Effect of pH on an In Vitro Model of Gastric Microbiota in Enteral Nutrition Patients

Graeme A. O'May,1* Nigel Reynolds,2 and George T. Macfarlane1

Microbiology and Gut Biology Group, University of Dundee,1 and Department of Digestive Disease and Clinical Nutrition, Ninewells Hospital,2 Dundee, United Kingdom

Received 6 December 2004/Accepted 28 February 2005

Patients with dysphagia due to oropharyngeal disease or cerebrovascular accident require long-term nutritional support via enteral feeding, which often results in microbial overgrowth in the upper gastrointestinal (GI) tract. Gastric acid is the primary innate defense mechanism in the stomach and has been assumed to provide an effective barrier to microbial colonization at pH values of < 4. To evaluate the efficacy of gastric acid as a barrier to overgrowth, the microbiota of gastric and duodenal aspirates was assessed by culturing methods. Additionally, a fermentor-based model incorporating enteral nutrition tubing of the gastric microbiota of enteral nutrition (EN) patients was constructed to assess the effect of pH on the microbiota. Results showed that gastric acidity had a relatively small effect on the numbers of microorganisms recovered from intestinal aspirates but did influence microbiota composition. Similarly, at pH 3 in the fermentor, a complex microbiota developed in the planktonic phase and in biofilms. The effect of pH on microbiota composition was similar in aspirates and in the fermentors. Candidas and lactobacilli were aciduric, while recoveries of Escherichia coli and Klebsiella pneumoniae decreased as pH was reduced, although both were still present in significant numbers at pH 3. Only Staphylococcus aureus and Bifidobacterium adolescentis persisted at higher pH values both in vitro and in vivo. Lactate and acetate were the main organic acids detected in both aspirates and fermentors. These data show that the simulator used in this investigation was capable of modeling the effects of environmental influences on the upper GI microbiota of EN patients and that gastric pH of < 4 is not sufficient to prevent microbial overgrowth in these individuals.

Dysphagic patients suffering neurological or oropharyngeal disease require long-term nutrition via enteral feeding. Enteral nutrition (EN) is the preferred method of delivery; it is safer and more effective and maintains gut function more efficiently than parenteral nutrition, which can lead to bowel dysfunction. Percutaneous endoscopic gastrostomy (PEG) feeding, a variant of EN in which the tube passes through the abdominal wall into the gastric lumen, is increasingly being used for long-term patient support (21). Diarrhea is the most common complication of EN, although sepsis, aspiration pneumonia, and stoma infections also occur (1).

In health, the stomach is generally devoid of a significant microbiota, containing only low numbers of lactobacilli (ca. 102 CFU/ml of contents) and, in a subset of the human population, Helicobacter pylori (12, 28). The normal duodenum (27) mainly contains lactobacilli and streptococci (ca. 102 to 104 CFU/ml of contents). Microbial cell population densities increase along the jejunum and ileum; colonic contents harbor up to 1012 culturable bacteria/g, the majority of which are strict anaerobes. Bacteroides spp. and bifidobacteria are the main culturable organisms in this part of the intestine (19), although molecular analyses indicate that eubacteria, clostridia, and other gram-positive bacteria predominate in the microbiota (29, 30).

Low pH is generally considered to be important in preventing significant microbial colonization of the stomach (32). In deed, a gastric pH of < 4 is considered to be an effective barrier to microbial overgrowth (8). However, a number of potential pathogens have evolved multiple acid resistance mechanisms to increase their survival during gastric transit (2). Other innate defenses of the upper gastrointestinal (GI) tract include enterosalivary nitrate circulation (34) and peristalsis. Lack of normal mastication leads to reduced peristalsis and reduced production of gastric acid and saliva. Saliva contains nitrate concentrations typically in the region of 1 mM (3), around 30% of which is converted to nitrite by oral facultative anaerobes (20), including lactobacilli, on the tongue (33). When swallowed, nitrite is converted to nitric oxide and other compounds (20) and exerts an antimicrobial effect in the stomach (34). All of these mechanisms may break down in EN patients. The result is microbial overgrowth in the upper GI tract. Additionally, the feeding tube itself provides a conduit through which allochthonous microorganisms can gain access to the stomach and duodenum.

