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Spontaneous bacterial cell lysis and biofilm formation in the colon of the Cape Dune mole-rat and the laboratory rabbit

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Abstract

A wide range of techniques, including highthroughput DNA sequencing methods, have been applied to the evaluation of the normal intestinal flora. However, the inability to grow many of those species in culture imposes substantial constraints on the techniques used to evaluate this important community. The presence of biofilms in the normal gut adds further complexity to the issue. In this study, a flow cytometric analysis was used to separate intact bacterial cells, cell debris, and other particulate matter based on bacteria-specific staining and particle size. In addition, an analysis of biofilm formation using fluorescent light microscopy was conducted. Using these approaches, the ratio of bacterial cell debris to intact bacterial cells as a measure of

spontaneous lysis of bacterial cells in the gut of the Cape dune mole-rat (*Bathyergus suillus*) and the laboratory rabbit (*Oryctolagus cuniculus*) was examined, and the degree of biofilm formation was semi-quantitatively assessed. The results suggest that the degree of spontaneous cell lysis was greater in the appendix than in the cecum in both the mole-rat and the rabbit. Further, the results point toward extensive epithelial-associated biofilm formation in the proximal mole-rat and rabbit large bowel, although the biofilms may be less structured than those found in laboratory rodents and in humans.

Keywords

Biofilm; Cecal appendix; Flow cytometry; Mole-rat; Rabbit; Spontaneous lysis

Introduction

A variety of issues impede the study of microbial communities in the mammalian gut. Many of the species are difficult to grow isolated in culture, and indeed the long co-evolution of many species together in the gut may have rendered some species entirely dependent upon other bacterial species or upon specific conditions found only within the gut. Further, the normal gut ecology entails epithelial-associated biofilms, and the culture of such biofilms in vitro is only in its infancy (Bollinger et al. 2005). In this study, we demonstrate the utility of flow cytometry to evaluate properties of the intestinal flora, and fluorescent light microscopy to evaluate biofilm formation on the epithelium.

Flow cytometry has been used on occasion to evaluate the number of intact bacteria in fecal samples (van der Waaij et al. 1994; Zoetendal et al. 2002). Although this approach provides few details regarding the qualitative nature of the organisms involved, it has the advantages of good quantification and of independence from culture methods, which have proven difficult to apply to many enteric species. In a recent study (Lee et al. 2010), flow cytometry has been used to evaluate the degree of spontaneous cell lysis of *Escherichia coli* populations grown in culture and in mice monoassociated with the bacteria. In cultured populations, the degree of spontaneous cell lysis was directly related to the age of the population, with older populations showing more lysis (Lee et al. 2010). Further, resistance to spontaneous lysis was apparently conditional on the environment for which the bacteria were adapted (Lee et al. 2010). For example, bacteria grown for multiple generations in culture exhibited a resistance to cell lysis when grown in culture, but not when grown in the gut of previously germ-free (mono-associated) mice. By the same token, bacteria grown for thousands of generations in the gut apparently developed resistance to spontaneous lysis while growing in the gut, but exhibited a relatively high rate of lysis when returned to culture in the lab.

The cecal appendix is a structure that has long been the subject of speculation with regard to its function, and provides an interesting case for evaluating spontaneous cell lysis by flow cytometry. The cecal appendix is a narrow projection extending from the cecum of a variety of rodents, lagomorphs, and a range of primates including man. The appendix in humans as well as the apex of the cecum in other animals for which studies have been performed is rich in adherent colonies of mutualistic bacteria (Smith et al. 2009; Bollinger et al. 2007a), or biofilms. The occurrence of the structure in humans has often been heralded as one of the most evident vestiges of evolution, although this claim has long been questioned (Keith 1912), and was eventually refuted by a study utilizing cladistics and comparative anatomy (Smith et al. 2009). Rather than a vestige of evolution, the cecal appendix has apparently evolved more than once (Smith et al. 2009) as a “safe-house” for symbiotic bacteria (Bollinger et al. 2007a), potentially playing an important role in cultures where

gastrointestinal infection following consumption of contaminated drinking water or food contributes significantly to child mortality. The proposed function is based on a deductive proof which, given that biofilms are abundant in the appendix and that biofilms are protective of bacteria, states that the narrow lumen of the appendix must be protective of the bacteria within that structure (Bollinger et al. 2007a). Although this proposed function is consistent with observations made in the fields of epidemiology, microbiology, immunology, and medicine, it remains unproven by direct experimental tests.

