Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2005.02811.x

Enhanced clearance of *Candida albicans* from the oral cavities of mice following oral administration of *Lactobacillus acidophilus*

S. Elahi,* G. Pang,† R. Ashman‡ and R. Clancy†

*Vaccine and Infectious Disease Organization, University of Sasketchewan, Sasketoon, Canada, †Discipline of Immunology and Microbiology, Faculty of Health, University of Newcastle, NSW, Australia, and ‡School of Dentistry, University of Queensland, Brisbane, Queensland, Australia

Accepted for publication 10 March 2005
Correspondence: Professor Robert Clancy, Discipline of Immunology and Microbiology, Faculty of Health, David Maddison Clinical Science Building, Royal Newcastle Hospital, Newcastle, NSW 2300, Australia.
E-mail: robert.clancy@newcastle.edu.au

Summary

Orally administered live *Lactobacillus acidophilus* was assessed for its capacity to enhance clearance from the oral cavity of DBA/2 mice shown previously to be 'infection prone'. *L. acidophilus* fed to DBA/2 mice significantly shortened the duration of colonization of the oral cavity compared to controls. Enhanced clearance of *Candida albicans* correlated with both early mRNA gene expression for interleukin (IL)-4 and interferon (IFN)-γ and expression of their secreted products in cultures of cervical lymph nodes stimulated with *Candida* antigen. In addition rapid clearance correlated with higher levels of IFN-γ and nitric oxide in saliva. Delayed clearance, less pronounced levels of the cytokine response, saliva IFN-γ and nitric oxide, and later mRNA expression for IL-4 and IFN-γ relative to feeding with the *L. acidophilus* isolate were noted in mice fed a different *Lactobacillus* isolate (*L. fermentum*). These observations indicate significant variations in individual isolates to activate the common mucosal system.

Keywords: *C. albicans*, cytokine, lactobacilli, nitric oxide

Introduction

Colonization of mucosal surfaces by *Candida albicans* is a major cause of disease, with vulvovaginal and oral–pharyngeal infection being particularly prevalent [1–4].

Mucosal infection in the upper gastrointestinal tract is a precedent for systemic spread in subjects with compromised immunity [3,5] although the mechanisms of protection that contain mucosal colonization, on one hand, and systemic invasion on the other hand, appear to differ [2,6]. Clinical models of vulvovaginal candidiasis reflect recurrent vulvovaginal candidiasis poorly, while models of oral infection have proved to be more valuable in the analysis of host immune mechanisms [7,8]. *Candida* infection of the oral mucosa in mice triggers an inflammatory response and stimulates cellular immunity [7,9]. A murine model that measures clearance following oral challenge with *C. albicans* has proved valuable as strains differ in their capacity to clear the oral inoculation of fungus. Thus BALB/c and DBA/2 mice, while sharing a genetic background (H-2^d), have been shown to be relatively infection-resistant (BALB/c) or infection-prone (DBA/2) [8], respectively. The study of this paradigm has shown that both interferon (IFN)-γ and interleukin (IL)-4 secretion from T lymphocytes correlate with optimal clearance [8], and that nitric oxide (NO) in mucosal secretions is a major effector mechanism [10]. Accelerated oral clearance following ingestion of killed *C. albicans* blastospores showed that these protective mechanisms participated within a framework defined by the common mucosal system [11].

Probiotic bacteria have been used extensively within the food industry, largely in fermented milk products. Claims of probiotic promotion of host defence have been supported by reports of enhanced phagocytosis [12,13], stimulation of production of various cytokines (including IL-2, IL-4, IL-10, IL-12, IL-18 and INF-γ) [14–19] and increased mucosal and secretory antibody levels in response to gut infection with rotavirus [20–22] or *Salmonella typhi* Ty21a [20,23], supporting the notion that some probiotics can enhance mucosal immunity. Feeding adult or neonatal nude mice probiotics prolonged their survival following intestinal challenge with live *C. albicans* by enhancing both antibody and cell-mediated immunity [24,25]. These latter studies, however, are not good models for the more restricted mucosal infection found in man. Reports of protection against clinical mucosal infection following ingestion of yoghurt containing *L. acidophilus* are encouraging [26], but these studies

fail to identify mechanisms of protection. This study was designed to examine the mechanism of protection induced by orally administered live *Lactobacillus* species using a model in which the host–parasite relationship had been studied extensively.

Materials and methods

Animals

Male DBA/2 mice (H-2^d), 6–8 weeks old, were purchased from the Animal Resource Centre, Perth, Western Australia. They were housed in groups of five under pathogen-free conditions.

Fungal culture

C. albicans (isolate no. 3630) was obtained from the National Reference Laboratory, Royal North Shore Hospital, Sydney, Australia. The yeast cells were cultured in Sabouraud dextrose broth (Oxoid, Hampshire, UK) for 48 h at 25°C in a shaking waterbath. The blastospores were transferred into fresh medium and cultured at 25°C for a further 18 h. The blastospores were collected by centrifugation, washed twice with phosphate-buffered saline (PBS) and then adjusted to 10^8 blastospores per ml in PBS until use.

