

A Full Flora, but not Monocolonization by *Escherichia coli* or Lactobacilli, Supports Tolerogenic Processing of a Fed Antigen

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Abstract

Fed protein undergoes processing and coupling to major histocompatibility complex (MHC) II molecules during passage through the intestinal epithelium, generating a tolerogenic form of the antigen in serum. Transfer of this factor to naïve animals induces tolerance in the recipient. In this study, we investigate what impact colonization with Gram-positive (*Lactobacillus plantarum*) or Gram-negative (*Escherichia coli*) bacteria has on tolerogenic processing in the gut. Germ-free (GF), monocolonized or conventional mice were fed ovalbumin (OVA), and their serum was collected and transferred to naïve conventional recipients that were tested for delayed-type hypersensitivity against OVA after parenteral immunization. A transferable tolerogenic factor was produced by conventional mice, but not by mice that were germ free or monocolonized with either *E. coli* or *L. plantarum*. Conventional, but neither GF nor monocolonized mice showed upregulation of MHCII expression in the epithelium of small intestine. The results suggest that a complex intestinal microflora is needed to support oral tolerance development.

Introduction

Several diseases, characterized by a failure of the immune system to develop tolerance to harmless antigens, are on the increase in Western countries. This has been most clearly demonstrated for allergies [1, 2], but can also be seen with inflammatory bowel diseases [3–5] and autoimmune disorders such as insulin-dependent type I diabetes and multiple sclerosis [6–8]. The reasons for the increase in these diseases are not known, but they may all be related to the increased hygienic lifestyle in the modern Western society. Good housing standard, small families [2, 9–11] and absence of infections [12] have all been linked to the high risk of developing allergies, while exposure to early day-care [13–15], pets [16], or a life-stock farming environment [17] all protect against allergies. These observations support the hygiene hypothesis, according to which insufficient exposure to microbes leads to faulty maturation of the immune system and failure to develop tolerance to innocuous antigen [18].

Proteins that enter the body via the gastrointestinal tract normally induce specific tolerance to themselves, a phenomenon called oral tolerance. It has been suggested that oral tolerance operates via induction of antigen-specific regulatory T cells that suppress immune activation upon renewed encounter with the same antigen [19]. The process by which regulatory T cells are induced is not known. However, it has been shown that passage of dietary antigen across the intestinal epithelium induces a tolerogenic factor present in the circulation [20–23]. The tolerogenic serum factor was first observed in 1983 by Strobel *et al.* [20]. Serum was collected from ovalbumin (OVA)-fed mice and injected into naïve mice. These mice became tolerant to OVA, and it was shown that gut 'processing' of the antigen was crucial for the production of the tolerogenic serum factor. The tolerogenic factor was later associated with the so-called tolerosomes, namely major histocompatibility complex (MHC) class II-positive exosome-like structures assembled in and released from the small intestinal epithelial cells [23].

Oral tolerance is not as easily induced in germ-free (GF) as in conventional animals and is also of shorter duration [24–26]. The quality and quantity of microflora needed to restore the tolerance-inducing capacity and the maturation of the intestinal immune system remains unknown.

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In this study, we have examined the effect of the intestinal microflora on tolerogenic processing. GF mice were colonized with either the Gram-positive bacterial species *Lactobacillus plantarum* or the Gram-negative *Escherichia coli*. The ability to process protein antigen into tolerosomes and the expression of MHC class II in the mucosa following the different colonization procedures were studied and compared to that of GF and conventional animals.

Materials and methods

Bacterial strains. A strain of *E. coli* was isolated from fecal samples of a 3-month-old child with symptoms of atopic eczema. The child participated in a prospective birth-cohort study aiming to examine the relation between intestinal colonization pattern and allergy development. The *L. plantarum* strain 299v was originally isolated from the human intestine and has the capacity to colonize the human gut [27]. *Escherichia coli* was cultured on blood agar plates aerobically in 37°C for 24 h, while *L. plantarum* was cultured anaerobically for 3 days. The bacteria were harvested and suspended at a concentration of 10⁹ colony-forming units (CFU)/ml.

Animals. Male and female GF NMRI mice were bred and maintained in stainless steel isolators at the Department of Medical Microbial Ecology at the Karolinska Institute, Stockholm, Sweden. They were 6–10 weeks old at the start of the experiment.

