

Stimulation of Immunity Without Alteration of Oral Tolerance in Mice Fed With Heat-Treated Fermented Infant Formula

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ABSTRACT

Objectives: Little information is available on the properties of fermented milk formula intended to healthy infants. This study analyzes the effect of long-term ingestion of a heat-treated, fermented milk formula on the development of oral tolerance or systemic immune response to soluble antigens in mice.

Materials and Methods: The C3H/HeN mice, fed with a heat-treated fermented (*Bifidobacterium breve* C50 and *Streptococcus thermophilus* 065) infant formula (htFF) or a matched control diet (control), were immunized with ovalbumin (OVA) with or without gavage of 20 mg OVA to induce tolerance or immunity, respectively. Systemic and local anti-OVA immune responses and intestinal barrier function were measured after 5 to 6 weeks.

Results: Oral tolerance to OVA developed similarly in htFF- and control-fed mice, attested to by the downregulation of OVA-specific immunoglobulin (Ig) G and IgE after oral OVA

administration. In contrast, immunization with OVA led to significantly higher titers in htFF-fed mice than in control-fed mice (log₂ IgG titers, 16.45 ± 1.24 and 15.46 ± 0.79, respectively; $P = 0.012$). Jejunal interferon gamma, interleukin 12p40 and interleukin 10 expressions were significantly higher in tolerized mice fed with htFF compared with those fed with the control diet. Mucosal to serosal intact horseradish peroxidase fluxes were lower in htFF-fed mice than in control-fed mice (39 ± 8 and 118 ± 38 ng/h.cm², respectively; $P < 0.0001$), indicating that the htFF diet reinforces intestinal barrier capacity to macromolecules.

Conclusions: In mice, htFF strengthens intestinal barrier and enhances systemic immune responses to antigens without interfering with the development of oral tolerance, suggesting a potential beneficial effect in host defence and vaccination. *JPGN* 43:451–458, 2006. **Key Words:** Fermented milk—Infant nutrition—Lactic acid bacteria—Heat-treated fermented infant formula. © 2006 Lippincott Williams & Wilkins

INTRODUCTION

When maternal milk is not available, adapted cow's milk formulas have proven effective in promoting normal growth in human infants. Recently, the concept of functional foods has led to the development of a fermented milk formula intended to potentially become an interesting alternative. A beneficial effect on colic irritability has been observed in infants fed with formula containing live probiotic bacteria *Bifidobacterium lactis* and *Streptococcus thermophilus* (1), and a heat-treated fermented (*Bifidobacterium breve* C50 and *S. thermophilus* 065) infant formula (htFF) was reported to reduce the severity of acute diarrhoea (2). Although the importance of gut flora-derived stimulation of the intestinal function has been underscored (3), as well as its involvement in intestinal homeostasis (4), the potential beneficial effects of fermented infant formula

and their impact on the modulation of the immune system remain debatable.

It is known that lactic acid bacteria stimulate the immunoglobulin (Ig) A secretory immune response to intestinal pathogens; in mice, the ingestion of yoghurt containing *Lactobacillus acidophilus* and bifidobacteria potentiated IgA responses to cholera toxin (5). A positive effect on the IgA secretory response to rotavirus was also documented in infants supplemented with live rather than heat-killed *Lactobacillus rhamnosus* GG (6,7). In addition, the poliovirus-specific intestinal IgA production was increased after vaccination of infants fed with htFF compared with infants fed with a control formula (8). In healthy children fed with a formula containing viable bifidobacteria, fecal levels of total IgA and antipoliovirus IgA were significantly higher than those recorded before formula ingestion (9). In parallel to these effects on secretory immunity, a number of studies have underlined the capacity of lactic acid bacteria to stimulate innate immune responses, including phagocytosis and proinflammatory cytokine secretion by monocytes/macrophages either in vitro (10,11) or in vivo (12,13). However, the gut mucosal

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immune system must fulfill dichotomic functions; although mounting potent immune responses to eliminate enteric pathogens, it also has to develop tolerance mechanisms to suppress responses to certain ingested food antigens (oral tolerance). Because the dietary and microbial environments in which a specific antigen is presented to the gut mucosa have a strong impact on the resulting immune response and on the development of tolerance versus immunity (14), we felt it important to evaluate the impact of lactic acid bacteria and their secretion products on the development of oral tolerance and conversely, to analyse the potential adjuvant effect of long-term consumption of fermented products on immune responses.

