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Original Article

Differences in the gut microbiota of healthy children and those with type 1 diabetes

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Abstract ***Background***: Intestinal barriers, intestinal flora, and mucosal immunity are the main factors responsible for the development of various allergic and autoimmune diseases. The aim of this study was to investigate the relationship between the intestinal flora of children and the presence of type 1 diabetes, and to determine if gut microbiota could partly explain the etiology of the disease.

Methods: Fecal flora analysis was done using quantitative cultures on selective and non-selective media with different thermal and atmospheric conditions for bacterial and fungal growth. The study group consisted of 35 patients (16 female, 19 male; mean age, 10.73 ± 4.16 years), who had been followed by the University of Istanbul, Cerrahpasa Medical Faculty, Department of Pediatrics, and were newly diagnosed with type 1 diabetes. The control group consisted of 35 healthy subjects (15 female, 20 male; mean age, 9.96 ± 4.09 years), who were randomly selected and had similar demographics.

Results: *Bifidobacterium* colonization was lower in patients with type 1 diabetes compared to the control group, whereas *Candida albicans* and *Enterobacteriaceae* other than *Echerichia coli* colonization was increased.

Conclusion: A decrease in beneficial anaerobic bacteria levels and a concomitant increase in *Enterobacteriaceae* other than *E. coli* and *C. albicans* colonization may lead to a disturbance in the ecological balance of intestinal flora, which could be a triggering factor in type 1 diabetes etiology.

Key words autoimmune disease, *Candida albicans*, gut flora, gut microbiota, type 1 diabetes mellitus.

Diabetes mellitus is a group of metabolic and endocrinologic diseases characterized by significant hyperglycemia resulting from defects in insulin secretion, insulin efficacy, or both. The vast majority of diabetes cases can be divided into two broad etiopathogenetic categories: in type 1 diabetes, loss of the insulin-secreting beta cells leads to disease; in type 2 diabetes, which is much more prevalent, the causative factor is a combination of insulin resistance and deficiency of compensatory insulin secretory response.[1,2]

Individuals at a high risk of developing type 1 diabetes can often be identified by serological evidence of an autoimmune pathologic process in the pancreatic islets or by genetic markers. The different variations of type 1 diabetes are numerous and varied, and a variety of factors, including chemicals, viruses, and immunity mediated by antibodies or cells, have been implicated as possible reasons for the disorder.[2,3]

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The gastrointestinal system must make distinctions between pathogenic and non-pathogenic organisms and food antigens. It must also tolerate the commensal flora, which maintain intestinal epithelial homeostasis by controlling inflammation and sensing potentially harmful pathogens. It is becoming increasingly clear that the nexus of the intestinal microbiota composition, intestinal barrier, and mucosal immunity plays a very important role in the development of a variety of allergic and autoimmune diseases.[4] In the case of type 1 diabetes, there is evidence of synergism among aberrant intestinal microbiota, a "leaky" gastrointestinal–epithelial barrier, and altered mucosal immunity in the pathogenesis of the disorder. Subclinical intestinal abnormalities have also been reported in human type 1 diabetes. In addition, recent studies show that subclinical enteropathy is associated with type 1 diabetes and is detectable before clinical onset of the disease, which suggests that the small intestine participates in the pathogenic process of type 1 diabetes.[5,6] In studies on the relationship between gut immunology and type 1 diabetes, increased expression of cytokines (e.g. interleukin [IL]-4, IL-1α, interferon-γ) and antigen-presenting and adhesion molecules (e.g. human leukocyte antigen [HLA] DR, DP, HLA class II, intercellular adhesion molecule-1) was seen in the small intestinal biopsies from patients with type 1 diabetes.[7,8]

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Type 1 diabetes leads to increased activation of the cellular and humoral immune system, thereby increasing the risk of the development of the disease. Early increased intestinal permeability may allow unregulated passage of environmental antigens, which could potentially trigger the autoimmune response, leading to type 1 diabetes.[9–12]

This relationship among tolerance, microbiota, and permeability has led to increased use of probiotics in children with atopic dermatitis, and these have been shown to alleviate the signs and symptoms of this condition.[10,13]

