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Biofilms of the Ruminant Digestive Tract

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Introduction

Direct observations of microbial growth in a large number of natural ecosystems have shown that the predominant populations are attached to surfaces where they grow in glycocalyx enclosed microcolonies that develop into adherent biofilms (Costerton *et al.* 1987; Cheng *et al.* 1991; Lappin-Scott *et al.* 1992). In various digestive tracts, this adhesion takes the form of very specific associations with insoluble nutrients (Cheng *et al.* 1977; Akin 1979; Cheng *et al.* 1991) to form particle associated microbial populations, and with tissue surfaces to form tissue associated populations (Cheng & Costerton 1980; Cheng *et al.* 1981a, b). A variation of this latter mode of growth is the association of a major microbial population with the mucous blanket of secretory intestinal tissue (Roze *et al.* 1982). Bacteria of the ruminant digestive tract can be divided into three distinct subpopulations, (i) those associated with the digesta, (ii) those associated with gastrointestinal tissue and (iii) those associated with gastrointestinal fluid (Cheng & Costerton 1980; Cheng *et al.* 1981a; Czerkowski & Cheng 1988).

General principles of microbial ecology dictate that any organism will establish itself in a favourable nutrient niche and proliferate within a physiologically integrated community. Our observations of the growth of bacteria associated with feed particles indicate that each species adheres to its own particular insoluble substrate (e.g. cellulose, protein, starch) and produces enzymes to degrade insoluble substrates to soluble nutrients (Cheng *et al.* 1984; McAllister *et al.* 1990b). Because most digestive processes involve the

coordination of physiologically related activities, we often see the development of structured consortia (Fig. 13.1) within which the metabolic products of the primary digestive organisms are the substrates for sequential members of a layered biofilm (Wolin & Miller 1988). The digestible dead epithelial cells of some tissues concentrate bacterial nutrients (for example urea) at their surfaces (Wallace *et al.* 1979; Cheng & Wallace 1979; Dinsdale *et al.* 1980) and constitute well defined niches in which taxonomically distinct microbial populations develop. These populations often play an important role in the overall function of the digestive tract (Cheng &



Fig. 13.1. Transmission electron micrograph (TEM) of a ruthenium red stained preparation of barley straw incubated in a continuous culture of a mixed natural population of bacteria from the bovine rumen. Note the extensive pit formation by rod-shaped Gram negative bacteria resembling *Fibrobacter succinogenes* and the presence of spirochaetes (s) in the same adherent consortium. Bar = 1 μ m. (From Cheng & Costerton 1986.)

Costerton 1980). The free microbial cells in gastrointestinal fluid can be largely available for attachment to new nutrients or freshly exposed tissue and this population contains some cells from the adherent population that are in transit between surfaces.

Generally, the presence of well adapted microbial biofilms on a tissue surface is beneficial in that these cells cooperate physiologically and the biofilm precludes the adhesion of pathogenic bacteria and viruses. Thus, an objective of successful management should be to encourage the development of beneficial biofilms on feed particles and on tissues at all levels of the digestive tract (Cheng *et al.* 1991). Modification of the physiological activity of the digestive tract populations of bacteria in mature animals can be achieved either through chemical agents or processing of the feed.

Biofilms on the ruminant tongue

The tongue is exposed to air, food, saliva and contents of the reticulo-rumen during regurgitation. The dorsum of the tongue, where particulate feed is chewed and compressed, is very highly abraded during regurgitation. Accordingly, the adherent biofilm of this tissue surface must persist in spite of massive shear forces. Resistance to mechanical displacement is exemplified by the tightly adherent growth of trichome forming bacteria and members of the *Alysiella* and *Simonsiella* genera (Fig. 13.2a, b) which predominate in particularly high abrasion areas (McCowan *et al.* 1979). However, many other chain forming bacteria and rod shaped organisms are present in less abraded areas. *Alysiella* and *Simonsiella* genera adhere firmly to the tongue tissues by fibrillar glycoprotein structures formed on only one side

