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Probiotics induce resistance to enteropathogens in a re-nourished mouse model

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Nutritional deficiency is commonly associated with impaired immune response and the relation between infection and malnutrition is synergic. Probiotics, especially lactic acid bacteria are immunomodulatory. The aim was to determine whether optimal doses of *Lactobacillus casei* and yogurt, used as adjuvants in a re-nutrition diet in a non-severe malnutrition experimental model, protect against *Salmonella typhimurium* and *Escherichia coli*. Groups of malnourished mice were used, which were re-nourished with milk for 7 or 14 d. After that, both groups of mice received the optimal doses of *Lb. casei* and yogurt supplements. We measured IgA+ and IgG+-B cells and phenotypic markers of T lymphocytes; CD3+, CD4+ and CD8+ cells. We also determined αβ and γδ T cell receptor (TCR). The ability to protect against *Sal. typhimurium* and *Esch. coli* infections and specific S-IgA were assessed. Probiotics complemented the effects of the re-nutrition diet, by stimulating recuperation of the activity of immune cells that improved protection against infections.

Keywords: Probiotics, malnutrition, *Lactobacillus casei*, yogurt, enteropathogens.

Bacterial pathogens colonize a host and produce adverse effects. As these bacteria enter a host, a variety of interactions determine the success of the infection process (Jensen et al. 1998). After entering the host, organisms must avoid host defence mechanisms, and they have evolved numerous strategies to exploit the cellular processes of the host so that they can survive and persist. Frequently, a bacterium must adhere very tightly to the cells and mediate its effects extracellularly, or it must find a way to invade the host cells and survive intracellularly (Goosney et al. 1999). The host responds to these actions with non-specific phagocytic cells and an inflammatory response as well as by activating specific cellular and humoral responses (Jones & Stanley, 1996). The outcome of immune responses in the gut may be affected by changes in the intestinal microenvironment induced by several factors, including infections, malnutrition, imbalances in endogenous microflora and mucosal injury. Nutritional deficiencies are commonly associated with impaired immune responses (Chandra, 1997; Woodward, 2001). Protein-energy malnutrition and infection are often synergic, as seen in the malnutrition-infection cycle. Causes of malnutrition are multiple and complex, and infection is a common precipitating factor; malnutrition can also make the animal susceptible to infection and the two interact, with each factor often making the other worse (Scrimshaw & San Giovanni, 1997).

Several reports show disease prevention or enhanced immune function from oral administration of certain members of the intestinal microflora. Much of the protection, which is attributed to probiotics, may be due to the resulting resistance to colonization. A probiotic is a microbial dietary adjuvant that beneficially affects the host by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract (Fuller, 1992). The probiotic spectrum of activity can be divided into nutritional, physiological, and antimicrobial effects. Probiotics can be used as innovative tools for treating dysfunctions of the gut mucosal barrier including acute gastroenteritis, food allergy, and inflammatory bowel disease.

Lactobacillus casei induces a secretory immune response and protection against enteric infections and the effect is dose-dependent (Perdigón et al. 1990, 1991, 1995b). In an experimental model of severe malnutrition, however, yogurt and *Lb. casei* do not increase the protective intestinal mechanisms to the levels found in the well-nourished host (Perdigón et al. 1995a; Agüero et al. 1996; Perdigón & Oliver, 2000). Therefore, we used *Lb. casei* and yogurt in the malnourished mice after mucosal recovery by adequate

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refeeding and selected optimal doses to avoid harmful effects at an intestinal level from over-stimulating the immune system (Gauffin Cano et al. 2002a, b).

The present work examined whether optimal doses of *Lb. casei* and yogurt, used as adjuvants in a re-nutrition diet in a non-severe malnutrition experimental model, induce protection against enteropathogens, such as *Salmonella typhimurium* and *Escherichia coli*.

Material and Methods

Animals

Weaned BALB/c mice from the closed colony of the breeding unit kept at CERELA were malnourished for 21 d by being fed with corn flour (protein-free diet, PFD). The diet was supplemented to meet nutritional requirements and was formulated as follows: vitamin mixture (2·2%) (ICN, Biomedicals, Inc-1263, Aurora, OH 44202), salt mixture (4%) (ICN, Biomedicals), and lipids (5%). At the end of this period, the animals that weighed 35–55% less than a group of well-nourished control were selected for the experiments. The well-nourished group (W-N) consumed *ad libitum* a balanced conventional diet (dry matter 88%, protein 23%, crude fibre 6%, minerals 10%, calcium 1·3%, phosphorus 0·8%, vitamins 5%, water 12%). The malnourished mice were re-nourished with the PFD but with the inclusion of skim milk (100 g/l of non-fat-milk (NFM)) for 7 d (Re7d) and for 14 d (Re14d). At the end of the malnutrition or the re-nutrition stages, five mice from each group were taken for evaluation.