The vast majority of endogenous bacteria are completely anaerobic. The intestinal flora, intestinal epithelium, and enteropathy-associated lymph tissue form a complex ecosystem. The microorganisms in the intestine can be classified into two main groups. The first group forms a permanent colony in the intestine and performs important functions. The second group (transient microorganisms) originates from external sources, such as food that is digested, and therefore changes daily. These microorganisms may be pathogenic bacteria and may not normally colonize the intestinal system. A well-balanced fecal flora contributes to the regulation of the intestinal passage, penetration of potentially harmful antigens and prevention of the proliferation of enteric pathogens. A typical bacterial colonization of the intestinal flora leads to increased intestinal permeability and degradation.[14–18] Therefore, intestinal flora and mucosal immunity could be responsible for the development of various allergic and autoimmune diseases (i.e. type 1 diabetes). Furthermore, several studies suggest an altered gut microbiota in type 1 diabetes.[19,20] Thus, if the effects of the intestine on autoimmunity could be better understood, the unexplained etiology of type 1 diabetes would be clarified, leading to novel nutritional and pharmacological treatments.

Methods

The study group consisted of 35 patients (16 female, 19 male) with a mean age of 10.73 ± 4.16 years, who were followed at the University of Istanbul, Cerrahpasa Medical Faculty, Department of Pediatrics, Division of Nutrition, and were newly diagnosed with type 1 diabetes. Written parental informed consent was obtained from each participating family, and the study protocol was approved by Istanbul University Cerrahpasa Medical Faculty Ethics Committee (date: 9 May 2011; no.: 16370).

The healthy control group consisted of 35 healthy subjects (15 female, 20 male) with a mean age of 9.96 ± 4.09 years, who were randomly selected and had similar demographics. The study started on 15 July 2011 and was completed on 15 March 2012.

Working procedure

Stool quantification of intestinal flora was conducted as a routine diagnostic test for all patients. The transient and permanent fecal flora of physiological bacteria and fungi were analyzed as described in Beckman and Rüffer.[14] Bacteria and fungi were cultured using both selective, quantitative methods and non-selective methods under different thermal and atmospheric conditions. After 3 days of incubation, colonies were counted, and the results expressed as colony-forming units per gram feces (c.f.u./g feces). The increase or decrease in colony count of each species of microorganism was determined according to several thresholds defined in Table 1.[14] Yeast was detected at a concentration of 10^3 c.f.u./g; at this concentration, the reproduction pattern is unique.

Patients with the following characteristics were considered during selection of a control group: (i) no abdominal distension and no complaints of constipation and dyspepsia; (ii) absence of a history of allergic disease, such as bronchial asthma or atopic dermatitis; (iii) absence of active infection, such as upper respiratory tract infection or acute gastroenteritis; (iv) absence of a history of known genetic (e.g. ataxia telangiectasia or phenylketonuria) or chronic degenerative illness (e.g. systemic lupus erythematous or rheumatoid arthritis); and (v) no intake of antibiotics or other drugs for the previous month.

Materials used for sample collection: each stool sample was collected in the appropriate container.

Table 1 Reference values for permanent and temporary bacteria and fungi

	Bacteria	Fungi	Normal range (c.f.u./g)	Decreased (c.f.u./g)	Increased (c.f.u./g)
Facultative anaerobic bacteria	*Escherichia coli*		10^6–10^7	$<10^6$	$>10^7$
	Enterococcus spp.		10^6–10^7	$<10^6$	$>10^7$
	Enterobacteria		$\leq 10^5$		$>10^5$
	Proteus spp.				
	Klebsiella spp.				
	Citrobacter spp. *Enterobacter* sp				
	Bacillus spp.		$\leq 10^4$		$>10^4$
	Pseudomonas Staphylococcus				
Microaerophilic bacteria	*Lactobacillus* spp.		10^6–10^7	$<10^6$	$>10^7$
Anaerobic bacteria	*Prevotella* spp. *Bifidobacterium*		10^8–10^{10}	$<10^8$	$>10^{10}$
	Bacteroides				
	Clostridium spp		$\leq 10^5$		$>10^5$
Fungal colonization		*Candida* spp.	$\leq 10^2$		$>10^2$
		Saccharomyces Aspergillus spp.			
		Mucor spp. *Geotrichum* spp.			

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The following recommendations were carefully applied in the laboratory to ensure the quality of the samples: (i) all subjects were selected from groups who did not consume any pancreatic enzymes or probiotics; (ii) care was taken to avoid contact between fecal samples and urine or disinfectants; (iii) to ensure the viability of anaerobic bacteria, each sample was filled to 75% of the tube; and (iv) the mouth of the basic stool carrier was considered closed, and in each case, the stool sample was tested individually.

