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Fermentation in the large intestine of single-stomached animals and its relationship to animal health

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The phasing out of antibiotic compounds as growth promoters from the animal industry means that alternative practices will need to be investigated and the promising ones implemented in the very near future. Fermentation in the gastrointestinal tract (GIT) is being recognized as having important implications for health of the gut and thus of the host animal. Fermentation in single-stomached animals occurs to the largest extent in the large intestine, mainly because of the longer transit time there. The present review examines the micro-ecology of the GIT, with most emphasis on the large intestine as the most important site of fermentative activity, and an attempt is made to clarify the importance of the microfloral activity (i.e. fermentation) in relation to the health of the host. The differences between carbohydrate and protein fermentation are described, particularly in relation to their endproducts. The roles of volatile fatty acids (VFA) and NH_3 in terms of their relationship to gut health are then examined. The large intestine has an important function in relation to the development of diarrhoea, particularly in terms of VFA production by fermentation and its role in water absorption. Suggestions are made as to feeds and additives (particularly those which are carbohydrate-based) which could be, or are, added to diets and which could steer the natural microbial population of the GIT. Various methods are described which are used to investigate changes in microbial populations and reasons are given for the importance of measuring the kinetics of fermentation activity as an indicator of microbial activity.

Gastrointestinal tract: Fermentation: Volatile fatty acids: Carbohydrates

Introduction

Intensification of the pig and poultry industries has brought increased risks of both clinical and sub-clinical enteric disease. Animals are vulnerable to potentially harmful micro-organisms

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Abbreviations: GIT, gastrointestinal tract; RS, resistant starch; VFA, volatile fatty acids.

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such as rotavirus, *Escherichia coli*, *Salmonella* spp., *Clostridium perfringens* and *Campylobacter sputorium*. In an attempt to control some of these problems there has been widespread use of in-feed antibiotics at both a therapeutic level (to combat disease) and at a sub-therapeutic level (as growth promoters). However, as a result of various public health scares associated with animal product consumption there is increasing consumer pressure to reduce such use of antibiotics. This has led to increased interest in alternatives to enhance both animal performance and disease resistance. Hence, there has been an increasing investment of time and money to investigate alternatives to maintain growth and performance of farm livestock, while at the same time considering the health, safety, and acceptability of the resultant animal products for the human diet (Ewing & Cole, 1994).

The large intestine is the most heavily colonized region of the gastrointestinal tract (GIT) and it is thought that interactions between bacteria are the most important factors which control the microflora of single-stomached animals (Drasar, 1988). The nature of bacterial interactions can be either antagonistic or synergistic. They can affect either the population level of a given strain, or its metabolic activity. Genetic transfers can also occur between strains within the gut. However, both the host (e.g. age, immune status, stress) and the diet can modulate the expression of the bacterial interactions (Raibaud, 1992) thus leading to changes in the activity of the whole flora.

Interest is now focusing on one of the natural defence mechanisms of the body which was first referred to as 'colonization resistance' by Van der Waaij *et al.* (1971). This mechanism is related to the population of non-pathogenic bacteria which are normally and naturally present within the GIT of all domestic animals and birds. It is defined as the resistance to colonization of the alimentary canal by newly ingested micro-organisms (Van der Waaij, 1989). There is a delicate balance between beneficial and pathogenic bacteria in the GIT, and many symbiotic and competitive interactions occur between them (Ewing & Cole, 1994).

After the initial colonization of the newborn animal at birth the bacterial population of the GIT remains fairly stable during suckling, although qualitative changes may occur (Drasar, 1989). However, the introduction of solid food causes major qualitative and quantitative alterations in flora as the strict anaerobes such as *Bacteroides* gain ascendancy with a concomitant decline in levels of the facultative organisms (Savage, 1977; Mallett & Rowland, 1988). Hence, the weaning process can leave young animals vulnerable to the presence of potentially pathogenic micro-organisms.

There is increasing evidence to suggest that, in a healthy adult host, the composition of the GIT microflora (as measured by molecular genetics techniques such as polymerase chain reaction–denaturing gradient gel electrophoresis) remains remarkably stable (Zoetendaal *et al.* 1998). However, there is also evidence to suggest that changes in the diet (particularly in relation to the fermentable fractions) can lead to changes in microbial activity (Djouzi & Andrieux, 1997). For example, it has been shown that an excess of protein reaching the large intestine can lead to increased NH_3 concentrations in the colon and diarrhoea in early weaned piglets (Dong *et al.* 1996).

The present review examines the micro-ecology of the GIT, with most emphasis on the large intestine as the most important site of fermentative activity, and an attempt is made to clarify the importance of the microfloral activity (i.e. fermentation) in relation to the health of the host. Suggestions are then made as to feeds and additives which could be or are added to diets and which could steer the natural microbial population of the GIT. Various methods are also described which are used to investigate changes in microbial populations and reasons are given for the importance of measuring the kinetics of fermentation activity as an indicator of microbial activity.

Large-intestinal fermentation in single-stomached animals

The alimentary tract extracts nutrients from the ingested diet, after which they are absorbed into the bloodstream. It therefore represents an interface between the metabolism of the animal and the external environment. Hydrolysis by enzymes is the most important mechanism for digestion in the small intestine. However, digestion also occurs by fermentation, an aspect of digestion most relevant to the large intestine of single-stomached animals (Ewing & Cole, 1994).

The large intestine comprises the colon and caecum. Until recently, the principal physiological functions of the large intestine have been considered to be the conservation of water and electrolytes (Dobbins & Binder, 1981; Ramakrishna *et al.* 1990), provision of a controllable route for the excretion of waste products of metabolism and toxic substances, and safe containment of the micro-organisms present (Cummings, 1983). However, it is now realized that one of the principal functions must include the salvage of energy and nutrients through its symbiotic relationship with the GIT microflora. Whereas the time taken for the intestinal contents to travel the length of the small intestine is only 2–4 h in man, the large bowel transit time is normally 20–80 h, so there is ample time for development and activity of the microflora. Although digestion by endogenous enzymes and absorption from the small intestine are very effective, there is still a constant supply of nutrients to the large intestine in the form of undigested dietary components, host enzymes, and desquamated gut mucosal cells. The absence of glucose means that inducible enzymes are produced by the microflora, which facilitates utilization of indigestible nutrients such as resistant starch (RS) and NSP (Bugaut, 1987).