Lactobacillus isolates

L. acidophilus LAFTI L10 and *L. fermentum* used in this study were human isolates obtained from DSM Food Specialties, Australia and the School of Microbiology Culture Collection, University of NSW, Australia, respectively. The bacteria were grown in Man–Rogosa–Sharpe (MRS) broth medium (Difco, Detroit, MI, USA) in a shaking incubator at 37°C for 20 h after which time the bacteria were washed twice with sterile PBS following centrifugation. Bacterial counts were obtained using an improved Neubauer counting chamber. A total of five fields per sample were counted using a phase contrast microscope (Olympus BX40, Japan) at 40 × magnification. The culture was adjusted to 5×10^9/ml and then stored at 4°C until use. The viability of bacteria was assessed by plating in serial dilutions aliquots of bacteria on MRS agar plates.

Oral feeding

Mice (five per group) were fed 1×10^9 lactocbacilli in 0·2 ml PBS by gastric intubation using a feeding needle every day for 2 weeks. Control mice were fed PBS. One day after the last feed, all mice were orally challenged with 1×10^8 *C. albicans* blastoconidia by topical application. Feeding was continued for an additional 6 and 14 days after challenge in BALB/c and DBA/2 mice, respectively. At each time-point, mice were killed to determine the level of colonization by *C. albicans*.

Candida antigen

Freshly cultured *C. albicans* (isolate no. 3630, National Reference Laboratory, Royal North Shore Hospital, Sydney, Australia) were washed in PBS and then resuspended in PBS at 1×10^{10}/ml. The cells were sonicated in an MSE Soniprep set at 10 amplitude for 30 cycles with intermittent cooling and sonication. The sonicate was centrifuged for 10 min at 2000 ***g*** after which time the supernatant was collected and dialysed against PBS. After protein estimation, the solution was filtered-sterile and stored in aliquots at 20°C until use.

Oral challenge

Mice were anaesthetized by intraperitoneal (i.p.) injection with 75 µl of ketamine : xylazil (100 mg/ml : 20 mg/ml). They were inoculated orally with the blastospores according the method described by Chakir *et al.* [7]. Briefly, 10^8/ml of blastospores in PBS were centrifuged at 14 000 ***g*** for 5 min. The pellet was recovered on a fine-tip sterile swab (Corsham, Wiltshire, UK) which was then used for oral inoculation by topical application.

Assessment of oral infection

Groups of mice (five per group) were killed at various time-points to determine the number of *C. albicans* in the oral mucosa. The oral cavity (i.e. cheek, tongue and soft palate), was completely swabbed using a fine-tipped cotton swab. After swabbing, the cotton end was cut off and then placed into an Eppendorf tube containing 1 ml of PBS. The yeast cells were resuspended by mixing on a vortex mixer before culture in serial 10-fold dilutions on Sabouraud dextrose agar (Oxoid, UK) supplemented with chloramphenicol (0·05 g/l) for 48 h at 37°C.

Saliva collection

Mice were anaesthetized by intraperitoneal injection with 75 ml of ketamine : xylazil (100 mg/ml : 20 mg/ml) at different time-points following infection with *C. albicans.* Saliva was collected from individual mice using a glass pipette after cholinergic stimulation by i.p. injection of isoprocarpine (2·5 mg/20 g of body weight).

Cytokine gene expression by reverse transcription-polymerase chain reaction (RT-PCR)

RNA extraction and amplification of synthesized cDNA from lymphoid cells have been described previously [26,27]. Briefly, 10 µl of total RNA extracted from 4×10^6/ml of cervical lymph node (CLN) cells was added to 20 µl of RT mix containing 6 µl of 5× RT reaction buffer (250 mM Tris-HCl, 375 mM KCl and 15 mM $MgCl_2$), 3 µl of 100 mM dithiothreitol, 1·5 µl of deoxynucleotide (10 mM), 1 µl of RNAse

inhibitor (40 U/ml), 0·5 μl of MMLV-RT (200 U/ml), 3 μl of oligo-(dT)15, 3 μl of acetylated bovine serum albumin (BSA) (1 mg/ml) and 2 μl of diethylpyrocarbonate(DEPC)-treated water. The cDNA synthesis was carried out at 42°C for 1 h followed by heating at 72°C for 10 min. PCR amplification was carried out by adding 5 μl of the first-strand cDNA to the PCR mix containing: 1 μM of each primer (20 μM), 1 μl of 4 mM dNTP mix, 5 μl of 10 × PCR buffer, 1·2 μl of 1·5 mM $MgCl_2$, 0·2 μl *Taq* DNA polymerase (50 U/ml) and 31 μl of DEPC-treated water. The mixture was subjected to amplification using a thermal cycler (Hybaid, Middlesex, UK) set at 94°C for 1 min (IL-4 and G3DPH) and 30 s for IFN-γ, 60° for 2 min (IL-4 and G3DPH) and 62°C for 1 min (IFN-γ), and 72°C for 3 min (IL-4 and G3DPH) and 90 s for IFN-γ with a final elongation step at 72° for 10 min. PCR amplification was carried out for 35–40 cycles. PCR fragments were separated on a 2% agarose gel electrophoresis, stained with ethidium bromide and then viewed under a UV transilluminator. The primer sequences were as follows: IL-4, sense GAA TGT ACC AGG AGC CAT ATC; antisense CTC AGT ACT ACG AGT ATT CCA; IFN-ã, sense TCT CTC CTG CCT GAA GGA C; antisense ACA CAG TGA TCC TGT GGA A. The amplified DNA products for IL 4 and IFN-ã were 399 base pairs (bp) and 460 bp, respectively.

