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Conflict of interest statement
None declared.

Acknowledgments
GRASP is a collaborative initiative between surveillance, microbiology, and genitourinary physicians and specialists. We thank the collaborating laboratories and clinics for their ongoing involvement in the programme and Alan Herring and Andrew Turner of the former Genitourinary Infections Reference Laboratory (GUIRL) in Bristol, who were previous partners in this programme and were involved in the collection of isolates in the 2002 collection. GRASP is funded by a grant from the Department of Health, England. The sponsor had no role in study design, data collection, data analysis, data interpretation, or in the writing of this report.

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Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial

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Perinatal administration of the probiotic *Lactobacillus rhamnosus* strain GG (ATCC 53103), reduces incidence of atopic eczema in at-risk children during the first 2 years of life (infancy). We have therefore assessed persistence of the potential to prevent atopic eczema at 4 years. Atopic disease was diagnosed on the basis of a questionnaire and a clinical examination. 14 of 53 children receiving lactobacillus had developed atopic eczema, compared with 25 of 54 receiving placebo (relative risk 0·57, 95% CI 0·33–0·97). Skin prick test reactivity was the same in both groups: ten of 50 children previously given lactobacillus compared with nine of 50 given placebo tested positive. Our results suggest that the preventive effect of lactobacillus GG on atopic eczema extends beyond infancy.

Lancet 2003; **361:** 1869–71

Improved hygienic conditions reducing early-life exposure to microbes have been associated with a heightened risk of allergic diseases. The hygiene hypothesis has been extended to suggest that the collective composition of the microbial gut colonisation early in life is especially crucial for healthy maturation of the naive immune system.[1] Probiotics provide a microbial stimulus by means of cultures of beneficial live microorganisms characteristic of the healthy infant gut.[1] Results of a randomised double-blind placebo-controlled study[2] showed that perinatal administration of probiotics (*Lactobacillus rhamnosus* strain GG; ATCC 53103) halved development of atopic eczema in children at high risk during the first 2 years of life (infancy).

To investigate whether the preventive effect of lactobacillus on atopic disease extends beyond infancy, we re-examined the cohort at the age of 4 years. The original study design has been described previously.[2] Briefly, 159 mothers were randomly allocated to receive two capsules of placebo (microcrystalline cellulose) or 1×10^{10} colony-forming units of lactobacillus GG (Valio, Helsinki, Finland) daily for 4 weeks before expected delivery. After delivery, capsules were taken postnatally for 6 months; during breastfeeding, either the mother or the infant consumed the preparation.

We invited all 132 (83%) of the 159 children who completed the 2-year follow-up to the 4-year follow-up visit. The invitation letter included a questionnaire about possible allergic symptoms and drugs during the previous 2 years. The diagnosis of atopic disease, made by a researcher (TP) who was unaware of the treatment allocation, was based on the questionnaire and a clinical examination (done by MK), which included inspection of eyes, ears, nose, and skin, auscultation of heart and lungs, palpation of abdomen, and assessment of growth. In particular, diagnosis of atopic eczema was confirmed if the child had itchy eczematous lesions with typical location and with relapsing or chronic course during the last 12 months; and allergic rhinitis with nasal discharge, blockage, sneezing, and itching related to allergen exposure; or asthma with chronic or recurrent cough, wheeze, or shortness of breath requiring regular inhaled corticosteroids.

We also did a skin-prick test in 100 of the 107 children who participated in the 4-year follow-up to milk, egg white, wheat flour (diluted 1 to 10 weight/volume with 0·9% weight/volume sodium chloride), gliadin (diluted 1 to 1000

	Placebo (n=50)	Lactobacillus GG (n=50)
Antigens		
Dietary		
Peanut	1	1
Cod	1	0
Environmental		
Cat	6	2
Alder	3	5
Birch	3	4
Dog	1	2
Local grasses	1	0

Values are number of patients. Data are presented for allergens that tested positive. Other antigens tested (see the text) did not elicit skin prick test reactivity.

Skin prick test reactivity

weight/volume with 0·9% weight/volume sodium chloride), cod, soya bean, hazel nut, peanut, birch, mugwort, alder, six local grasses, cat, dog, and *Dermatophagoides pteronyssimus* allergen Der p1, (ALK, Abello, Denmark); and latex (Stallergens, Marseille, France) as previously described.[2] The test was judged positive if a wheal of 3 mm or larger was seen in response to any of the allergens in the presence of an appropriate response to the positive control (10 g/L of histamine dihydrochloride; ALK) and no response to the negative control (allergen diluent; ALK). During the previous follow-up, we did double-blind placebo-controlled challenges to cow's milk when symptoms, clinical signs, or skin-prick test responses had been suggestive of cow's milk allergy. At 4 years, cow's milk challenges were repeated if persistence of the cow's milk allergy was suspected.