Feeding procedure of test groups

Different groups of the Re7d or Re14d mice were fed with the supplemented PFD and with optimal doses of yogurt (Yo) and *Lb. casei* (Lc) previously selected (Gauffin Cano et al. 2002a, b). Yogurt was prepared with 100 g/l of NFM fermented at 42 °C for 4 h with a stock culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp *bulgaricus*, from the CERELA culture collection. The total number of bacteria in the fermented product was 2×10^8 cfu/ml. The optimal dose was yogurt administered diluted (1 : 1) for 5 consecutive days ($\frac{1}{2}$ Yo) (Gauffin Cano et al. 2002b). We chose this dose as optimum because it caused no histological alteration of the small intestine.

The other bacterial strain used for experiments was *Lb. casei* var *casei* CRL 431 from CERELA culture collection. It was cultured for 8 h at 37 °C in MRS (De Man & Sharpe, 1960) broth (Oxoid Ltd, Ogdensburg, NY 13669, USA) and harvested by centrifugation at 5000× ***g*** for 10 min, and washed three times with sterile saline solution. Mice were given the optimal dose of this culture of *Lb. casei* daily at a concentration of 10^7 (cfu)/animal for 5 consecutive days ($Lc10^7$). The optimal dose was determined in studies *in vivo* as being a daily dose of between 10^6 and 10^7 cfu/mouse; we chose the dose that did not produce secondary effects such as translocation. Cultures were given at a concentration of 20% (v/v) in 10% NFM to the Re7d or Re14d group (Gauffin Cano et al. 2002a).

A well-nourished control group received a conventional diet (W-N), a M-N control group was fed with PFD for 21 d, and Re7d and Re14d controls were re-nourished for 7 and 14 d with 10% NFM respectively.

Table 1. Effect of supplementation of the re-nutrition diet with the optimal doses of *Lb. casei* and yogurt on numbers of IgA and IgG secreting cells associated with the small intestine of Re7d and Re14 d mice

Values are means ± SD for $n=5$

Control and experimental animals	Fluorescent cells/10 fields (magnification × 100)	
	IgA+cells	IgG+cells
Control		
W-N	86·0±2·8	43·7±1·4
M-N	43·7±3·0[1]	22·0±5·1[1]
Re7d	79·6±2·7[2]	27·7±4·2
Re14d	95·2±1·2[2]	35·7±2·0[2]
Re7d		
5d $Yo\frac{1}{2}$	84·8±3·6	29·9±2·3
5d Lc 10^7	70·1±2·9	25·5±2·5
Re14d		
5d $Yo\frac{1}{2}$	89·0±4·6	17·7±3·3[3]
5d Lc 10^7	94·9±6·4	10·9±3·1[3]

W-N: well-nourished control; M-N: malnourished control groups; Re7d: re-nourished for 7 d control groups; Re14d: re-nourished for 14 d control groups; $Yo\frac{1}{2}$: diluted yogurt; $Lc10^7$: *Lb. casei* administered to a concentration of 10^7 cells/d per mouse

[1] Significantly different from W-N control ($P<0·01$)

[2] Significantly different from M-N control ($P<0·01$)

[3] Significantly different from Re14d control ($P<0·01$)

Tissue sections

Small intestines from five mice from each group were removed at the end of each treatment period and processed by a modified Saint-Marie's technique (Saint-Marie, 1962). Briefly, tissues were fixed in 95% ethanol for 24 h at 4 °C, and dehydrated in three changes of absolute alcohol and cleared by passing through three consecutive baths of xylene at 4 °C each for 45 min. The tissue was embedded in paraffin at 56 °C for 3–6 h. Tissue sections (3–4 μm) were placed on glass slides.