Male and female conventional NMRI mice (6–10 weeks old at the start of the experiment) were either from the Department of Medical Microbial Ecology, Karolinska Institute, Stockholm, Sweden, or purchased from B&K Universal (Uppsala, Sweden). We term the latter strain NMRI BK and the former NMRI KI. The conventional NMRI KI mice are continuously checked for the presence of a functionally active intestinal microflora and are also checked for the absence of potentially pathogens according to an internationally established standard, while the GF animals are checked for the absence of microbes. The NMRI KI mice had free access to a steam-sterilized standard mouse food and to sterilized water.

Colonization. GF mice were monocolonized while kept in the isolators. Bacterial aliquots of *L. plantarum* or *E. coli* were dispensed into sterile ampoules, which were heat-sealed and sterilized with chromsulfuric acid on the outside. The ampoules were transferred into the isolator, where they were broken, and approximately 0.8 ml (10⁹ CFU/ml) of the contents was given orally to each animal using a syringe; the remaining contents (0.2 ml) were spread on the furs and bedding material. Two groups ($n=28$) were monocolonized and allowed to stabilize for 3 weeks before the tolerization experiment. One additional group of NMRI KI mice ($n=28$) was kept under GF conditions and another group ($n=28$) was kept under conventional conditions.

Bacterial counts. After sacrifice of the mice, 5 µl of caecal and proximal small intestinal contents (approximately 4 cm from pylorus) was obtained using a calibrated loop, serially diluted in sterile saline and spread on TSA plates (for enumeration of *E. coli*), Rogosa agar plates (for enumeration of lactobacilli) and blood agar plates to ensure absence of contaminants. Samples from GF mice were only plated nondiluted. After culture, the number of colonies was counted and the results are expressed as CFU/g of intestinal contents.

Antigen. OVA (Grade V; Sigma Chemicals, St Louis, MO, USA) was used throughout this experiment. For delayed-type hypersensitivity (DTH) testing, the OVA solution (2 mg/ml) was denatured by heating at 80 °C in a water bath for 1 h.

Adoptive transfer of serum. All four individual groups of NMRI KI mice (*E. coli*-colonized, *L. plantarum*-colonized, GF and conventional) were split into two groups of 14 animals in each, which were fed by gavage either with 50 mg OVA dissolved in phosphate-buffered saline (PBS) (at a concentration of 100 mg/ml) or with PBS alone. This was performed inside the stainless steel isolators. One hour after feeding, the mice were taken out and blood was collected by cardiac puncture. The blood was pooled for each group, allowed to clot and then centrifuged at 2500 × *g* for 10 min at room temperature. The serum collected from each group was injected intraperitoneally into seven conventional recipient NMRI BK mice, each recipient receiving 0.6–0.7 ml of serum.

Immunization of recipients. One week after serum transfer, the recipients were immunized intramuscularly in the hind legs with 100 µg OVA emulsified in 0.05 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, MI, USA). Three weeks after the first immunization, all animals received a subcutaneous booster immunization with 100 µg OVA emulsified in Freund's incomplete adjuvant (Difco Laboratories) at the base of the tail.

DTH reaction. One week after booster immunization, all recipient mice were challenged with 100 µg denatured OVA given subcutaneously in the hind footpad. The footpad thickness was measured before and 24 h after challenge with a micrometer calliper (Oditest; Kroplin, Hessen, Germany). The difference between the two measurements gave an index of footpad swelling, which was used for group comparisons.

Mixed lymphocyte reaction (MLR). Single-cell suspensions of splenocytes from NMRI KI and NMRI BK were prepared by squeezing the spleen through a nylon filter (Falcon; Becton, Dickinson, NJ, USA). Erythrocytes were lysed with ammonium chloride buffer (pH 7.6) for 5 min in 37°C. The cells were then centrifuged for 5 min at 400 × *g*. After repeated washes in PBS, cells were suspended in complete medium. Target cells were irradiated (2500 rad) and incubated for 72 h in 37 °C and 5% CO₂, with responder cells at a ratio of 1:1 (4 × 10⁵ of each

population). Cells were pulsed with 1 μ Ci/well [^{3} H]-thymidine (Amersham International, Amersham, UK) for 10 h. Incorporated [^{3} H]-thymidine was measured in a β -counter.