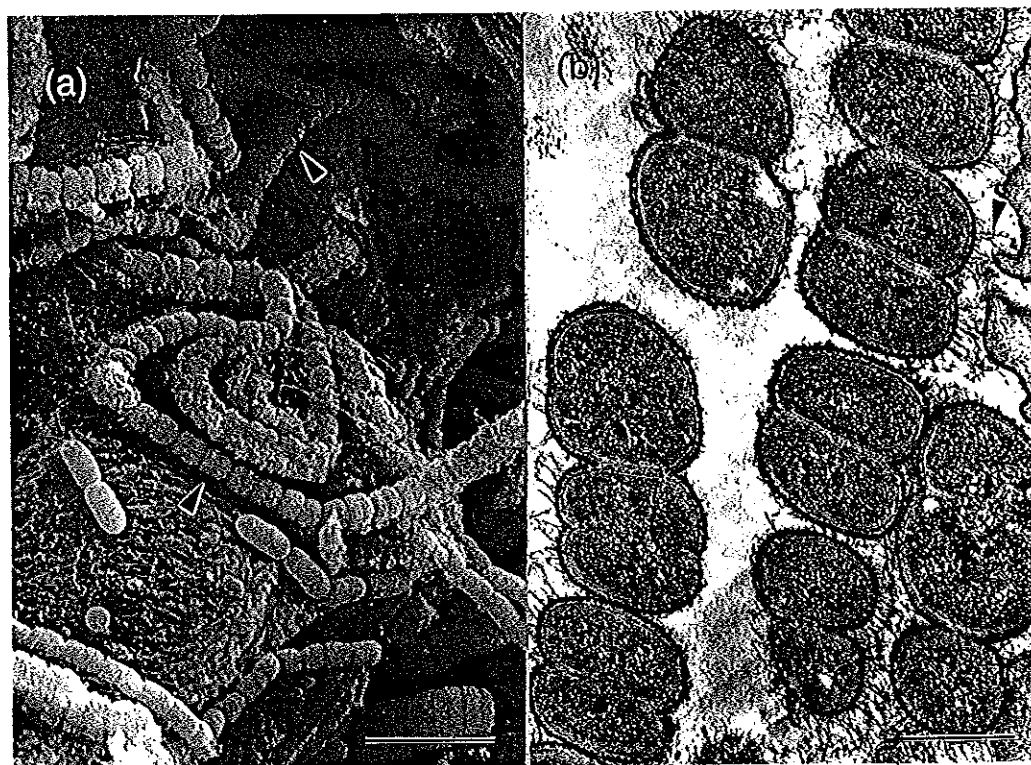


Fig. 13.2. (a) Scanning electron micrograph (SEM) of a mixed population on the tissue of the tongue. Almost all the bacteria are either filamentous or chain forming, and many follow the contours of the tissue cells, often appearing wedged between them (arrows). (From McCowan *et al.* 1979). (b) TEM showing the adhesion of these trichome forming cells to tissues by means of fibres (arrow) found only on one side of the bacterial cells. Bars = 5 µm. (From McCowan *et al.* 1979.)

Table 13.1. Proportion of bacteria associated with plant cell wall material and liquor in rumen digesta of cows fed straw based diets^a

Genera	Composition (%)	
	Plant cell wall material	Rumen liquor
<i>Butyrivibrio</i>	32	7
<i>Selenomonas</i>	14	10
Unnamed spirochaete	8	0
<i>Lachnospira</i>	8	1
<i>Megasphaera</i>	0	11
<i>Streptococcus</i>	3	12
<i>Ruminococcus</i>	16	6
<i>Bacteroides</i>	11	38
Others	8	15
Total number of isolates	368	292
Viable count (CFU ^b ml ⁻¹ × 10 ⁷)	230 ± 38	201 ± 13

^aData from these animals each sampled twice (Latham 1980).^bcfu, colony forming units.

of these bacterial cells. Adhesion is so avid that disruption of the host tissue is usually necessary to detach these cells (McCowan *et al.* 1978, 1979). The adherent biofilm of the bovine tongue remains remarkably consistent in both morphological balance and numbers throughout the life of the animal, and it is notably resistant to the influences of different diets.

Biofilms within the reticulo rumen

Approximately 20–30% of the bacteria in the reticulo-rumen are found in the rumen fluid, while 70–80% are associated with feed particles (Forsberg & Lam 1977; Craig *et al.* 1987), and approximately 1% are associated with the epithelial surface. The fluid population contains detached representatives from the surfaces of feed particles and tissue, in addition to organisms that are not normally adherent and subsist on soluble nutrients in the rumen fluid (McAllister *et al.* 1994). The rumen fluid population has a distinct composition (Table 13.1; Latham 1980) with some shared genera in both the particle and tissue associated populations (Table 13.2). Within minutes of ingestion, feed particles (e.g. legumes, grass, cereal grains) are colonized by organisms associated with the rumen fluid (Bonhomme 1990). Starch granules (Minato & Suto 1979; McAllister *et al.* 1990b) and cellulose (Akin 1986; Bowman & Firkins 1993) rapidly