Numbers of IgA, IgG secreting cells; CD3+, CD4+ and CD8+ T lymphocytes and αβ- or γδ TCR

Numbers of IgA, IgG secreting cells; CD3+, CD4+ and CD8+ T lymphocytes and αβ- and γδ T cell receptor (TCR) were determined by direct immunofluorescence on samples from small intestines of five animals from each group. It was performed using the monospecifc antibodies (α- and

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Table 2. Effect of supplementation of the re-nutrition diet with the optimal doses of *Lb. casei* and yogurt on the numbers of CD3+, CD4+ and CD8+ T lymphocytes associated with the small intestine of Re7d and Re14d mice

Values are means ± SD for $n=5$

Control and experimental animals	T lymphocytes (fluorescent cells/10 fields; magnification × 100)		
	CD3+	CD4+	CD8+
Control			
W-N	56·1 ± 1·5	48·9 ± 4·3	37·0 ± 5·3
M-N	15·5 ± 1·5[1b]	20·0 ± 2·5[1b]	17·9 ± 1·4[1b]
Re7d	26·2 ± 3·8[2a]	21·5 ± 3·7	19·3 ± 2·5
Re14d	32·5 ± 2·6[2b]	28·5 ± 5·1[2a]	29·9 ± 4·4[2a]
Re7d			
5d Yo$\frac{1}{2}$	45·0 ± 2·3[3b]	38·6 ± 5·0[3b]	31·0 ± 3·5[3b]
5d Lc 10^7	46·3 ± 5·0[3b]	32·9 ± 6[3a]	29·4 ± 2·7[3b]
Re14d			
5d Yo$\frac{1}{2}$	36·2 ± 2·7	36·2 ± 4·7	30·3 ± 3·0
5d Lc 10^7	39·8 ± 3·2[4a]	39·1 ± 2·7[4a]	33·3 ± 2·3

W-N: well-nourished control; M-N: malnourished control groups; Re7d: re-nourished for 7 d control groups; Re14d: re-nourished for 14 d control groups; Yo$\frac{1}{2}$: diluted yogurt; Lc10^7: *Lb. casei* administered to a concentration of 10^7 cells/d per mouse

[1] Significantly different from W-N control
[2] Significantly different from M-N control
[3] Significantly different from Re7d control
[4] Significantly different than Re14d control
[a] $P<0·05$, [b] $P<0·01$

Table 3. Effect of supplementation of the re-nutrition diet with the optimal doses of *Lb. casei* and yogurt on the numbers of αβ- and γδ TCR T lymphocytes associated with the small intestine of Re7d and Re14d mice

Values are means ± SD for $n=5$

Control and experimental groups	T lymphocytes (fluorescent cells/10 fields; magnification × 100)	
	αβ TCR	γδ TCR
Control		
W-N	44 ± 3·0	43·0 ± 3·3
M-N	14·9 ± 2·2[1b]	19·2 ± 2·2[1b]
Re7d	14·5 ± 1·0	15·1 ± 2·5
Re14d	24·4 ± 2·4[2b]	30·3 ± 2·7[2b]
Re7d		
5d Yo$\frac{1}{2}$	27·0 ± 3·0[3b]	37·4 ± 5·8[3b]
5d Lc 10^7	30·2 ± 0·6[3b]	37·3 ± 2·8[3b]
Re14d		
5d Yo$\frac{1}{2}$	37·7 ± 1·2[4b]	40·5 ± 4·5[4a]
5d Lc 10^7	39·0 ± 6·4[4b]	40·7 ± 3·7[4a]

W-N: well-nourished control; M-N: malnourished control groups; Re7d: re-nourished for 7 d control groups; Re14d: re-nourished for 14 d control groups; Yo$\frac{1}{2}$: diluted yogurt; Lc10^7: *Lb. casei* administered to a concentration of 10^7 cells/d per mouse

[1] Significantly different from W-N control
[2] Significantly different from M-N control
[3] Significantly different from Re7d control
[4] Significantly different from Re14d control
[a] $P<0·05$, [b] $P<0·01$

γ-chain specific) conjugated with fluorescein isothiocyanate (FITC) (Sigma, Saint Louis, Missouri 63103, USA) for IgA and IgG. FITC conjugates of monoclonal antibodies specific for CD3+ (GIBCO BRD Life Technologies, Neutro Química SA, Buenos Aires, Argentina), CD4+ and CD8+ (Sigma, Saint Louis, MO 63103, USA) and αβ- and γδ TCR (ACCURATE Chemical & Scientific Corporation, Westbury, NY 115900, USA). Histological samples were incubated in a humidified chamber with 0·1 ml of different antibodies at 1/60 dilutions for IgA, and 1/100 for IgG for 30 min at 37 °C; at 1/100 for CD3+, CD8+, αβ- and γδ TCR and 1/200 for CD4+ for 30 min at 4 °C. They were washed three times with 0·01 M-phosphate-buffered saline (PBS), pH 7·2. Slices were mounted in glycerol:PBS. In all determinations, histological slices from small intestine of mice that did not receive any treatment were used as control. They were W-N or M-N and they were double-blind. The number of positive fluorescent cells was expressed as the number of fluorescent cells/10 fields (magnification ×100).

