Microflora Persistence on Duodenojejunal Flat or Normal Mucosa in Time After a Meal in Children

MARIO CIAMPOLINI,*1 STEFANIA BINI* AND ALESSANDRA ORSI†

*Department of Pediatrics, University of Florence, Via L. Giordano 13, 50132 Florence, Italy
and †Microbiological Laboratory, SMN Hospital, Florence, Italy

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CIAMPOLINI, M., S. BINI AND A. ORSI. Microflora persistence on duodenojejunal flat or normal mucosa in time after a meal in children. PHYSIOL BEHAV 60(6) 1551–1556, 1996.—A pathogenic role for high numbers of bacteria in the small intestine had been suggested previously by bacterial counts on luminal aspirates, but these investigations were flawed by the sampling device “contamination” in the mouth and the changing nature of fluent intestinal content. A procedure was developed to sterilize the Watson biopsy capsule with HCl in the upper portion of the duodenum. Bacteria were counted in the mucosal homogenate of the first (diagnostic) duodenojejunal biopsy in 80 untreated celiac children, and in 46 children with irritable bowel syndrome (IBS) in a four-cell, controlled, randomized investigation. Persistence of bacteria on the mucosa for 20 h after the last meal was investigated in 62 subjects, and for 26 h after the last meal in 64 subjects. Bacteria, mainly streptococci and staphylococci, persisted at a concentration of $10^6$ per gram of mucosa 20 h after the last meal. The number of bacteria per gram of mucosa was 24 times higher in all 62 children of the 20-h fast groups than in all 64 children of the 26-h fast groups ($p < 0.001$). The bacteria count in celiac children was 39 times higher in the 20-h fast group than in the 26-h one. This difference was significantly higher than the 11 times difference that was found on the normal mucosa between the 20- and 26-h fast IBS groups ($p < 0.001$), which was still significant. The number of bacteria on duodenojejunal mucosa depends on nutrient absorption and persists longer than the intermeal interval in these subjects.

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INTESTINAL flow and absorption of nutrients is rapid, and very few bacteria, if any, are cultivated in duodenojejunal aspirates of healthy children and adults examined in the fasting condition. Thus, 33 luminal aspirates were sterile and 83 of 92 contained less than $10^5$ CFUs (colony-forming units) per ml in 9 studies in “healthy” children (4.6–8,13,23,24,43,47). Conversely, only 32 were sterile and 76 of 187 showed less than $10^5$ CFUs per ml in 9 studies in children with chronic or persistent diarrhea (6,8,11,13,18,24,29,32,49). Both differences are highly significant and the high bacterial concentration, above $10^5$ CFUs per ml (including gram-positive cocci), may either affect the pathogenesis or represent an index in the pathogenesis, or be a consequence of the disease. Studies on luminal fluid are flawed by possible contamination of sampling devices in the mouth and throat (30) and by the flushing out of luminal content (38). The same strains and similar bacterial density occur in simultaneous cultures of mucosa and luminal aspirate (22,51,55). This mucosal flora might more directly affect disease than the luminal kind. Microbiology counts on duodenojejunal mucosa have been reported world-wide in only about 20 children in two investigations (6,22), although persistence over time and changes with meals have not been investigated.

METHODS

Subjects

Between 1981 and 1987, 140 children from 8 months to 16 years of age were consecutively selected for intestinal biopsy and randomized by use of a random-number table (5) into the experimental and control groups at the Pediatric Gastroenterology Unit of Florence University. A definitive diagnosis of irritable bowel syndrome (IBS) or celiac disease was made in 126 of the 140 biopsied children after a 2-year follow-up and the 126 children were included in a four-cell, prospective, controlled, randomized investigation. At the end of the investigation, 62 children were assigned to a 20-h fast group and 64 to a 26-h fast group (Table 1). No child had intestinal symptoms in the 5 days before biopsy, or had febrile disease, or had used any drug or medication in the previous 3 weeks, or had been on a gluten-free diet in the last few years. The study was reviewed and approved

1 To whom requests for reprints should be addressed. E-mail: CIAMPOLINI@CESIT1.UNIFI.LIT

1551
by the Departmental Human Experimentation Committee. Informed consent was obtained from the children’s parents.

**Diagnostic Criteria**

Celiac disease was diagnosed according to the criteria of the “European Society of Pediatric Gastroenterology and Nutrition” (59). Subtotal villous atrophy (i.e., mucosa flattening) was originally found in the small intestine at presentation and on gluten challenge in all subjects after a period of about 6 months of gluten-free diet. A decrease in antigliadin antibodies and rapid nutritional recovery on a gluten-free diet were also a requirement.