Immunoperoxidase staining of sections from the small intestine. Immediately following the sacrifice of the monocolonized, GF and conventional mice, biopsies from the proximal small intestine (approximately 4 cm from pylorus) were removed and put in specimen moulds (Tissue-Tek Cryomold Biopsy; Miles Inc., Elkhart, IN, USA) with freeze medium (Tissue-Tek O.C.T., Sakura, the Netherlands). The biopsies were instantly frozen in isopentane cooled by liquid nitrogen and then stored at -70°C . Cryostat sections (6 μ m) were fixed in cold acetone (30 s in 50% acetone followed by 5 min in 100% acetone) and then air dried for 5 min and washed three times in PBS–Tween (0.05% Tween, 3×5 min). Endogenous peroxidase activity was blocked by incubating the slides for 20 min in a solution of 1 U/l glucose oxidase (Type V-S; Sigma), 10 mM glucose and 1 mM NaN_3 . This solution was preheated to 37°C for 15 min before use. The slides were incubated overnight at 4°C with biotinylated mouse MoAb directed against I-A^{b,k,u}, I-A^d, I-A^b and I-A^q and (Pharmingen, San Diego, CA, USA) diluted 1/500 in PBS–Tween. The slides were then incubated with avidin-conjugated peroxidase (ABC-complex, Dako A/S, Glostrup, Denmark) for 30 min. Finally, the peroxidase staining was visualized with amino-ethyl-carbazole followed by a light counterstaining with Mayer's hematoxylin. The slides were mounted with aqueous solution (aquatex, E Merck, Darmstadt, Germany) and cover slides were applied. The assessment of MHC class II-positive cells was done with a Leica Q500MC microscope (Leica, Cambridge, UK). For each individual mouse, 15 villi were examined.

NMRI KI and NMRI BK mice have haplotype H-2^q. In order for the adoptive serum-transfer model to function, donor and recipient mice must have the same MHCII haplotype (unpublished data). We used the outbred strain NMRI KI as donor mice and the outbred strain NMRI BK as recipient mice in the serum-transfer experiment. Therefore, it was necessary to investigate whether the two outbred strains shared the same MHCII haplotype. An MLR, in which splenocytes from NMRI KI mice were mixed with NMRI BK, was performed. Only a weak MLR reaction was detected when cells from the different NMRI mice strains were mixed. Mixing NMRI KI mice splenocytes with splenocytes from C57BL/6 mice with haplotype H-2^b gave rise to a much stronger MLR. However, only a weak MLR reaction could be detected between NMRI KI mice and DBA mice with haplotype H-2^q (Fig. 1). These data indicate that both NMRI KI and NMRI BK have the same haplotype, namely H-2^q.

Immunohistochemistry investigations confirmed this observation. Small intestinal biopsies were collected and frozen. Cryosections were stained with antibodies against different MHCII haplotypes (I-A^{b,k,u}, I-A^d, I-A^b and I-A^q), and exclusively, I-A^q antibody stained positive on both NMRI KI and NMRI BK (data not shown).

Results

Bacterial levels in intestinal contents

Four different colonization groups were studied. One group was kept under conventional conditions and one group was kept under GF conditions. The other two groups were monocolonized with either the Gram-negative bacterium *E. coli* or the Gram-positive bacterium *L. plantarum*. After 3 weeks of colonization, all mice were sacrificed and bacterial contents in caecum and small intestine were quantified. The monocolonization was well established in all mice, with approximately 10 times more bacteria in caecal contents compared to small intestinal contents. *Escherichia coli* reached colonization levels of $10^{10.3}$ in the caecum and $10^{9.0}$ in the small intestine of mice monocolonized by this bacterium. *Lactobacillus plantarum* colonized at $10^{8.9}$ CFU/g in the caecum and $10^{7.9}$ in the small intestine of monocolonized animals (Table 1). *Escherichia coli* bacteria could not be detected in *L. plantarum*-colonized animals and vice versa. There was also no contamination with other bacterial species in the monocolonization groups. GF animals showed no bacterial growth in the caeca or small intestines. Mice kept under conventional conditions had *Lactobacillus* levels similar to mice monocolonized with *L. plantarum* in both caecum and small intestine. In contrast, *E. coli* could neither be isolated from the caeca nor from the small intestines of conventional animals (Table 1).