Microbial biofilms can be defined as matrix-enclosed microbial accretions which adhere to biological and nonbiological surfaces (14). They are implicated in the pathogenesis of many infectious conditions (4), particularly those involving indwelling medical devices (13). Such communities are particularly problematic because of their inherent recalcitrance to antimicrobial agents (7) and their abilities to act as reservoirs in which pathogens can survive during antibiotic therapy. Biofilms are known to form on PEG tubes. Such communities are composed of both bacteria and yeasts (6, 10, 11) and are known to be a cause of tube deterioration (9).

Previous work in our laboratory has demonstrated that patients receiving EN harbor an abnormal gastric microbiota,
comprised mainly of candidas, gram-positive facultative anaerobes, enterobacteria, and enterococci, with lower numbers of anaerobic genera such as bifidobacteria and clostridia. Similar profiles have been reported in other studies (5, 6, 9–11).

To model potential therapeutic interventions in EN patients, we developed a continuous culture-based model of the gastric microbiota which consisted of 11 microbial strains representative of those isolated most commonly in the endoscopy clinic. This system also facilitates quantitation and visualization of PEG tube biofilm communities. The aims of the present study work were twofold: the first objective was to investigate the response of the microbiota in the model to changing pH, in terms of its composition and fermentation product output. The second aim was to assess the effect of pH on the structure and composition of microbial biofilms formed on PEG tube surfaces in the gastric simulator.

MATERIALS AND METHODS

Patient aspirates. Gastric and duodenal material was obtained from patients (n = 20) undergoing PEG tube placement at Ninewells Hospital, Dundee, United Kingdom. The Tayside Research Ethics Committee gave ethical permission for the work. All patients received nasogastric feeding prior to PEG tube insertion. Samples were aspirated at endoscopy from the stomach or duodenum and analyzed immediately upon receipt in the laboratory. All endoscopes were sterilized, as per the manufacturer’s instructions, and were checked for sterility on a weekly basis. Duodenal fluid was aspirated into a sterile trap, and the endoscope was flushed with sterile water (20 ml) prior to aspiration of fluid from the stomach.

Samples were serially diluted to 10⁻⁵ in prerduced half-strength peptone water, and aliquots (each, 100 µl) of each dilution from 10⁻¹ to 10⁻⁵ were spread onto agar plates. (i) For aerobic incubation, these were nutrient agar CM3 (for detection of aerobes and facultative anaerobes), MacConkey agar no. 2 (enterobacteria), and yeast and mould agar (yeasts and moulds). (ii) For anaerobic incubation, these were Wilkins-Chalgren agar (anaerobes/facultative anaerobes), MRS agar (lactic acid bacteria), Clostridium perfringens agar (C. perfringens and other clostridia), Rogosa agar (lactobacilli), blood agar (fastidious anaerobes), brain heart infusion agar containing 5% (vol/vol) defbrinated horse blood (fastidious anaerobes/facultative anaerobes), azide blood agar (streptococci and enterococci), and Bacteroide minimal salts agar (Bacteroides spp.) (24).

Anaerobic incubation was done with a MACS MC-1000 Anaerobic Workstation (Don Whiteley Scientific Ltd., Shipley, West Yorkshire, United Kingdom) under a 10% H₂–10% CO₂–80% N₂ atmosphere at 37°C for 72 h. Aerobic plates were incubated at 37°C with the exception for yeasts, which were incubated at 30°C for 48 h. Following incubation, colony morphologies were recorded, and colonies representative of each type were subcultured onto appropriate solid media. Subcultures were transferred aseptically to 1.0 ml of cryogenic storage medium (comprising, per liter of distilled water, Wilkins-Chalgren anaerobe broth, 33 g; porcine gastric mucin [Sigma type III], 20 g; and glycerol, 100 ml) and stored at −85°C until required.