The diagnosis of IBS was made in infants with chronic or intermittent diarrhea less than 3 years of age (12,58), and in older subjects with chronic or intermittent abdominal pain associated with 3 of the following symptoms or signs: relief of abdominal pain with defecation; loose stools with or without the onset of pain; more frequent stools with the onset of pain; abdominal bloating; feelings of incomplete evacuation; passage of mucus via the rectum; urgency (42,50,60). Normal hemogram, sedimentation rate, urinalysis, urine culture, stool tests for occult blood, and ultrasound abdomen examination (including kidney) were required, as well as absence of ova and parasites and pathogenic bacteria, milk or egg allergy; IV dehydration therapy was not needed. Organic disorders, including lactose intolerance, cystic fibrosis, pancreatitis, inflammatory bowel disease, liver and peptic ulcer diseases, and lower respiratory or urinary tract infections were excluded. Children with IBS eventually recovered in 1 to 3 months and maintained good health in the following year.

**Procedure**

We were able to investigate the bacterial count per gram of mucosa in untreated children with celiac disease and in those with IBS. These children were studied during the course of intestinal biopsy for diagnostic purposes. Both subject groups were studied a few days after abatement of intestinal symptoms, diarrhea, or abdominal pain. Children with celiac disease were eating gluten-containing foods and had subtotal atrophy of the mucosa. Children with IBS showed normal mucosa and were considered as healthy controls. A sterile method of sampling was developed. Persistence of bacteria on mucosa was investigated either 20 or 26 h after the last meal. The postmeal-time interval was assigned at random.

**Experimental Comparison**

Intestinal biopsy was performed in the afternoon after feeding cessation of either 20 h (i.e., since the previous day’s dinner at 1900 h) or 26 h (i.e., since the previous day’s lunch at 1300 h). The 20-h fast children were also allowed a small snack between lunch and dinner, whereas the 26-h fast children were not. A day’s abstinence from food was useful for accelerating progression of the capsule and avoiding failures in its advancement.

**Intestinal Biopsy and Microbiological Procedures**

Intestinal biopsy was performed in every case by means of the Watson capsule at the Treitz level, or just after this level with fluoroscopic examination. The biopsy capsule was aseptically mounted, and introduced into the stomach. The pH was measured to exclude hypochlorhydric subjects with gastric juice over 6.0 (20). The capsule was advanced into the duodenum, and sterilized with 1 ml of HCl 0.1 N in the second third of the duodenum and flushed with 10 ml saline after 1 min. Oxygen-free CO₂ gas was introduced. The jejunal mucosa was sampled after a further 30 cm progression (i.e., at the Treitz level) after 15–45 min. Care was taken to avoid contamination of the biopsy after withdrawal, by cleansing the closed capsule with dry, sterile gauze. The capsule was opened and the mucous layer was smears on a slide, stained with Methylene Blue, and observed under a microscope. A 2–8 mg portion of the biopsy without the mucous layer was immediately weighed, washed, homogenized in 0.1 ml Columbia CO₂ broth, and diluted 4 times with 1:10 dilution. Five microliters were plated in duplicate on nonselective blood agar. This procedure was repeated under anaerobic conditions to ensure cultivation of anaerobic organisms (3). The CFUs were counted and at least 10 random colonies were kept for 2-day incubation for the assessment of the cultural, microscopic, and biochemical characteristics of the bacteria (27). Diplococci were cultured with streptococci, and Sarcinae and micrococci with staphylococci (27). The lower limit was about 10⁵ per g for the total CFU counts and 1% of the total CFU count for the isolation of less numerous strains. The variation coefficient (SD and mean) was 4.5% in 10 measurements on the same sample.

**Validations**

HCl sterilization and equilibration procedures were performed in duplicate samples of mouth saliva in 10 fasting children with IBS. The tightness of the Watson capsule was verified in the mouth saliva of 35 subjects (22 IBS and 13 celiac) after an overnight fast. Five microliters were plated in blood agar. CFU counts lower than 200 bacteria per ml could not be visualized and the sample appeared sterile. The saliva contained over 10⁵ CFUs per ml in 10 subjects.

**HCl sterilization procedure.** Saliva was sucked into the Watson biopsy capsule in the mouth. The tube and capsule were injected with 1 ml of HCl 0.1 N, and flushed with 10 ml of saline after 1 min. The pH was over 6.0 at this time and no growth of bacteria was observed in the juice obtained from inside the capsule in 10 subjects.