We assessed the concentration of exhaled nitric oxide as a marker of bronchial inflammation. The expired air with dead space was collected in bags by the tidal breathing method.[3] The concentration of exhaled nitric oxide was assessed within 4 h after collection by a Sievers 280 chemiluminescence analyser (Sievers, Boulder, CO, USA). The mean concentration of exhaled nitric oxide in healthy age-matched children was 10·1 ppb (range 7·3–18·2) (Tuomas Jartti, personal communication). 80 of the 107 children participating at the 4-year follow-up co-operated in the collection. Since acute respiratory infectious diseases increase and inhaled corticosteroids reduce the concentrations of exhaled nitric oxide,[3] the four children with asthma, who all required regular inhaled corticosteroids, and the 19 children with signs of acute respiratory infectious disease were excluded from the analysis of exhaled nitric oxide concentrations. Consequently, we assessed the concentration of exhaled nitric oxide in 32 children previously given placebo and 25 children given probiotics.

We compared between-group differences by unpaired *t* test or χ^2 test. 54 of 68 children in the placebo group and 53 of 64 in the lactobacillus group completed the 4-year follow-up. Atopic eczema was diagnosed in 14 of the 53 children on lactobacillus, compared with 25 of the 54 on placebo (relative risk 0·57, 95% CI 0·33–0·97). Five of 54 children in the placebo group and ten of 53 in the lactobacillus group had developed seasonal allergic rhinitis (p=0·15), and one in the placebo group and three in the lactobacillus group had developed asthma (p=0·30).

The mean concentration of exhaled nitric oxide was greater in the placebo group (14·5 ppb, 95% CI 12·0–17·1), than in the lactobacillus group (10·8 ppb, 8·6–13·0; p=0·03). The same trend was seen even when we excluded the 20 children with a history of acute respiratory infectious disease during the past 3 weeks but no signs at the clinical examination (13·8 ppb [10·3–17·3] and 10·3 ppb [8·0–12·6], respectively; p=0·09). Of the 100 children in whom we did a skin-prick test, nine of 50 in the placebo group and ten of 50 in the lactobacillus group tested positive for an allergen (p=0·80), of whom five in each group reacted to more than one allergen (table). Allergies to cow's milk had been diagnosed previously by double-blind placebo-controlled cow's milk challenge in 6 of 54 children on placebo and 11 of 53 on lactobacillus (p=0·17); in two children in each group (p=0·98), the symptoms persisted up to the age of 4 years.

Our findings show that the preventive effect of lactobacillus GG on atopic eczema in at-risk children extends to the age of 4 years. This age, however, does not yet allow a final assessment of any effect on respiratory allergic diseases, since these typically manifest themselves at an older age.[4] Although the number of children with allergic rhinitis and asthma did not differ between groups, the concentration of exhaled nitric oxide was significantly greater in children receiving placebo than in those receiving lactobacillus, suggesting the possibility of more underdiagnosed or subclinical cases of respiratory allergic diseases in the placebo group.[3]

The immunological basis of the hygiene hypothesis suggests a counter-regulatory balance between T helper (Th) 1-type and Th2-type immune responses.[1] The collective composition of the bifidobacterium microbiota might be crucial for maturation of human immunity to a non-atopic state; infants later developing antigen-specific IgE antibodies have more clostridia and fewer bifidobacteria in their stools during the postnatal period than those who did not.[1] Specific strains of the lactobacillus microbiota, including lactobacillus GG, generate anti-inflammatory interleukin 10 and transforming growth factor β,[1] suggesting a mechanism by which the risk of atopic eczema could be decreased, since the concentration of antigen-specific IgE antibodies was not reduced, at least by age 4 years. In fact, the cytokine production patterns induced by intestinal microbiota might be strain-specific.[1] The IgE-independent protective effect of bacterial components on atopic disease we identified might be mediated by activation of the innate immune system via evolutionarily highly conserved pattern-recognition receptors.[5]

Thus, distinct causes and pathogenic mechanisms underlie the heterogeneous manifestations of allergy. The immunological effects of probiotic strains also vary, even within the same species.[1] Since the probiotic approach was promising and safe, future studies should focus on detection of new potential successful probiotic strains to be applied in combinations when combating allergic diseases.

Contributors

E Isolauri, S Salminen, M Kalliomäki, and H Arvilommi developed and designed the study and contributed to the writing of the report. M Kalliomäki did the clinical examinations. T Poussa analysed and interpreted the data.

Conflict of interest statement

None declared.

Acknowledgments

We thank Eino Hietanen (deceased), Department of Clinical Physiology, Turku University Hospital, for supervision of eNO assays and Johanna Hvitfelt-Koskelainen for participation in the follow-up of the participants. The study was supported by grants from the Academy of Finland and Turku University Hospital (EVO Fund). The funding sources had no role in study design, data collection, data analysis, data interpretation, or in the writing of the report.