Table 13.2. Genera recovered from epithelial tissue at 13 sites in the bovine rumen

Genus	Number of sites yielding this genus ^a
<i>Fusobacterium</i>	12
<i>Propionibacterium</i>	12
<i>Eubacterium</i>	8
<i>Bacteroides</i>	6
<i>Selenomonas</i>	5
<i>Butyrivibrio</i>	4
<i>Streptococcus</i>	4
<i>Peptococcus</i>	3
<i>Bifidobacterium</i>	3
<i>Lactobacillus</i>	3
<i>Micrococcus</i>	2
<i>Flavobacterium</i>	2
<i>Hemophilus</i>	2

^aBlocks of tissue were removed, washed, homogenized, and cultured to recover these adherent organisms.

become heavily colonized by amylolytic and cellulolytic bacteria, respectively. These initial colonizing bacteria multiply and are joined by additional bacteria from the ruminal fluid to form the mature biofilms that are frequently observed on the surfaces of feed particles and epithelial tissue (McAllister *et al.* 1994).

Direct examination of the leaves of forage legumes exposed to rumen fluid shows that certain bacterial species invade the intercellular spaces and colonize pectin rich surfaces (Cheng



Fig. 13.3. (a) TEM of a ruthenium red stained preparation of sterilized barley straw incubated with a pure culture of strain BL-2 of *Bacteroides succinogenes*. Note the 'pits' and discontinuities of the cellulose where these bacterial cells have adhered, the vesicular residues of bacterial cell envelope (arrow) which are still adherent to the plant cell wall, and the fibrous bacterial product (g) that mediates bacterial adhesion. (b) TEM of a ruthenium red stained preparation of barley straw incubated with a continuous culture of a mixed natural population of bacteria from the bovine rumen. Note the extensive 'pit' formation by cells resembling *Ruminococcus* species (arrows). Bars = 1 μ m. (Authors' unpublished data.)

& Costerton 1980). When a particular bacterium has adhered to its specific nutrient substrate (e.g. cellulose) and finds itself in a favourable nutrient niche, it proliferates to form a functional microcolony in which specific digestive enzymes are produced in especially high concentrations. These enzymes may remain cell associated, but they remain active even after bacterial cell breakdown (Stewart *et al.* 1979; Forsberg *et al.* 1981; Groleau & Forsberg 1981). Cell envelope fragments of *Fibrobacter succinogenes* concentrate sufficient cellulase activity to form deep pits in the surface of cellulose fibres (Fig. 13.3a). The adherent bacterial biofilm can be thought of as constituting a local 'critical mass' of enzyme activity that focuses its attack on cellulose to produce distinct localized pits (Fig. 13.3b). This process is characteristic of the bacterial digestion

of feeds such as legumes, grass and straw (Cheng *et al.* 1991).

The population of bacteria associated with the epithelial tissues of the reticulo-rumen is relatively small (i.e. less than 1%: Fig. 13.4a, b, c). Taxonomically, this tissue associated microbial population is unique in that it contains large numbers of *Fusobacterium* species and Gram positive cocci (Table 13.2). These latter organisms are ideally suited to the epithelial environment, because many of them are facultative anaerobes and produce urease, which hydrolyses urea and generates the ammonia required for bacterial metabolism within the rumen (Cheng & Wallace 1979). Bacteria contained within this biofilm are unique in producing an essential enzyme (i.e. urease) lacking in the animal tissue (Cheng & Wallace 1979; Wallace *et al.* 1979) and this