Cytokine enzyme-linked immunosorbent assay (ELISA)

Cervical lymph node cells in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) were cultured at 4×10^6 cells per well for 3 days in the presence of *C. albicans* antigen (2·5 μg/ml) in a 24-well culture plate. The culture supernatants were collected and assayed for IL-4, IL-12 and IFN-γ concentrations by ELISA using matched-antibody pairs and recombinant cytokines as standards (Pharmingen, San Diego, CA, USA). Briefly, immuno-polysorb microtitre plates (Nunc, Denmark) were coated with capture rat monoclonal anti-IL-4 (IgG1), IL-12 (IgG2a) or IFN-γ (IgG1) antibody at 1 μg/ml in sodium bicarbonate buffer (pH 9·6) overnight at 4°C. The wells were washed and then blocked with 1% BSA before the culture supernatants and the appropriate standards were added to each well. Biotinylated rat monoclonal anti IL-4, IL-12 or IFN-γ antibody at 2 μg/ml was added as the second antibody. Detection was performed with streptavidin peroxidase (Chemicon, Melbourne, Australia) and tetramethylbenzidene (TMB) (Sigma-Aldrich). The sensitivities of the assay were 31 pg/ml for IFN-γ and 15 pg/ml for IL-12 and IL-4, respectively.

Nitric oxide determination

Saliva was collected from mice after subcutaneous injection of pilocarpine to stimulate saliva flow [9]. NO in saliva was determined by measuring nitrite accumulation, a stable metabolic product of NO with oxygen as determined by the Griess reaction [28]. Briefly, equal volumes of saliva and Griess reagent (0·5% sulphanilamide and 0·05% N^{-1}-napthylethelenediamide hydrochloride in 2·5% phosphoric acid) were mixed at room temperature. After 5 min, the absorbance was measured at 550 nm using an ELISA microplate reader. The concentration of nitrite was determined by a standard curve prepared with sodium nitrite (1–100 mM).

Statistical analysis

The data were compared using the non-parametric Mann–Whitney *U*-test. *P*-values < 0·05 were considered significant. All calculations were performed using a statistical software program (StatView; Abacus Concepts, CA, USA).

Results

Kinetics of oral infection by *C. albicans* in mice fed lactobacilli

The results in Fig. 1 show that the levels of colonization in the oral cavity in DBA/2 mice fed lactobacilli were similar to control (PBS-fed) mice 1 day after infection. This was followed by a rapid decline in colonization levels at day 2 in the groups of mice fed *L. acidophilus* or *L. fermentum* [compared with a 3-log higher number of yeasts in control mice ($P < 0·05$)]. By day 6, mice fed *L. acidophilus* had undetectable numbers of yeast in the oral cavity. Colonization persisted to day 8 in mice fed *L. fermentum*, although at significantly lower levels than those found in the control group ($P < 0·05$).

The protective effect of lactobacilli against colonization with *C. albicans* was observed in DBA/2 mice (Fig. 2b) 2 weeks after the cessation of oral feeding with *L. acidophilus*.

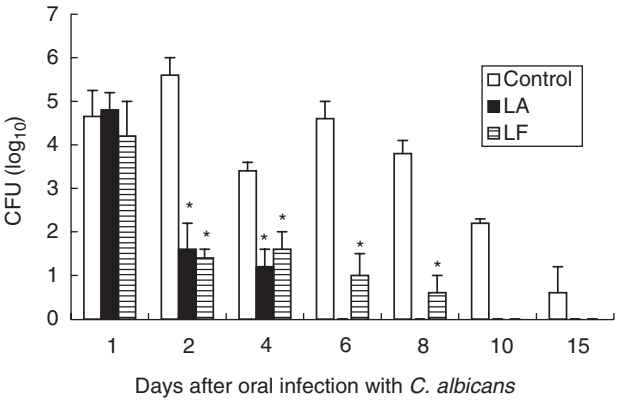

Fig. 1. Enhanced clearance of *Candida albicans* from oral cavities of mice fed lactobacilli. DBA/2 mice ($n = 5$ per group) were fed 5×10^9 *Lactobacillus acidophilus* (LA) or *L. fermentum* (LF) bacteria each day for 20 days after which time they were infected with 1×10^8 *C. abicans* by swabbing the oral cavities. Control mice were fed phosphate buffered saline (PBS). The clearance rate of *C. albicans* from the oral mucosa was determined on the days indicated. The results shown are the mean colony-forming units ± s.e.m. *$P < 0·05$.