Challenge

After 5 d of yogurt or *Lb. casei* feeding for Re7d or Re14d, animals were orally challenged with 2×10^6 cfu/ml *Sal. typhimurium* in the smooth phase or 1×10^5 cfu/ml *Esch. coli* enteroinvasive using an oral catheter. W-N, M-N, Re7d and Re14d control group, which had not received the feeding treatment, were challenged in the same way.

Colonization assays

Mice were killed by cervical dislocation on days 2, 5 and 7 after *Sal. typhimurium* challenge and on days 2 and 5 after *Esch. coli* challenge. Their spleens, liver and small intestines were removed aseptically. Numbers of viable bacteria in the liver and spleen were determined in both the control and in each experimental group. Livers and spleens were homogenized in a final volume of 5 ml peptone-water (0·1% peptone from meat; Merck, Darmstadt, Germany) with a teflon homogenizer. Homogenized tissues were serially diluted in peptone-water and 0·1 ml was plated in duplicate on MacConkey agar plates. The number of colonies (cfu) was counted after 48-h incubation at 37 °C. Pathogens were identified by biochemical tests (indole, Voges-Proskauer, methyl red, citrate utilization) for *Esch. coli* and serology for *Sal. typhimurium*. Results were expressed as cfu per g of liver or spleen. We only determined *Sal. typhimurium* and *Esch. coli* because it is not possible that *Lb. casei* and yogurt favour bacterial translocation. This effect has been studied (Allori et al. 2000).

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Table 4. Effect of supplementation of the re-nutrition diet with the optimal doses of *Lb. casei* and yogurt on protection against *Sal. typhimurium*. Determination of the number of viable bacteria in liver and spleen of Re7d and Re14d mice

Values are means ± SD for $n=5$

	Log cfu/g of liver or spleen†					
	Days post challenge					
	2		5		7	
Control and experimental animals	Liver	Spleen	Liver	Spleen	Liver	Spleen
Control						
W-N	0	0	2·0 ± 0·6	2·2 ± 0·6	2·8 ± 0·4	3·6 ± 0·4
M-N	1·5 ± 0·3[1b]	0	3·3 ± 0·3[1a]	4·4 ± 0·5[1a]	4·6 ± 0·6[1a]	4·9 ± 0·6[1a]
Re7d	0	0	2·7 ± 0·3[2a]	2·7 ± 0·3[2a]	3·9 ± 0·2	5·0 ± 0·2
Re14d	0	0	3·0 ± 0·6	3·5 ± 0·5	3·7 ± 0·5	3·6 ± 0·6
Re7d						
5d $Yo\frac{1}{2}$	0	0	3·6 ± 0·7	4·4 ± 0·6[3a]	4·4 ± 0·1[3a]	5·8 ± 0·3[3a]
5d Lc 10^7	0	0	2·8 ± 0·3	1·2 ± 0·3[3b]	2·7 ± 0·2[3b]	3·3 ± 0·3[3b]
Re14d						
5d $Yo\frac{1}{2}$	0	0	3·8 ± 0·5	4·0 ± 0·6	4·1 ± 0·5	3·3 ± 0·3
5d Lc 10^7	0	0	0[4b]	0[4b]	0[4b]	0[4b]

W-N: well-nourished control; M-N: malnourished control groups; Re7d: re-nourished for 7 d control groups; Re14d: re-nourished for 14 d control groups; $Yo\frac{1}{2}$: diluted yogurt; Lc10^7: *Lb. casei* administered to a concentration of 10^7 cells/d per mouse

† The number of viable bacteria in liver and spleen was measured on 2, 5 and 7 d post challenge of each of five experimental and control mice

[1] Significantly different from W-N control

[2] Significantly different from M-N control

[3] Significantly different from Re7d control

[4] Significantly different from Re14d control

[a] $P<0·05$, [b] $P<0·01$

Specific secretory IgA. Enzyme-linked immunosorbent assay (ELISA)

Intestinal fluid for determination of specific antibodies was obtained 2, 5 and 7 d after challenge for *Sal. typhimurium* infection and on days 2 and 5 after *Esch. coli* challenge. Intestinal fluid was collected by a modification of the method described by Lim et al. (1981): intestinal contents were washed out using 1 ml of PBS (pH 7·2) and then centrifuged at 2000 ***g*** for 30 min. The supernatant was collected and stored at −70 °C until ELISA assay.