Using a mouse model, we therefore evaluated the long-term effects of htFF on the intestinal barrier function and on the development of oral tolerance to the food type protein ovalbumin (OVA) versus local and systemic immunization.

MATERIALS AND METHODS

Female C3H/HeN mice, ages 3 to 4 weeks at the beginning of the experiment, were used. They were divided into 2 dietary groups ($n = 12$ mice per group); the first group received ad libitum a pellet formula containing 50% of htFF, whereas the second group received a control formula.

Heat-treated Fermented Infant Formula

The infant formula used in this study was a follow-on formula, prepared from an adapted cow's milk-based formula, fermented by *B. breve* C50 and *S. thermophilus* 065 (Danone, France). The fermentation is started in the presence of 10^7 bacteria, a population reaching 10^9 colony-forming units/mL after a 7-hour incubation at 37°C. After heating at 80°C for 15 seconds, in view of inactivating the live bacteria, the formula is supplemented with various nutritive elements, homogenized at 250 bars and spray-dried at 200°C. The efficacy of the inactivation process is controlled using a standard plate count method, showing the absence of living bacteria. The final formula, consisting of a fermented heat-treated cow's milk-based infant formula containing bacterial bodies and fermentation products, was provided by Blédina-sa (Steenvoorde, France). The nutrient composition of this infant formula is as follows (grams per 100 g): proteins, 12.4; lipids, 21; lactose, 46.6; maltodextrin, 14; vitamins and minerals.

Preparation of the Control and htFF Diets

Both formulae (control and htFF) were prepared as pellets at the INRA/APAE, Jouy en Josas, France. The htFF formula was a mixture of 50% htFF and 50% standard rat/mice chow Ref. 04 (SAFE-UAR, Villemoisson, France). We did not feed the mice with 100% of the htFF because of its high-lactose content (46.6 g/100 g), which is not suitable for rodents. The matched control diet was formulated to reach protein, lipid, sugar, vitamin and mineral concentrations similar to those present in the htFF diet. Table 1 shows the composition of the 2 diets.

TABLE 1. Composition of diets

Ingredients (g/100 g)	htFF formula	Matched control formula
Heat-treated fermented (<i>B. breve</i> C50 and <i>S. thermophilus</i> 065) infant formula	50	0
Rat/mice standard chow (SAFE-UAR Ref. 04)	50	0
Casein	0	12.5
Corn oil	0	6.75
Colza oil	0	6.75
Corn starch	0	27.75
Potato starch	0	5
Saccharose	0	2.5
Lactose	0	23.3
Cellulose	0	4
Malto-dextrins	0	7
Mineral mixture	0	4.45
Vitamin mixture	0	0.034

The htFF diet is composed of 50% of a heat-treated milk-based fermented infant formula and 50% standard mice chow.

The control formula was made of a mixture of proteins, oil, lactose, vitamins and minerals to obtain a composition similar to the htFF diet.

Experimental Protocol

All mice were fed with their respective diets (control or htFF) 1 week before commencement of the tolerance or immunization protocols and throughout the 5 weeks of the experiment.

All together, 4 groups of mice ($n = 12$ per group) were tested, comprising mice tolerized or sensitised to OVA and fed with either the control or the htFF formula. The experimental schedule and protocols for tolerance induction and immunization are summarized in Table 2. Briefly, all the mice received their respective diet for 7 days before htFF mice were gavaged with 20 mg OVA in 200 μ L bicarbonate buffer, pH 8.0, at day 0, and control mice were gavaged with bicarbonate buffer alone. All mice were then primed subcutaneously with 25 μ g OVA in Freund adjuvant and boosted 2 weeks later by subcutaneous injection of 10 μ g OVA. After 1 additional week, mice were bled at the retroorbital plexus to measure humoral OVA-specific response (IgG, IgE). Spleen and jejunal fragments and rectal feces were also collected.