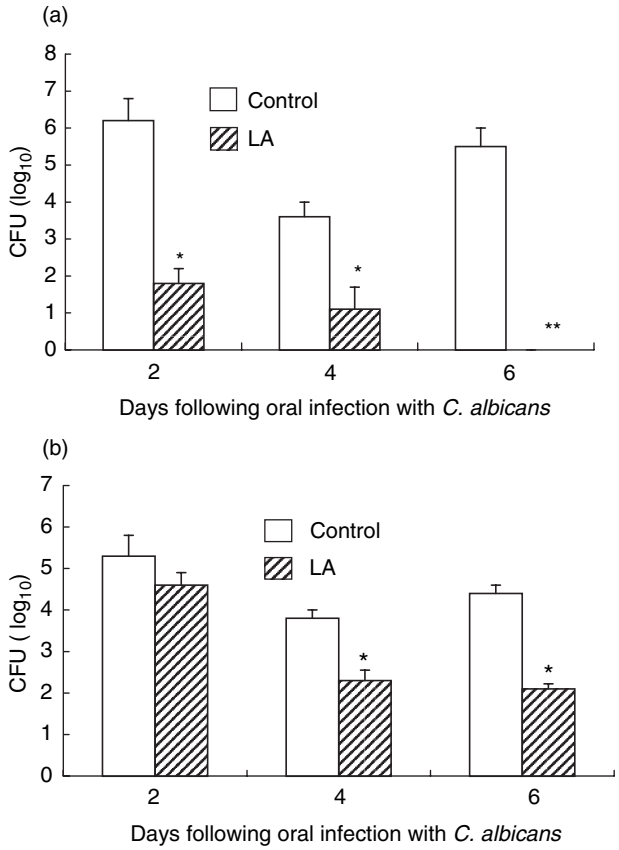

Fig. 2. Protective effect of *Lactobacillus acidophilus* against *Candida albicans* infection after cessation of feeding (a) DBA/2 mice fed every two days for 34 days with 5×10^9 *L. acidophilus* bacteria or phosphate buffered saline (PBS) (control) or (b) mice whose feeding had been ceased for 2 weeks from day 20 before challenge with *C. albicans*. At 2, 4 and 6 days after infection, the rates of clearance of *C. albicans* from the oral cavity were determined in both groups. The results shown are the mean colony-forming units ± s.e.m. for five mice. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$ compared with control mice.

However, the rate of oral clearance was less compared to that in mice that were fed continuously with *L acidophilus* (Fig. 2a). By day 6, while higher colonization levels were found in the mice where oral feeding had ceased, colonization was undetectable in mice continuously fed *L. acidophilus* (Fig. 2a).

IL-4 and IFN-γ mRNA gene expression in mice fed lactobacilli

The effect of lactobacilli on mRNA expression of IL-4 and IFN-γ in CLN cells was determined by RT-PCR. As shown in Fig. 3, IFN-γ gene expression was detected at day 0 and continued to be expressed until day 15 in mice fed lactobacilli. In contrast, it was not expressed in control mice fed PBS until the day following infection with *C. albicans*. IL-4 gene expression on day 1 in *L. acidophilus*-fed mice was comparable to the levels of expression at day 6 in the control group. IL-4 mRNA could not be detected after the yeasts were eliminated in lactobacilli-fed mice, while gene expression for IFN-γ remained up to day 15. There was a significant difference in the pattern of IL-4 gene expression between mice fed *L. acidophilus* and those fed *L. fermentum* (Fig. 3). In *L.* acidophilus-fed mice, IL-4 message was expressed early from day 1 to day 8, whereas in *L. fermentum*-fed mice it was expressed late from day 4 to day 8. The signal intensities of gene expression for IL-4 and IFN-γ were weaker in CLN cells from *L. fermentum*-fed mice than those from *L. acidophilus*-fed mice following oral infection with *C. albicans* (Fig. 3).

Cytokine secretion by antigen-stimulated cervical lymph node cells from mice fed lactobacilli

To determine the effect of lactobacilli on the pattern and the kinetics of cytokine production in mice infected with *C. albicans*, CLN cells were cultured in the presence of *Candida* antigens for 72 h, following which time the levels of IL-4, IL-12 and IFN-γ in the culture supernatants were measured. As shown in Fig. 4 (top panel), significantly higher levels of IL-4 were produced at day 2, peaking at day 4 in mice fed *L. acidophilus* (but not PBS or *L. fermentum*). Mice fed *L. acidophilus* showed a decline in IL-4 levels by day 6.

By contrast, high levels of IFN-γ were detected in *L. acidophilus*-fed mice compared to PBS- or *L. fermentum*-fed mice at day 0 ($P < 0{\cdot}01$) with maximal levels occurring at day 2 ($P < 0{\cdot}01$) and day 6 ($P < 0{\cdot}05$) compared with controls (Fig. 4, middle panel). In *L. acidophilus*-fed mice, the levels of IFN-γ remained high before declining to levels seen in control and *L. fermentum*-fed mice at day 15.

IL-12 (a regulatory cytokine secreted by macrophages known to promote a Th1 response) was measured, particularly to note any correlation with levels of IFN-γ. Higher levels of IL-12 were detected at day 0 ($P < 0{\cdot}05$) in *L. acidophilus*-fed mice compared with control or *L. fermentum*-fed mice (Fig. 4, bottom panel). While an increase in IL-12 was detected in all groups at day 1 following infection with *C. albicans,* higher levels of IL-12 were detected in *L. acidophilus*- compared with *L. fermentum*-fed mice ($P < 0{\cdot}05$), but when compared with the control group there was no significant difference. In control mice, levels of IL-12 secretion increased in response to infection, but with a different time-course (possibly reflecting antigen load). Mice fed *L. acidophilus* or *L. fermentum* continued to secrete high levels of IL-12 in the late stages following infection, although the elevated secretion of day 1 may well reflect the oral infection.