The typical model used in the laboratory to evaluate the role of the cecal appendix is the laboratory rabbit (*Oryctolagus cuniculus*). However, the absolute size of the rabbit's appendix is several times greater than that of the human appendix, and vastly larger when the size of the structure is considered relative to body size. Thus, the rabbit's appendix is sufficiently large such that it can aid in digestion, making it functionally distinct from the much smaller human appendix. This relatively large appendix is similar to the appendices found in a few other species, including the common brushtail possum (*Trichosurus vulpecula*) and the brush-tailed porcupine (*Atherurus africanus*; Smith et al. 2009). Unlike the rabbit, some rodents possess a cecal appendix with a lumen that is too small to contribute significantly to digestion, as in humans and some other primates. The Cape dune mole-rat (*Bathyergus suillus*), for example, is an herbivorous subterranean rodent found in the sandy soils of the western and southern Cape provinces in South Africa. They feed on grasses and grass roots as well as the below ground storage organs of plants (Bennet and Faulkes 2000). They have an appendix that is only about 4 mm in diameter (Kotzé et al. 2010), just wide enough to contain some measurable amount of fecal material and still possess the relatively thick walls and immune tissue that are typically associated with cecal appendages (Berry 1900). Thus, the cecal appendix found in mole-rats, like the structure found in humans, is too small to serve as a fermentation chamber during digestion. With this in mind, the rabbit appendix and the mole-rat appendix could be viewed as representative examples of two different morphotypes of cecal appendices that might be functionally distinct.

In the present study, flow cytometry is used to evaluate the degree of spontaneous cell lysis in microbial communities in the appendix, cecum, and large bowel of the Cape dune mole-rat and the rabbit. This approach, which involves measurement of the relative levels of intact bacterial cells and bacterial cell debris in a given sample, has been applied to the intestinal contents of mice associated with a single species of bacteria (monoassociated mice; Lee et al. 2010), but not to the intestinal contents of fully colonized animals. The present study thus extends the application of a previously developed technique to provide insight into the nature of bacterial populations in the cecal appendix to two mammals, both homologs of the human vermiform appendix (Smith et al. 2009).

Although spontaneous cell lysis is an interesting parameter that might be correlated with appendix function, the formation of biofilms is the hallmark of the appendix function (Bollinger et al. 2007a; Parker and Thomas 2010). For this reason, in addition to the evaluation of spontaneous cell lysis, we also evaluated biofilm formation using fluorescent light microscopy. An established approach (Bollinger et al. 2007b; Palestrant et al. 2004) using a minimally disruptive staining procedure with acridine orange was utilized, in combination with a novel approach evaluating the presence of shed biofilm fragments in the fecal stream.

Materials and methods

Sample procurement

All procedures were approved by the appropriate Institutional Animal Care and Use Committee. Ethanol-fixed *B. suillus* intestinal tract samples were obtained from the Zoology

Department, University of Cape Town (UCT), South Africa. These specimens were collected by UCT when the Airports Company of South Africa launched an eradication program at the Cape Town International Airport. The reason for the eradication was that the mole-rat's burrow systems proved to be a threat to the safety of emergency vehicles using the areas next to the runways. The samples of the intestinal tracts were fixed in ethanol, stored at room temperature, and made available to various researchers for study. Samples were taken of the cecum, the cecal appendix, and proximal part of the ascending colon. Samples were taken from two females and three males, ranging in weight from 244 to 307 g (average 277 g). The length of the cecal appendix in the animals ranged from 10–12 mm with a diameter between 2.5–3.5 mm.

Laboratory rabbits (*O. cuniculus*) were purchased from Charles River Laboratories (Wilmington, MA). The animals were fed ad libitum prior to euthanization, and were euthanized by administration of sodium pentobarbital followed by exsanguination. The intestine was dissected free, placed on ice, and the location of fecal material in the colon was documented. For assessment of biofilm formation along the gut epithelium, bulk stool was removed from sections of intestine, and the sections were placed in an embedding medium for frozen tissue specimens (Tissue-Tek O.C.T. compound, Sakura Finetechnical Co., Ltd. Tokyo, Japan) so that the luminal side of the sample, with remaining stool material, was face up and covered with the O.C.T. compound. Fecal pellets were obtained from the most distal portion of the colon. Samples were then flash-frozen in liquid nitrogen and sectioned on a cryostat. In addition to tissue samples, luminal contents were removed, flash-frozen using liquid nitrogen, and stored at -80°C until use. The length of the cecal appendix in the animals ranged from 12 to 19 cm with a diameter of 1 to 2 cm, which was generally dependent on the amount of luminal contents.