Measurement of OVA-specific IgE and IgG Circulating Antibodies (Humoral Response)

Plasma IgG and IgE titers were measured using an enzyme-linked immunosorbent assay as follows: 96-well flat-bottomed plates (Immulon II; Thermo LabSystems, Courtaboeuf, France) were coated overnight at 4°C with 100 μ L of 25 μ g/mL OVA (Sigma-Aldrich Co, Saint Quentin Fallavier, France) in phosphate-buffered saline (PBS). After washes with PBS-0.05% Tween 20 (PBS-Tween), nonspecific binding sites were blocked for 2 hours at 37°C with 100 μ L per well of PBS-1% bovine serum albumin (BSA). The plates were then incubated for 2 hours at 37°C with 100 μ L of plasma samples serially half-diluted in PBS-0.1% BSA. Peroxidase-conjugated rat anti-mouse IgG or IgE (Serotec, Cergy-Saint-Christophe, France) diluted 1:1000 in PBS-0.1% BSA was

TABLE 2. *Experimental protocol*

Mice	Day -7	Day 0	Day 7	Day 21	Day 28	Day 35
htFF and control	←htFF or control diet during 6 weeks (n = 12 mice in each group)→					
Tolerized	—	OVA gavage 20 mg in bicarbonate buffer, 0.2 mol/L	OVA in CFA priming 25 µg sc	OVA boost, 10 µg sc	Blood sampling (anti-OVA IgG, IgE)	Sacrifice, intestinal permeability splenocyte response
Immunized	—	Bicarbonate buffer gavage				

CFA, Complete Freund adjuvant; sc, subcutaneous.

added (100 µL per well) and incubated for 2 hours at 37°C. Finally, 100 µL of citrate buffer, pH 4.0, containing 10 mg orthophenylenediamine (Sigma-Aldrich Co) and 30 µL H₂O₂ were added to each well. The reaction was stopped with 50 µL per well 6N H₂SO₄ and plates read at 492 nm using an automatic Multiskan microplate reader (Thermo LabSystems). Titers were expressed as log₂ of the highest plasma dilution giving an optical density at least twice the blank value.

Measurement of Splenocyte Activation (Cellular Response)

Spleens were removed at the end of the experimental protocol, and splenocytes were isolated through a 30-µm nylon mesh in RPMI containing 2% fetal calf serum. After washing, the cells were seeded on to 96-well round-bottomed culture plates at 4 × 10⁵ cells per well in Cerottini culture medium (Dulbecco modified Eagle medium glutamax supplemented with 8% heat-inactivated fetal calf serum, 36 mg/L asparagine, 116 mg/L arginine, 10 mg/L folic acid, 1 g/L 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, 0.05 mmol/L β-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL streptomycin and 1 µg/mL fungizone) in the presence or absence of the specific immunizing antigen OVA (160 µg per well). After 6 days of OVA stimulation, 1 µCi of ³H-thymidine was added to each well for 16 hours; cell nuclei was extracted using a cell harvester, and accumulated radioactivity was measured using a 1450 microbeta Wallac trilux counter (PerkinElmer, Inc, Courtaboeuf, France). Results are expressed as ³H-thymidine incorporation in cells, expressed in counts per minute.

Measurements of Jejunal Cytokine Expression

Jejunal fragments from 6 mice per group (3–5 mm taken at 5–10 cm from the stomach) preserved in “RNA later” (Ambion, Inc, Austin, TX) were homogenized using a FastPrep apparatus. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. The 3 µg of total RNA was reverse transcribed for 75 minutes at 37°C in a reaction mixture containing 50 µg/mL of Random hexamers (Promega Corporation, Charbonnières-les-Bains, France), 10 IU/µL of Moloney murine leukemia virus reverse transcriptase, 1 × reverse transcriptase (RT) buffer (250 mmol/L Tris-HCl, pH 8.3, 375 mmol/L KCl, 15 mmol/L MgCl₂), 0.01 mol/L of dithiothreitol, 125 µmol/L of each deoxyribonucleotide triphosphate and 2 UI/µL of RNaseOUT (Invitrogen Corporation, Cergy Pontoise, France). After denaturation of the RT, complementary DNAs were stored at -20°C. The interleukin

(IL) 12p40, IL-4, IL-10, FoxP3 and TATA box binding protein messenger RNAs (mRNAs) were quantified using real-time RT-polymerase chain reaction (PCR) using gene-specific fluorogenic probes and the Taqman Universal PCR Master Mix kit (Assay-on-Demand; Applied Biosystems, Courtaboeuf, France). Interferon gamma (IFN-γ) expression was quantified using SYBRgreen PCR Master Mix (Applied Biosystems) and 900 nmol/L of the IFN-γ forward and reverse primer set (15).