Bacterial isolates were identified by cellular fatty acid-methyl ester profiling using the MIDI system in combination with colonial and cellular morphology and gram reaction. Fatty acid methyl esters (FAMEs) were extracted from bacterial pellets obtained from approximately 40 ml of culture in anaerobic peptone yeast extract broth (17), supplemented with glucose (10 g/liter) for anaerobic isolates or from 40 mg of bacteria grown on BBL tryptcose soy broth agar (Becton Dickinson, Ltd., Oxford, United Kingdom) for aerobic isolates by saponification, methylation, and extraction, as described previously (26). These FAMEs were separated using a model 5898A Microbial Identification System (Microbial ID, Inc., Newark, Del.), which consisted of a Hewlett-Packard model 6890 gas chromatograph (GC) fitted with a 5% phenylmethyl silicone capillary column (0.2 mm by 25 m), a flame ionization detector, a Hewlett-Packard model 7683A automatic sampler, and a Hewlett-Packard Vectra XM computer (Hewlett-Packard Co., Palo Alto, CA). GC parameters were as follows: carrier gas, ultra high-purity hydrogen; column head pressure, 60 kPa; injection volume, 2 µl; column split ratio, 100:1; septum purge, 5 ml/min; column temperature, 170 to 270°C; injection port temperature, 300°C; and detector temperature, 300°C. Peaks were automatically integrated; fatty acid names and percentages were calculated with numerical analysis done with the standard MIDI Library Generation Software (Microbial ID, Inc.). Bacterial isolates were identified by comparing FAME profiles to known cultures in the MIDI aerobe and anaerobe standard libraries. The system was calibrated by using a standard MIDI FAME calibration mixture before each run, and it was validated by using the type strains Stenotrophomonas maltophilia ATCC 13637, Bacteroides fragilis ATCC 25285, and Clostridium perfringens ATCC 13124. Yeasts were identified by using the API 20 C AUX biochemical identification system (API, bioMerieux, Basingstoke, Hampshire, England).

Continuous culture microbiota. The microbiota introduced into the fermentation system consisted of 11 strains belonging to species most commonly isolated from EN patients. These were Candida albicans D1/GA/Y2, Candida famata D1/GA/Y1, Staphylococcus aureus D1/GA/N1, Escherichia coli A2/DA/MAC1, Klebsiella pneumoniae A1/DA/MAC1, Streptococcus parasanguis A5/DA/M2, Streptococcus intermedius A5/DA/C3, Streptococcus agalactiae D4/GA/W2, Lactobacillus paracasei A1/DA/M1, Lactobacillus shapara A1/DA/M2, and Bifidobacterium adolescentis A5/DA/W3. These strains were isolated from gastric and duodenal aspirates and were identified by using morphological and chemotaxonomic criteria, as described previously. Each taxon was the most commonly isolated for that particular genus at the time in vitro work was started. Subsequent data from EN aspirates confirmed this selection.

The chemostats were of all glass construction with a working volume of 500 ml. The growth medium contained (per liter) porcine gastric mucin (Sigma type III), 0.1 g; casein, 0.1 g; peptone, 1.0 g; yeast extract, 1.0 g; tryptone soy broth, 0.1 g; NaCl, 1.0 g; KCl, 1.5 g; KH₂PO₄, 1.0 g; MgCl₂, 0.25 g; CaCl₂, 0.15 g; and hemin, 0.2 mg. The growth medium was set at pH 6.5 using 1.0 M HCl or 1.0 M NaOH, as required. Chemostat pH was controlled over a range of 6.0 to 3.0 (lower limit of the controller) by the addition of 1.0 M HCl, using a New Brunswick Scientific (St. Albans, Herts, United Kingdom) pH 1000 pH system attached to a Thermo-Russell (Auchterarder, United Kingdom) CW711/EXT/250 pH electrode. The chemostats were maintained at 37°C by using a Haake B3 recirculating water bath and operated at a dilution rate of 0.2/h under putatively aerobic conditions.