ELISA was performed essentially as described by Engwall & Perlmann (1971, 1972) but modified as follows: the wells of polystyrene microtitre plates were coated with 200 µl of kill suspension of *Sal. typhimurium* or *Esch. coli* at an optical density (OD) of 0·65 ($1·9\times10^6$ cells/ml) and 0·9 (2×10^7 cells/ml) respectively in 0·06 M-sodium carbonate-bicarbonate buffer (pH 9·6) for 18 h at 4 °C. The non-adherent microorganisms were removed by washing three times with PBS, pH 7·2, containing 0·05% (v/v) Tween 20 (PBS-T). Non-specific protein binding sites were blocked with PBS containing 5% of non-fat dry milk for 30 min at room temperature. Plates were washed three times with PBS-T solution.

Different dilutions of intestinal fluid were assayed, and 200 µl of sample dilutions were added in triplicate to wells coated with *Sal. typhimurium* and *Esch. coli*. Three wells were used as a reaction control to each of which was added 200 µl of PBS. Plates were incubated for 60 min at 37 °C, and then washed three times with PBS containing 0·05% Tween-20 (PBS-T). Peroxidase-conjugate goat anti-mouse IgA (α-chain specific) (Sigma, Saint Louis, Missouri 63103, USA) was diluted 1/2000 in PBS and 200 µl was added to each well. Plates were incubated for 60 min at 37 °C and washed three times with PBS-T. The reaction took place at room temperature for 30 min in the dark and was stopped by addition of 1M-H_2SO_4. OD was measured at 493 nm and the antibody concentration in each sample was expressed as OD/200 µl.

Statistical analysis

Results were summarized using descriptive statistics such as the mean and SD by Student's test. Statistical comparisons of the treatment *v.* control groups were analysed by ANOVA.

Results

Effect of optimal doses of Lb. casei *and yogurt on the number of IgA and IgG secreting cells from small intestine*

Malnutrition decreased ($P<0·01$) the number of IgA and IgG secreting cells compared with W-N control. The re-nutrition diet, without feeding treatment, increased

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Table 5. Effect of supplementation of the re-nutrition diet with the optimal doses of *Lb. casei* and yogurt on protection against *Esch. coli*. Determination of the number of viable bacteria in liver and spleen of Re7d and Re14d mice

Values are means ± SD for $n=5$

	Log cfu/g of liver or spleen†			
	Days post challenge			
Control and experimental groups	2		5	
	Liver	Spleen	Liver	Spleen
Control				
W-N	0	0	$2{\cdot}4 \pm 0{\cdot}3^{1b}$	$3{\cdot}3 \pm 0{\cdot}6$
M-N	$2{\cdot}4 \pm 0{\cdot}3^{1b}$	$3{\cdot}4 \pm 031^{1b}$	$2{\cdot}7 \pm 0{\cdot}3$	$3{\cdot}7 \pm 0{\cdot}5$
Re7d	$2{\cdot}0 \pm 0{\cdot}7$	$1{\cdot}1 \pm 0{\cdot}3^{2b}$	$2{\cdot}4 \pm 0{\cdot}6$	$2{\cdot}0 \pm 0{\cdot}6^{2a}$
Re14d	0^{2b}	0^{2b}	$2{\cdot}0 \pm 0{\cdot}6^{2b}$	$1{\cdot}8 \pm 0{\cdot}2^{2b}$
Re7d				
5d Yo$\frac{1}{2}$	$1{\cdot}9 \pm 0{\cdot}2$	0^{3b}	0^{3b}	0^{3b}
5d Lc 10^7	$2{\cdot}5 \pm 0{\cdot}7$	$1{\cdot}4 \pm 0{\cdot}1$	$2{\cdot}9 \pm 0{\cdot}6$	$3{\cdot}2 \pm 0{\cdot}3^{3a}$
Re14d				
5d Yo$\frac{1}{2}$	0^{4b}	0^{4b}	0^{4b}	0^{4b}
5d Lc 10^7	0^{4b}	0^{4b}	0^{4b}	0^{4b}

W-N: well-nourished control; M-N: malnourished control groups; Re7d: re-nourished for 7 d control groups; Re14d: re-nourished for 14 d control groups; Yo$\frac{1}{2}$: diluted yogurt; Lc10^7: *Lb. casei* administered to a concentration of 10^7 cells/d per mouse

† The number of viable bacteria in liver and spleen was measured on 2, 5 and 7 d post challenge of each of five experimental and control mice

[1] Significantly different from W-N control

[2] Significantly different from M-N control

[3] Significantly different from Re7d control

[4] Significantly different from Re14d control

[a] $P<0{\cdot}05$, [b] $P<0{\cdot}01$

Table 6. Effect of the supplementation of re-nutrition diet with the optimal doses of *Lb. casei* and yogurt on protection against *Sal. typhimurium*. Determination of the concentrations of a-Salmonella S-IgA in intestinal fluid of Re7d and Re14d mice