To avoid bias, stool samples were sent to the laboratory along with the patient number labeled with the patient's initials. Quantitative analysis of bacteriological and fungal intestinal flora was performed by Laboratoires Réunis in Luxembourg (ISO 15189; certified since 2007). Stool samples of six patients in the study did not reach the laboratory on time due to weather conditions and therefore could not be evaluated.

Statistical analysis

NCSS 2007 and PASS 2008 (Kaysville, UT, USA) were used for statistical analysis. To compare quantitative data, chi-squared test, Yates continuity correction, and Fisher's exact test were used. The significance level was evaluated at $P < 0.05$ and $P < 0.01$.

Results

In total, 35 type 1 diabetic patients, consisting of 16 female and 19 male patients with an average age of 10.73 ± 4.16 years, and a control group of 35 healthy children with similar demographic features, composed of 15 female and 20 male children with an average age of 9.96 ± 4.09 years, were selected. There were no significant differences between the study and control groups with respect to age and gender.

Selective and quantitative cultures were performed under different thermal and atmospheric conditions on non-selective media.

Within the study and control groups, no colonies of *Proteus mirabilis*, *P. vulgaris*, *Klebsiella oxytoca*, *K. pneumoniae*, *Citrobacter* spp., *Serratia* spp., *Hafnia alvei*, *Morganella morganii*, *Providencia* spp., *Pseudomonas* spp., *Enterococcus*, beta-hemolytic *Streptococcus*, *S. aureus*, or *Geotrichium* species of fungus and molds ($\leq 10^4$ c.f.u./g) were observed.

A decrease in *E. coli* colonization in three of the type 1 diabetes patients (8.6%) and an increase in colonization in 15 patients (42.9%) were identified. Within the control group, a decrease in colonization in two individuals (5.7%) and an increase in colonization in 11 individuals (42.9%) were identified. There were no statistically significant differences between the two groups.

In the type 1 diabetes group, *Enterobacteriaceae* colonization was observed in 13 patients (37.1%) and in three individuals (8.6%) in the control group; this difference was statistically significant.

A decrease in *Enterococcus* colonization was identified in 10 of the type 1 diabetes patients (28.4%) and in eight of the controls (22.9%). Conversely, an increase in colonization was observed in two of the patients with type 1 diabetes (5.7%) and in four of the controls (11.4%). No statistically significant difference was identified between the two groups.

A decrease in *Bacteroides* spp. colonization was identified in seven of the patients with type 1 diabetes (20.0%) and in one of the controls (2.9%). Despite the large numerical difference between the groups, no statistically significant difference was identified.

A decrease in *Bifidobacterium* spp. colonization was identified in eight of the type 1 diabetes patients (22.8%) and in one of the controls (2.9%). There was a statistically significant difference between the groups ($P < 0.05$).

A decrease in *Lactobacillus* colonization was observed in 18 of the type 1 diabetes patients (56.3%) and in 14 of the controls (43.8%). Only one type 1 diabetes patient and none of the controls had increased *Lactobacillus* colonization. No significant difference was identified between the groups.

Candida albicans colonization was identified in 14 of the type 1 diabetes mellitus patients (40%) and in five of the controls (14.3%). A statistically significant difference was identified between the two groups. Additionally, colonization by unclassified *Candida* spp. other than *C. albicans* was observed in seven of the patients in the type 1 diabetes group (20%) and in six of the controls (17.1%), but no significant difference was identified between the groups. The distribution of bacteria and fungi in the control and study groups is given in Table 2.

The stool flora of the patients and the controls are listed in Table 3.

Discussion

Type 1 diabetes is among the most extensive chronic diseases of children, and the frequency of this disease is rising worldwide.[21] Although there have been several studies focused on the role of the intestine in type 1 diabetes development in animal models and in humans, the exact cause of type 1 diabetes remains unknown..[22] The latest pediatric type 1 diabetes prevalence data in a large pediatric population in Istanbul, Turkey, showed that the prevalence of type 1 diabetes was 0.67 per 1000 individuals. This prevalence is 2.5-fold higher than that in the capital of Turkey (Ankara), in 1993, suggesting that type 1 diabetes prevalence is increasing in Turkey, as in other parts of the world.,[23]