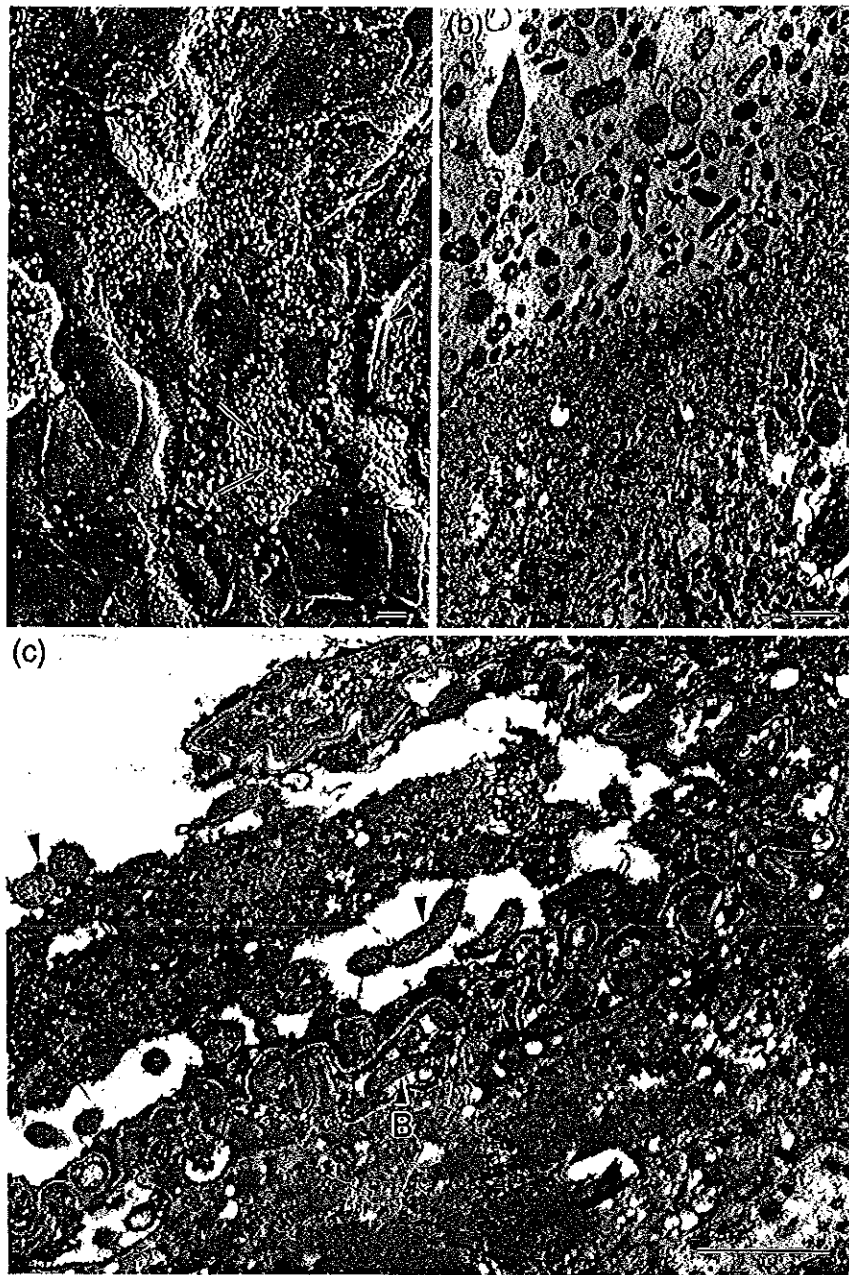


Fig. 13.4. (a) SEM of the bacterial population of the rumen epithelium from a milk-fed Holstein calf. The number of adherent bacteria varies from one tissue cell to the next. One tissue cell (small arrows) may be completely covered by adherent bacteria while adjacent cells carry very low numbers. Bacteria can be seen colonizing the undersurface of a shedding epithelial cell (large arrow). (From Cheng *et al.* 1981b.) (b) TEM of a ruthenium red stained population of the rumen tissue showing the thick and complex bacterial biofilm that develops on this organ wall. These cells are enclosed in a delicate fibrous network within the biofilm. (Cheng *et al.* 1979.) (c) TEM of a section of a ruthenium red stained preparation of the tissue of the bovine rumen showing the digestion of a dead tissue cell by adherent bacteria (arrows). Note that one of these bacteria (B) appears to have penetrated the intercellular space under the cell undergoing digestion. Bars represent: (a), (b) 5 μ m; (c) 1 μ m. (From Cheng *et al.* 1981b.)

association between animal and ruminal bacteria is an excellent example of the mutualistic benefits of biofilms.

The tissue associated biofilm is acquired early (within 2 days of birth) in the life of the ruminant (Fonty *et al.* 1987, 1988; Stewart *et al.* 1988; Cheng *et al.* 1991). Whole animal infusion studies have shown that this population is maintained even if no feed is ingested (Wallace *et al.* 1979; Dinsdale *et al.* 1980). High numbers of tissue associated bacteria have been found at 14 sites on the epithelia of cattle fed hay and concentrates. Milk-fed animals (up to 200 kg) also possess microbial cells on their epithelial surfaces, indicating that the development of mature biofilms does not require the presence of solid feed in the digestive tract (Wallace *et al.* 1979).