For some experiments utilizing bacteria from fresh ceca of rabbits, bacteria were cultured from cecal contents according to the following protocol: a 10–20 mg sample of cecal contents were cultured at 37°C in 10 ml of liquid medium (Minimal Essential Media with 1.0 mM HEPES, 0.1 mM non-essential amino acids, and 1 mM sodium pyruvate) with constant shaking for 17 h. Particulate fiber was allowed to settle for 15 s and aliquots of bacteria were frozen at -80°C until needed. At various times, bacteria samples were thawed quickly and 100 μl was added to 10 ml of fresh liquid medium. These cultures were incubated at 37°C for 17 h with constant shaking. After samples were removed from the shaker, they were either used immediately (Time=0) or allowed to sit at 37°C with no change in media (without addition of nutrition) for 24 to 96 h, and the ratio of intact bacterial cells to cell debris was assessed by flow cytometry as described below.

Mice (strain C3H, Jackson Laboratories) were fed ad libitum prior to euthanization, and were euthanized by exsanguinations under isoflurane anesthesia. Luminal contents were removed, flash-frozen using liquid nitrogen, and stored at -80°C until use. In addition, fresh fecal pellets were collected from the cage, frozen using liquid nitrogen, and stored at -80°C until use.

Quantification of bacterial cells and bacterial cell lysis by flow cytometry

Quantification of intact bacterial cells and cell debris was carried out by flow cytometry based on a previously established method (Lee et al. 2010). The identity of various populations identified by flow cytometry (e.g., intact bacterial cells versus cell debris) was confirmed by sorting and examination under a fluorescence microscope. To perform the flow cytometry, samples of intestinal contents were diluted to a concentration of 10 mg/ml in a “counting buffer” consisting of phosphate buffered saline with 10 mg/ml bovine serum albumin and 0.2 g/L NaN_3 . Large fiber particles were allowed to settle and 15 μl of sample were suspended in an additional 3 ml of counting buffer. Any remaining large particles were

then removed from the suspension using a 35 µm strainer (BD Biosciences, San Jose, CA) to avoid clogging of the 70 µm nozzle on the flow cytometer. For quantification of cultured bacterial samples, 30 µl of bacterial cultures were suspended in 3 ml of counting buffer.

The “Bacteria Counting Kit” (Invitrogen Corp; Carlsbad, CA) was utilized to quantify the bacteria. Each sample of luminal contents was divided into three portions and assessed by flow cytometry as either unstained, stained without counting beads, and stained with counting beads (6 µm beads provided with the Kit). Filtered buffer only (background), buffer with counting beads only, and cultured bacteria (*E. coli* K12) were used as controls. Staining using the SYTO BC bacteria stain was assessed using the fluorescein isothiocyanate (515BP/20) channel. For staining, 0.5 µl SYTO BC and 2.5 µl beads were added per 500 µl sample. Beads were sonicated as directed before use. The suspension was allowed to incubate for 5 min or longer in the dark before assessment by flow cytometry.

Flow cytometry was performed in the Duke University Center for AIDS Research Flow Cytometry Core Facility. The machine used for flow cytometry was a BD FACSAriaII (FACSDiva 6.1.1), and all parameters were collected on a log scale with a time duration of 60 s. Calibration, or “quality control” particles of 0.3, 0.5, 0.8, and 1.0 µm in diameter (BD Biosciences; San Jose, CA) were used to provide approximate size standards for the analyses, and data were assessed using FlowJo 8.6.3.

The flow cytometric analysis was determined to be approximately 100% efficient in the detection of cultured bacteria with the settings and the tuning of the instrument utilized in this series of experiments. Although the technique could not be confirmed to be 100% efficient in the detection of bacterial cells from intestinal samples (because the number of cells could not be verified by counting colony forming units), the technique was effective at counting particles substantially smaller than bacterial cells, indicating that the counting of intact bacterial cells was well within the detection limits of the machine. A two-tailed *T* test was used to compare results from different groups.