Growth medium was introduced into the fermentors 18 h after inoculation. Samples for microbiological and chemical analyses were taken after steady-state conditions had been achieved after at least nine culture turnovers. Lengths (ca. 12 cm) of silicon PEG tube were suspended in the chemostats to study biofilm formation. The PEG tubes were sampled twice daily for 4 days by aseptic excision of a 5-mm length of tube. Excess fluid was removed, and adherent microorganisms were sampled by scraping both the exterior and interior surfaces with a sterile scalpel.

Enumeration of microorganisms was done by serial dilution in half-strength peptone water and spread plating onto the following solid culture media: MacConkey agar no. 2 (peptone water and spread plating onto the following solid culture media: Mac-

Conkey agar no. 2 (E. coli and K pneumoniae), rose fungal chloramphenicol agar with 0.1 mg ml⁻¹ tetracycline (C. albicans and C. famata), nutrient agar with 8.0% (wt/vol) NaCl (S. aureus), Rogosa agar (L. casei and L. paracasei), Beers agar (B. adolescentis), and kanamycin esculin azide agar (S. parasanguis, S. intermedius, and S. agalactiae).

Biofilm visualization. Sections of PEG tube (each, 5 mm in length) were removed from the fermentors and cut into 1- by 1-mm squares with a sterile scalpel. Care was taken to minimize disturbance of the surface communities during each procedure. Each square was immersed in BactLight Live/Dead staining solution (Molecular Probes Europe BV, Leiden, The Netherlands) for 15 min. Sections were then examined by fluorescence microscopy with a Zeiss microscope (Molecular Probes Europe BV, Leiden, The Netherlands) for 15 min. Sections were then examined by fluorescence microscopy with a Zeiss Axioskop fluorescence microscope connected to a Dell Optiplex GX110 PC, running C-Imagesystem Simple-PCI imaging software (Compix, Inc., Cranberry Township, PA).

Analysis of microbial fermentation products. Samples were centrifuged (13,000 × g, 15 min) to remove microbial cells. Short-chain fatty acids (SCFA) were measured by GC after extraction into ether, as described previously (23), with the addition of an internal standard (50 mM tert-butyl acetic acid). SCFA were separated on a HP-INNO wax cross-linked PEG (30 m by 0.25 mm) column (Agilent Technologies). Injector and detector temperatures were 250 and 300°C, respectively. The flow rate of the helium carrier gas was set at 1.8 liters/min. The oven temperature program was 120°C for one min, followed by 10°C min⁻¹ to 260°C, where it was maintained for 2 min. Lactate and succinate were measured by GC after extraction into chloroform (23), with the addition of an internal standard (100 mM oxalic acid), using the above GC settings.

Chemicals. Unless stated otherwise, all microbiological culture media were obtained from Oxoid, Ltd. (Basingstoke, Hants, United Kingdom). Other chemicals were purchased from the Sigma Chemical Co. (Poole, Dorset, United Kingdom).Downloaded from http://aem.asm.org/ on 2017-11-07 by guest
TABLE 1. Composition of aspirate microbiotas and microbial cell population densities in relation to pH