Studies with Svalbard reindeer suggest that the formation of tissue adherent biofilm may be related to energy intake. In the severe Norwegian winter, these animals are frequently starved and the epithelial tissue of the rumen is sparsely colonized by small bacteria (Cheng *et al.* 1993). In contrast, when feed is readily available in the spring and summer, the rumen epithelium is completely covered by a thick bacterial biofilm of several component species.

Biofilms and ruminant digestion and physiology

In addition to the primary digestion of food materials in the rumen, the three major microbial populations participate in a myriad of metabolic processes that help sustain the animal by the production of volatile fatty acids, vitamins and the microbial biomass that is itself digested in the lower digestive tract. These microbial activities can sometimes be located in the rumen by direct cytological identification of the enzymes concerned (Fay *et al.* 1979, 1983). It has been estimated that 75% of the protease activity (Brock *et al.* 1982), 70% of the amylase activity and 80% of the endoglucanase activity (Minato *et al.* 1966) is associated with the particulate fraction of rumen contents. These data indicate that the biofilms associated with feed particles are responsible for the majority of feed digestion in the rumen.

Biofilms associated with the omasum

The epithelium of the omasum is colonized by a biofilm that resembles that of the reticulo-rumen in both its density and its distribution (Fig. 13.5a). A large proportion of the surface of this tissue is occupied by a varied population of bacteria (Fig. 13.5a), but a mycelial fungus (Fig. 13.5b) and a bacterium that grows in especially coherent glycocalyx enclosed microcolonies (Fig. 13.6) are also observed. The bacteria of this adherent tissue associated population have a taxonomic composition similar to that on the epithelium of the reticulo-rumen, but some of the Gram positive species that colonize the rumen wall appear to be absent in the omasum (Table 13.3).

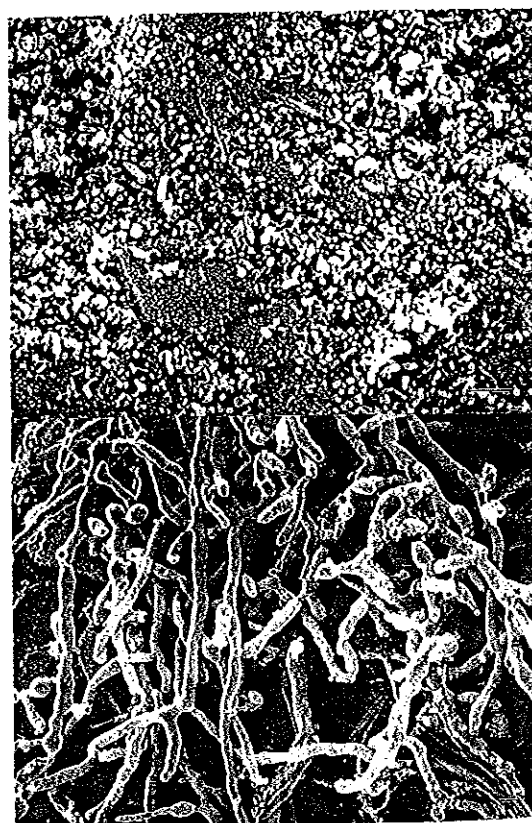


Fig. 13.5. (a) SEM of the tissue surface of the omasum of a 6 month old Holstein calf fed a grain diet. This tissue surface is colonized by a varied bacterial population similar to that of the reticulo-rumen. (b) SEM of the surface of the omasum of a calf fed as in Fig. 13.7 showing one of the areas of the tissue colonized by a fungus that produces elongated cells. Bars = 5 μ m.