Quantification of macroparticulate fiber

The luminal contents from mole-rats were dried by evaporation of the ethanol overnight at room temperature. Luminal contents from rabbits were thawed and dried using a lyophilizer. Dried samples were washed for 24 h with a solution of sterile phosphate buffered saline with 1% (w/v) bovine serum albumin and 0.2 g/L NaN₃ at a ratio of 10 mg dried luminal contents per 1 ml buffer to remove soluble material, bacteria and microparticulate fiber. After 24 h, the mixture was vortexed, macroparticulate matter was allowed to settle for 5 s, and the supernatant was removed. Samples were then freeze-dried overnight, and a final weight determined.

Quantification of biofilm fragments in digesta

For assessment of biofilm fragments in samples of digesta, 4.0 µl of SYTO BC stain were added to 2.5 mg of digesta suspended in 30 µl of H₂O. Samples were incubated in the dark for 15 min, placed on a slide, coverslipped, and viewed under a fluorescent microscope. Slides were semi-quantitatively scored by adding the total number of “biofilm fragment scores” for each fragment (1=small, 2=medium, and 3=large biofilm fragments) in a given high power (40×) field. For each sample, ten high-powered fields were evaluated and the average taken.

Assessment of biofilm formation on the gut epithelium

Biofilms adjacent to epithelium in normal bowel from rabbits were evaluated using a confocal laser microscope following cryosectioning and staining of the tissue with acridine

orange as previously described (Palestrant et al. 2004). In summary, 4- μ m cryosections were prepared from frozen biopsies, mounted on microscope slides, and stored at -80°C until needed. Slides were allowed to warm to room temperature and fixed in 95% ethanol, gradually hydrated to distilled water, rinsed with 1% acetic acid for 6 s and then washed twice in phosphate buffered saline, pH 7.4. Slides were incubated with 0.1% acridine orange (Sigma) in 67 mM phosphate buffer, pH 6.0 for 3 min, washed with phosphate buffer for 1 min and differentiated in 100 mM CaCl_2 for 30 s. Slides were coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and evaluated using a Zeiss 410 confocal light microscope. Photos were taken of the areas at the border between the epithelium and the lumen, and a false-color image was constructed from the black and white images to approximate the color of the acridine orange fluorescence in the biofilms and to differentiate microbial biofilms from the rabbit tissue.

Results

Spontaneous decay of cultured enteric bacteria

To examine the utility of our flow cytometric technique for the evaluation of bacterial cell lysis in complex mixtures of bacteria, the spontaneous lysis of enteric bacteria cultured from the ceca of rabbits was monitored by flow cytometry as described in the “Materials and methods” section. Immediately following overnight culture, the ratio of intact bacteria to bacterial cell debris was approximately 3.5 to 1. Within 24 h, the ratio was about 1.2, and had dropped to 0.45 within 96 hours (Fig. 1). These results are not necessarily expected to reflect processes that occur in vivo, but do demonstrate the spontaneous lysis of cultured bacteria in culture over time, and indicate the feasibility of monitoring that lysis by flow cytometry.

Bacterial cell/cell debris ratio in the mole-rat

The flow cytometric profile of the luminal contents from the mole-rat was very similar to that previously reported for cultured *E. coli* (Lee et al. 2010), with populations of intact cells and populations of cell debris evident (Fig. 2a). However, the size distribution of the intact cells was substantially greater than that previously reported for *E. coli*. The appendix of the mole-rat, on average, contained less bacteria per gram of luminal contents than did the cecum (Fig. 2b), although the difference in the means was not statistically significant ($p=0.17$). On average, the appendix contained a lower ratio of intact bacterial cells to cell debris than did the cecum (Fig. 2c), indicating that more spontaneous cell lysis had occurred in the appendix than in the cecum, potentially suggesting that the population of bacteria in the appendix may be older than the population in the cecum. Similarly, the ratio of intact cells to cell debris in the large bowel was less than that in the cecum, suggesting the possibility of an older population of bacteria in the large bowel than in the cecum.