<table>
<thead>
<tr>
<th>Genus</th>
<th>pH range</th>
<th>0-2</th>
<th>3-6</th>
<th>&gt;6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida</td>
<td></td>
<td>4.0 ± 0.2</td>
<td>3.2</td>
<td>4.7</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td></td>
<td>4.0 ± 0.2</td>
<td>4.7</td>
<td>4.7</td>
</tr>
<tr>
<td>Escherichia</td>
<td></td>
<td>3.1 ± 0.2</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Klebsiella</td>
<td></td>
<td>4.2 ± 0.2</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td></td>
<td>4.9 ± 0.2</td>
<td>4.9</td>
<td>4.9</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td></td>
<td>5.4 ± 0.2</td>
<td>5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>5.4 ± 0.2</td>
<td>5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Total counts</td>
<td></td>
<td>4.5 ± 0.2</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>No. of genera</td>
<td></td>
<td>2.3 ± 0.2</td>
<td>2.3</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Data are expressed as log_{10} CFU/ml and means ± standard deviation (number of patients).

RESULTS

Effect of pH on gastric and duodenal aspirate microbiotas. The effect of pH was investigated by examining gastric and duodenal aspirates together, because individually neither contained sufficient samples with a wide enough range of pH values to make a valid comparison. pH did not have a marked effect on total microbial counts of gastric and duodenal aspirates (Table 1). In contrast, the species composition of aspirates was affected by gastrointestinal pH. For example, bifidobacteria were only detected in aspirates with a pH of 3 or higher, as were staphylococci and klebsiellas. Streptococci, candidas, and lactobacilli were found throughout the pH range. Although E. coli was present in aspirates with pH values of 0 to 2, the organism was detected at a lower frequency and in fewer numbers than in aspirates of higher pH. The mean number of genera detected was similar between pH 3 to 5 and ≥6 (4.3 ± 3.2 and 4.5 ± 2.7, respectively), although it was lower in those aspirates with a pH of <3 (2.3 ± 1.2).

Effect of pH on the gastric microbiota in vitro. Chemostat pH was varied from 6.0 to 3.0, and effects on the composition of planktonic microbiotas were assessed by traditional culturing techniques to determine microbial viabilities (Fig. 1). Bifidobacterium adolescentis was detected only at pH 6.0 (8.1 ± 0.2 CFU/ml). Staphylococcus aureus was found over a pH range of 6.0 to 5.0 (2.9 ± 0.1 and 3.6 ± 0.1 CFU/ml, respectively). Viable counts of E. coli and K. pneumoniae decreased from ca. 10^2 CFU/ml to 10^7 CFU/ml as culture pH was reduced from 6.0 to 3.0 (Fig. 1A). In contrast, candidas occurred in the highest numbers at pH 4.0 and were present in higher numbers at pH 3.0 than at pH 6.0. Lactobacilli were also able to persist under acidic growth conditions, with maximum recoveries occurring at pH 4.0 and 5.0 (Fig. 1B). Streptococcal numbers at pH 6.0 and 5.0 were 8.2 ± 0.1 and 7.4 ± 0.1 CFU/ml (S. paranginai) and 7.1 ± 0.2 and 6.6 ± 1.0 CFU/ml (S. intermedium), respectively. No streptococci were found at pH values of 3 or 4, due to overgrowth of lactobacilli on kanamycin esculin azide culture medium. Streptococcus agalactiae was never established in the chemostats.

The effect of pH on PEG tube biofilm communities was also assessed (Fig. 2). Bifidobacterium adolescentis and S. aureus were only found when culture pH was set at 6.0 (6.6 ± 0.5 and 0.9 ± 1.6 CFU/cm^2, respectively). In contrast, E. coli and K. pneumoniae were detected at all pH values in the biofilms. The highest numbers of E. coli and K. pneumoniae occurred at pH 5 (7.4 ± 0.3 and 7.3 ± 0.2 CFU/cm^2). Candidas were recovered from PEG tube biofilms at all pH values, although the highest cell numbers were recorded at pH 3.0 (4.5 ± 0.1 and 4.6 ± 0.0 CFU/cm^2 for C. albicans and C. famata, respectively). Lactobacilli were also detected throughout the pH range, with maximum recovery at pH 5.0 (6.0 ± 0.6 and 6.9 ± 0.6 CFU/cm^2 for L. paracasei and L. sharpeae, respectively). Streptococcus parasanguis and S. intermedium were found in the biofilm at pH 6.0 and 5.0, with maximal recoveries of both taxa at pH 6.0 (7.5 ± 0.3 and 6.7 ± 0.3 CFU/cm^2).