Production of IFN-γ and NO in saliva

As shown in Fig. 5, significant levels of IFN-γ were detected in the saliva of *L. acidophilus* fed mice at days 2 ($P < 0{\cdot}01$), 4 ($P < 0{\cdot}01$) and 8 ($P < 0{\cdot}05$) compared with control mice or

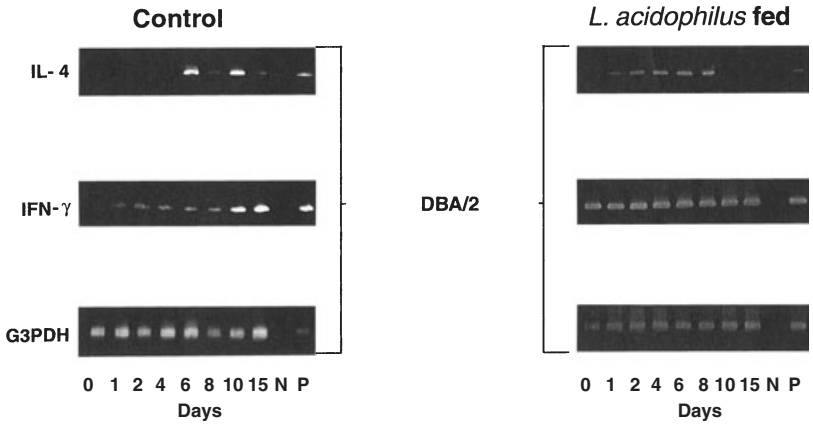

Fig. 3. Cytokine mRNA gene expression in the regional lymph nodes of *Lactobacillus acidophilus*-fed DBA/2 mice following oral infection with *Candida albicans*. Total RNA was extracted from cervical lymph node (CLN) cells of mice and analysed by reverse transcription-polymerase chain reaction (RT-PCR) for interleukin (IL)-4 and interferon (IFN)-γ gene expression in mice fed *L. acidophilus*, *L. fermentum* or phosphate buffered saline (PBS) mice at different time-points following oral *Candida* infection. Equivalent loading of each sample was determined by the G3DPH message shown below. The amplified DNA products for IL-4 and IFN-γ were 399 base pairs (bp) and 460 bp, respectively.

mice fed *L. fermentum*. By day 10, the levels in *Lactobacillus*-fed and control mice were not significantly different. At day 15, the levels in lactobacillus-fed mice were still detectable, whereas in control mice the levels were lower or undetectable.

There was an early and higher level of salivary NO in *L. acidophilus*-fed mice than in the control group ($P < 0{\cdot}05$) or in mice fed *L. fermentum* ($P =$ n.s.). NO levels peaked at days 1 and 2 following infection with *C. albicans* and remained detectable until the yeasts were cleared from the oral cavity in *L. acidophilus* mice. In contrast, there was a delayed production of NO in the controls which correlated with a delayed clearance of yeast. NO production in mice fed *L. fermentum* remained low throughout.

Discussion

This study demonstrates that feeding live *L. acidophilus* to an 'infection-prone' DBA/2 mouse strain significantly shortens the duration of colonization of the oral cavity following inoculation with *C. albicans*. When compared to control mice, the accelerated clearance of *C. albicans* from the oral cavity of DBA/2 mice fed *L. acidophilus* correlated with an early appearance of mRNA for both IL-4 and IFN-γ, and their secreted products, from stimulated cervical node lymphocytes, and with the appearance of the effector molecules IFN-γ and NO in saliva.

The limitation of oral colonization by *C. albicans* did not involve direct interference by *L. acidophilus* as the live bacteria were given into the stomach. Oral cultures grew no lactobacilli and enhanced clearance was detected for at least 2 weeks after ceasing oral feeding with *L. acidophilus*. Considerable variation existed between different *Lactobacillus* isolates in the induction of mucosal protection [28,29]. Data presented show that the *L. acidophilus* isolate was more effective than the *L. fermentum* isolate in terms of protection and local production of IL-4, NO and IFN-γ. These results support the concept that certain probiotic bacteria have a valuable capacity to drive the common mucosal system to enhance protection at distant mucosal sites [30,31]. Given that 'infection-resistant' BALB/c mice do not enhance clearance following ingestion of lactobacillus (unpublished data), the enhanced immunity in DBA/2 mice suggests that the protective effect is primarily restorative rather than a booster effect associated normally with secondary immunization. Thus the variations in the capacity of different isolates to stimulate immunity, on one hand, and in host capacity to respond on the other hand, most probably explain much of the variability seen in uncontrolled human studies [32]. The importance of careful isolate selection in clinical studies used to develop therapeutic strategies must be emphasized.