Bacterial cell/cell debris ratio in the rabbit

Some portions of the flow cytometric profile of the luminal contents from the rabbit were very similar to that of cultured *E. coli*, with populations of intact cells and populations of cell debris evident (Fig. 3a). However, a substantial number of particles roughly the size of intact bacterial cells, but which did not stain effectively with the bacteria-specific dye, were identified (Fig. 3a). Based on microscopic examination of the sorted particles, this fraction appears to consist in large part of microparticulate fiber. The appendix of the rabbit, on average, contained significantly less bacteria per gram of luminal contents than did the cecum (Fig. 3b). Consistent with results observed in the mole-rat, the appendix of the rabbit contained, on average, a lower ratio of intact bacterial cells to cell debris than did the cecum (Fig. 3c), suggesting that the population of bacteria in the appendix may be older than the population in the cecum. Similarly, the ratio of intact cells to cell debris in the large bowel

was less than that in the rectum, suggesting the possibility of an older population in the rectum than in the cecum.

Macroparticulate fiber in the appendix, cecum, and large bowel

To provide further insight into possible differences in the luminal contents of the appendix, cecum, and large bowel, the amount of particulate fiber which rapidly (within 1 min) sedimented from aqueous solution was assessed. Particles larger than about 50 μm were typical of those assessed using this approach. A wide range of fiber concentration was observed in all compartments of the mole-rat, and no substantial difference was observed between those compartments (Fig. 4a). In the rabbit, on the other hand, a substantially higher concentration of large fiber particles were observed in the appendix compared to the cecum (Fig. 4b). These differences between the laboratory rabbit and the mole-rat may reflect substantial differences between the two species with regards to the circulation of fecal matter through the large bowel.

Fragments of biofilms in the mole-rat and rabbit digesta

While flow cytometry is efficient at the detection of monodisperse microorganisms, it is not designed to detect fragments of biofilms, which are expected to be regularly shed from the normal mammalian gut (Bollinger et al. 2007a). Fluorescence light microscopy, on the other hand, has proven useful for the detection of biofilms (Bollinger et al. 2007b; Palestrant et al. 2004), and was thus used to evaluate the possible presence of biofilms in the colon of mole-rats and rabbits. Using this approach, pieces of irregularly shaped material 50 to 200 μm in diameter consisting of aggregates of bacteria associated with an extracellular matrix were observed within the digesta obtained from the ceca of both mole-rats and rabbits (Fig. 5). The observation of densely packed bacteria within an extracellular matrix was consistent with the idea that the structures were indeed fragments of biofilms. However, the orientation of the bacteria within the fragments tended to be disordered, suggesting that the fragments did not originate from well-structured biofilms. Further, fragments of biofilms with spatially oriented bacteria were not observed in the fecal pellets of either species, suggesting that they were either not incorporated into fecal pellets or were degraded following incorporation.

Laboratory mice and rats are known to harbor wellformed biofilms in their proximal large bowel, and were thus utilized as a control for the above studies. Staining of digesta taken from the ceca of C3H mice revealed numerous fragments of well-formed biofilms (Fig. 5), some of which possessed a wave-like character potentially reflecting the epithelial surface upon which the biofilms formed. In addition, unlike biofilm fragments obtained from mole-rats and rabbits, the bacteria within the biofilm fragments from the cecal contents of mice exhibited an obvious degree of orientation, perhaps reflecting an orientation with respect to the direction of flow that is often observed in biofilms and has been observed in the laboratory rodent gut and in humans (Bollinger et al. 2007b; Palestrant et al. 2004). Further, in contrast to the lack of biofilm fragments in the fecal samples of mole-rats and rabbits, fragments of biofilms were observed in the fecal pellets of mice. When comparing the relative amount ("biofilm fragment score" per high-powered field, see "Materials and methods" section) of biofilm fragments in the cecal contents and in the fecal pellets of the mice, the fecal pellets averaged only 10% less fragments than that found in the cecum, which was not a significant decrease (paired, twotailed T test; $p=0.4876$). Further, the number of biofilm fragments in the digesta of the cecal contents was correlated with the number of fragments in the fecal pellets within the same mouse ($r^2=0.61$, $p=0.022$), suggesting that the biofilm fragments present within the fecal pellets of mice may reflect the conditions found in the cecum.