Analysis of microbial fermentation products. Low concentrations of lactate, succinate, and acetate were present in both
gastric and duodenal aspirates (Table 2), but propionate was only detected in duodenal aspirates. Other SCFA such as butyrate, isobutyrate, valerate, isovalerate, caproate, or isocaproate were not found. Lactate accumulated in chemostats at pH 3.0 and 4.0 (1.4 ± 0.13 and 2.3 ± 0.18 mM) but not at higher pH values (Table 3). Succinate was detected at low levels (<0.5 mM) from pH 4.0 to 6.0 but not at pH 3.0. Acetate concentrations were maximal at pH 5.0 (3.7 ± 0.03 mM), but this SCFA was not found at pH 3.0. Propionate was only detected in trace amounts at pH 5.0 and 6.0 (0.13 ± 0.01 and 0.18 ± 0.03 mM, respectively). No other SCFA or organic acids were present in the chemostats at any pH value.

Visualization of PEG tube biofilms. Live/Dead staining was used in conjunction with fluorescence microscopy to visualize the spatial organization of PEG tube biofilm communities (Fig. 3). Dead organisms were colored red, and live cells were colored green. The results showed substantial colonization of the PEG tube surfaces by bacteria and yeasts. At all pH values, surface growth was characterized by microcolonies of various sizes, interspersed with sparsely colonized regions (Fig. 3A to D). Microcolonies were generally composed of both bacterial and yeast cells. As pH was reduced in the fermentors, yeast cell mass increased. Additionally, at pH of ≤4, elongated yeast and bacterial cells were observed (Fig. 3C and D). Yeast pseudohyphae were frequently observed to pass through microcolonies that contained both bacteria and other yeast forms (Fig. 3C). This phenomenon was increasingly evident at low pH. Additionally, bacteria in the microcolony surrounding these intrusive filaments usually stained red, in contrast to their immediate neighbors, which were predominantly green (Fig. 3C).

**DISCUSSION**

Patients receiving EN have been reported to harbor an abnormal microbiota in their upper GI tract, which mainly consists of enterobacteria, streptococci, staphylococci, lactobacilli, and candidas (5, 6, 10). To investigate environmental factors affecting the growth of these organisms, we developed a continuous culture model of the EN patient upper GI tract microbiota to study the effects of pH on microbial community structure and metabolism.

Microbiological counts done of gastric and duodenal aspirates (Table 2), but propionate was only detected in duodenal aspirates. Other SCFA such as butyrate, isobutyrate, valerate, isovalerate, caproate, or isocaproate were not found. Lactate accumulated in chemostats at pH 3.0 and 4.0 (1.4 ± 1.6 and 2.3 ± 2.5 mM) but not at higher pH values (Table 3). Succinate was detected at low levels (>0.5 mM) from pH 4.0 to 6.0 but not at pH 3.0. Acetate concentrations were maximal at pH 5.0 (3.7 ± 4.1 mM), but this SCFA was not found at pH 3.0. Propionate was only detected in trace amounts at pH 5.0 and 6.0 (0.13 ± 0.01 and 0.18 ± 0.03 mM, respectively). No other SCFA or organic acids were present in the chemostats at any pH value.