Transferable oral tolerance could be induced in conventional, but not in GF or monocolonized mice

NMRI KI mice were colonized with either *L. plantarum* or *E. coli* for 3 weeks or kept under GF or conventional conditions. Mice in each colonization group were given

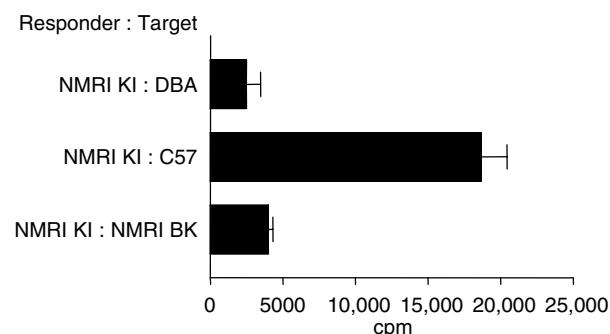


Figure 1 No mixed lymphocyte reaction (MLR) between splenocytes from NMRI KI and NMRI BK mice. Target splenocytes were irradiated and mixed with responder splenocytes at a ratio of 1:1. Target splenocytes from the mouse strains of DBA, C57BL/6 and NMRI BK were used. After 3 days of incubation, [^{3} H]-thymidine was added and counts per minute were measured. Bars show mean values and standard deviation of triplicates.

Table 1 Bacterial levels in caecal and small intestinal contents

Colonization	Luminal population levels ($^{10}\log$ CFU/g contents)			
	Caecum		Small intestine	
	<i>E. coli</i>	<i>Lactobacilli</i>	<i>E. coli</i>	<i>Lactobacilli</i>
<i>E. coli</i>	10.3	<2	9.04	<2
<i>L. plantarum</i>	<2	8.90	<2	7.90
Conventional	<2	8.72	<2	8.53
Germ free	<2	<2	<2	<2

Bacterial population levels in intestinal contents were determined by viable counts on TSA (for *Escherichia coli*) or Rogosa (for *Lactobacillus plantarum*) after 3 weeks of colonization. The numbers are expressed as mean values for each group ($n=14$). The level of detection was $2^{10}\log$ (1 CFU) from an inoculate of 10 μ l.

either 50 mg OVA or PBS perorally and sacrificed after 1 h. Serum was obtained and injected into recipient NMRI BK mice, which were later immunized and challenged with OVA. Serum from conventional mice given OVA perorally contained a tolerogenic transferable factor. This was evident from the fact that DTH (Fig. 2) to OVA in this group was significantly ($P<0.05$) reduced compared to the DTH reaction in the group which received serum from control conventional animals given PBS. Production of tolerogenic factor could not be demonstrated in GF mice (Fig. 2). Thus, when serum was transferred from GF animals fed OVA to conventional recipients, OVA-specific DTH was not significantly suppressed in the recipients, although there was a tendency towards lower responsiveness in animals receiving serum from GF mice fed OVA. More remarkably, there was even less evidence of production of tolerogenic factor in mice monocolonized with either *E. coli* or *L. plantarum*, a Gram-negative and Gram-positive member of the normal intestinal microflora (Fig. 2). In fact, there was even an increase in the lymphocyte proliferative response to OVA in mice receiving serum from *L. plantarum*-colonized OVA-fed mice. However, this increase was not observed on the DTH response. OVA-specific IgG, IgG1, IgG2a and IgE were also determined. However, these antibody titres were either too low to be detected (IgE and IgG2a) or there were no differences between groups of mice (IgG and IgG1) (data not shown).

MHCII is equally expressed in intestinal lamina propria, but not in the epithelium, of mice kept under different colonization conditions

Biopsies were collected from small intestine at sacrifice and cryosections were stained with anti-MHCII antibody. All immunohistochemistry analyses were performed blindly. The small intestinal epithelium stained positive for MHCII in all examined sections from conventional animals. In contrary, no MHCII expression could be detected in the epithelium of GF animals, or in animals

monocolonized with *E. coli* or *L. plantarum* (Fig. 3). Approximately 6–8% of the intestinal lamina propria area stained positive for MHCII, and this remained constant regardless of the colonization conditions (Fig. 4).

Discussion

Bacteria start to colonize the skin and mucosa of the newborn child as soon as it leaves the sterile uterus. A complex microflora will soon develop and it is difficult to study the influence of single bacterial species on the development of the immune system in humans. However, mice may be kept under GF conditions in isolators and may be colonized inside the isolators with defined bacterial species. The development of the immune system in response to colonization with a specific single bacterial strain or group of bacteria may, thus, be studied.