Table 13.3. Bacteria isolated from the lower digestive tract of cattle

Omasum	Abomasum	Duodenum, small intestine and caecum
<i>Propionibacterium acnes</i>	<i>Propionibacterium acnes</i>	<i>Staphylococcus</i> sp.
<i>Propionibacterium</i> sp.	<i>Fusobacterium prausnitzii</i>	<i>Propionibacterium acnes</i>
<i>Fusobacterium prausnitzii</i>	<i>Fusobacterium</i> species	<i>Propionibacterium</i> sp.
<i>Butyrivibrio fibrisolvens</i>	<i>Eubacterium aerofaciens</i>	<i>Clostridium</i> sp.
<i>Eubacterium aerofaciens</i>	<i>Eubacterium</i> species	
<i>Clostridium clostridioforme</i>	<i>Clostridium</i> species	
<i>Peptostreptococcus intermedius</i>	<i>Peptostreptococcus intermedius</i>	
	<i>Staphylococcus</i> species	
	Gram positive cocci	
	Gram positive bacillus	



Fig. 13.6. SEM of one of the rare areas of the tissue of the omasum which is colonized by bacteria that form discrete ball-like adherent microcolonies. Bar = 5 μ m. (From Cheng & Costerton 1986.)

The microbiology of the contents of the omasum has not been examined to date, but the direct flow of digesta from the reticulo-rumen into this organ leads us to anticipate no major differences in the particle associated and fluid populations.

Biofilms within the abomasum

A major change in the ruminant digestive system occurs at the abomasum, where the epithelium becomes secretory and the pH of the lumen is reduced to between 2 and 4. These conditions are not as conducive to the formation of the complex biofilms that are observed in other regions of the digestive tract. In cattle and sheep, direct observation shows relatively few tissue associated bacteria in the mucous blanket at the epithelial surface of

Table 13.4. Bacterial urease production

Area sampled	Urease activity ^a		
	Holstein	Hereford	Sheep
Reticulum	8.08	2.10	1.15
Rumen	4.72	1.04	0.96
Omasum	0.52	0.71	0.35
Abomasum	0.01	3.15	0.02
Duodenum	0.07	-	0.02

^a μ moles N min⁻¹ in⁻² of epithelial tissue.

the abomasum. In addition, the low level of urease associated with tissue from the abomasum suggests that this organ is sparsely colonized by ureolytic bacteria in Holstein cattle and sheep (Table 13.4). Bacteria are seen within the mucous blanket at the tissue surface and particular types appear to predominate in certain areas (Fig. 13.7). The species of bacteria within this limited biofilm are not markedly different from those of the upper tract, except for the presence of relatively high numbers of Gram positive cocci and of an unidentified group of Gram positive bacilli (Table 13.3). An exception to this sparse bacterial colonization of the abomasum is seen in certain breeds of beef cattle (Angus, Hereford) in which greater colonization and high levels of bacterial urease are detected on the tissue surface (Table 13.4).

Biofilms of the lower digestive tract

A thick and coherent mucous blanket on the tissue surfaces of the small intestine, caecum and

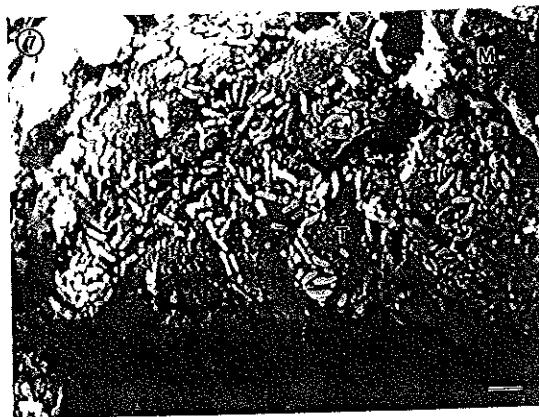


Fig. 13.7. SEM of the tissue of the bovine abomasum showing 'buried' bacteria where the copious mucous layer (M) is intact and clear outlines of adherent native bacteria where the tissue surface (T) is exposed by loss of the covering mucus. The diversity of bacterial forms seen in the rumen and omasum is sharply reduced in the abomasum. Bar = 5 μ m. (From Cheng *et al.* 1981b).

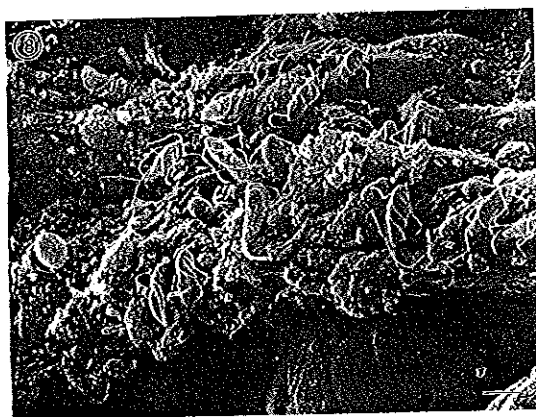


Fig. 13.8. SEM preparation of the mouse ileum in which the mucous blanket has been retained. Note the association of very large numbers of bacteria and protozoa with the mucous blanket and very few with the actual tissue surface (arrow). Bar = 5 μ m. (From Costerton *et al.* 1985.)