The colonic microflora is a complex ecosystem largely consisting of anaerobic bacteria, which outnumber the facultative organisms at least 100:1. The total bacterial count in faeces is 10^{10} to 10^{12} colony-forming units per millilitre (Eastwood, 1992). The microflora of a single individual can consist of more than 400 bacterial species (Holdeman *et al.* 1977). There is substantial evidence to suggest that the indigenous microflora of the GIT is, in large part, influenced by the genotype of the host, be it between species (Hackstein *et al.* 1995), or even between individuals within a species (Van der Waaij, 1987).

Carbohydrate fermentation

Bacteria in the colon metabolize available carbohydrate to obtain energy for their own growth and maintenance (including motility, enzyme synthesis, maintenance of ionic and osmotic gradients, and active ion transport). Such an anaerobic fermentation has been summarized by the following general equation (Ewing & Cole, 1994):

$$57.5\ C_6H_{12}O_6 + 45\ H_2O \rightarrow$$
$$65\ \text{acetate} + 20\ \text{propionate} + 15\ \textit{n}\text{-butyrate} + 140\ H_2 + 95\ CO_2 + 288\ \text{ATP}.$$

A general molar ratio of these acids is 1 acetate:0·31 propionate:0·23 *n*-butyrate, though the proportions can and do vary, depending on the type of substrate available, the composition of the anaerobic flora, and the prevailing pH. However, the equation shows that 73 % of the C units and 68 % of the energy value of fermented carbohydrate can reappear in the form of completely metabolizable volatile fatty acids (VFA). Such a preservation of metabolic energy has major implications for the maintenance of the colonic bacterial population, the metabolic needs of the colonic epithelium and for the 'energy salvage' from malabsorbed carbohydrate by means of colonic VFA absorption (Soergel, 1994).

The colon rapidly absorbs free VFA (Ruppin *et al.* 1980), thus conserving energy and

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reducing the osmotic load. Henning & Hird (1972), McNeil *et al*. (1978) and Heneghan (1988) have shown that bacterial VFA are absorbed in the colon along a concentration gradient from lumen to serosa without evidence of active transport. VFA are an important fuel for large intestinal colonocytes, with butyric acid being the most important (Heneghan, 1988). Disregarding regional differences along the length of the colon, epithelial cells prepared from the entire length of the colon revealed a preference of metabolic fuels in the following order: VFA, ketone bodies, amino acids, glucose (Roediger, 1982). This is in contrast to the cells of the small intestine, where the preference for metabolic fuels is: glutamine, glucose, and ketones in ruminants (Britton & Krehbiel, 1993). These differences are not surprising given that the cells of both regions have undoubtedly adapted to the fuels available. For example, while glucose is potentially available from the intestinal blood supply it is unlikely that significant amounts of glucose will reach the large intestine via the digesta.

Dietary protein

As carbohydrate sources (starch and other fermentable carbohydrates) become depleted due to fermentation in the large intestine, carbohydrate:N of the caecum decreases and the fermentation becomes more and more proteolytic (Piva *et al.* 1995). Hence, some of the intestinal VFA may originate from polypeptides which are apparently the major source of the mainly branched-chain VFA (isobutyrate, valerate, and isovalerate) which are formed by the metabolism of branched-chain amino acids such as valine, leucine and isoleucine (Macfarlane *et al.* 1992). A proteolytic fermentation can also lead to the formation of such potentially toxic metabolites as NH_3, amines, volatile phenols and indoles (Yokoyama *et al.* 1982; Russell *et al.* 1983; Macfarlane *et al.* 1992) which are found only in small amounts in the healthy colon (Rasmussen *et al.* 1988). In fact, deamination of amino acids from both dietary or endogenous protein is the main source of NH_3 in the colon (Wrong & Vince, 1984). NH_3 generated in the colon readily passes across the gut wall thereby gaining access to other tissues of the body (Rowland, 1992). NH_3 can disturb the development of the mucosa of the intestine (Visek, 1984) and has been reported to be negatively correlated to reduced villus height (Nousiainen, 1991). Furthermore, NH_3 produced and absorbed must be excreted as urea with an energy cost of about 7 % of total energy expenditure in single-stomached as well as in ruminant animals (Eisemann & Nienaber, 1990). Sub-acute levels of NH_3 may influence metabolism of the host and result in reduced animal performance (Piva *et al.* 1995). Microbially generated amines can alter the amount of acid and bicarbonate added from the gut mucosa, and the rate of transit through the bowel (Calloway, 1968).

However, microbial growth is stimulated by the presence of fermentable carbohydrate. This leads to an increased demand for amino acids by micro-organisms (Hawe *et al.* 1991), as indicated by reduced protein catabolism in the hindgut and hence lower NH_3 concentrations and urinary N excretion (Mason *et al.* 1977; Misir & Sauer, 1982). Therefore, the production of potentially harmful compounds, such as tryptophan catabolism to skatole, would be less likely in the presence of a carbohydrate source. Similarly, there is also evidence which suggests that N excretion as urea in urine can be shifted to N excretion as bacterial protein in the faeces by the inclusion of fibrous feedstuffs in the diet (Kirchgessner *et al.* 1993). The bacterial requirement for N for growth can even result in the diffusion of urea from the blood of the animal into the GIT lumen. Work by Canh *et al.* (1997) showed that an increased dietary NSP content in the diet influenced the partitioning of N between the urine and faeces, with important implications for the intensive pig industry in terms of the N contents of piggery slurry.

The influence of host diet on gastrointestinal tract fermentation

The microbiological environment found within the GIT is extremely complex. The host, its diet and the micro-organisms themselves all influence the life and activities of the GIT microflora. Factors which can influence the GIT microflora include: the concentration of endogenous nutrients, antibodies, bacterial interactions and metabolites, disease, redox potential, drugs, pH, the age of the host.

