions will allow a better understanding of the possible action of these nutrients on host resistance or susceptibility to infectious agents.

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