Biofilm formation on the gut epithelium of rabbits

To probe the possibility that the putative biofilm fragments present in the digesta of the rabbit reflected biofilm fragments shed from the surface of the rabbit gut epithelium, we evaluated the presence of biofilms on the epithelium of the rabbit. Previous studies have demonstrated that formation of dense biofilms is limited to the cecum of both laboratory rats and laboratory mice and to the cecal appendix and cecum of humans. In contrast, the rabbit gut showed dense epithelial-associated biofilm formation throughout the appendix, cecum and proximal large bowel until the point of bolus formation at the end of the haustrated gut (Figs. 6, 7 and 8). In contrast to studies in humans and in laboratory rats, the bacteria in the epithelial-associated biofilms of the rabbit showed no evident orientation within the biofilm. This observation is consistent with the idea that the biofilm-like fragments observed in the digest in the cecum of the rabbit were indeed fragments of biofilms shed from the epithelial lining of the rabbit gut. Although similar studies in the mole-rat are warranted, evaluation of biofilm formation on the gut epithelium requires fresh, flash-frozen samples (Bollinger et al. 2007b; Palestrant et al. 2004), which were not available in the mole-rat. Further, the presence of numerous long, filamentous bacteria, many with spirochete-like morphology, in the mole-rat gut (Fig. 5) made assessment of bacterial growth patterns extremely difficult using cross-section slides of the mole-rat gut.

Discussion

The first application of flow cytometry to evaluate spontaneous cell lysis in a complex bacterial community provided encouraging results. Most importantly, assessment of the complex mixture of bacteria cultured from the rabbit gut demonstrated that the technique was indeed useful in detecting spontaneous lysis of bacterial cells over time. Assessment of samples harvested directly from the gut also provided interesting results, regardless of the preservation method used, indicating the robust nature of the approach. The effect of microscopic fiber fragments on the analysis was of interest. Sorting experiments followed by examination under a light microscope revealed that fiber particles generally populated the flow diagram with an apparent size comparable to that of intact bacterial cells, but, as expected, did not take up bacteria-specific dye to the extent that the bacteria did. However, it was also observed that, upon aging, a subset of bacterial cells lost their propensity to adsorb dye and thus tended to overlap with microscopic fiber fragments in the flow diagram. This effect prevented effective quantification of microscopic fiber particles using the flow cytometer using our approach, suggesting that further work in this area is warranted.

Despite the fact that the absolute and relative size of the appendix is much smaller in the mole-rat compared to the rabbit, the results obtained from the two species were strikingly similar in some regards. The mole-rat appendix is more than 50-fold smaller in size than the rabbit appendix, occupying a volume of about 0.2 cm³ compared to the more than 10 cm³ occupied by the typical rabbit appendix (Smith et al. 2009). The cecal appendixes of mole-rats and rabbits contained, on average, a lower ratio of intact bacterial cells to cell debris than did the cecum, suggesting that the population of bacteria in the appendix may be older than the population in the cecum.

Although the mole-rats tended to have slightly less bacteria than did the rabbits, the absolute concentrations of bacteria from the mole-rats and from the rabbits may not be directly comparable for technical reasons. The samples from mole-rats were taken from animals captured in the field and were stored in ethanol at room temperature, whereas samples from the rabbits were harvested from laboratory specimens and stored at -80 °C. It remains unknown how preservation and subsequent long-term storage under these conditions might have affected the results. However, the morphology of the bacteria in the mole-rats was profoundly different than that found in the rabbit (and indeed in any other mammal

evaluated to date), and may be responsible in part for the reduced number of bacteria found in the rodents.

One limitation to the flow cytometry approach is that, to the extent that bacteria growing in biofilms cannot be removed from those biofilms, the measurement of bacteria growing in biofilms is not possible by that technique. Further, to the extent that growth of biofilms is dependent on adherence to the mucosal epithelia (Swidsinski et al. 2005; Palestrant et al. 2004; Bollinger et al. 2007a), microbial growth in biofilms is expected to be more abundant in areas such as the appendix, which have a higher surface/volume ratio. Thus, the flow cytometric technique may underestimate the presence of bacteria in the appendix, although it is expected to provide a good measure of bacteria growing in the planktonic (free swimming) state in the appendix. However, analysis of biofilm formation by fluorescence light microscopy directly revealed biofilm formation on the epithelium of the rabbit appendix. Further, observation of biofilm-like fragments in the digesta taken from the ceca of the mole-rats indicated that biofilms were also a characteristic feature of the mole-rat gut ecology. Although technical limitations prevented direct observation of epithelial-associated biofilms on the mole-rat gut, the overall appearance of biofilms in both fermenting guts appeared to be less structured than those found in humans and in laboratory rodents. In addition, biofilm formation in the rabbit gut appeared to be far more extensive than that found in laboratory rodents or in humans. With this in mind, biofilm formation in the fermenting gut may be fundamentally different than that found in omnivores such as laboratory mice and humans. However, the observation of biofilms in the gut of mole-rats and rabbits is consistent with the idea that the cecal appendices of these animals protect adherent bacterial colonies that are capable of re-inoculating the gut in the event that the normal flora are depleted.