It has earlier been shown that it is difficult to induce oral tolerance in GF animals [24, 25, 28, 29]. In this study, we confirm these results and extend them by showing that GF animals are less capable of producing a

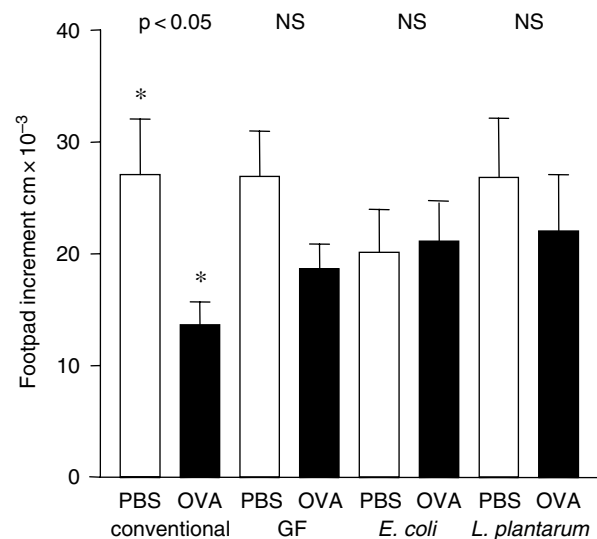


Figure 2 Systemic delayed-type hypersensitivity reactions could be suppressed in recipient mice injected with serum from conventional, but not from germ-free or monocolonized, ovalbumin (OVA)-fed donor mice. Donor NMRI mice (14 per group) were either kept under conventional or germ-free conditions or colonized with *Escherichia coli* or *Lactobacillus plantarum* for 3 weeks. One half of each colonization group received an oral administration of 50 mg OVA and the other half of the group received only PBS. One hour later, all mice were sacrificed and serum was withdrawn from them and pooled in eight groups. Approximately 0.7 ml serum was injected into naïve recipient NMRI mice. Recipient mice were systemically primed with 100 μ g OVA/Freund's complete adjuvant 1 week later. Three weeks after priming, animals received a booster immunization subcutaneously with 100 μ g OVA/Freund's incomplete adjuvant. One week after booster, mice were challenged with 100 μ g heat-aggregated OVA in the footpads. Specific increments in footpad swelling were determined by measuring footpad thickness before and 24 h after challenge. Data are expressed as mean values $\pm$ 1 SEM. * $P<0.05$. NS, not significant.

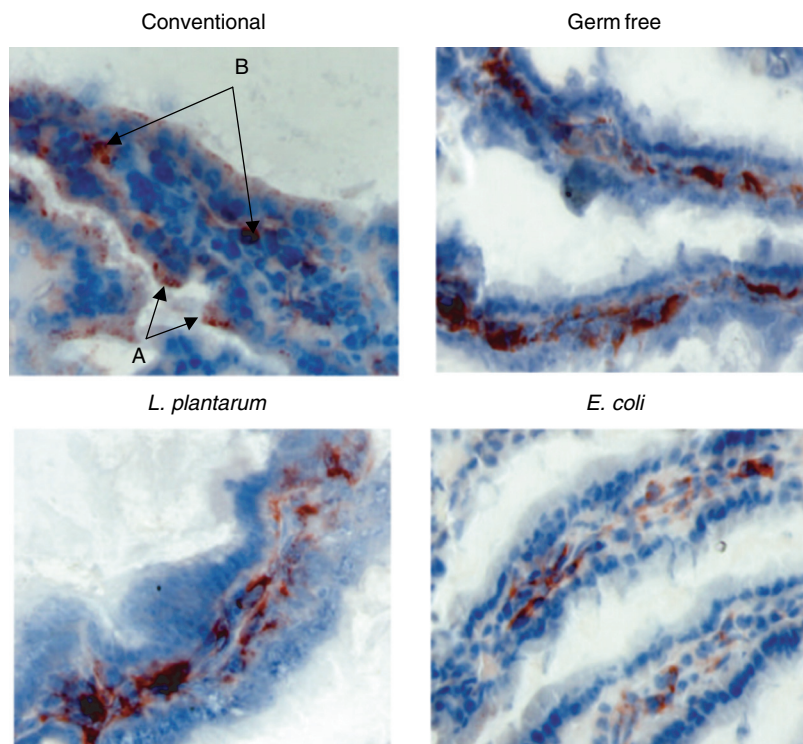


Figure 3 Mice kept under conventional conditions expressed major histocompatibility complex (MHC) class II in the epithelium. NMRI mice, seven to eight per group, were either kept under conventional or germ-free conditions or colonized with *Escherichia coli* or *Lactobacillus plantarum* for 3 weeks. Cryosections of small intestine were stained with anti-MHCII antibody and the sections were evaluated blindly for the expression of MHCII in the epithelium. Panel A shows MHCII-positive staining in the intestinal epithelium and panel B shows MHCII-positive staining in lamina propria.

tolerogenic serum factor after an antigen feed. Further, transferable tolerogenic factor could be produced by conventional mice, but not by mice monocolonized with either *E. coli* or *L. plantarum*.