Values are means ± SD for $n=5$

	a-*Salmonella* S-IgA/200 µl†		
Control and Experimental animals	Days post challenge		
	2	5	7
Control			
W-N	$142{\cdot}8 \pm 15{\cdot}1$	$159{\cdot}5 \pm 13$	$175{\cdot}4 \pm 16$
M-N	$9{\cdot}8 \pm 3{\cdot}6^{1b}$	$17{\cdot}1 \pm 3{\cdot}5^{1b}$	$19{\cdot}9 \pm 2{\cdot}7^{1b}$
Re7d	$11{\cdot}3 \pm 1{\cdot}5$	$22{\cdot}5 \pm 3{\cdot}1$	$19{\cdot}7 \pm 6{\cdot}0$
Re7d	35 ± 12^{2a}	41 ± 10^{2a}	$40{\cdot}3 \pm 9^{2a}$
Re7d			
5d Yo$\frac{1}{2}$	$61{\cdot}5 \pm 5^{3b}$	$81{\cdot}5 \pm 4^{3b}$	$85{\cdot}7 \pm 5^{3b}$
5d Lc 10^7	$135{\cdot}7 \pm 4^{3b}$	$151{\cdot}3 \pm 4^{3b}$	$161{\cdot}3 \pm 3^{3b}$
Re14d			
5d Yo$\frac{1}{2}$	$223{\cdot}8 \pm 17^{4b}$	$244{\cdot}2 \pm 19^{4b}$	228 ± 20^{4b}
5d Lc 10^7	$330{\cdot}2 \pm 18^{4b}$	$302{\cdot}6 \pm 18^{4b}$	$291{\cdot}4 \pm 19^{4b}$

W-N: well-nourished control; M-N: malnourished control groups; Re7d: re-nourished for 7 d control groups; Re14d: re-nourished for 14 d control groups. Yo$\frac{1}{2}$: diluted yogurt; Lc10^7: *Lb. casei* administered to a concentration of 10^7 cells/d per mouse

† Levels of a-*Salmonella* S-IgA were measured by ELISA in intestinal fluid obtained 2, 5 and 7 d post challenge of each of five experimental and control mice

[1] Significantly different from W-N control

[2] Significantly different from M-N control

[3] Significantly different from Re7d control

[4] Significantly different from Re14d control

[a] $P<0{\cdot}05$, [b] $P<0{\cdot}01$

($P<0{\cdot}01$) IgA+ cells, and after only 14 d of re-nutrition diet, IgG+ cells increased with respect to the M-N control group. Giving probiotic cultures did not significantly alter these values compared with re-nourished control groups and the number of IgG cells fell significantly after *Lb. casei* and yogurt feeding in the Re14d group (Table 1).

Effect of optimal doses of Lb. casei *and yogurt on the number of CD3+, CD4+ and CD8+ T lymphocytes from small intestine*

Malnutrition decreased ($P<0{\cdot}01$) numbers of CD3+, CD4+ and CD8+ T lymphocytes compared with W-N control. We observed a significant increase ($P<0{\cdot}05$) of CD3+ cells after 7 d and 14 d of re-nutrition diet and the administration of *Lb. casei* or yogurt produced an increase greater than re-nourished controls. Re-nutrition for 14 d increased the number of CD4+ and CD8+ T lymphocytes slightly. When the probiotics were given CD4+ cells increased ($P<0{\cdot}05$) relative to Re7d and Re14d control groups. CD8+ cells increased significantly ($P<0{\cdot}05$) after 14 d of re-nutrition but probiotics did not affect this cell population (Table 2).

Effect of optimal doses of Lb. casei *and yogurt on the number of αβ- or γδ TCR of T lymphocytes from small intestine*

Malnutrition decreased ($P<0{\cdot}01$) numbers of αβ- and γδ TCR relative to W-N control. The re-nutrition diet after 14 d produced an increase in both TCR types, with respect to the M-N control group. *Lb. casei* and yogurt feeding increased ($P<0{\cdot}01$) αβ- and γδ TCR compared with Re7d and Re14d control groups, being higher for γδ TCR (Table 3).

Colonization assays

According to previous findings (Perdigón et al. 1991) the effect of previous feeding with probiotic bacteria on the protection of mice must be taken into account until day 7 after *Sal. typhimurium* challenge and day 5 after *Esch. coli* challenge.