The digestive tracts of infants are devoid of bacteria at birth, and an infant delivered by normal spontaneous vaginal delivery encounters *Enterobacteriaceae*, *Enterococcus*, and *Streptococcus*, leading to the development of the infant's first bacterial flora. Anaerobic microorganisms develop later as a result of nutritional and environmental factors. Only at the age of 2 does an infant develop bacterial flora similar to those of adults.[24]

Enterobacteriaceae constitutes a large family of bacterial species that includes *E. coli, Klebsiella* spp., *P. mirabilis, P. vulgaris, Oxytoca* spp., *K. pneumoniae, Citrobacter* spp., *Serratia* spp., *M. morganii, Providencia* spp., and *Pseudomonas* spp. These species are found in limited numbers within human and animal flora and are capable of rapidly proliferating in aerobic and anaerobic environments. In humans, these species

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Table 2 Distribution of bacteria and fungi

Bacterium or fungus	Status	Control group (n = 35)		Study group (n = 35)		P
		n	%	n	%	
Echerichia coli	No colonization	22	62.9	17	48.6	0.483†
	Increased colonization	11	31.4	15	42.9	
	Decreased colonization	2	5.7	3	8.6	
Enterobacteriaceae	Normal	32	91.4	22	62.9	**0.010**‡
	Increased colonization	3	8.6	13	37.1	
Enterococcus	Normal	23	65.7	23	65.7	0.641†
	Increased colonization	8	22.9	10	28.6	
	Decreased colonization	4	11.4	2	5.7	
Bacteroides spp.	Normal	34	97.1	28	80	0.055§
	Decreased colonization	1	2.9	7	20	
Bifidobacterium spp.	Normal	34	97.1	27	77.1	**0.028**§
	Decreased colonization	1	2.9	8	22.9	
Lactobacillus spp.	Normal	21	60	16	45.7	0.337†
	Decreased colonization	14	40	18	51.4	
	Increased colonization	0	0	1	2.9	
Candida albicans	Normal	30	85.7	21	60	**0.032**‡
	Increased colonization	5	14.3	14	40	
Candida spp.	Normal	29	82.9	28	80	0.792‡
	Increased colonization	6	17.1	7	20	

†Chi-squared test; ‡Yates continuity correction test; §Fisher's exact test.

account for the majority of urinary tract infections and a fraction of diarrhea cases and they can also cause severe infections in immunodeficient patients.

Enterobacteriaceae synthesize numerous microbiocide substances with toxic effect against enteropathogenic bacteria. In addition, the lipopolysaccharides on the bacterial cell wall contribute to stabilization of the intestinal barrier through group-specific antigens and low-molecular-weight peptides that directly affect the immune system in the intestines. In the present study, when *Enterobacteriaceae* spp. other than *E. coli* were evaluated as a group, increase in colonization was seen in 37.1% of the type 1 diabetes groupand in 8.6% of the control group, respectively. A statistically significant difference was observed between the groups.

Echerichia coli was long assumed to be the most important intestinal bacterial species, but *E. coli* actually represents <0.1% of the bacterial mass of the colon.[14,17] *Echerichia coli* converts carbohydrates to short-chain fatty acids under aerobic or anaerobic conditions. Short-chain fatty acids fulfill an essential role in enterocyte nutrition. A decrease in *E. coli* in the stool flora could adversely affect enterocyte nutrition. In this context, situations in which intestinal integrity cannot be maintained can lead to the migration of antigens into the lymphatic system on the intestinal surface.[25,26]

In the present study, *E. coli* colonization in patients with type 1 diabetes was detected as decreased in 8.6% and as increased in 42.9%, whereas in the control group this was 5.7% and 31.4%, respectively. No differences in *E. coli* colonization were identified between the groups (P > 0,05), eliminating the possibility that *E. coli* is one of the causes of progression to type 1 diabetes.

In 1989, Strachan formulated the hygiene hypothesis in an attempt to describe the increase in immune-mediated diseases. According to this hypothesis, low exposure to microorganisms leads to insufficient stimulation of T-helper 1 cells, which in later years contributes to the development of hypersensitivity-related diseases, such as allergic rhinitis and asthma.[18]

Staphylococcus aureus and beta-hemolytic *Streptococcus* are ubiquitous in the oral cavity and on the skin and rarely temporarily colonize the gastrointestinal system. These bacteria inhibit the growth of fungi and yeast on the skin by synthesizing fatty acids.[27] Neither *S. aureus* nor beta-hemolytic *Streptococcus* colonization was identified in the present study or control groups.