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Association between tuberculosis and a polymorphic NFκB binding site in the interferon γ gene

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Interferon γ is believed to be crucial for host defence against many infections. To test the hypothesis that a polymorphism in the gene for interferon γ (*IFNG*) is associated with susceptibility to tuberculosis, we did two independent investigations. In a case-control study of 313 tuberculosis cases, we noted a significant association between a polymorphism (+874A→T) in *IFNG* and tuberculosis in a South African population (p=0·0055). This finding was replicated in a family-based study, in which the transmission disequilibrium test was used in 131 families (p=0·005). The transcription factor NFκB binds preferentially to the +874T allele, which is over-represented in controls. This preferential binding suggests that genetically determined variability in interferon γ and expression might be important for the development of tuberculosis.

Lancet 2003; **361:** 1871–72

Tuberculosis accounts for the largest number of deaths worldwide that are due to a sole infectious agent. Interferon γ is thought to be essential for the immune response to infection in man, but genetic variability in the interferon γ gene (*IFNG*) itself has not previously been associated with susceptibility to infectious disease in the general population. Indeed, the importance of the interferon γ response in tuberculosis is unclear because the pathogen seems to have evolved strategies to disrupt the activity of the interferon γ molecule.[1]

A microsatellite polymorphism in the first intron of *IFNG* has been associated with several autoimmune and chronic inflammatory conditions.[2] One particular allele of this microsatellite (the 12 CA repeat) is associated with increased production of interferon γ in vitro,[3] and with allograft fibrosis in recipients of lung transplant. This link suggests a functional role in vivo for either the microsatellite itself, or, more probably, a functional polymorphism in linkage disequilibrium with the 12 CA repeat. A single nucleotide polymorphism (+874A→T) is situated directly adjacent to the CA repeat region in the first intron of *IFNG*. There was an absolute correlation between the presence of the +874T allele and the 12 CA repeat allele.[3] The +874A→T polymorphism lies within a binding site for the transcription factor NFκB and electrophoretic mobility shift assays showed specific binding of NFκB to the allelic sequence containing the +874T allele.[3] Since this transcription factor induces interferon γ expression, the +874T and +874A alleles probably correlate with high and low interferon γ expression, respectively.

In two studies, we investigated the role of the +874T→A polymorphism in tuberculosis. In a case-control study, participants were unrelated, and from a community in which all members were of similar socioeconomic status, have the same ethnic background (South African coloured) and have a high annual incidence of tuberculosis.[4] Informed consent was obtained from all participants, and the study was approved by the ethics committee of the Faculty of Health Sciences, Stellenbosch University (Tygerberg, South Africa). To look for the +874A→T polymorphism, we genotyped DNA samples from 313 patients and 235 controls older than 15 years with no history of tuberculosis. The affected group consisted of 241 patients in whom pulmonary tuberculosis was bacteriologically confirmed, and 72 patients with tuberculous meningitis.

Allele frequencies were the same in both affected groups and they were therefore analysed as one group. Cases were screened for HIV-1 and excluded from analysis if positive. Women accounted for 60% of both the disease and control groups. The control group was in Hardy-Weinberg equilibrium, and the sample size provided at least 95% confidence and 80% power (Epi Info 2000 version 1.1) for an allele with an odds ratio of 1·65 or higher. Genotyping was done by PCR as previously described.[3]

Statistical analysis by the χ^2 and Fisher's exact tests showed a significant association between +874A→T and tuberculosis for both the genotype (p=0·017) and the allele frequencies (p=0·0055) (table 1). The +874T allele seemed to confer protection against the disease, since comparison of +874T carriers versus non-carriers shows a significant difference between the disease and control groups (table 1).

We designed a second, family-based study to test the findings of the first one and to control for any possible population stratification and other environmental factors. We did a transmission disequilibrium test (TDT) in 131 families in which one or more child was affected with tuberculosis, and had at least one parent. Families were from the same community as in the first investigation, with no duplication between the cases of tuberculosis analysed in both studies. Of 287 patients, 274 consented to HIV testing, of whom ten proved positive for HIV-1. Affected children were excluded if HIV-positive (2·4%). 65 families had both parents available, whereas 66 had only one parent. There were 44 families with more than one affected child. Paternity of all the siblings was checked with highly polymorphic microsatellite markers, and instances of non-paternity were excluded from analysis. Data for 612 individuals were analysed with TRANSMIT (version 2.5.2). This program

	TB (n=313)	Control (n=235)	Odds ratio (95% CI)	p
Genotype				
AA	186 (59%)	111 (47%)	..	
AT	102 (33%)	98 (42%)	..	
TT	25 (8%)	26 (11%)	..	
Total	313	235	..	0·017*
Allele				
A	474 (76%)	320 (68%)	..	
T	152 (24%)	150 (32%)	1·64 (1·16–2·30)	0·0055*
T allele				
Absent	186 (59%)	111 (47%)	..	
Present	127 (41%)	124 (53%)	1·46 (1·12–1·91)	0·0062*

TB=tuberculosis. *p value indicates the significance of over-representation of the A allele in patients with TB

Table 1: **Case-control study: association of +874A→T with tuberculosis**