**Equilibration procedure.** The capsule was flushed with 10 ml of air, and saliva sucked from the tube after the HCl sterilization. The first 2 ml of saliva were discarded and 5 μL were plated. The pH of this sample was approximately 7.0, and the CFU count was over 10⁵ per ml in all subjects.

**Tightness verification.** The capsule was HCl-sterilized, equilibrated with saline, dried, filled with sterile saline, immersed in the mouth saliva for 2 s, and cleansed with sterile gauze. From the fluid inside the capsule, 3.5 ± 2.9 × 10⁵ CFUs per gram were counted.

The HCl sterilization was twice performed in 13 children with celiac disease and 5 with IBS during biopsy procedure, in the antrum and the duodenum. Fluid was collected through a biopsy tube before and after sterilization plus equilibration with antral juice. Up to 10⁵ CFUs were found per ml of juice before sterilization, whereas only 1–2 colonies were grown from 3 of 18
samples after sterilization and equilibration. The same bacteria were grown in the same numbers in anaerobic cultures.

These results were interpreted as showing that contamination of the open capsule was unavoidable in the mouth and throat and persisted down to the stomach. The antral juice was practically devoid of bacteria at the time of biopsy (i.e., 20–26 h after the last meal). The HCl sterilization was highly effective, as was the equilibration procedure, but the closed capsule allowed slight leakage of salivary fluid during withdrawal.

Assessments

The presence of symptoms was assessed in an interview by a validated method (12). Diaries were kept by the nursing staff for a week before the biopsy. Intake records and computerized food composition tables were used to estimate energy intake. The anthropometric, biochemical and hematological measurements have been previously described (12).

Statistical Analyses

The CFU count was expressed in log(10) as mean ± SD, but ± SEM was used in the figure. Differences between groups were evaluated with Student’s unpaired t-test or by Chi square analysis on logarithms.

Group sizes were estimated to detect 1 half logarithm difference in the total CFU count between groups at a significance level of 0.05 and a power of 0.90 (5).

RESULTS

Subtotal villous atrophy of the intestinal mucosa, which is diagnostic of celiac disease, was found in 80 of 126 children. Of these, 42 subjects were in the 20-h fast group and 38 in the 26-h fast group. A further 46 children showed normal mucosa and fit the diagnosis of IBS; 20 of these were in the 20-h fast group and 26 in the 26-h fast group. No difference was found in gender, age, clinical symptoms, or biochemical tests between the 20- and the 26-h fast groups (Table 1). Malabsorption and malnutrition were present in children with celiac disease, and those with IBS were in the range of normality in the investigated anthropometric and biochemical parameters. The tricipital and quadricipital skinfolds were significantly thinner in all 4 investigated groups than the local reference for the same age.

The antral juice pH was between 1.4 and 4.3 with a mean of 2.3. Bacteria were located on intestinal epithelial cells (not inside) and in the nearby mucus in microscopic observations of surface mucus immediately after biopsy (Figs. 1 and 2). No difference emerged in the CFU counts of children with celiac disease and IBS who were in the same fasting-time group. Total CFU count was $6.4 \pm 1.4 \text{log}(10)$ per gram of intestinal mucosa in celiac disease and $6.2 \pm 1.0 \text{log}(10)$ in IBS among those who were in the 20-h fast group. Total CFU counts were $4.8 \pm 2.2$ and $5.1 \pm 2.1 \text{log}(10)$ per gram of mucosa respectively, in the 2 groups in the 26-h fast group (Figs. 3 and 4). Neither count showed any significant difference from CFUs counted inside the closed Watson capsule after immersion in saliva: $4.5 \pm 0.6 \text{log}(10)$ (see Validations). The bacteria count per gram of mucosa was 24 times higher in all 62 children of the 20-h fast groups than in all 64 children of the 26-h fast groups ($p < 0.001$). The bacteria count in celiac children was 39 times higher in the 20-h fast group than in the 26-h one (Fig. 3). This difference was significantly higher than the 11 times difference that was found on the normal mucosa between the 20- and 26-h fast IBS groups (Chi square, $p < 0.001$), which was still significant (Fig. 4). Only 1 child from the 20-h fast group had no bacteria in the biopsy, compared to 8 of those from the 26-h fast group (Chi square, $p < 0.05$). Jejunal flora was mainly composed of streptococci, which were isolated more than twice as often as staphylococci (Figs. 3 and 4). Biopsies showing growth of these two genera were compared in the 20- and 26-h fast groups. Both genera had higher CFU counts in the biopsies of the 20-h fast groups than in those of the 26-h fast groups, although the differences were significant only in celiac disease. The difference in staphylococci maintained significance after inclusion of biopsies showing no growth of this genus ($p < 0.05$). Differences in alpha, beta, or gamma streptococci, coagulase-positive or -negative staphylococci isolations, and CFU counts in the 4 investigated groups resembled those found in total streptococcal and staphylococcal spp, without reaching significance. A further 31 strains were isolated in 23 total biopsies, with no difference between the 20- and 26-h fast groups, although these additional strains were more frequently found in biopsies with low CFU counts: 9 were isolated from 19 biopsies with lower counts than $10^7$ per gram and 14 from 98 biopsies with higher counts (Chi square, $p < 0.05$). Neisseria spp were found 20 times; Esche-
between 10^3 and 10^7 per gram of mucosa (6). Three studies in healthy volunteer adults or in adults at surgery found 5 sterile biopsies. Avigad et al. found about 10^5 bacteria in 7 biopsies showing growth of these genera (see notes). The difference in log(10) CFUS per gram of normal mucosa in 2 of 4 children was significant also after inclusion of biopsies showing no growth of this genus. a, geometric mean CFUs in 31 of 42 biopsies; b, geometric mean CFUs in 33 of 38 biopsies; c, geometric mean CFUs in 18 of 42 biopsies; d, geometric mean CFUs in 10 of 38 biopsies; e and f, geometric mean CFUs in all biopsies performed (42 and 38, respectively).