The addition of prebiotics (additives which stimulate the beneficial micro-organisms in the host) to feeds is increasingly being practised in both the food and feed industries. Stewart *et al*. (1993) summarized the potential beneficial effects of prebiotics as being: antagonism towards pathogens, competition with pathogens, stimulation of enzyme reactions, decreases in NH_3 and phenol production, increased colonization resistance.

Anti-microbial growth promoters

Supplementation of animal feeds with sub-therapeutic levels of antibiotics has been used extensively in animal production as it is purported to increase growth rate, especially in chickens and pigs. The exact mechanism by which anti-bacterial growth promoters exert their stimulatory effect is still unknown. However, Yokoyama *et al*. (1982) proposed that primary involvement of the GIT microflora could occur via the following mechanisms: (l), suppression of pathogens responsible for sub-clinical infections; (2), reduction of bacterial production of growth-depressing toxins; (3), reduction of bacterial destruction of essential nutrients. This led to the suggestion that the stimulation of animal growth could be due to a beneficial shift in intestinal bacterial metabolism.

Exposure of animals to antibiotics such as penicillin and tetracycline also exerts a selective pressure in the GIT environment which allows the proliferation of drug-resistant strains of bacteria, particularly *E. coli* (for review, see Savage, 1980). In most cases, the drug resistance is encoded on plasmids which may be transferred to other bacteria even in the absence of selection pressure by antibiotics. The implications of antibiotic-induced transferable drug resistance in intestinal bacteria for human and animal health and for treatment of disease are obvious and much discussed (Savage, 1980) and have led to restrictions on the use of anti-bacterial growth promoters in animal feeds (Mallett & Rowland, 1988).

It is becoming increasingly clear that other substances can also stimulate the beneficial microflora of the GIT and therefore the host's own immune system, which can then act to inhibit any potential pathogenic species.

Dietary carbohydrates

The amount and composition of substances reaching the large intestine can be readily modified by diet and, in terms of bacterial substrates, it is probably the carbohydrate fraction (oligosaccharides, NSP and starches) which is most important (Mathers & Annison, 1993). It is thought that the carbohydrate fraction can stimulate the growth of certain micro-organisms which produce VFA and use NH_3 as their source of N as described above. Some specific compounds can have more precise effects on particular microbial species. For example, substances such as mannose (Ofek *et al.* 1977) and galactan (Mathew *et al.* 1993) have been found to block adherence of pathogenic *E. coli* strains, though the exact mechanism for this action is still poorly understood.

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Oligosaccharides. Recently, it has been shown that oligosaccharides may have the ability to specifically promote the proliferation of *Bifidobacteria* in the colon (Djouzi & Andrieux, 1997) which are believed to be beneficial to the health of the GIT (Crittenden & Playne, 1996). Oligosaccharides vary in the dose required to stimulate the *Bifidobacteria* though most have been demonstrated to increase *Bifidobacteria* numbers in the human colon at doses of <15 g/d (Ito *et al.* 1993; Tamura *et al.* 1993; Gibson *et al.* 1994; Kaneko *et al.* 1994; Tomomatsu, 1994). Apart from stimulation of specific bacterial species it has also been suggested that oligosaccharides in the feed may mimic those present on the gut wall in terms of microbial attachment. The idea is that if pathogenic species attach to the oligosaccharide which is not part of the gut wall the organism will then be passed out with the digesta (Ewing & Cole, 1994).

Lactulose is a disaccharide of galactose and fructose (0-β-D-galactopyrdanosyl-1-4-D-fructofuranose) used in studies of gut function in man (Pomare *et al.* 1985). It largely escapes digestion in the small intestine but is completely fermented in the colon (Saunders & Wiggens, 1981) unless given in doses of 20 g or more. The data of Florent *et al.* (1985), investigating the chronic addition of lactulose to the diet, showed that this ingredient led to a shift in microbial activity of the GIT. This was suggested to be by a quicker bacterial catabolism of lactulose. H_2 breath excretion (used to measure colonic fermentation *in vivo*) decreased whereas the molar ratio acetic acid:total VFA and the concentration of lactic acid in caecal contents increased significantly. Such a decrease in the pH of caecal contents has been shown to favour the growth of lactic acid-producing bacteria such as *Lactobacillus*, *Bifidobacterium*, and *Eubacterium* species (Gottschalk, 1979; Hill, 1983).

There are four other classes of oligosaccharides which are frequently considered:

(i) Transgalactosylated oligosaccharides which are a mixture of tri-, tetra-, penta- and hexasaccharides (of galactose and glucose) obtained when lactose is enzymically hydrolysed by *Aspergillus oryzae*. This sugar can be utilized by all *Bifidobacterium* species tested and by some *Lactobacilli*, *Bacteroides*, *Streptococci* and *Enterobacteria* (Tanaka *et al.* 1983).
(ii) Soyabean oligosaccharide extract is prepared from defatted soyabean whey and comprises a mixture of sucrose (44 %), stacchyose (23 %), raffinose (7 %) and monosaccharides. The efficiency of utilization of soyabean oligosaccharide extract by intestinal bacteria *in vitro* was greatest for *Bifidobacterium* compared with species of *Lactobaccillus*, *Mitsuokella* and *Bacteroides* (Hayakawa *et al.* 1990).
(iii) Fructo-oligosaccharides. The commercial preparation is a mixture of tri-, tetra- and pentasaccharides (glucose and fructose) produced from sucrose using the enzyme β-fructofuranoside (obtained from the fungus *Aspergillus niger*). It is utilized by most *Bifidobacteria*, *Bacteroides* and some *Streptococci*, *Lactobacilli* and *Enterobacteria*, but not *E. coli* (Hidaka *et al.* 1986).
(iv) Mannan-oligosaccharides. Mannan sugars may be linked to form oligosaccharides which are thought to have growth-enhancing properties for poultry (Savage & Zakrzewska, 1996). It has also been suggested that these derivatives of yeast cell walls can enhance the function of the non-specific immune system (Spring & Privulescu, 1998) of single-stomached animals, though the mechanisms for this are not yet clear.