A previous study of the DBA/2 and BALB/c murine strains with respect to oral challenge with *C. albicans* identified a timely and balanced IFN-γ/IL-4 cytokine response to antigen stimulation of lymphocytes in regional lymph nodes, and an early appearance of NO within the oral cavity, as key parameters of effective immunity [8,10]. Preliminary studies in subjects having a dental prosthesis fitted show low levels of NO in saliva as a marker of 'proneness to infection' (unpublished observations); *L. acidophilus* induced a more vigorous initial increase in IFN-γ and IL-12 secretion from the cervical nodes and higher early levels of salivary NO and IFN-γ than did control mice or mice fed *L. fermentum*, yet similar accelerated levels of clearance were induced in the first 4 days following challenge in mice fed either isolate of *Lactobacillus*. The complete eradication of *C. albicans* at day 4 following

low NO in saliva ~ decr immune function

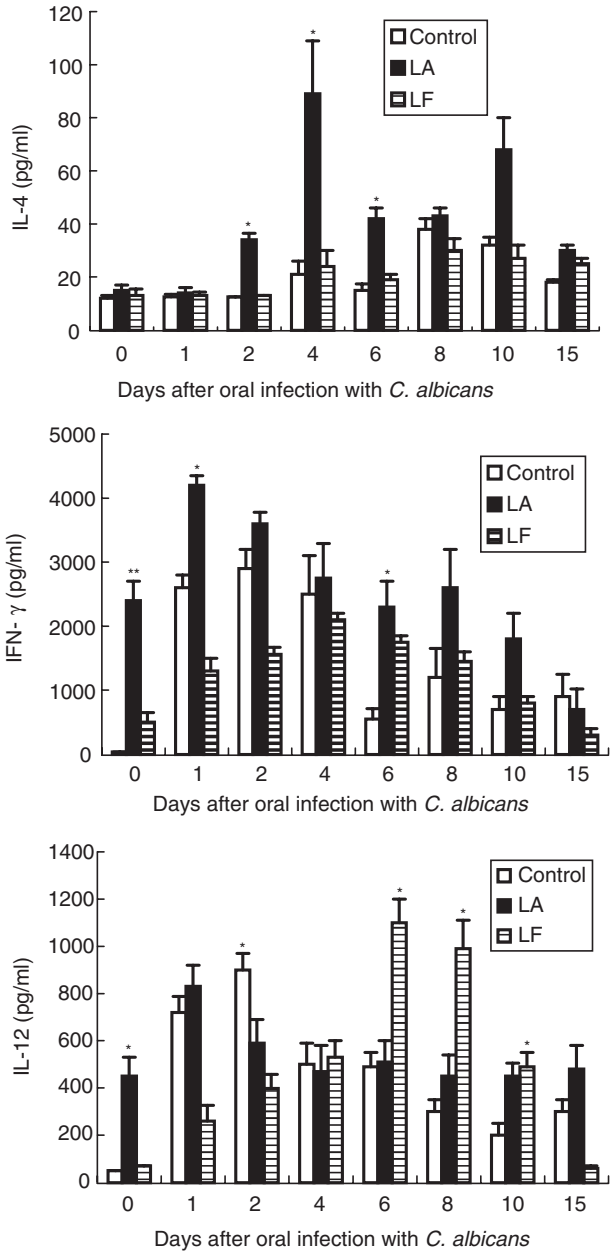

Fig. 4. Cytokine secretion by cervical lymph node cells stimulated with *Candida* antigen DBA/2 mice fed lactobacilli were orally infected with *C. albicans*. At the days indicated, cervical lymph node cells were isolated (4×10^6 cells/ml) and stimulated with or without *Candida* antigen (2·5 μg/ml). Cell-free culture supernatants were collected and assayed for interleukin (IL)-4, interferon (IFN)-γ and IL-12 secretion by enzyme-linked immunosorbent assay (ELISA). The results shown are the mean ± s.e.m. for five mice in each group. $^*P < 0{\cdot}05$. LA, *Lactobacillus acidophilus*; LF, *L. fermentum*.