FIG. 3. Number of streptococci, staphylococci, and total CFU counts in atrophic jejunal biopsies of untreated children with celiac disease 20 h after last meal (white columns), or 26 h after last meal (dashed columns). Geometric mean of CFU counts shown in log(10) by height of columns. SEM shown by vertical bars. Significance vs. group with dinner consumption shown by asterisk: p < 0.005, < 0.005, < 0.001, respectively, from the left. Mean streptococcal and staphylococcal growth calculated only in biopsies showing growth of these genera (see notes).

Ritchia coli 4 times; Propionibacteria and yeasts twice; Proteus, Clostridium perfringens and Chromobacterium once.

Lower counts were found in anaerobic than aerobic cultures in children fasting 20 h, without any significant differences. Anaerobic CFU counts were significantly lower in children fasting for 26 h than in those fasting for 20 h in the celiac and overall groups. (p < 0.001 and p < 0.01). The difference observed in celiac disease (18 times) was significantly wider than that in healthy children (4 times), p < 0.01. The same spps with similar CFUs were isolated in aerobic and anaerobic cultures.

Mean daily intake was 6.7 ± 1.2 MJ in the week investigated before the biopsy. The intakes at lunch and dinner were 32.4% and 32.2% of the total daily intake, and 35.4% was consumed at breakfast and in 1–2 optional snacks. No intake difference was significant among groups. The snack between lunch and dinner was consumed by 90.5% of all children fasting 20 h.

**DISCUSSION**

In the present study, bacteria were still found on mucosa at a density of 10^6 per gram of mucosal homogenate after 20 h without meals in IBS and untreated children with celiac disease who had been diarrhea-free for a few days. Evans et al. found 7.6 log(10) CFUs per gram of normal mucosa in 2 of 4 children investigated for suspected celiac disease (22). The other 2 had sterile biopsies. Avigad et al. found about 10^6 bacteria in 7 biopsies in children who had recovered from diarrhea, with a range between 10^5 and 10^7 per gram of mucosa (6). Three studies in healthy volunteer adults or in adults at surgery found 5 sterile samples, 7 with counts lower than 10^4, and 4 with counts up to 10^6 CFUs per gram of mucosa (46,48,51). CFU counts on celiac mucosa have been reported in 3 children (22). Two subjects had over 10^6 CFUs per gram and the third showed no growth (22). In the lumen of children and adults with untreated celiac disease 10^6–10^9 CFUs have been observed, but the samples were sterile or of low growth in other subjects (4,15,21,22,31,52).

Bacteria persisted on both atrophic and normal mucosa up to 20 h after the previous meal, and rapidly decreased in number in the following 6-h fast. These are the most important findings of this report. Mucosa-associated bacteria may stimulate intestinal immune cells (1,2,9,16,33,39,44,54,57), and high bacterial counts in the lumen are associated with diarrhea relapses in infants (4,6–8,11,13,18,23,24,29,32,43,47,49). The decrease was significantly higher on flat mucosa (39-fold) than on the normal mucosa in children with IBS (11-fold; significant). CFUs on biopsies obtained 26 h after the last meal were not significantly different from CFUs contaminating saline inside the closed capsule after immersion in mouth saliva in validation experiments. These biopsies may have been almost sterile before withdrawal through the mouth.