There is increasing evidence to suggest that some oligosaccharides are completely fermented either in the terminal ileum (fructo-oligosaccharides) or the proximal large intestine (transgalactosylated oligosaccharides), and so are unavailable for micro-organisms in the distal colon (Houdijk, 1998).

Resistant starch. RS consists mainly of amylose and is, by definition, that fraction of starch which escapes enzymic digestion in the small intestine (Englyst & Cummings, 1985; McBurney *et al.* 1988; Blakeney, 1993; Marsono *et al.* 1993; Asp *et al.* 1996). Such starch is

potentially a good substrate for colonic fermentation (Ramakrishna, 1996). The presence of RS in feeds is related to many factors including amylose:amylopectin, the granule structure of the starch, the physical form of the feed, the effects of processing and the presence of NSP, amylase inhibitors, lectins and phytate (Cummings & Englyst, 1987). For example, the RS content of legumes is high (Goodlad & Mathers, 1990).

Mason & Just (1976) found that feeding potato starch to pigs, which is known to be digested by large-intestinal bacteria (Baker *et al.* 1950), resulted in a higher proportion of butyric acid in the total VFA than for ingested maize starch, which is almost completely hydrolysed in the small intestine. Mallett *et al.* (1988), using two different RS supplements in the diets of rats, found a decrease in the caecal content pH of 1–2 units and an associated reduction in NH_3 concentration. Potato starch significantly decreased the concentration of VFA in the rat hindgut while amylomaize supplementation increased propionic and butyric acids but led to a decrease in the occurrence of minor, branched-chain fatty acids. There is now also evidence that giving such a RS orally to human patients with cholera increases faecal VFA concentrations and shortens the duration of diarrhoea (Ramakrishna *et al.* 2000). However, it has been shown that the dose of added RS can also be important in terms of determining the pattern of fermentation endproducts (Mathers *et al.* 1997), whereby the addition of small doses of raw potato starch (80 g/kg) to rat diets led to increased butyrate while higher doses (>80 g/kg) led to the opposite effect.

NSP. NSP are principally the polysaccharides of the plant cell wall and include all non-α-glucan polymers in the diet such as cellulose, pectin and hemicellulose (Cummings & Englyst, 1987). The Englyst procedure measures dietary fibre as NSP in plant foods. Starch is removed enzymically and NSP is measured as the sum of its constituent sugars released by acid hydrolysis. The sugars may, in turn, be measured by GLC, giving values for individual monosaccharides. A value is obtained for total dietary fibre and, if required, for soluble and insoluble dietary fibre (Cummings & Englyst, 1987).

Many studies using NSP have been carried out using growing pigs, though different parameters have been evaluated by different workers. For example, Annison & Topping (1994) showed that short-chain fatty acid concentrations were increased by feeding NSP-containing feeds. The concentration of VFA in the contents of the upper part of the colon of pigs given different types of fibre was also shown to vary with the type of fibre fed by Stanogias & Pearce (1985). Von Glawischnig (1990) fed different sources of NSP to stimulate the indigenous microflora of the piglets. They found that 25–30 % wheat bran in the creep feed led to the best results in terms of reduced incidence of diarrhoea and recommended this level for routine use in pig herds.

Feeding isolated NSP sources (Tulung *et al.* 1987; Walter *et al.* 1988) or diets rich in NSP and RS (Cheng *et al.* 1987; Goodlad & Mathers, 1990; Mathers *et al.* 1990; Key & Mathers, 1993) readily provoked substantial changes in caecal VFA pattern in the rat. In some cases, there appears to be a strong linear relationship between the amount of substrate supplied and the molar proportions of the major VFA. This supports the contention derived largely from *in vitro* studies, but with *in vivo* support, that the chemical composition of the substrates does control, to some extent, the pattern of bacterial endproducts. Edwards (1996), in a microbiological study, found that the use of fermentable fibres in piglet feeds enhanced intestinal populations of *Lactobacilli* and reduced the incidence and severity of diarrhoea.

Pectins are structurally based on a polymer of galacturonic acid residues with additional rhamnose and arabinose substituents, in which a variable proportion of the uronic carboxyl groups are esterified with methanol (Adrian, 1976). The physical properties of pectin include its water-holding capacity as well as its ability to absorb bile salts, to form gels and to distend the

intestine (Roth *et al.* 1995). Pectins are extensively degraded within the GIT and are a ready source of fermentable carbohydrate for GIT micro-organisms (De Wilde, 1980; Holloway *et al.* 1983). The increase in available energy within the gut may result in changes in the diversity of bacterial species or in their metabolic capabilities (Mallett & Rowland, 1988). Roth *et al.* (1995) showed that the addition of pectin to the diet improved colonic water absorption, probably as a result of the VFA produced.

Inulin is a naturally-occurring storage oligomer of fructose found in many plants such as onion, garlic, artichoke and chicory (Yazawa *et al.* 1978). The degree of polymerization of inulin may range from 2 to 60 with oligofructose defined as the fraction of inulin with a degree of polymerization below 20 (Wang & Gibson, 1993). These substances have been shown to be resistant to the degradative properties of the GIT and therefore reach the colon in an intact form (Rasmussen *et al.* 1988). They may therefore be available as substrates for the large intestinal microflora. Bacterial growth data showed that both oligofructose and inulin exerted a preferential stimulatory effect on colony counts for the genus *Bifidobacterium*, whilst maintaining populations of potential pathogens (*E. coli*, *Clostridium*) at relatively low levels (Wang & Gibson, 1993). Roland *et al.* (1995) reported that inulin consumption led to high amounts of H_2 but no CH_4, to a four-fold greater caecal concentration of butyrate than for other fibres and to a decrease in caecal pH, all of which indirectly also suggested a change in the GIT microflora.

Benefits of gastrointestinal tract fermentation for animal health

The microflora as a whole has a trophic effect on the epithelium which can lead to a faster turnover rate. It also provides a continuous stimulus to the local lymphoid tissue and protects against pathogenic bacterial species which have to compete with the indigenous microflora for nutrition and binding sites (Liebler *et al.* 1992).