Livers from M-N control mice were significantly colonized by *Sal. typhimurium* at day 2 after challenge, compared with W-N control mice. For Re7d and Re14d re-nutrition treatments, there was no colonization in the liver and spleen up to the day 5 post-infection; from then

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Table 7. Effect of supplementation of the re-nutrition diet with the optimal doses of *Lb. casei* and yogurt on protection against *Esch. coli*. Determination of the levels of a-*Esch. coli* S-IgA in intestinal fluid of Re7d and Re14d mice

Values are means ± SD for $n=5$

Control and experimental animals	a-*Esch. coli* S-IgA/200 µl† Days post challenge 2	5
Control		
W-N	189 ± 20	206·8 ± 18
M-N	30·6 ± 9[1]	27·7 ± 10[1]
Re7d	107·9 ± 17[2]	111·7 ± 20[2]
Re14d	140 ± 21[2]	142·3 ± 19[2]
Re7d		
5d $Yo\frac{1}{2}$	113·8 ± 19	123·8 ± 20
5d Lc 10^7	115·4 ± 17	107·5 ± 18
Re14d		
5d $Yo\frac{1}{2}$	317·2 ± 20[3]	378·5 ± 19[3]
5d Lc 10^7	410·3 ± 22[3]	338·4 ± 21[3]

W-N: well-nourished control; M-N: malnourished control groups; Re7d: re-nourished for 7 d control groups; Re14d: re-nourished for 14 d control groups; $Yo\frac{1}{2}$: diluted yogurt; $Lc10^7$: *Lb. casei* administered to a concentration of 10^7 cells/d per mouse

† Levels of a-*Esch. coli* S-IgA were measured by ELISA in intestinal fluid obtained 2 and 5 days post challenge of each of five experimental and control mice

[1] Significantly different from W-N control ($P<0·01$)

[2] Significantly different from M-N control ($P<0·01$)

[3] Significantly different from Re14d control ($P<0·01$)

on, colonization started, although to a lesser extent than in the M-N control. When the probiotic bacteria were given, *Lb. casei* were more effective than yogurt feeding and in the Re14d group, *Sal. typhimurium* were not found in the liver or the spleen of treated mice (Table 4).

We observed colonization from 2 d after infection with *Esch. coli* in the M-N and Re7d control groups, and in the Re14d groups on day 5 after challenge compared with the W-N control. Yogurt supplementation of the re-nutrition diet showed a better protective effect after 7 d of re-nutrition than did *Lb. casei* feeding. Probiotic bacteria did not show colonization in any organs under study after 14 d of re-nutrition diet (Table 5).

Specific secretory IgA

Concentrations of *Sal. typhimurium* and *Esch. coli* S-IgA in the intestinal fluid of M-N control groups were significantly ($P<0·05$) lower than for W-N control. With the re-nutrition diet these values increased slightly after *Sal. typhimurium* challenge and significantly ($P<0·05$) after *Esch. coli* infection compared with M-N control groups. Both probiotic supplements increased the concentration of *Sal. typhimurium*-specific IgA, and the effect of *Lb. casei* on these values was better than for yogurt feeding in both Re7d and Re14d groups (Table 6). Yogurt and *Lb. casei* supplements increased ($P<0·05$) the concentration of *Esch. coli*-specific IgA in the Re14d groups (Table 7).

Discussion

The mucosal immune system is a critically important protective barrier, which allows the gastrointestinal tract to interface safely with the body's internal milieu and with the external environment of the intestinal lumen. In addition, cell-mediated immune responses evoked at a mucosal level are important mechanisms of local protection.

Regulation of mucosal immunity is clearly carried out for induction and terminal differentiation of B cells with preference for IgA production. The result is a first-line defence mediated mainly by S-IgA antibodies providing immune exclusion. Phenotypic markers expressed by mucosal T cells are of interest because they provide clues to their function *in vivo*. T cells are characterized by the surface expression of the CD3+ T cell receptor complex. TCR and CD3+ are usually acquired in the thymus with accessory molecules such as CD4+ and CD8+. A large proportion (65–80%) of CD3+ T cells reside in the lamina propria of the intestinal epithelium. In the lamina propria, most are CD4+ (Abreau-Martin & Targan, 1996); compared with cells from other sites, the immunoregulation of these cells is weighted heavily in favour of helper function for immunoglobulin production. Lymphocytes that appear in the intestinal villous epithelium (intraephitelial lymphocytes, IEL) preferentially include CD8+ T cells (Klein & Mosley, 1993). Two functions have been ascribed to these latter cells, namely, cytotoxicity and suppression of mucosal hypersensivity reactions (i.e., mediation of oral tolerance), although as yet there is no definitive evidence that they are cytolytically active under physiological or pathological conditions (Abreu-Martin & Targan, 1996). Approximately 95% of the lamina propria T cells bear αβ TCR and only 3% bear γδ TCR complex. In the intraepithelial compartment 50% of lymphocytes express γδ TCR and the remainder αβ TCR. In contrast to the extrathymic derivation of IEL, lamina propria T cell ontogeny involves the thymus followed by induction in Peyer's patches, which are lymphoid follicles within the mucosa (De Geus et al. 1990; Kiyono et al. 1996). γδ T cells respond to antigens that are not recognized by αβ T cells, and they can also respond to pathogens, damaged tissue, and cells directly and initiate immune response without the requirement for antigen processing or dedicated antigen-presenting cells. This gives γδ T cells greater flexibility than the more classical type of αβ T cell in mediating cellular immunity (Chien, 1996; Boismenu & Havran, 1998). Resistance to infection may be depressed by impaired mucosal immunity, such as depressed S-IgA production by malnutrition, leading to colonization of the small intestine and clinical infections of the gut with the risk systemic spread.