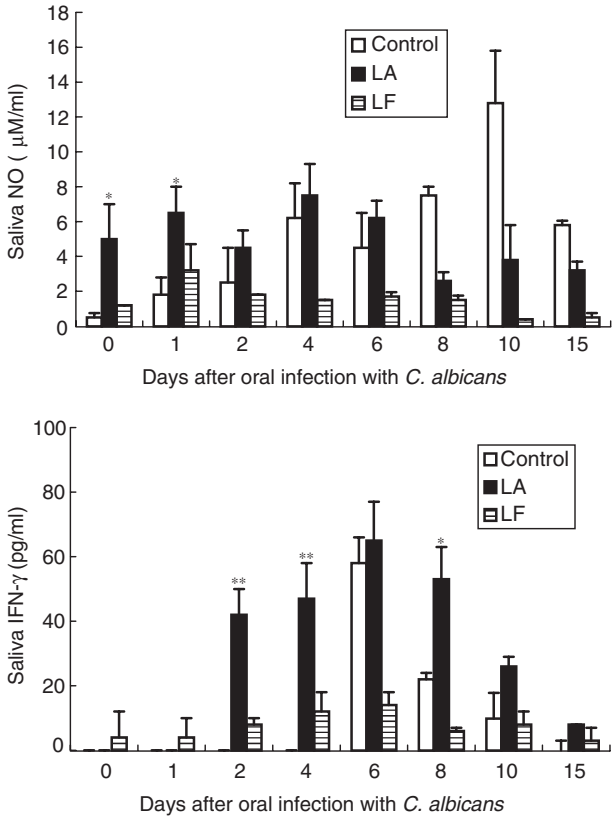

Fig. 5. Nitric oxide and interferon (IFN)-γ secretion in saliva from DBA/2 mice fed lactobacilli and infected with *Candida albicans*. Saliva was collected from control or probiotic-fed mice at the times indicated following oral infection with *C. albicans*. The amounts of salivary NO and IFN-γ were measured by the Griesse test and enzyme-linked immunosorbent assay (ELISA), respectively. The results shown are the mean ± s.e.m. for five mice in each group. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$. LA, *Lactobacillus acidophilus*; LF, *L. fermentum*.

feeding with *L. acidophilus* coincided with a spike of IL-4 secretion. These results confirm that a complex mix of effector mechanisms are responsible for clearance of yeast from the oral mucosa. The variability in the capacity of different *Lactobacillus* bacteria to stimulate cellular and humoral parameters of mucosal protection is noted, particularly in terms of salivary NO and IFN-γ levels. Previous studies in this yeast-infected murine model demonstrated a protective positive paracrine feedback loop between NO and IL-4 production [10]. The raised saliva levels of NO prior to *C. albicans* challenge, following ingestion of *L. acidophilus*, suggests that *L. acidophilus* stimulates additional mechanisms, possibly involving macrophages, as enhanced secretion of IL-4 from stimulated T cells was not detected at the time of the infection challenge.

Several bacterial species have been shown to induce non-specific protection against experimental infection in mice [19,32–34] by enhancing the host immune response [35,36]. For example, adult and neonatal immune deficient nude mice fed *L. acidophilus* are protected against orogastric challenge with live *C. albicans* [24,33,34], whereas heat-killed *L. acidophilus* were not effective, suggesting that either colonization or actively secreted factors were protective [25]. The mechanism of 'non-specific' protection by fed bacteria is

unclear, but may involve activation of antigen-presenting cells by bacterial DNA containing CpG motifs through Toll-like receptor 9 (TLR9) [35], which promotes a Th1 response characterized by production of IL-12 and IFN-γ [36,37]. Recent studies have shown that lactobacilli induce cytokine and NO secretion from peritoneal macrophages, spleen and Peyer's patch lymphocytes in mice fed probiotic bacteria [16,19]. Increased levels of IgA in milk, saliva, intestinal secretions of mice and humans and a reduction of rotavirus infection in children receiving probiotic-supplemented formula [21,38] all support the concept that certain probiotics activate the common mucosal system in a non-specific way. In the current studies, the time-course of effector mechanisms activated by *L. acidophilus* best fits stimulation of both lymphoid and non-lymphoid cells.

References

1 Epstein J, Truelove E, Izutau K. Oral candidiasis: pathogenesis and host defence. Rev Infect Dis 1984; **6**:96–106.

2 McCarthy GM. Host factors associated with HIV-related oral candidiasis. A review. Oral Surg Oral Med Oral Pathol 1992; **73**:181–6.

3 Odds FC. Candida and candidosis. Baltimore, MD: University Park Press, 1988:104–10.

4 Samaranayake LP. Host factors and oral candidiasis. London: Wright, 1990:66–103.

5 Myerowitz RL, Pazin GJ, Allen CM. Disseminated candidiasis. changes in incidence, underlying diseases, and pathology. Am J Clin Pathol 1977; **68**:29–38.

6 Greenfield RD. Host defence system interaction with *Candida*. J Med Vet Mycol 1992; **30**:89–104.

7 Chakir J, Cote L, Coulombe C, Delasriers N. Differential pattern of infection and immune response during experimental oral candidiasis in BALB/c and DBA/2 (H-2^d) mice. Oral Microbiol Immunol 1994; **9**:88–94.

8 Elahi S, Pang G, Ashman RB, Clancy R. Cellular and cytokine correlates of mucosal protection in a murine model of oral candidiasis. Infect Immun 2000; **68**:5771–7.

9 Lacasse M, Fortier C, Chakir J, Cote L, Deslaurieres N. Acquired resistance and persistence of *C. albicans* following oral candidiasis in the mouse: a model of the carrier state in humans. Oral Microbiol Immunol 1993; **8**:313–18.

10 Elahi S, Pang G, Ashman RB, Clancy R. Nitric oxide enhanced resistance to oral candidiasis. Immunology 2001; **104**:447–54.

11 Elahi S, Clancy R, Pang G. A therapeutic vaccine for mucosal candidiasis. Vaccine 2001; **19**:2516–21.

12 Perdigon G, de Macias EN, Alvarez S, Oliver G, Pesce de Ruiz Holgado A. Effect of perorally administered *Lactobacilli* on macrophage activation in mice. Infect Immun 1986; **53**:404–10.

13 Schiffrin EJ, Rochat F, Link-Amster H, Aeschlimann JM, Donnet-Hughes A. Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. J Dairy Sci 1994; **78**:491–7.