**TABLE 2. Microbial fermentation products detected in gastric and duodenal aspirates**

<table>
<thead>
<tr>
<th>Aspirate</th>
<th>Lactate (mM)</th>
<th>Succinate (mM)</th>
<th>Acetate (mM)</th>
<th>Propionate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric</td>
<td>0.70 ± 1.2 (13)</td>
<td>0.20 ± 0.2 (13)</td>
<td>0.20 ± 0.2 (21)</td>
<td>ND* (21)</td>
</tr>
<tr>
<td>Duodenal</td>
<td>0.52 ± 0.83 (14)</td>
<td>0.34 ± 1.27 (14)</td>
<td>0.39 ± 0.94 (14)</td>
<td>0.05 ± 0.19 (14)</td>
</tr>
</tbody>
</table>

* Data are means ± standard deviation (number of aspirates assayed). n varies because insufficient volume was available in some aspirates to undertake solvent extractions.

**TABLE 3. Microbial fermentation products detected in chemostats run at different pH values**

<table>
<thead>
<tr>
<th>pH</th>
<th>Lactate (mM)</th>
<th>Succinate</th>
<th>Acetate (mM)</th>
<th>Propionate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>1.37 ± 1.58</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4.0</td>
<td>ND</td>
<td>0.04 ± 0.06</td>
<td>0.17 ± 0.25</td>
<td>ND</td>
</tr>
<tr>
<td>5.0</td>
<td>ND</td>
<td>0.28 ± 0.09</td>
<td>3.68 ± 4.12</td>
<td>0.13 ± 0.0</td>
</tr>
<tr>
<td>6.0</td>
<td>ND</td>
<td>0.25 ± 0.03</td>
<td>2.02 ± 0.22</td>
<td>0.18 ± 0.0</td>
</tr>
</tbody>
</table>

* Data are means ± standard deviations (n = 8).

ND, not detected.
rates showed that pH had a marked effect on species composition and the number of microbial genera isolated per aspirate, but not on overall cell numbers in these ecosystems (Table 1). Acid suppression therapy is common in the treatment of EN patients, and these results suggest that its use will significantly affect the composition of microbial communities in the upper GI tract, for example, by encouraging the growth of candidas. The potential consequences of the ecological effects of acid suppression therapy should therefore be considered when it is used in EN patients, especially in those individuals who are immunocompromised and so particularly vulnerable to infection.

The effect of acidity on the upper GI microbiota was examined by varying the pH of steady-state chemostat cultures from 6.0 to 3.0 in incremental steps of 1 pH unit. The composition of planktonic and PEG tube biofilm microorganisms was assessed using traditional culturing techniques so that cell viability could be determined (Fig. 1 and 2). As pH was reduced, viable counts of *E. coli* and *K. pneumoniae* decreased, although both organisms were still present in significant numbers at pH 3.0. A similar effect was seen in the biofilm populations (Fig. 2A), showing that these clinical strains were tolerant of the levels of acidity found in the stomach of EN patients. *Staphylococcus aureus* and *B. adolescentis* were never detected in gastric or duodenal aspirates when the pH was <4.0 (data not shown), and these organisms could only be established at relatively high pH values in the chemostats. In contrast, acid tolerant lactobacilli and candidas were detected at all pH values in the aspirates and upper GI simulator (Fig. 2A and B), demonstrating that they were adapted to growth in the upper GI environment.

*Escherichia coli* is autochthonous to the lower GI tract, where it is present at levels of approximately $10^9$ CFU/g of gut contents, representing about 0.2% of the total microbiota as determined by fluorescent in situ hybridization (15). Like other colonic microorganisms, the bacterium must survive passage through gastric acid in the upper GI tract to reach the large bowel. For this reason, *E. coli* possesses a number of acid resistance mechanisms (2, 16, 25) that allow it to survive in environments with a pH of as low as 2 (32), although the minimum pH for it to be able to multiply has been reported to be 4.4 (22). Results obtained in this study show that in mixed culture, *E. coli* was able to multiply in both planktonic and biofilm environments where the pH was 3.0. This apparent ambiguity may be due to ecological or metabolic interactions with other microorganisms in the ecosystem. The more-acid-tolerant microorganisms within the microbiota (lactobacilli, candidas) may provide a protected niche within which less aciduric microorganisms (*E. coli* and *K. pneumoniae*) can grow. The existence of protected niches in biofilms is a well-recognized phenomenon (31), and similar mechanisms may occur in the planktonic population through the formation of bacterial aggregates. These data suggest that the typical levels of acidity found in the stomach of EN patients, together with the presence of an abnormal microbiota containing acid-tolerant microorganisms, is not a barrier to colonization by opportunistic pathogens such as *E. coli*. The presence of such a microorganism in the stomachs of a vulnerable group of patients may be a cause for concern and shows that there is a need for studies of interventions aimed at controlling microbial overgrowth in these individuals.