Unlike term infants, the stool flora of preterm neonates, who spend their first 4 weeks in hospital, have been identified as containing potentially pathogenic *Enterococcus, K. pneumoniae* and *E. coli*; in addition, *Bifidobacterium*, which is an important constituent of the intestinal flora in preterm neonates, is decreased due to prophylactic antibiotics. Thus, intestinal *Bifidobacterium* and *Bacteroides* are decreased. Furthermore, the development of the intestinal flora in infants born by cesarean section is delayed until they are 1 year old. Many studies have identified a decrease in *Bifidobacterium* and *Bacteroides* due to cesarean section.[28,29] Numerous studies have also identified cesarean section delivery as a risk factor for the progression to type 1 diabetes mellitus,[30,31] although other studies have not observed a link.[32,33] Children with autoantibody-positive type 1 diabetes have been found to have a higher rate of *Bacteroides* spp. colonization compared with those who are autoantibody negative.[19,22] In the present study, a decrease in *Bacteroides* spp. colonization was identified in 20.0% of type 1 diabetes patients versus 2.9% of control subjects, respectively. Despite the large numerical difference between the two groups, no statistically significant difference was identified (P = 0.055).

A statistically significant decrease, however, was detected in *Bifidobacterium spp*. colonization in patients with type 1 diabetes compared with the control group (22.8% vs 2.9%, respectively;

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Table 3 Stool flora list

Patient ID no.	Sex	Age (years)	*Echerichia coli*	*Enterobacteriaceae*	*Enterococcus*	*Bacteroides* spp.	*Bifidobacterium* spp.	*Lactobacillus* spp.	*Candida albicans*	*Candida* spp.
Type 1 diabetic patients										
1	F	15.9	N	↑	N	N	N	N	↑	Ø
2	F	17.5	N	↑	N	N	↓	↓	Ø	Ø
3	M	15.8	N	Ø	↓	N	N	N	Ø	Ø
4	F	5.0	N	Ø	N	N	N	↑	↑	↑
5	F	7.1	N	↑	↓	N	N	N	Ø	Ø
6	F	7.8	↑	↑	N	N	N	↓	Ø	Ø
7	M	14.0	N	Ø	N	↓	N	N	↑	Ø
8	M	8.8	↑	Ø	N	N	N	N	↑	Ø
9	F	11.2	↑	Ø	N	N	N	N	Ø	Ø
10	M	12.8	↑	Ø	N	N	N	↓	↑	Ø
11	M	5.4	N	Ø	N	N	N	↓	↑	Ø
12	M	9.8	↓	Ø	↓	N	N	N	Ø	Ø
13	F	5.2	N	Ø	↓	N	N	N	↑	Ø
14	F	12.0	N	↑	N	↓	N	N	Ø	Ø
15	F	10.0	↓	↑	N	N	N	N	Ø	Ø
16	F	14.5	↑	Ø	N	N	N	N	Ø	↑
17	M	6.2	↑	↑	N	N	N	↓	↑	Ø
18	M	13.6	↑	Ø	N	↓	N	↓	↑	↑
19	M	13.2	N	↑	N	N	N	N	Ø	Ø
20	M	13.7	↑	Ø	↓	↓	↓	↓	Ø	Ø
21	M	13.3	N	↑	↓	↓	↓	N	↑	Ø
22	F	13.8	↑	↑	N	N	N	↓	Ø	Ø
23	M	13.0	↑	Ø	N	N	N	↓	Ø	Ø
24	M	15.9	↑	Ø	N	↓	↓	↓	Ø	Ø
25	M	15.0	↑	Ø	↓	↓	↓	↓	Ø	Ø
26	M	5.2	N	↑	↑	N	↓	N	↑	Ø
27	M	4.5	N	Ø	↓	N	N	↓	↑	Ø
28	F	14.2	N	↑	N	N	↓	↓	Ø	Ø
29	M	13.5	↑	Ø	N	N	N	N	Ø	↑
30	F	11.0	N	Ø	N	N	N	N	↑	Ø
31	F	12.2	N	Ø	↑	N	N	↓	Ø	Ø
32	M	5.9	↑	↑	↓	N	↓	↓	Ø	Ø
33	M	2.6	↓	Ø	↓	N	N	↓	Ø	↑
34	F	3.7	N	Ø	↓	N	N	↓	Ø	↑
35	F	12.5	↑	Ø	N	N	N	↓	Ø	↑