14 Heumann D, Barras C, Severin A, Glauser MP, Tomasz A. Gram-positive cell walls stimulate synthesis of tumour necrosis factor alpha and interleukin-6 by human monocytes. Infect Immun 1994; **62**:2715–21.

15 Kato I, Tanaka K, Yokokura T. Lactic acid bacterium potently induces the production of interleukin-12 and IFN-gamma by mouse splenocytes. Int J Immunopharmacol 1999; **21**:121–31.

16 Marin ML, Tejada-Simon MV, Lee JH, Murtha J, Ustunol Z, Pestka JJ. Cytokine production in macrophage and T-cell models by *Streptococcus thermophilus*: comparison with *Bifidobacterium sp*. and *Lactobacillus bulgaricus*. J Food Prot 1998; **61**:859–64.

17 Miettinen M, Matikainen S, Vuopio-Varkila J *et al.* Lactobacilli and streptococci induce IL-12, IL-18 and gamma interferon production in human peripheral blood mononuclear cells. Infect Immun 1998; **66**:6058–62.

18 Nicaise P, Gleizes A, Forestier F, Quero A, Labarre M. Influence of intestinal bacterial flora on cytokine (IL-1, IL-6 and TNF-alpha) production by mouse peritoneal macrophages. Eur Cytokine Netw 1993; **4**:133–8.

19 Tejada-Simon MV, Ustunol Z, Pestka JJ. *Ex vivo* effects of lactobacilli, streptococci, and bifidobacteria ingestion on cytokine and nitric oxide production in a murine model. J Food Prot 1999; **62**:162–9.

20 Link-Amster H, Rochat F, Saudan K, Mignot O, Aeschlimann JM. Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. FEMS Immunol Med Microbiol 1994; **10**:55–63.

21 Phaupradit P, Varavithya W, Vathanophas K *et al.* Reduction of rotavirus infection in children receiving bifidobacteria-supplemented formula. J Med Assoc Thai 1999; **82**:43–8.

22 Saavedra JM, Bauman NA, Oung I, Perman JA, Yolken RH. Feeding of *Befidobacterium bifidum* and *Streptococcus thermophilis* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. Lancet 1994; **344**:1046–9.

23 Perdigon G, Alvarez S, Pesce de Ruiz Holgado A. Immunoadjuvant activity of oral *L. casei*: influence of dose on the secretory immune response and protective capacity in intestinal infections. J Dairy Res 1991; **58**:485–96.

24 Wagner RD, Pierson C, Warner T *et al.* Biotherapeutic effects of probiotic bacteria on candidiasis in immunodeficient mice. Infect Immun 1997; **65**:4165–72.

25 Wagner RD, Pierson C, Warner T, Dohnalek M, Hilty M, Balish E. Probiotic effects of feeding heat-killed *L. acidophilus* and *L. casei* to *C. albicans*-colonized immunodeficient mice. J Food Prot 2000; **63**:638–44.

26 Hilton E, Isenberg HD, Alperstein P, France K. Ingestion of yogurt containing *L. acidophilus* as prophylaxis for candida vaginitis. Ann Intern Med 1992; **116**:353–7.

27 Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate and nitrite in biological fluids. Anal Biochem 1982; **126**:131–44.

28 Fernandez MF, Boris S, Barbes C. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. J Appl Microbiol 2003; **94**:449–55.

29 Mercenier A, Pavan S, Pot B. Probiotics as biotherapeutic agents: present knowledge and future prospects. Curr Pharm Des 2003; **9**:175–91.

30 Clancy R. Immunobiotics and the probiotic evolution. FEMS Immunol Med Microbiol 2003; **38**:9–12.

31 Cross ML. Immune-signalling by orally-delivered probiotic bacteria: effects on the common mucosal immune responses and protection at distal mucosal sites. Int J Immunopathol Pharmacol 2004; **17**:127–34.

32 Shanahan F. Probiotics: a perspective on problems and pitfalls. Scand J Gastroenterol 2003; **38**(Suppl. 237):34–36.

33 De Petrino SF, De Jorrat MEBB, Meson O, Perdigon G. Protective ability of certain lactic acid bacteria against an infection with *C. albicans* in a mouse immunosuppression model by corticoid. Food Agric Immunol 1995; **7**:365–73.
34 Wagner RD, Warner T, Farmer J, Roberts L, Balish E. Colonization of congenitally immunodeficient mice with probiotic bacteria. Infect Immun 1997; **65**:3345–51.
35 Rachmilewitz D, Katakura K, Karmeli F *et al.* Toll-like receptor 9 signalling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. Gastroenterology 2004; **126**:520–8.
36 Roman M, Martin-Orozco E, Goodman JS *et al.* Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. Nat Med 1997; **3**:849–54.
37 Horner AA, Ronaghy A, Cheng PM *et al.* Immunostimulatory DNA is a potent mucosal adjuvant. Cell Immunol 1998; **190**:77–82.
38 Majamaa M, Isolauria E, Saxelin M, Vesikari T. Lactic acid bacteria in the treatment of acute rotavirus gastroenteritis. J Pediatr Gastroenterol Nutr 1995; **20**:330–8.