Previously, it has generally been accepted that bacterial overgrowth will not occur if gastric pH is <4. In vivo and in vitro studies have demonstrated that pH <4 results in killing of
99.9% of bacteria within about 90 min (8). Evidence obtained in this investigation suggests that this may not be the case. Data from both in vitro and in vivo studies showed that a variety of microorganisms, bacteria, and yeasts, including some potentially pathogenic taxa, are capable of multiplying in environments with pH values as low as 3. Future studies on the ability of potentially pathogenic microorganisms to overcome the gastric acid barrier should, therefore, take account of the likely ecologic and physiologic effects of gastric microbionts in patients likely to be exposed to the pathogen.

Fluorescence microscopy demonstrated significant biofilm growth on PEG tube surfaces (Fig. 3). Mature surface growth occurred at all pH values in the form of discrete microcolonies surrounded by sparsely colonized interstitial voids. Microcolonies contained both live and dead rod and coccal forms, together with yeasts of various morphologies. As pH was reduced in the modeling studies, yeasts could be seen to constitute a greater proportion of the biofilms, which correlated with the viable count data (Fig. 2A). At pH ≤5, elongated bacterial and yeast cells were observed, which was indicative of a stress response to the increasingly acidic conditions in the fermentors. Moreover, yeast pseudohyphae were usually found to be protruding into the interior of bacterial microcolonies (Fig. 3C), while the bacterial cells surrounding these protrusions were dead. Whether this phenomenon was caused by a direct yeast killing mechanism, such as production of antimicrobial substances, or indirectly by competition for nutrients or changing local environmental conditions is unknown. Other studies have also demonstrated C. albicans aggregation with bacteria, particularly streptococci in oral biofilms (18).

Analysis of microbial fermentation products showed that very low amounts of organic acids were produced in the chemostats (Table 3). Depending on culture pH, lactate or acetate predominated, which correlated with the preponderance of lactic acid bacteria (streptococci and lactobacilli) in the fermentors. When the concentration of fermentation products in gastric and duodenal aspirates was measured, similar fermentation profiles were found (Table 2), showing that the model reproduced the metabolism of the microbiota in the upper GI tract of EN patients.

pH strongly affected fermentation in the chemostats (Table 3). At the higher pH values (i.e., pH 5 and 6), acetate predominated, while lactate was not detected under these growth conditions. As culture pH was reduced, however, acetate formation was replaced by lactate production. Cell counts of lactobacilli in the fermentors were similar at low and high pH values; the increase in lactate production at low pH may have been due to increased streptococcal numbers. However, the reduction in lactate-utilizing enterobacteria could have resulted in this metabolite accumulating in the fermentors. These results show that the metabolic responses of the microbiont in the fermentors to changing pH were similar to those seen in pH-dependent microbial communities in the upper GI tract. In fact, the profiles of fermentation products detected in the chemostats were indistinguishable from those in gastric and duodenal aspirates. Taken together, these results show that the chemostat-based system used in this investigation could effectively model environmental influences on the upper GI microbiont of EN patients. Future studies will use this simulator to assess the effects of therapeutic interventions on the gastric microbionts in these individuals.

ACKNOWLEDGMENT

We thank Nestlé, who provided funding for this work.

REFERENCES