The production of fermentation endproducts such as VFA are not the only benefits of GIT fermentation in terms of animal health. The use by the microflora of compounds such as NH_3 also has an important effect as it leads to decreased amounts of those compounds both in the intestinal contents and in the blood. It is also known that the presence of an active microflora can discourage colonization by potentially pathogenic species by the mechanism known as 'colonization resistance'.

'Colonization resistance'

In combination with the host, the indigenous microflora of the whole tract plays an important role in the development of colonization resistance (Van der Waaij, 1989). The effect of colonization resistance differs between microbial species and may even differ between strains of the same species. There are several mechanisms involved, both of microbial and host origin. It is not yet clear which bacterial groups in the indigenous flora are involved and the biochemical mechanisms are still mostly unknown. However, factors such as microbial production of bacteriocins, VFA, extra-cellular enzymes and competition for nutrients and attachment sites, all appear to be of some importance (Hentges, 1986). The host factors contributing to colonization resistance involve peristaltic movement, the secretion of mucus, epithelial cell desquamation, the 'gut associated lymphoid tissue' producing IgA and non-specific adherence blocking factors, and the nutrient composition and flow of digesta (Stewart *et al.* 1993). Several of these host factors can also be modulated by the indigenous microflora (Van der Waaij, 1989). The

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review by Erickson & Hubbard (2000), describing immunomodulation by probiotics in human diets, provides a valuable overview of some of the effects that manipulation of the GIT microflora can have upon the immune system, a research subject which will be of vital importance in the future.

Volatile fatty acids

Acetate, propionate and *n*-butyrate, and the gases H_2, CH_4 and CO_2, which can be detected in breath (Tsuji *et al.* 1992), are the major endproducts of fermentation in the large intestine (Bugaut & Bentéjac, 1993). Formate, valerate, caproate and the branched-chain acids isobutyrate and isovalerate (Macfarlane *et al.* 1992) also occur in the large intestine, though in smaller proportions (10–20 mol/100 mol total VFA), usually as a result of the fermentation of protein which has escaped digestion in the small intestine. VFA are present at total concentrations ranging from about 30 to 190 mM in the faeces of individuals consuming an 'ordinary' diet (Savage, 1986), irrespective of the hindgut size and the herbivorous status of mammalian species (Bugaut, 1987; Rechkemmer *et al.* 1988; Bergman, 1990). They are the predominant anions in the large intestine of mammals and create a slightly acidic pH level (6·0–7·0) (Bugaut, 1987; Cummings *et al.* 1987). Lactic acid is an intermediary product of carbohydrate fermentation and accumulates only when VFA production is inhibited in an acidic milieu of pH less than 5·5 (Soergel, 1994).

The diet can lead to large differences in colonic VFA production (McBurney *et al.* 1988). For example, starch fermentation has been shown to yield high butyrate proportions (Englyst *et al.* 1987; Goodlad & Mathers, 1988; Scheppach *et al.* 1988), in contrast to fermentation of the more oxidized substrate pectin, where acetate is the major endproduct (Englyst *et al.* 1987; Goodlad & Mathers, 1988). Englyst *et al.* (1987) fermented a number of different polysaccharides and reported the quantities and proportions of VFA produced from starch, arabinogalactan, xylan and pectin.

Substrate redox state and availability, fermentation rate, transit time through the colon and the composition of the bacterial population can all affect the percentage amounts of VFA produced (Allison & Macfarlane, 1989). VFA can have an antibacterial effect, thereby preventing the establishment of pathogenic bacteria, such as *Salmonella* spp. (Cummings, 1983). AL Sutton, AB Scheidt, DM Forsyth, AG Mathew, JA Patterson, DT Kelly and KA Meyerholz (unpublished results) showed that the addition of propionic acid to the diet of weaning piglets led to reduced *E. coli* counts in ileal contents, while counts for *Lactobacilli* remained unaffected. However, Hentges (1983) suggested that the evidence for an effect of VFA is controversial. Freter *et al.* (1983) showed that much of the controversy could be resolved by interpreting published data to show that fatty acids prolong the lag phase of bacterial multiplication but have little or no effect on the subsequent growth rate exhibited during colonization. In later studies (e.g. Que *et al.* 1986), the *in vivo* antibacterial mechanisms did allow *in vivo* multiplication to constant populations after an initial lag phase. Moreover, in broth containing fatty acids there was a prolonged lag phase followed by logarithmic growth; the latter was not affected by the added fatty acids (Freter, 1992). The data of Rolfe (1984), on the other hand, did show a rapid death of *Clostridium difficile* in broth containing VFA.

The production of VFA has several possible actions on the gut mucosa. All VFA are readily absorbed by the colonic mucosa but only acetic acid reaches the systemic circulation in appreciable amounts via the liver (Cummings & Englyst, 1987), from whence it can be metabolized by muscle (Knowles *et al.* 1974). Butyric acid is metabolized by the colonocytes but has also

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been measured in the portal blood (Goodlad & Mathers, 1990). Propionic acid is metabolized in the liver (Eastwood, 1992) where it inhibits gluconeogenesis. VFA have a stimulatory effect on Na (between 50 and 80 % of total absorption; Roediger, 1989*b*) and water absorption from the colonic lumen, which is thought to be related to the recycling of H ions (Cummings & Englyst, 1987).

The large quantities of VFA formed by fermentation of carbohydrates are also thought to induce HCO_3^- secretion into the lumen, which is an important buffer for the regulation of luminal pH. In fact it has been shown that under certain pathological conditions a low luminal pH was associated with decreased VFA and increased lactate concentration in the lumen (Vernia *et al.* 1988; Roediger, 1989*a*) which occurred due to a shift to lactic acid-producing bacteria. Dohgen *et al.* (1994) showed that, in contrast with VFA, lactate does not stimulate HCO_3^- secretion.