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Table 3 Continued

Patient ID no.	Sex	Age (years)	*Echerichia coli*	*Enterobacteriaceae*	*Enterococcus*	*Bacteroides* spp.	*Bifidobacterium* spp.	*Lactobacillus* spp.	*Candida albicans*	*Candida* spp.
Control patients										
1	F	6.2	N	Ø	N	N	N	N	↓	Ø
2	M	8.4	N	Ø	↑	N	↓	N	Ø	↑
3	F	8.4	↑	↑	↑	N	N	N	Ø	Ø
4	M	14.0	↑	↑	N	N	N	↓	Ø	Ø
5	F	6.8	↑	↑	N	N	N	↓	Ø	Ø
6	F	17.9	N	Ø	↓	N	N	↓	Ø	Ø
7	M	5.8	↑	Ø	↓	N	N	N	Ø	Ø
8	M	8.1	N	Ø	N	N	N	N	↑	Ø
9	M	5.8	N	Ø	N	N	N	N	Ø	Ø
10	M	7.8	↑	Ø	N	N	N	N	Ø	Ø
11	F	8.4	N	Ø	↓	N	N	N	Ø	Ø
12	M	15.9	N	Ø	N	N	N	N	Ø	Ø
13	M	16.1	↑	Ø	↓	N	N	↓	Ø	Ø
14	F	6.8	↑	Ø	↓	N	N	↓	Ø	Ø
15	M	8.1	N	Ø	N	N	N	↓	Ø	Ø
16	M	15.1	N	Ø	↓	N	N	↓	↑	Ø
17	F	3.2	↑	Ø	↑	N	N	↓	Ø	Ø
18	F	10.0	N	Ø	↑	N	N	N	Ø	↑
19	M	12.1	N	Ø	N	N	N	N	Ø	Ø
20	M	8.9	N	Ø	N	N	N	↓	Ø	Ø
21	M	7.0	N	Ø	N	N	N	↓	Ø	↑
22	M	11.0	N	Ø	N	N	N	N	Ø	↑
23	M	9.5	↓	Ø	N	N	N	↓	Ø	↑
24	M	6.3	N	Ø	N	N	N	N	↑	Ø
25	M	3.5	↓	Ø	N	N	↓	N	Ø	Ø
26	F	2.8	N	Ø	N	N	N	↓	Ø	Ø
27	M	8.4	N	Ø	↓	N	N	N	Ø	Ø
28	F	8.2	↑	Ø	N	N	N	N	Ø	Ø
29	F	13.2	N	Ø	N	N	N	N	↑	Ø
30	F	14.4	N	Ø	N	N	N	N	Ø	Ø
31	M	13.1	↑	Ø	N	N	N	N	Ø	Ø
32	M	14.2	N	Ø	N	N	N	↓	Ø	Ø
33	F	15.7	N	Ø	↓	N	N	↓	Ø	↑
34	F	12.0	↑	Ø	N	N	N	N	Ø	Ø
35	F	15.8	N	Ø	N	↓	N	N	↑	Ø

Ø, no colonization; ↑, increased colonization; ↓, decreased colonization; N, normal colonization.

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$P < 0.05$). This finding was consistent with the de Goffau *et al*. study, which concluded that butyrate-producing bacteria, including *Bifidobacterium*, were important with respect to the development of beta-cell autoimmunity.[19]

Lactobacillus spp. are part of the flora of the human mouth, intestine, and vagina. Although anaerobic, *Lactobacillus* spp. can proliferate in aerobic environments. In addition, *Lactobacillus* spp. do not have the catalase enzyme, but they can eliminate peroxidase radicals by using superoxide dismutase and peroxidase enzymes, thereby creating a suitable environment for the reproduction of *Bifidobacterium*.[24,34] In the present study, *Lactobacillus* colonization decreased in both the study group and the control group, and no significant change in *Lactobacillus* colonization was identified.

Yeasts, such as *C. albicans*, are also part of the normal gut microflora, but cell counts do not normally exceed $10^{4.5}$ c.f.u./g feces.[33] Disturbances to the microbial ecosystem within the gut may result in *Candida* overgrowth. *Lactobacillus*, *Bifidobacterium* and *Bacteroides* species within this ecosystem elicit this inhibitory activity via production of short-chain fatty acids and antimicrobial compounds (i.e. bacteriocins).[35,36] In the present study, colonization of *Bacteroides* and *Bifidobacterium* in type 1 diabetic patients group was found to be decreased. Consequently, we have suggested that the significantly increase in *C. albicans* colonization identified in patients with type 1 diabetes (40%) compared with the control group (14.3%) may be attributed to the decrease in both these bacteria.