In both the mole-rat and the rabbit, the ratio of intact cells to cell debris in the large bowel (mole-rat) or the rectum (rabbit) tended to be less than that in the cecum. Although assessment of more samples is necessary to confirm this trend, the trend suggests that the technique is valid, since the bacterial population in the rectum is expected to be older than that in the cecum. In addition, the cecal appendix of the mole-rat and the rabbit contained less bacteria, on average, than did the cecum, although evaluation of a larger number of animals will be needed to confirm this observation in the mole-rats.

Interestingly, both the mole-rat and rabbit practice coprophagy (Björnhag 1981; Bennet and Faulkes 2000). Björnhag described a retrograde transport of ingesta along the wall of the haustra in the rabbit colon. This colonic separation mechanism (CSM) was later classified the “wash back” type by Cork et al. (1999) where bacteria-rich digesta is transported back to the cecum by a series of retroperistaltic movements. *B. suillus* has a colonic groove or furrow (Kotzé et al. 2010) which may relate more to the classification of a “mucus trap” type of CSM (Cork et al. 1999) seen in the guinea pig and the chinchilla (Holtenius and Björnhag 1985). While the morphology of the CSM differs in the two species, they both have the potential to increase the retention time of bacteria in the cecum (Cork et al. 1999). However, the impact, if any, of the CSM on the retention of bacteria in the appendix remains unknown, and several African mole-rat species have a colonic groove but no appendix (Kotzé et al. 2010). Thus, additional studies on the relationship between the CSM and the cecal appendix are warranted.

The possibility that bacterial communities in the appendix are somewhat older than those present in the cecum, as suggested by the present results, is consistent with the view that the cecal appendix is a safehouse for symbiotic intestinal bacteria. That is, bacteria within the appendix may reside in that location longer than do bacteria growing in the cecum, an indicator of the relative protection afforded by the narrow lumen of the appendix and the

relative isolation of the appendix from the main flow of the fecal stream. On the other hand, the potentially older age of bacterial colonies in the cecum may be a reflection of other processes, such as a faster intrinsic rate of lysis in the appendix compared to the cecum. Nevertheless, the apparent presence of biofilms in the gut of both the mole-rat and the rabbit strongly support the idea that the gut ecology in animals with fermenting guts is, like that of humans and of laboratory rodents, strongly dependent on biofilm formation for homeostasis.

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Fig. 1.

Autolysis of bacterial cells over time as monitored by flow cytometry. **a** Flow cytometric profile of standard size particles. **b** Flow cytometric profile of cultured bacteria from a rabbit cecum after 17 h of culture. The area of the profile indicated by the small box on the left corresponds to events associated with cell debris, and the larger boxed area on the right of the profile encloses the events associated with intact bacterial cells. Samples were stained with a fluorescent dye as described in the “Materials and methods”. **c** Flow cytometric profile of cultured bacteria from a rabbit cecum after 96 h of culture with no fresh media added. Some events, both large and small, stained at levels approximately one log lower

than did the starting bacteria. These events were apparently due to bacteria or bacteria cell components, but were not counted because the nature of the components (e.g., intact cells versus cell debris) was not known. **d** Change in ratio of bacterial cells/cell debris as a function of time

Fig. 2.

Assessment of bacterial distribution in the mole-rat gut and changes in the ratio of intact cells to cell debris. **a** Representative flow cytometric profile of the luminal contents from the cecum of a mole-rat. The area of the profile indicated by the small box on the left corresponds to events associated with cell debris, and the larger boxed area on the right of the profile encloses the events associated with intact bacterial cells. Samples were stained with a fluorescent dye as described in the “Materials and methods”. **b** Total number of intact cells. **c** Ratio of bacterial cells/cell debris as a function of the location in the gut. Although the large bowel tended to have a lower ratio of intact cells to cell debris, this difference was not statistically significant ($p=0.0504$)

Fig. 3.