The colonic epithelium can actively absorb Na against electrochemical gradients, giving it the capacity to dehydrate the faeces. Some of the major stimuli for Na absorption in the mammalian colon are VFA. They have been shown to enhance Na absorption from the normal human colon both during *in vivo* perfusion of the whole colon (Roediger & Moore, 1981) and in a system where the intact rat colon was perfused *in vitro* (Radcliffe *et al.* 1987). In the latter, VFA increased Na absorption approximately five-fold over the control. Of the VFA, butyrate is more effective than acetate or propionate in enhancing Na absorption (Roediger & Moore, 1981). This capacity is of considerable importance in limiting fluid losses in those acute diarrhoeal illnesses which are primarily characterized by small intestinal fluid secretion (Ramakrishna, 1996). K, on the other hand, may be either absorbed or secreted by the colon, depending on the blood to lumen gradient (Salas-Coll *et al.* 1976).

Butyric acid. Butyrate is preferentially metabolized by colonocytes (Roediger, 1982) resulting in portal butyrate levels that are much lower relative to concentrations in colonic contents (Cummings *et al.* 1987; Bourquin *et al.* 1993). It accounts for about 70 % of total energy consumption of the colon (Smith & German, 1995). Acetate, on the other hand, has been shown to effectively increase colonic blood flow, O_2 uptake and stimulation of motility in isolated ileal loops (Scheppach, 1994).

Additionally, in a feeding study in pigs reported by Gálfi & Bokori (1990), a daily diet containing 0·17 % sodium *n*-butyrate increased the blood plasma content of *n*-butyrate from 13·9 μmol/l to 27·1 μmol/l compared with animals fed unsupplemented diets. *n*-Butyrate also appeared in the stomach (2·9 μmol/l) and increased the average daily body mass gain of pigs by 23·5 %, with a concomitant feed consumption increase of 8·9 % (Gálfi & Bokori, 1990). In a later *in vivo* study with weaning piglets, Gálfi & Neogrády (1996) found that increasing butyrate concentration in the feed led to increased numbers of *Lactobacilli* while *E. coli* decreased, in all sections of the GIT. Consequently, the authors concluded that there was a selective antimicrobial effect of *n*-butyrate. They classified the butyrate sensitivity of microbes, as follows: (1), *n*-butyrate sensitive microbes: *Clostridium acetobutylicum*, *E. coli*, *Streptococcus cremoris*, *Lactococcus lactis* and *L. cremoris*, and *Salmonella* spp.; (2) *n*-butyrate- and pH-less-sensitive microbes: *Lactobacillus* spp. and *Streptococcus bovis*. It was also found that butyrate has a growth promoting action on the wall of the digestive tract.

Given that the salvage function of the colon for absorption of unabsorbed Na and water from the small intestine depends on the metabolic integrity of the colonic epithelial cells (Roediger, 1994), it is not unreasonable to conclude that butyric acid has an important role to play in the prevention of diarrhoea.

Diarrhoea and gastrointestinal tract fermentation

A major problem of the intensive pig industry is the occurrence of diarrhoea in weaning piglets associated with increased mortality (Bruininx & van der Peet-Schwering, 1996). Pathologists are often tempted to account for the occurrence of diseases by the presence or absence of some specific microbiological agent. Much useful information has been gained in this way. For example, Sutton & Patterson (1996) reported that within 2 d of weaning, numbers of *Lactobacilli* in the intestines had decreased and *E. coli* numbers had increased. The numbers of pathogens present can indeed be important when a major infectious agent is introduced into a susceptible herd. However, the relationship between the infection and the disease is rarely simple. One can distinguish two kinds of diarrhoeic disease: the monofactorial (e.g. transmissible gastroenteritis virus) and the multifactorial (e.g. Colibacillosis or rotavirus infection, especially after weaning). For the multifactorial diseases (Vannier *et al.* 1983) the clinical signs and lesions are induced by an infectious agent which can be considered as a final 'effector' whose multiplication depends on factors on the farm which disturb physiological or immunological mechanisms of regulation (e.g. humoral immunity, clearance, and intestinal motility). Table 1 shows a list of the potential causes of diarrhoea in piglets cited by various authors.

Diarrhoea is an excessive loss of fluid in the faeces. For many years it was considered that this fluid loss originated primarily from the small intestine, either due to excessive secretion of fluid and electrolytes (as in cholera in humans), or to impaired absorption due to reduced villus absorptive area (as in rotavirus diarrhoea). The close interrelationship between colonic absorptive capacity and development of diarrhoea led to the concept of diarrhoea as a 'failure of colonic salvage' (Read, 1982). Drochner *et al.* (1987) showed that piglets of 8 weeks or more absorbed water at a rate of 18·5 ml water/kg body weight per h, while other workers (Skadhauge, 1985) have shown that this is much less for newborn piglets. The reserve capacity of the colon to absorb fluid is critical in determining the magnitude of fluid losses in diarrhoeal disease. This means that diarrhoea would occur only if the small bowel outflow into the caecum was greater than the maximum capacity of the colon. On the other hand, if colonic absorption were decreased in acute diarrhoeal illness, then diarrhoea would occur with much lower levels of small bowel secretion. This can happen in diseases such as *Shigella* dysentery where small bowel outflow is essentially normal, but colonic absorption is poor.

Table 1. Suggested causes of post-weaning diarrhoea in pigs

Suggested cause	Age of pig affected	Reference
Environmental conditions		
Large litter size		Madec & Josse (1983)
Infectious		
Escherichia coli	Neonate and post-weaning	Linton *et al.* (1978); Renault *et al.* (1982)
Clostridia	Neonate	Taylor & Bergeland (1992)
Rotavirus	Neonate and post-weaning	Paul & Stevenson (1992)
Coronavirus (transmissible gastroenteritis)	Neonate	Saif & Wesley (1992)
Nutritional		
Age or weight at weaning		Svensmark *et al.* (1989)
Excess protein in the diet		Vannier (1983)
Hypersensitivity to dietary antigens		Newby *et al.* (1984)
Overfeeding		Makkink (1993); Bertshinger *et al.* (1978)

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Colonic VFA formation from fermentable carbohydrate is important for the maintenance of morphological and functional integrity of the colonic epithelium. Carbohydrate-induced diarrhoea can only occur when the amount of carbohydrate entering the colon exceeds its fermentation capacity (Soergel, 1994). When this happens, intact carbohydrate appears in the faeces; the resulting increase in the osmotic load leads to additional water in the colonic contents and diarrhoea can ensue. While enterotoxin-mediated diarrhoea is commonly considered to originate in the small intestine, *E. coli* heat-stable toxin and the cholera toxin also induce colonic fluid secretion. In the rat, addition of VFA to the lumen reverses the secretory response to cholera toxin and *E. coli* heat-stable toxin in the colon but not in the ileum (Ramakrishna *et al.* 1990). Clinical recovery is paralleled by a steep rise in stool VFA concentration and increased colonic Na absorption (Ramakrishna & Mathan, 1993). Infection with *Salmonella typhimurium* is one case where lesions are mainly confined to the caecum, colon and rectum.