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We found that re-nutrition for 14 d is important to improve immunity by increasing IgA+ cells. In our re-nourished model, probiotics as supplements to the re-nutrition diet helped to maintain the number of IgA+ cells in the optimal dose (Gauffin Cano et al. 2002a, b) and provide better protection against infections that are increased by malnutrition. Feeding with *Lb. casei*, but not yogurt, prevented *Sal. typhimurium* infection (100% protection) after 14 d of the re-nutrition diet (Table 4). This protection could be mediated partly by the increment of the concentration of specific S-IgA secreted into the intestinal fluid and the increase in the number of IgA+ cells observed in our model after *Lb. casei* treatment. Similarly, we did not observe a positive effect after the supplementation of the re-nutrition diet with yogurt (Table 4). In *Sal. typhimurium* infection, even when we determined high levels of specific S-IgA (Table 6), the role of antigen specific S-IgA in the intestinal lumen depended on its ability to inhibit the absorption of soluble and particulate antigens by forming intraluminal immune complexes, to neutralize biologically active antigens (toxins and viruses) and to interfere with microbial adherence to epithelial cells (Mestecky & Elson, 1999). We cannot, however, exclude cross reaction between S-IgA and other bacterial strains of the gut. This might be why yogurt was not effective against *Sal. typhimurium* infection. Perhaps, in our malnutrition model, the interaction of bacteria from yogurt and the peptides released during fermentation increased the inflammatory response that favoured the passage of pathogen. In the healthy host, however, no inflammatory response is observed. Other mechanisms may be involved in the inhibitory interaction between *Sal. typhimurium* and *Lb. casei*. How enteric pathogens pass through the intestinal epithelium has become a subject of considerable interest. Enteric pathogens appear to use different strategies to interact with M cells and infect the host. Invasion of M cells by *Sal. typhimurium* is particularly striking (Jensen et al. 1998). For *Lb. casei* the pathway of internalization is via the M and FAE (follicle associated epithelial) cells of Peyer's patches, and it has been suggested that the different effects of LAB on the mucosal immunostimulation relate to the different pathways of gut internalization used to make contact with the immune cells associated with the lamina propria (Perdigón et al. 2000).

Both of our treatments induced 100% protection against *Esch. coli* in the group after 14 d on the re-nutrition diet. One of the mechanisms of protection against this enteropathogen might be the specific S-IgA because this immunoglobulin increased with both probiotics (Table 7).

Differentiation and maturation of lymphoid cells depend on a series of intracellular biochemical events, which are affected by malnutrition. To maintain maximum IgA response in mucosa-associated tissue, intimate cell communication among γδ T cells, CD4+ αβ T cells, and IgA B cells may be a prerequisite. Therefore, we evaluated the effect of the re-nutrition diet and the subsequent administration of probiotics on different markers of the immune cells, because they are necessary to obtain an appropriate immune response. Our results suggest a significant influence of both probiotics on CD3+, CD4+ and CD8+ T populations after 7 d of re-nutrition, which would tend to improve the resistance to enteric infection (Table 2).

Immune responses to foreign antigens are exquisitely specific, largely because of antigen receptors expressed by B and T lymphocytes. Thus a key step in lymphocyte maturation is the acquisition of a functional antigen receptor. When we analysed the effect on TCR we found significant recuperation of αβ- and γδ T cells after probiotic treatment after 14 d of re-nutrition (Table 3); γδ TCR cells reached approximately the values of the well-nourished control groups Re14d+Yo and Re14d+Lc. We showed the influence of the nutritional supplements on the recovery of the intestinal mucosa necessary for the extrathymic maturation of T lymphocytes.

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