Sampling contamination from mouth bacteria was ruled out before biopsy by highly effective sterilization with HCl 0.1 N. Contamination of the closed capsule by saliva (see Validations) had a negligible influence (about 2%) on counts performed after 20 h of fasting. Permanent spillover of bacteria with fasting gastric juice from the upper respiratory tract into the duodenojejunum might be suggested by four papers (10,11,32,43). The subjects in all these reports were different from those of the present study in regard to country, age, background illness, presence of symptoms, fasting period, and gastric pH. The investigated gastric juices had a low pH, compatible with the common finding of sterility or few bacteria (less than 1000 bacteria per ml) (20,26,28,34,45). The samples of prepyloric juice were practically sterile in 18 of the present subjects investigated for validation purposes after HCl capsule sterilization and equilibration with juice in the antrum during biopsy. Moreover, bacteria spill-
over from the stomach could not explain the significant decrease in flora that was found 26 h after the last meal.

Upper respiratory tract bacteria are often brought down with ingesta into the duodenum at a concentration of over 10^9 per ml during meals (20). This wave of bacteria is cleared from duodenal aspirates in normal adults after complete stomach emptying, within 1–2 h (19,20). These bacteria might accumulate on mucosa at the last meal, reach the concentration of over 10^9 within 20 h, and decrease to significantly lower concentrations between 20 and 26 h from the last meal in the investigated children.

Bacteria are able to multiply in the nutrients inside the small bowel, and rapid proliferation has been widely shown both in experimental animals and in humans after mechanical impairment to progression or adhesion to the intestinal surface (35). The deep location of many bacteria on the cell surface, in long chains or aggregates with the same morphologies as were observed in the present investigation, the significantly lower number of isolations when the CFU count was high, as well as the significant difference observed between children with celiac malabsorption and those (IBS) with normal mucosa, all support local proliferation on the mucosa during absorption time. A positive correlation has been shown between carbohydrate malabsorption, severity of diarrhea, and the CFU count in the small intestinal juices of children with protracted diarrhea (1,29).

With a few exceptions, bacteria in the intestinal lumen tend to mirror in number and strain those found on the mucosa in simultaneous cultures of luminal aspirate and mucosal biopsy at the same level (22,51,55). Five studies were repeatedly conducted on the small intestinal juice at different levels or at the same level after a few days (14,23,37,41). Three were performed in healthy volunteer adults (37,41,51), and two in diarrheic children or adults (14,23). CFU numbers and bacterial types were similar in the luminal samples repeated in the same subject, despite normal renewal of small intestinal content within 90 min (36), or even less in diarrheic subjects (40,53,56). This consistency of the microflora in the renewal of luminal content confirms our finding of a rather stable colonization of duodenojejunal mucosa, and suggests both meal-to-meal accumulation and shedding of bacteria into the lumen, as also shown for uroepithelial cells and urine infection (17).

High numbers of bacteria were associated with flat and also normal duodenojejunal mucosa in children with recent intestinal symptoms, under usual feeding habits. The high number persisted for a period (20 h) that is longer than the usual inter-meal time for children having 4 meals a day. The number of bacteria on the mucosa depended on meals, because the number was significantly lower in children fasting for 26 h (i.e., those who did not consume a further meal, the last dinner).

What’s the meaning of the rapid growth of bacteria on intestinal mucosa with meals? Is it generalized to the whole population? No. It was observed in people in frequent symptoms, such as diarrhea, abdominal pain, anorexia, or vomiting, and promotes development of these symptoms, as well as more complex pathological conditions. Mucosa-associated bacteria stimulate intestinal immune cells in correlation with the extent of bacterial growth (1,2,9,16,33,39,44,54,57). This stimulation accounts for two thirds, on average, of the global stimulation of the immune system (1,9). The intestinal microflora proliferation largely depends on absorption rate, and increases with persistence of nutrients in the lumen, as in celiac malabsorption. The absorption rate depends on insulin sensitivity, and decreases with insulin resistance (25). There is indirect evidence that subjects with IBS develop this diffuse condition of insulin resistance during symptomatic periods (12). High insulin sensitivity has to be maintained on these grounds to preserve an efficient and healthy immune system (12). Conversely, the suggested chain of events explains the many pathologic associations of insulin resistance.

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REFERENCES