GF mice lack MHCII expression in the small intestinal epithelium [24, 25]. This implies that the constitutive MHCII expression in the intestinal epithelial cells of normal mice is an effect of the constant exposure of microbial products from the normal gut flora [24, 25, 30, 31]. It has

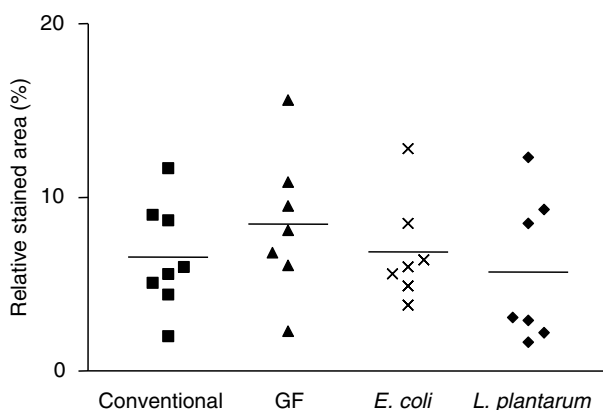


Figure 4 No difference in the expression of major histocompatibility complex (MHC) class II could be detected in the lamina propria of mice. NMRI mice, seven to eight per group, were either kept under conventional or germ-free conditions or colonized with *Escherichia coli* or *Lactobacillus plantarum* for 3 weeks. Cryosections of small intestine were stained with anti-MHCII antibody and the relative stained area was calculated. Individual values and group mean values are expressed.

been shown that SCID mice, which do not express MHCII in the intestinal epithelium, lack the ability to produce the tolerogenic factor [32, 33]. Treatment of SCID mice with interferon- γ (IFN- γ) induces MHCII expression and enables SCID mice to produce the tolerogenic factor (unpublished data). Monocolonization with either *E. coli* or *L. plantarum* was insufficient to induce MHCII expression in the intestinal epithelium. These mice were colonized for 3 weeks, which has been shown to be enough to induce MHCII expression in ex-GF mice colonized with a 'conventional' flora [34]. We therefore suggest that the absence of MHCII expression in our monocolonized mice is one factor contributing to the lack of oral tolerance induction. The fact that a complex flora was required in order to establish a tolerogenic promoting intestinal environment is in accordance with the finding that children who develop allergies have lower levels of most types of short-chain fatty acids in their intestinal contents [35]. Such fatty acids are end products of many different strictly anaerobic species, and the findings indicate that children who become allergic have a less complex anaerobic microflora than those who remain healthy.

It has been shown that colonization of GF mice with *L. paracasei* made them more susceptible to oral tolerance induction [29], but we could not demonstrate any effect of *L. plantarum* alone. *Lactobacillus paracasei* is a very strong inducer of IL-12 [36] and would therefore probably be an efficient inducer of IFN- γ from intraepithelial cells, which would lead to upregulation of the expression of MHCII in

the epithelium. We found *L. plantarum* to be inefficient in this respect in the present study, but in a previous study on gnotobiotic rats colonized with both a Gram-positive (*L. plantarum*) and a Gram-negative (*E. coli*) bacteria, it led to an increased expression of MHCII in small intestinal epithelium. However, this was only seen when the rats were colonized with both species. In addition, an increase in CD25⁺ cells could be detected in the lamina propria of these rats [37]. This may have an implication for oral tolerance induction since regulatory CD4⁺ T cells, which are important for downregulation of immune responses, express CD25 [38].

In conclusion, neither *E. coli* nor *L. plantarum* could alone reproduce the capacity of a full flora in promoting the generation of a tolerogenic factor from ingested protein. Delayed acquisition of a fully complex anaerobic flora may impair the development of tolerance to environmental antigens and thus underlie the rising incidence of allergies in Western countries.

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