The PCR reaction was carried out using an ABI PRISM 7700 sequence detection system (Applied Biosystems, Courtaboeuf, France). The enzyme was heat-inactivated at 95°C for 10 minutes. A 2-step PCR procedure of 15 seconds at 95°C and 60 seconds at 60°C was applied for 40 cycles. The results were referenced to the expression of the TATA box binding protein housekeeping gene by calculating 2^{-ΔCT}, with ΔCT being the difference in threshold cycles of target and housekeeping genes. All results are expressed as arbitrary units (AU).

Electrical Parameters and Intestinal Permeability to Macromolecules (Horseradish Peroxidase) and Small Molecules (Mannitol) in the Jejunum

Jejunal fragments were mounted in Ussing chambers exposing a surface area measuring 0.2 cm². They were bathed on each side with 1.2 mL of oxygenated thermostated Ringer's solution and short-circuited using an automatic voltage clamp (World Precision Instruments, Ltd, Hertfordshire, UK) after appropriate correction for fluid and system resistance. Electrical parameters, including potential difference, short-circuit current (I_{sc}) and total ionic conductance (G), were recorded at regular intervals during the 2-hour period of experimentation. A change in ionic conductance was considered an index of altered paracellular permeability. Horseradish peroxidase (HRP) transport was measured as an index of macromolecular permeability, and mannitol transepithelial passage was measured as a marker of paracellular permeability to small molecules. After equilibration of electrical parameters, HRP was added to the mucosal compartment at a final concentration of 0.4 mg/mL (10 µmol/L), and mannitol (final concentration, 5 mmol/L) was introduced on both sides of the jejunum with ¹⁴C-mannitol (12.2 kBq/mL) on the apical side only. The 2 markers were applied simultaneously in the mucosal compartment, and their transport was monitored by sampling 800 µL of the basal compartment at 30-minute intervals for 120 minutes, with replacement with fresh Ringer's solution. Each sample was analysed for mannitol fluxes by counting ¹⁴C-radioactivity in 500-µL samples by liquid scintillation photometry. The rate of intact HRP transport was determined by an

enzymatic assay (16) on 200 μL of samples. The HRP and manitol fluxes were expressed in $\text{ng/h}^{-1}\cdot\text{cm}^{-2}$ and $\mu\text{mol/h}^{-1}\cdot\text{cm}^{-2}$, respectively. Flux values are given at steady state (ie, during the second hour of the experiments).

Statistical Analyses

Statistical analyses were performed using the SAS software (SAS Institute, Cary, NC). The results are expressed as mean $\pm$ SD. Comparison of means was performed using analysis of variance and nonparametric Wilcoxon tests. The general linear model procedure was also used for multiple group-to-group comparisons. The differences were considered significant when $P < 0.05$.

RESULTS

At the end of the experimental protocol, mice in both dietary groups had a similar growth rate, as attested to by similar body weights (22–23 g).

Systemic Immune Response to OVA as a Function of the Diet

After immunization with OVA in the absence of prior OVA ingestion, plasma OVA-specific IgG titers were significantly higher in mice receiving the htFF compared with control mice ($\log_2$ titers, 16.45 ± 1.24 and 15.46 ± 0.79 , respectively; $P = 0.012$) (Fig. 1). In contrast, the anti-OVA IgE response was similar in both diet groups ($\log_2$ titers, 7.23 ± 0.9 and 6.99 ± 1.59 in htFF and control groups, respectively), suggesting that their T helper (Th) 1 but not Th2 response was

stimulated by the htFF. This Th1 response was further corroborated by a rise in plasma IgG2a response in htFF mice ($\log_2$ titer, 10.58 ± 1.93) compared with control mice ($\log_2$ titer, 8.88 ± 2.00 ; $P = 0.047$).

Oral tolerance developed similarly in mice fed with either diet (Fig. 1). The OVA-tolerized mice showed a significant decrease ($P < 0.0001$) in anti-OVA IgG and IgE titers ($\log_2$ IgG titers, 7.54 ± 1.66 and 8.04 ± 1.64 ; $\log_2$ IgE titers, 2.65 ± 0.68 and 3.01 ± 1.37 in htFF and control mice, respectively) compared with OVA-immunized mice ($\log_2$ IgG titers, 16.45 ± 1.25 and 15.46 ± 0.79 ; $\log_2$ IgE titers, 7.23 ± 0.90 and 6.99 ± 1.59 in htFF and control mice, respectively).