Much time has passed since Metchinkov's description of beneficial bacteria and the immunology studies that led to her receiving the Nobel Prize in Medicine. It has only recently been established, however, that probiotic bacteria, which Metchinkov suggested were important for a long and healthy life, are involved not only in the synthesis of vitamins needed by our organisms but also in the control of gene expression in humans.[37]

In summary, we identified notable differences among the anaerobic digestive flora (*Bifidobacterium*), *C. albicans* colonization, and *Enterobacteriaceae* other than *E. coli* colonization of the type 1 diabetic group and the control group. Thus, the disruption of the intestinal flora may play a crucial role in the physiopathology of type 1 diabetes.

One limitation of this study was the bacterial culture methods used for analysis of the intestinal microbiota. Nowadays several molecular-based methods, such as quantitative polymerase chain reaction (PCR) targeting rRNA, real-time quantitative PCR, and so on, are widely used because of high specificity and sensitivity.

A review of the literature indicates that few studies have described the physiopathology of type 1 diabetes based on disturbance of the intestinal flora and the resulting intestinal leak, in humans. In the present study, the decrease in beneficial anaerobic bacteria and the increase in colonization by *C. albicans*, which is known to disrupt the integrity of the intestinal flora, appear to be factors that may contribute to the development of this disease. The use of probiotic and prebiotic food products may have the potential to prevent the development of type 1 diabetes. Double-blind placebo-controlled studies are needed to determine if probiotic and prebiotic food products can be used to protect against type 1 diabetes.

Conclusion

A decrease in beneficial anaerobic bacterial levels and a concomitant increase in *Enterobacteriaceae* other than *E. coli* and *C. albicans* colonization may lead to a disturbance in the ecological balance of the intestinal flora, which could be a triggering factor in type 1 diabetes etiology.

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References

1 American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2011; **34**: 62–9.
2 Soyucen E, Yilmaz S, Celtik C *et al*. Seroprevalence of autoimmune thyroiditis and celiac disease in children with insulin-dependent diabetes mellitus in the Thrace region of Turkey. *Turk. J. Gastroenterol.* 2010; **21**: 231–5.
3 Bodansky HJ, Staines A, Stephenson C, Haigh D, Cartwright R. Evidence for an environmental effect in the etiology of insulin dependent diabetes in a transmigratory population. *BMJ* 1992; **304**: 1020–22.
4 Vaarala O, Atkinson MA, Neu J. The "perfect storm" for type 1 diabetes: The complex interplay between intestinal microbiota, gut permeability, and mucosal immunity. *Diabetes* 2008; **57**: 2555–62.
5 Sapone A, De Magistris L, Pietzak M *et al*. Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. *Diabetes* 2006; **55**: 1443–9.
6 Bosi E, Molteni L, Radaelli MG *et al*. Increased intestinal permeability precedes clinical onset of type 1 diabetes. *Diabetologia* 2006; **49**: 2824–7.
7 Westerholm-Ormio M, Vaarala O, Pihkala P *et al*. Immunologic activity in the small intestinal mucosa of pediatric patients with type 1 diabetes. *Diabetes* 2003; **52**: 2287–95.
8 Auricchio R, Paparo F, Maglio M *et al*. In vitro-deranged intestinal immune response to gliadin in type 1 diabetes. *Diabetes* 2004; **53**: 1680–83.
9 Neu J, Reverte CM, Mackey AD *et al*. Changes in intestinal morphology and permeability in the biobreeding rat before the onset of type 1 diabetes. *J. Pediatr. Gastroenterol. Nutr.* 2005; **40**: 589–95.
10 Rook GA, Brunet LR. Microbes, immunoregulation, and the gut. *Gut* 2005; **54**: 317–20.
11 Yan F, Polk DB. Commensal bacteria in the gut: Learning who our friends are. *Curr. Opin. Gastroenterol.* 2004; **20**: 565–71.
12 Cani PD, Bibiloni R, Knauf C *et al*. Gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* 2008; **57**: 1470–80.
13 Moro G, Aslanoglu S, Stahl B *et al*. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. *Arch. Dis. Child.* 2006; **91**: 814–19.
14 Beckman G, Rüffer A. *Mikroökologie Des Darmes: Grundlagen, Diagnostik, Therapie*. Schlüttersche, Hannover, 2000 (in German).
15 Shimizu K, Ogura H, Hamasaki T *et al*. Altered gut flora are associated with septic complications and death in critically ill patients with systemic inflammatory response syndrome. *Dig. Dis. Sci.* 2011; **56**: 1171–7.