Assessment of bacterial distribution in the rabbit gut and changes in the ratio of intact cells to cell debris. **a** Representative flow cytometric profile of the luminal contents from the cecum of a rabbit. The area of the profile indicated by the *small box on the left* corresponds to events associated with cell debris, and the *larger boxed area* on the right of the profile encloses the events associated with intact bacterial cells. Samples were stained with a fluorescent dye as described in the “Materials and methods”. Poorly staining particles with a size similar to that of intact bacterial cells (i.e., associated with a similar degree of forward light scatter as intact cells) were sorted and identified as fiber particles by light microscopy. **b** Total number of intact cells. **c** Ratio of bacterial cells/cell debris as a function of the location in the gut. Although the large bowel tended to have a lower ratio of intact cells to cell debris, this difference was not statistically significant ($p=0.106$)

Fig. 4. Fraction (weight per total weight) of large fiber particles in the lumen **a** of the mole-rat gut and **b** of the rabbit gut

Fig. 5.

Fragments of biofilms shed into the fecal stream. Samples were taken from the ceca of either mole-rats (**a–b**), rabbits (**c–d**), or mice (**e–h**), stained with SYTO BC bacteria stain, and evaluated under a fluorescent microscope as described in the “Materials and methods”. The *bar* in panel **a** indicates 80 μm , except in panels **b**, **d**, and **h**, where the same *bar* represents 20 μm . Panels **b** and **h** are expanded views from sections of panels **a** and **g**, respectively. In panel **g**, the orientation of bacterial within the biofilm is readily apparent. Panels **e** and **f** show the same section in two different focal planes, illustrating the wave-like shape of the fragment. The *inset* in panel **a** shows planktonic microorganisms, also stained with SYTO

BC bacteria stain, from the ceca of mole-rats. The majority of the microorganisms found in the ceca of the molerats unexpectedly possessed an elongated and often spirochetelike morphology

Fig. 6.

False-color image of epithelial-associated biofilms (*orange color*) in the large bowel of the laboratory rabbit (*O. cuniculus*). Biofilms adjacent to epithelium in a normal rabbit large bowel ($n=2$) were observed using a confocal laser microscope following flash freezing, cryosectioning and rapid staining of the tissue with acridine orange as previously described (Palestrant et al. 2004). The *orange* (false-color approximates original fluorescent light color) regions with a speckled appearance are bacterial biofilms within the mucus layer in the lumen of the bowel, and the brightly stained blue areas (false color) are the nuclei of the epithelial cells that also stain with acridine orange. The *bar* represents 20 μm . Panel **a** the

appendix, showing a biofilm closely associated with the gut epithelium. Panel **b** the mid-cecum, showing a biofilm substantially thicker than that seen in the appendix, also closely associated with the gut epithelium. Panel **c** the mid colon (end of continuous stream of digesta), showing sporadic association of bacteria with the gut epithelium. Panel **d** the distal colon, showing little or no association of bacteria with the gut epithelium

Fig. 7.

Biofilm distribution in the rabbit GI-tract: decreased biofilm formation in regions with bolus formation. *Arrows* indicate various sites where tissue was taken and evaluated for the presence of biofilms. Biofilms were observed (Bio(+)) in regions proximal to the “transition zone” labeled in the photo, whereas biofilms were not observed (Bio(-)) in regions distal to the transition zone. The transition zone contains the termination of the haustrated, proximal colon (proximal end of transition zone), the end of the continuous stream of digesta (3.5 to 5 cm distal from the end of the haustrated colon, mid-transition zone), and the first distinct fecal bolus (remaining portion of the transition zone). The *bar* represents 5 cm

Fig. 8.

The transition between biofilm rich and biofilm poor regions of the rabbit gut. **a** Plot showing the rapid drop-off of biofilms at the end of the continuous digesta stream (see Fig. 7) in the rabbit gut. The degree of biofilm formation was graded subjectively on a scale of 0 (no biofilms) through 10 (continuous coverage of biofilms) in samples from three different rabbits. The scores from each rabbit are shown. The midpoint in the curve, 3 to 5 cm proximal to the end of the continuous digesta stream, corresponds to the end of the haustrated, proximal colon. **b** H&E stained cross-section of colon within 1 cm proximal of the end of the continuous digesta stream **c** H&E stained cross-section of colon 6 cm distal to the end of continuous digesta stream. Bar 500 μ m