Cellular response to OVA is presented in Figure 2. In basal conditions, cell proliferation (^3H -counts per minute) was identical in OVA-tolerized and OVA-immunized mice, but cells from the htFF mice presented a higher rate of basal proliferation (3081 ± 576 vs 1355 ± 1289 cpm in immunized mice, $P = 0.02$, and 2623 ± 1622 vs 708 ± 371 in tolerized mice, $P = 0.0082$). After stimulation with OVA, in immunized mice, cell proliferation was higher in htFF mice than in control mice (7533 ± 1718 vs 3539 ± 768 , $P < 0.0001$). In tolerized mice, cell proliferation was also higher in htFF than in control mice (3713 ± 1491 vs 1400 ± 254 , $P = 0.0043$).

Cytokine (IFN- γ , IL-12p40, IL-10 and IL-4) and FoxP3 Expression in the Jejunal Mucosa

As shown in Figure 3, no significant difference in IFN- γ expression was observed between OVA-tolerized

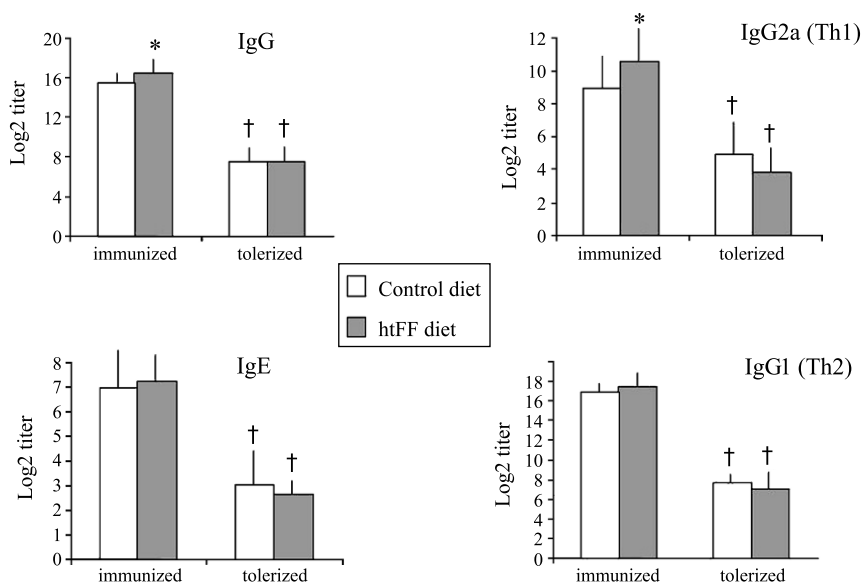


FIG. 1. The OVA-specific IgG, IgG1, IgG2a and IgE titers ($\log_2$) in tolerized or sensitised mice receiving either control or htFF diet. Tolerization was achieved in both dietary groups equally, but immunization was significantly higher in mice receiving the htFF diet and led to a Th1 response, as attested to by an increase in IgG2a response without modification of IgG1 or IgE response. *Significantly different from control diet, $P = 0.012$. †Significantly different from tolerized mice, $P < 0.0001$; $n = 12$ mice per group.

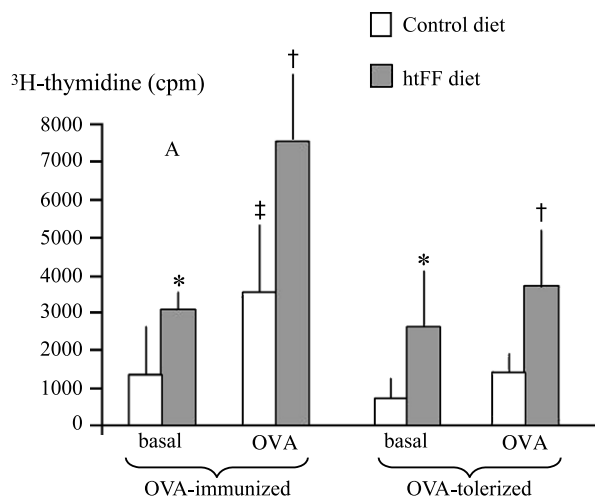


FIG. 2. The OVA-specific cellular immune response expressed as incorporation of ^3H -thymidine (counts per minute) by spleen cells. In basal conditions, proliferation (counts per minute) was higher in cells from htFF mice. In OVA-stimulated conditions, proliferation increased more in htFF than in control mice. Significantly different from control diet, * $P < 0.02$ in basal conditions and ‡ $P < 0.004$ in OVA-stimulated conditions. Significantly different from the basal condition in OVA-immunized mice, † $P < 0.006$ but not in OVA-tolerized mice ($P = 0.315$). cpm = counts per minute.