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16 Vaarala O. Is the origin of type 1 diabetes in the gut. *Immunol. Cell Biol.* 2012; **90**: 271–6.
17 Todar K. Lactic acid bacteria. Todar's Online Textbook of Bacteriology 2012; 1–5. Available from URL: http://textbookofbacteriology.net/lactics.html.
18 Strachan DP. Hay fever, hygiene, and household size. *BMJ* 1989; **299**: 259–60.
19 de Goffau MC, Luopajarvi K, Knip M *et al*. Fecal microbiota composition differs between children with β-cell autoimmunity and those without. *Diabetes* 2013; **62**: 1238–44.
20 Brown CT, Davis-Richardson AG, Giongo A *et al*. Gut microbiome metagenomics analysis suggests a functional model for the development of autoimmunity for type 1 diabetes. *PLoS ONE* 2011; **10**: e25792.
21 Kandemir N, Açikgöz E, Yordan N *et al*. The epidemiology of juvenile-onset insulin-dependent diabetes mellitus in Turkish children: A retrospective analysis of 477 cases. *Turk. J. Pediatr.* 1994; **36**: 191–5.
22 Vaarala O. Gut microbiota and type 1 diabetes. *Rev. Diabet. Stud.* 2012; **9**: 251–9.
23 Akesen E, Turan S, Güran T *et al*. Prevalence of type 1 diabetes mellitus in 6-18-yr-old school children living in Istanbul, Turkey. *Pediatr. Diabetes* 2011; **12**: 567–71.
24 Macie RI, Sghir A, Gaskins HR. Developmental microbial ecology of the neonatal gastrointestinal tract. *Am. J. Clin. Nutr.* 1999; **69**: 1035–45.
25 Murray PR. *Manual of Clinical Microbiology*, 7th edn. American Society for Microbiology, Washington DC, 1999.
26 Ryan KJ, Ray CG. *Sherris Medical Microbiology. Enterobacteriaceae*, 5th edn. McGraw-Hill, New York, 2010.
27 Ewing WN. *The Living Gut*, 2nd edn. Notthingam University Press, Nottingham, UK, 2008.
28 Schwiertz A, Gruhl B, Lobnitz M *et al*. Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full term infants. *Pediatr. Res.* 2003; **54**: 393–9.
29 Alderberth I, Lindberg E, Aberg N *et al*. Reduced enterobacterial and increased staphylococcal colonization of the infantile bowel: An effect of hygienic lifestyle. *Pediatr. Res.* 2006; **59**: 96–101.
30 Dahliquist G, Kallen B. Maternal-child blood group incompatibility and other perinatal events increase the risk for early onset type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 1992; **35**: 671–5.
31 Ahadi M, Tabatabaeiyan M, Mozzami K. Association between environmental factors and risk of type 1 diabetes-a case-control study. *Endokrynol. Pol.* 2011; **62**: 134–7.
32 Patteson CC, Waugh NR, Carson DJ, Cole SK, Hadden DR. A case-control investigation of perinatal risk factors for childhood type 1 diabetes in Northern Ireland and Scotland. *Diabetes Care* 1994; **17**: 376–81.
33 Dahlquist GG, Patterson C, Soltesz G. Perinatal risk factors for childhood type 1 diabetes in Europe. *Diabetes Care* 1999; **20**: 1698–702.
34 Mazmanian SK, Liu CH, Tzianabos AO *et al*. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* 2005; **122**: 107–18.
35 Bernhardt H, Wellmer A, Zimmerman K, Knoke M. Growth of Candida albicans in normal and altered faecal flora in the model of continuous flow culture. *Mycoses* 1995; **38**: 265–70.
36 Sonoyama K, Miki A, Sugita R *et al*. Gut colonization by Candida albicans aggravates inflammation in the gut and extra-gut tissues in mice. *Med. Mycol.* 2011; **49**: 237–47.
37 Hooper LV, Wong MH, Thelin A *et al*. Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 2001; **291**: 881–4.

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