Argenzio *et al*. (1984) showed that 3-d-old piglets with transmissible gastroenteritis had a complete absence of net fluid absorption from the colon. Similarly-affected piglets of 3 weeks of age, even with marked fluid secretion in the small intestine, had a six-fold increase in fluid absorption in the large intestine, with no detectable diarrhoea in the infected animals. This implies that if the large intestine functions to its maximum capacity it can readily absorb fluids so that diarrhoea need not occur. Analysis of luminal contents indicated that in older pigs unabsorbed carbohydrates from the small intestine were almost completely fermented to short-chain fatty acids in the colon whereas in younger pigs carbohydrate passed through the colon virtually unchanged. This demonstrated that the development of microbial fermentation, together with rapid VFA absorption, is a primary feature of colonic compensation in older pigs with transmissible gastroenteritis. It is a clear example which demonstrates that colonic absorption relies to a large extent on host–microfloral interactions.

Methods to measure fermentative activity

In the past, investigations of causes of diarrhoea were based on microbial counts of known pathogens. The weaknesses of this methodology became obvious, however, when it was realized that the pathogens could also be isolated from a healthy animal. It was concluded that the interaction between host and micro-organisms, and that between the micro-organisms, were probably of greater importance than simply the presence or absence of certain individual species.

It has been shown that the metabolic activity of the conventional flora can be sensitive to changes in diet (Rowland & Wise, 1985) though the species composition (according to viable counts) appears to be less affected by changes in the diet, at least with respect to the populations of the major genera. This is a major disadvantage of traditional counts; it is impossible to count more than eight to ten of the hundreds of species present at any one time (Vaughan *et al.* 2000). It has been concluded (Freter, 1992) that while the strict anaerobes are absolutely necessary for the proper functioning of the large-intestinal flora, it is unlikely that only one or a few of these anaerobes are of predominant importance. Rather, each strain has to fill its own distinct ecological niche. It has even been suggested that a few key micro-organisms present in comparatively small numbers may have key roles to fulfil in the function of the total microflora (RA Prins and CS Stewart, unpublished results). An example of why microbial counts are not always useful to investigate microflora activity was reported in a study by Florent *et al*. (1985). They measured the effects of an oral load of non-absorbable sugars, and failed to show any significant modification of bacterial flora populations, which was in agreement with findings of Finegold & Sutter (1978) and Hentges (1978). However, they pointed out that studies of

bacterial enzymes, on the other hand, had revealed marked changes (Hill, 1983; Simon & Gorbach, 1984). Consequently, there is now increasing interest in methods which can indicate microbial activity of a whole population.

Microbial counts. The bacteria found in the gut are of diverse types and the problems of their identification are the same as those for bacteria from other environments. However, some of the classical, even if arbitrary, divisions used to classify bacteria cannot be applied easily to them; for example, the gram stain is often of little value because some cultures lose their gram-positivity after as little as 2 h, and many isolates are very pleomorphic (Clarke, 1977). Colony and dilution counts on pure cultures of bacteria are invariably lower than total microscopic counts (Clarke, 1977). This was previously thought to occur because there were large numbers of dead cells in a culture. Certainly, direct counts of gut bacteria are usually considerably higher than viable counts (Hungate, 1966; Moore & Holdeman, 1974; Salanitro *et al.* 1974).

There are other explanations for the discrepancies. Considerable differences between counts may occur because any single medium will not grow all bacteria normally present in the gut. This is a major problem for the assessment of viable bacterial numbers in any natural environment. Colony counts of anaerobic rumen bacteria are influenced markedly by the methods and media used (Hungate, 1966; Grubb & Dehority, 1976). Another source of error is attachment of bacteria to solid particles. This is a particular problem in the GIT, where cellulolytic and other bacteria can be firmly attached to plant fragments. Further errors may arise because of clumping of bacteria. Bacteria in freshly voided swine faeces, for example, may occur as discrete pure colonies rather than as single cells (Rall *et al.* 1970).

Techniques from the field of molecular biology (such as comparative sequencing and molecular probes) are increasingly being used to explore the GIT microflora (Zoetendaal *et al.* 1998; Vaughan *et al.* 2000). These techniques have the advantage that by measuring DNA or RNA primers of different microbial species, one has a very powerful tool to gain a more complete picture of most, if not all, of the species present in the microflora, including those which are either difficult or impossible to culture. Such techniques are going to lead to a complete reassessment of the structure of the GIT microflora not only in relation to the host animal species, but also in terms of age, diet (S Konstaninov, W-Y Zhu, A Akkermans, BA Williams, S Tamminga and W de Vos, unpublished results), health and genome of the host (Zoetendaal *et al.* 1998). However, it is also recognized that, apart from the descriptive role of these techniques, it is also important to relate them to the dynamics of GIT processes (Ziemer *et al.* 2000).

Measurements of whole microbial population activity. Instead of counting micro-organisms, the magnitude of a whole mixed microbial population can also be determined by measuring microbial activity (Clarke, 1977). A measure of microbial activity may be obtained from production rates of microbial protein or fermentation endproducts, or from turnover rates of various chemical pools that involve the microbes. When studying metabolism of the whole gut microflora, information on the metabolic reactions performed by the individual species comprising the flora is often of little use. The colonic microflora of mammals is a highly complex ecosystem comprising over 400 different microbial species. Details of the metabolism of these species in terms of available nutrients are not known. Even if they were, it would be difficult to predict whether a reaction that occurs with a pure culture *in vitro* would also occur when the organism is interacting with other species *in vivo* (Rowland, 1992).