(1.33 ± 0.82 AU) and OVA-immunized (2.04 ± 1.19 AU) mice receiving the control diet. In contrast, there was a significant increase in IFN- γ expression in OVA-tolerized mice receiving the htFF-based diet (3.04 ± 1.59 AU) compared with OVA-immunized mice on the same diet (0.99 ± 0.46 AU, $P = 0.0042$) and with OVA-

tolerized mice receiving the control diet (1.33 ± 0.82 AU, $P = 0.014$). This increase in mRNA in tolerized mice was also observed for IL-10 and IL-12p40 but not for IL-4 and FoxP3.

In immunized mice, there was no modification whatsoever of cytokine expression observed in the dietary group.

Electrical Parameters and Intestinal Permeability to Macromolecules (HRP) and Small Molecules (Mannitol)

Ussing chambers allowed the analysis of the following: (1) the electrogenic activity of the intestine, as reflected by I_{sc} ; (2) the paracellular permeability to ions and small molecules, as reflected by ionic conductance and mannitol fluxes, respectively; and (3) the intestinal passage of intact antigens, as seen by intact HRP fluxes. As shown in Figure 4, there was a significant increase in I_{sc} in mice receiving the htFF diet compared with control mice ($31 \pm 27 \mu\text{A}/\text{cm}^2$ compared with $20 \pm 16 \mu\text{A}/\text{cm}^2$, $P = 0.039$).

Figure 5 indicates that there was an increase in paracellular permeability to ions but not to mannitol or HRP in htFF mice compared with control mice. Indeed, ionic conductance (G) was significantly increased ($21.5 \pm 9.3 \text{ mS}/\text{cm}^2$ vs $16.8 \pm 10.7 \text{ mS}/\text{cm}^2$, $P = 0.054$), but mannitol flux (also a marker of paracellular permeability) was not modified ($50.7 \pm 29.9 \mu\text{mol}/\text{h}^{-1}\cdot\text{cm}^{-2}$ and $40.5 \pm 33.7 \mu\text{mol}/\text{h}^{-1}\cdot\text{cm}^{-2}$, $P = 0.18$). Intact HRP fluxes were significantly decreased ($39.6 \pm 8.1 \text{ ng}/\text{h}^{-1}\cdot\text{cm}^{-2}$ vs $117.8 \pm 37.9 \text{ ng}/\text{h}^{-1}\cdot\text{cm}^{-2}$ in htFF and control mice, respectively; $P = 0.054$), attesting to a reinforcement

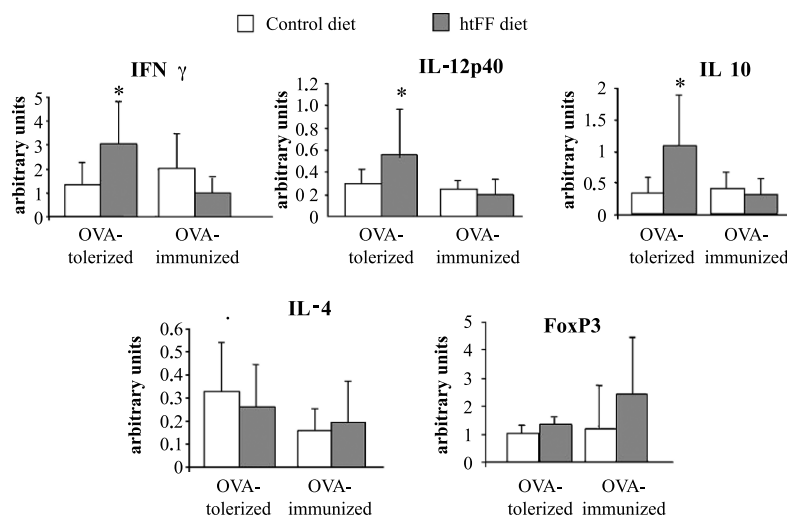


FIG. 3. Expression of IFN- γ , IL-12p40, IL-4, IL-10 and FoxP3 in jejunal mucosa, measured by quantitative RT-PCR. The expression of IFN- γ , IL-12p40 and IL-10 was increased in tolerized mice in the htFF group only compared with immunized mice. *Significantly different from control group, $P < 0.014$ ($n = 6$ mice per group).