large intestine of ruminants presents considerable technical problems in digestive tract microbiology. Routine preparation of tissues of monogastric animals for electron microscopy leads to the loss of the mucous blanket and, with it, the greater part of the biofilm on the tissue surface (Roze *et al.* 1982). Most of the autochthonous bacteria and protozoa that occupy the tissue surfaces of the intestine live in the mucous layer

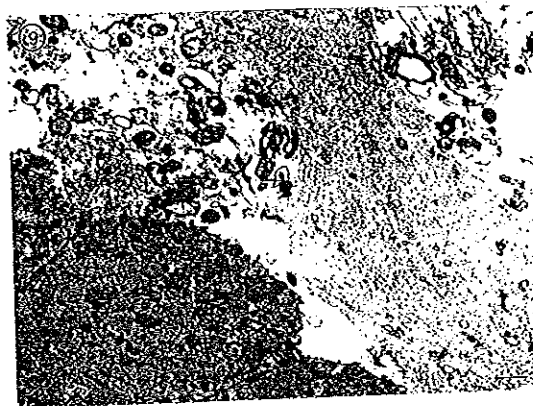


Fig. 13.9. TEM of a section of a ruthenium red-stained preparation of the bovine ileum showing the presence of bacteria associated with the mucous layer that is retained on the tissue surface in this preparation. Bar = 1 μ m. (From Cheng *et al.* 1981b).

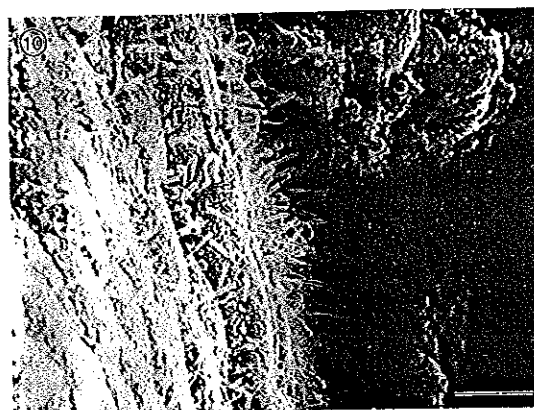


Fig. 13.10. SEM of a colon of a milk-fed Holstein calf showing the end-on association of rod shaped bacteria with the surface of this tissue. Bar = 1 μ m. (From Cheng & Costerton 1986).

(Fig. 13.8) and only a very small number of organisms grow immediately on the tissue surface (Savage 1977; Savage *et al.* 1968). Examinations of the lower digestive tract of ruminants have not yet been made using immunological methods for mucous retention, but some micrographs of these tissues show numerous bacteria in association with remnants of the mucous blanket (Fig. 13.9). In these preparations, small numbers of organisms are sufficiently adherent to, or embedded within, the tissue surface to remain associated with duodenal and colonic epithelia even after removal of the mucous blanket (Fig. 13.10).

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Bacterial recovery from these tissues has been plagued by the same loss of the mucous blanket and its epithelial embedded population, a technical problem that may account for the relatively low numbers and few species isolated from this region (Table 13.3).

Manipulation of biofilms to benefit ruminants

The digestive tract of the newborn ruminant constitutes a microbial 'vacuum' which may be colonized by beneficial autochthonous bacteria or by pathogens, such as the pathogenic *Escherichia coli* that cause neonatal scours (Fig. 13.11). Studies in our laboratory have shown that oral inoculation of newborn lambs with 36 autochthonous organisms, at 16 h and 14 days, resulted in complete bacterial colonization of the ruminal epithelium (Fig. 13.12). Inoculated animals exhibited a greater average weight gain (at 135 days), accelerated development of the upper digestive tract, and were more resistant to enterotoxigenic *E. coli* than uninoculated control animals reared under identical vivarium conditions (T. A. McAllister, K.-J. Cheng & J. W. Costerton, unpublished data). However, this laboratory inoculum failed to improve the formation of biofilms within the digestive tracts of inoculated lambs, compared with those in lambs that were allowed to develop