Growth of anaerobic bacteria under favourable conditions is directly related to the amount of ATP derived from fermentation, so cell yields are a function of both the amount of substrate fermented and of the fermentation endproducts produced (Clarke, 1977). Therefore, measures of both substrate utilization and endproduct production can be used to indicate growth of cells and cell mass. The endproducts most commonly measured include VFA and gas. Consequently,

a better approach to understanding the role of the gut microflora in nutrition and toxic events is to treat the microflora as a single entity (Rowland, 1992), ignoring its multi-organism composition. This approach has been used with some success by a number of researchers using a variety of functional assays on faeces or gut contents (Goldin & Gorbach, 1976; Midtvedt, 1989).

For example, Robinson *et al.* (1989) incubated contents from different areas of the pig GIT and measured production of gas and VFA to determine whether methanogenic activity was similar to that in man. They discovered that it was not, but also that the measurements of VFA were consistent with previously reported findings *in vivo*. They also found that the measurements for gas production, while underestimating the gas produced for the whole animal, were useful for comparative purposes. This was also found by Jensen & Jørgensen (1994) in a similar study investigating the effect of dietary fibre in pig diets.

Using human faeces as an inoculum (McBurney *et al.* 1990) it was shown that six different sources of starch, while having the same total VFA production, differed in their rates of gas production. They showed that the gas production reflected the fermentation rate *in vitro*. They concluded that an *in vitro* assessment of fermentative characteristics of raw starches was a useful means of evaluating the potential effects of starches on colonic function.

Cumulative gas production. The method of Theodorou *et al.* (1994) which measures the kinetics of fermentation, can also be used to assess activity of microbial populations (Williams *et al.* 2000*a*). This method involves measurement of accumulating gas during fermentation, so that one obtains a picture of the kinetics of microbial activity of the population acting as a whole. At the end of the fermentation period samples are taken for the measurement of VFA and NH_3, and substrate utilization. The technique is carried out under strictly anaerobic conditions and is being used to examine the activity of the microflora from many different sources, including the rumen (Williams *et al.* 1995), different sections of the GIT of pigs (Williams *et al.* 1997*a*) and the caecae of poultry (Williams *et al.* 1997*b*). By using different starting substrates it becomes possible to look at shifts in microbial populations which are associated with the fermentation of a particular feedstuff, e.g. RS, protein or fibre (Williams *et al.* 2000*a*). Alternatively, a standard substrate (such as ileal chyme) can be chosen and small quantities of potential feed additives can be added to look at the shifts in endproducts which result in order to predict what might happen in terms of fermentation in the large intestine of single-stomached animals (E Bauer, BA Williams, C Voigt, R Mosenthin and MWA Verstegen, unpublished results).

Gibson & Fuller (2000) described principles which were important for the development of both *in vitro* and *in vivo* techniques to identify probiotics and prebiotics as potential feed additives. According to these principles the cumulative gas production technique is also being used to test a range of different products in order to assess the endproducts of *in vitro* fermentation (Williams *et al*. 2000*b*; Bauer *et al*. 2001). However, while such a comparatively simple test can give a qualitative indication of the endproducts which may be found *in vivo*, further research is needed to determine what the quantitative relationship would be.

Reliability of faeces to represent the large-intestinal microflora. It could be questioned whether the use of faeces as an inoculum for *in vitro* studies of *in vivo* GIT events is truly representative of what occurs earlier in the GIT (i.e. the caecum and/or colon). Unlike the rumen, which is considered to resemble a chemostat with regular inputs and outputs, the large intestine of single-stomached animals more closely resembles a batch-flow culture. Drasar (1988) described faeces as the spent culture medium of the large gut fermenter. It has been studied because it is readily available and provides a source material for the major groups of intestinal bacteria. It can also be sampled from the same host at different times. In detailed microbial count studies, Moore *et al.* (1978) concluded that the composition of the bacterial flora of

faeces resembled that of the large intestine and concluded that freshly passed faeces collected under strictly anaerobic conditions could be considered as representative of the large-intestinal flora. In terms of VFA and cumulative gas production, some differences were found between inocula from the caecum, mid-colon and faeces (Williams *et al*. 1997*a*), but it was concluded that faeces did indeed give a reasonable estimate of activity higher in the tract.

Conclusions

One interesting possibility for the replacement of antibiotics as growth promoters in animal production involves changes in the animal diet which will stimulate development of the autochthonous microflora of the host animal. Stimulation of an 'appropriate' microflora can be achieved by stimulation of the fermentation of specific carbohydrates in the GIT. By doing so, not only is the production of VFA stimulated and 'colonization resistance' enhanced, it is also likely to lead to a reduced protein fermentation and thus less of the potentially toxic compounds which normally result from such fermentation.

The problem of post-weaning diarrhoea in piglets is an example of a situation where the inclusion of fermentable carbohydrates in the diet may have a positive effect. Given that this is an area where the removal of antibiotics from the diet is going to have an immediate effect, the search for alternatives is of immediate importance to pig farmers.

However, there are still many unknowns in terms of GIT fermentation and animal health, in terms of the host, the microflora and the interaction between the two. In terms of the host, many investigations are under way to unravel the complexities of the host immune system particularly in terms of its development in the young animal and in relation to the animal's microflora. In terms of investigating the microflora itself, new methodologies in molecular biology are casting a new light on its complexity and development. Such techniques provide better opportunities to investigate the whole microfloral population, particularly compared with the more traditional plating techniques. Interactions between the two are often the most difficult to assess and techniques need to be developed which can assist with this. One example measures *in vitro* fermentative activity using specific substrates to target specific groups of micro-organisms which allows changes in activity of the population as a whole to be detected. This kind of technique could be used in combination with others to detect the influence of diet on the microflora as well as other factors which alter the GIT microflora both in terms of specific species affected and activity. Such techniques might also prove useful in providing an *in vitro* assessment of the endproducts of large-bowel fermentation. However, the relationships between *in vitro* and *in vivo* results will still need to be evaluated.

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