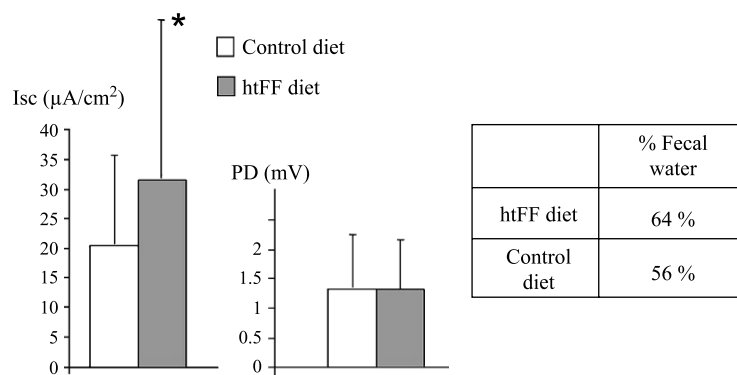


FIG. 4. Electrical parameters (Isc and potential difference) in jejunal fragments mounted in Ussing chambers, from mice fed with control or htFF diet. The Isc was significantly increased ($P < 0.04$) in mice receiving the htFF diet ($n = 31$ to 36 intestinal fragments). PD = potential difference. *Significantly different from control diet group.

of the intestinal barrier to macromolecules by the htFF diet.

DISCUSSION

The present results indicate that feeding mice at weaning with a heat-treated fermented milk formula is associated with 2 important modifications of intestinal and immune functions. First, it stimulates the antigen-specific IgG response after systemic immunization without altering the development of oral tolerance to soluble food antigens. Second, it strengthens the intestinal barrier to intact antigens, as exemplified by HRP—an effect that could be of help in digestive pathologies associated with compromised epithelial integrity.

The relevance of feeding healthy infants with fermented cow's milk-based infant formula during the neonatal period remains debatable. A first point for consideration, however, is the possible beneficial effects

in allergy-prone infants, whereas a second suggestion is their possible role as an immune adjuvant via the oral route during vaccination. The role of fermented milk in the prevention of allergy was addressed by Kalliomaki et al. (17), who showed that perinatal administration of *L. rhamnosus GG* decreased the subsequent occurrence of eczema in infants at risk of developing allergic diseases and extended this beneficial effect for up to the first 4 years of life (18). However, cow's milk allergy was diagnosed in 11% of infants receiving placebo versus 20% of the infants on *L. rhamnosus GG*, suggesting that treatment had no beneficial effect on the prevention of the development of cow's milk allergy. In another study, viable and heat-inactivated *L. rhamnosus GG* significantly alleviated eczema and subjective symptoms, although adverse gastrointestinal symptoms and diarrhoea were observed in the group receiving heat-treated bacteria (19). Few other reports have dealt with this question. A study on guinea pigs comparing the allergenicity of milk and fermented milk

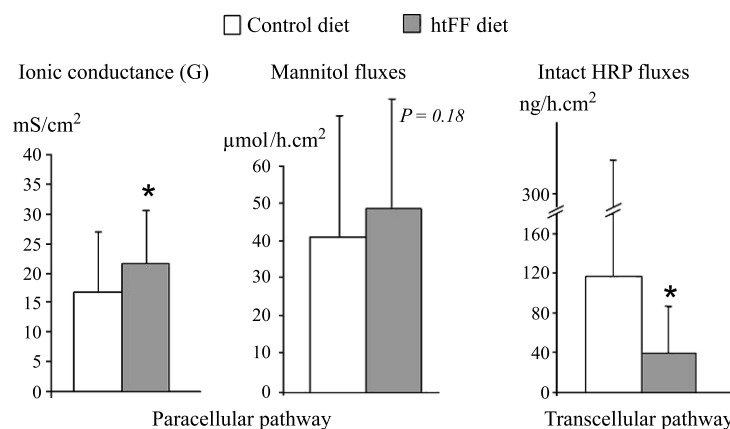


FIG. 5. Intestinal permeability as measured by ionic conductance and mannitol (paracellular) and intact HRP fluxes (transcellular). There was a slight but significant increase in ionic conductance and a tendency to increase mannitol fluxes in the htFF group compared with the control group, indicating mild modification of the paracellular pathway. In contrast, a significant decrease in intact HRP fluxes was observed in htFF mice, excluding paracellular leakage of macromolecules and indicating a reinforcement of the intestinal barrier to food type antigens. *Significantly different from control diet group, $P = 0.054$.