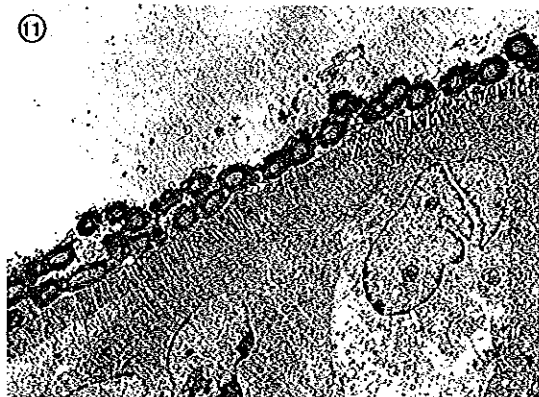


Fig. 13.11. TEM of a ruthenium red-stained preparation of the ileum of a newborn calf that had been infected with an enterotoxigenic strain of *E. coli* (ETEC). Note the heavy colonization of the microvillar (M) border of this tissue and the extensive matrix (X) surrounding the adherent bacterial cells (arrow).



Fig. 13.12. SEM of the rumen tissue surface of a newborn lamb reared for 4 months under vivarium conditions and inoculated orally at 16 h and 14 days after birth with a mixture of 36 strains of bacteria isolated from all regions of the bovine digestive tract. Bar = 1 μ m (From Cheng & Costerton 1986.)

natural microbial populations while suckling their mothers. Apparently these laboratory grown bacterial strains lose their ability to compete effectively with microorganisms that are adapted to forming functional biofilms under the more rigorous conditions of the natural environment.

Established biofilms are difficult to change by biological manipulation, but they can be altered by chemical manipulations that favour the induction of particular enzyme activities or the establishment of certain species within the digestive system. The production of nitrotoxin degrading enzymes may be induced by feeding nitrate and nitro-compounds (Majak *et al.* 1982), while methane production can be inhibited by halogenated compounds (Chalupa 1977; Demeyer & van Nevel 1975; Prins 1978). Ionophores are now routinely used to alter rumen fermentation to favour the production of propionate (van Nevel & Demeyer 1988).

Once a stable microbial population has been established, feed efficiency can be improved and digestive disturbances prevented by the manipulation of specific plant materials for digestion by existing microbial populations. For decades, physical and chemical processing have been used to improve the utilization of feed by ruminants. Many of these techniques were developed prior to an appreciation for the importance of microbial biofilms in feed digestion. When processing is examined in detail, it is evident that the

effectiveness of many processes is due to their ability to enhance the formation of biofilms on the surface of feed. Grinding and alkali treatment increase the number of adhesion sites, enhance the availability of nutrients for the biofilm (Latham *et al.* 1979; Kolankaya *et al.* 1985; Bowman & Firkins 1993) and increase the digestion of these feeds in the rumen. Biological delignification via white rot basidiomycetes has been shown to increase the *in vitro* digestibility of oat straw, presumably by breaking down microbial barriers and stimulating biofilm formation (Jung *et al.* 1992). In contrast, fat and formaldehyde inhibit the formation of biofilms and limit the microbial digestion of feeds (Stewart 1977; McAllister *et al.* 1990a). Many of the advances possible in physical and chemical processing of feed have probably already been made and future strategies to enhance the formation of digestive biofilms on plant tissues are likely to result from technologies in genetic engineering and plant breeding.

The too rapid release of nutrients that results from the bacterial digestion of bloat-causing legumes and finely ground cereal grains can be reduced by selecting plants for a slow initial rate of ruminal digestion and by processing grains to a coarse particle size. Conversely, the slow initial rate of ruminal digestion of grasses can be increased by breeding plants with cell walls that are more susceptible to digestion by rumen bacteria (Cherney *et al.* 1986). Selection for brown midrib mutants of maize, sorghum and pearl millet has resulted in plants that contain substantially lower (25–50%) lignin than normal genotypes and these plants have increased digestibilities *in vitro* and in dairy cows and sheep (Porter *et al.* 1978; Wedig *et al.* 1988). Recently, Watson (1990) has advocated using antisense RNA genes or catalytic RNA molecules as anti-lignin 'knock-out' genes to perturb the biosynthesis of lignin in alfalfa. The improved ruminal digestibility of these selected or genetically altered forages is most likely due to a reduction in the barriers to biofilm formation. Further characterization of natural plant compounds (e.g. condensed tannins) that inhibit the formation of digestive biofilms could provide additional selection criteria for plant breeding programmes or potential targets for genetic manipulation.

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