failed to demonstrate any difference in intestinal anaphylaxis or IgG1 (IgE) response to β -lactoglobulin, a major allergen in cow's milk (20). In the present study, feeding mice with fermented milk formula had no impact on the development of humoral systemic tolerance to OVA. It may, however, have promoted systemic T-cell tolerance because suppression of the splenocyte proliferative response to OVA only reached significance in mice fed with htFF. Assessment of mucosal responses according to the diet did not demonstrate any significant enhancement of the total or specific IgA response but showed a significant increase in jejunal levels of mRNA encoding IFN- γ , IL-10 and IL-12p40 but not IL-4 in tolerized mice while being fed with the fermented formula compared with tolerized mice being fed with the control diet. Although mRNA may not reflect the actual local concentration of cytokines in the intestinal mucosa, the results are compatible with a stimulation of local immune response. That IFN- γ may promote oral tolerance is suggested by 2 studies (21,22), although oral tolerance is often associated with the production of Th2 cytokines. Indeed, the effect of IFN- γ is ambiguous because exaggerated production of IFN- γ is deleterious to the intestinal mucosa and is thought to play a central role in the intestinal lesions secondary to the intolerance to gluten in celiac disease (23). Our results suggest that the milk formula containing heat-treated lactic acid bacteria used in this study can promote the production of both proinflammatory (IFN- γ and IL-12) and regulatory (IL-10) cytokines, without affecting the immune balance or the induction of immune tolerance. In this respect, the absence of modification of FoxP3 mRNA (forkhead box P3, a key element for CD25⁺ T regulatory cell function [24]) in the intestinal mucosa also indicates that the fermented milk formula did not alter tolerance. Moreover, htFF promoted Th1 responses in response to systemic immunization, suggesting a potential role of dietary fermented milk in enhancing host defence and favouring vaccination. The mechanism underlying this effect remains to be deciphered. Bacteria-derived products, such as DNA, may play a role because unmethylated CpG oligonucleotides derived from prokaryotes can stimulate immune responses (25) and act as adjuvants to switch on Th1 immunity (26). However, the effects of bacterial DNA on immunity are complex because in inflammatory conditions such as murine experimental colitis, they have been proven to be beneficial in inhibiting the development of such colitis through a mechanism involving Toll-like receptor 9 (27,28) and the release of type I IFN (29).

Other effects of fermented infant formula is the slight but significant increase in Isc and the strengthening of epithelial barrier function to food type antigens, as shown by the significant decrease in the transport of the macromolecular tracer HRP (MW 40 kd). The Isc increase may be caused by an increase in chloride

secretion from the serosa to mucosa, although we did not measure transepithelial sodium or chloride fluxes to determine the origin of this increase (ie, increase in sodium absorption or chloride secretion). This possibility is also suggested by the slight increase in fecal water observed in htFF mice, which could be related to water secretion passively after chloride secretion, although such hydroelectrolytic movements seem to remain in a physiological range.

Reinforcement of the intestinal barrier has already been reported in guinea pigs fed with milk fermented with the lactic acid bacteria used in the present work (20) and in mice receiving the probiotic mixture VSL#3 (30). It is noteworthy that, although IFN- γ expression in the intestinal mucosa was increased in tolerized htFF mice, mucosal IFN- γ release was probably small and without effect on epithelial integrity because IFN- γ is known to increase both transcellular and paracellular permeability (31). The reinforcement of the intestinal barrier to intact proteins in mice receiving the htFF diet may be explained by the different properties already reported for defined strains of probiotic bacteria, such as antiapoptotic effect (32) and stimulation of epithelial cell proliferation and repair (33).

Taken together, the present results indicate that in this animal model, htFF is capable of stimulating immunity without altering oral tolerance and of strengthening intestinal barrier function. Such a set of properties may be of significant interest in infant nutrition.

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