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Influence of dietary components on development of the microbiota in single-stomached species

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After birth, development of a normal microbial community occurs gradually, and is affected by factors such as the composition of the maternal gut microbiota, the environment, and the host genome. Diet also has a direct influence, both on composition and activity of this community. This influence begins with the milk, when specific components exert their growth-promoting effect on a beneficial microbiota, thereby suppressing potential pathogens. For example, breast-fed infants compared with formula-fed babies usually have a microbial community dominated by bifidobacteria. When solid food is introduced (weaning), dramatic changes in microbial composition occur, so pathogens can gain access to the disturbed gastrointestinal (GI) ecosystem. However, use of specific dietary components can alter the composition and activity of the microbiota positively. Of all dietary components, fermentable carbohydrates seem to be most promising in terms of promoting proliferation of beneficial bacterial species. Carbohydrate fermentation results in the production of SCFA which are known for their trophic and health-promoting effects. Fermentation of proteins, on the other hand, is often associated with growth of potential pathogens, and results in production of detrimental substances including NH_3 and amines. In terms of the GI microbiota, lipids are often associated with the antimicrobial activity of medium-chain fatty acids and their derivatives. The present review aims to provide deeper insights into the composition and development of the neonatal GI microbiota, how this microbiota can be influenced by certain dietary components, and how this might ultimately lead to improvements in host health.

Diet: Fermentation: Gastrointestinal tract: Microbial community: Single-stomached species

Introduction

There is considerable interest in probiotics, prebiotics and, ultimately, in the promotion of gastrointestinal (GI) health in human nutrition. Concomitantly, with the ban on dietary antibiotics as growth promoters within the European Union, animal nutritionists are urgently seeking alternatives, particularly for young animals. Consequently, both sciences are now looking to each other's results and methodologies to find solutions for these issues.

There has long been an interest in differences between breast and formula milk in nutrition of human babies, and much research has focused on their differences in terms of development of the GI microbial community. For example, significant differences were found in the bacterial composition along the GI tract (GIT) between formula- and breast-fed infants (Stark & Lee, 1982). For piglets, interest has focused on the time of abrupt weaning, which has always been a source of losses for the farmer. For both issues, it is only recently that new techniques in molecular biology are allowing dramatic advances in understanding the changes that occur in the whole microbial community, rather than only of selected species within that community.

In adult single-stomached species, a 'beneficial' commensal microbiota colonises the GIT in relationship with the host. One important aspect is the ability of this community to resist invasion by exogenous micro-organisms, which is known as 'colonisation resistance' (Van der Waaij *et al.* 1971). There

Abbreviations: FOS, fructo-oligosaccharide; GI, gastrointestinal; GIT, gastrointestinal tract; GOS, galacto-oligosaccharide; MCFA, medium-chain fatty acid; NDO, non-digestible oligosaccharide; TOS, transgalacto-oligosaccharide.

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are several mechanisms by which such resistance is achieved. For example, the action of antimicrobial metabolites (Walker & Owen, 1990), or the maintenance of a lower pH, may lead to reduced counts of pathogenic bacteria such as *Escherichia coli* (Sutton & Patterson, 1996). Competition for adhesion sites and nutrients (Van der Waaij *et al.* 1971) is another mechanism by which such resistance could work. Young animals, however, need time to develop a complex community as well as their immune system and, until such developments have taken place, are vulnerable to the presence of potential pathogens in their GIT.

Diet is recognised as an important factor controlling microbial composition and activity in the GIT of single-stomached animals (Macfarlane *et al.* 1992), as it largely determines substrate availability for microbial fermentation (Cummings & Englyst, 1987). For human infants, it has been suggested that a supply of complex carbohydrates might alter the colonic microbiota, or induce appropriate microbial enzymes, leading to improved fermentability of those substrates (Parrett *et al.* 1997). Rats, aged 6 weeks old, fed a high-protein diet, showed increased populations of coliforms and clostridia, whereas rats fed a diet based on maize, wheat flakes, wheat middlings and soyabean meal, had increased lactobacilli (Chung *et al.* 1977). A supply of appropriate substrates for microbial fermentation might therefore encourage development and maintenance of a beneficial GI microbial community, thereby supporting any health-promoting effects of that community. These effects include 'colonisation resistance', stimulation of SCFA production, and reduced production of potentially harmful substances. Fermentable carbohydrates are considered to be most promising in terms of a positive influence on the composition and activity of the indigenous microbiota of the GIT (Gibson & Roberfroid, 1995; Williams *et al.* 2001).

Dietary modulation of the GI microbiota is of special interest for the young animal or infant, at times of change or stress, such as the time of intestinal colonisation immediately after birth, or at the time of weaning. In the present review, aspects concerning the influence of maternal milk and/or colostrum are described, including the proteins, carbohydrates and fats which have been shown to have an effect on colonisation of the neonatal GIT. The effects of different nutrients on GI microbial composition are also described, especially that of fermentable carbohydrates. Reference is made to protein fermentation, and to the effects of some lipids on bacterial composition. The present review includes data mainly from pigs and human subjects, but also some from studies on rodents and dogs.

The gastrointestinal microbiota: a brief overview

The total number of microbial cells within the GIT of single-stomached animals, including man, exceeds that of the host cells by at least one order of magnitude (Savage, 1977). These bacteria are constantly interacting with each other, and with the host, comprising a highly complex ecosystem of which comparatively little is known. The colon contents support at least 400 different species, with numbers as high as 10^{10} and 10^{11} culturable bacteria/g digesta (Savage, 1977; Mackie & White, 1997). In pigs, the majority of the large-intestinal microbiota are obligate anaerobes, though some aerobic and facultative micro-organisms also exist (Varel & Yen, 1997). The emergence of new molecular techniques has shown that the culturable fraction of the GI microbiota is probably only 10 to 50 % of the total (Amann *et al.* 1995; Vaughan *et al.* 2000). These new methods enable the detection of microbial species that are either difficult or impossible to cultivate (O'Sullivan, 1999; Vaughan *et al.* 2000; Tannock, 2001). Methods based on genes encoding 16S rRNA have been used to examine differences in the GI bacteria in various host species, such as man (Tannock *et al.* 2000), pigs (Leser *et al.* 2002), chickens (Netherwood *et al.* 1999) and mice (Walter *et al.* 2000).

There is some interest in seeing the extent to which the lumen microbiota resembles that attached to the mucosa of the intestine, though there are often methodological difficulties in obtaining representative samples. Using 16S rRNA, Zoetendal *et al.* (2002) compared faecal communities with biopsy samples from the different parts of the human colon, and found differences between them. Differences in the structure of communities in human faecal and colonic contents were also observed using dot blot hybridisation and cultivation (Marteau *et al.* 2001). Zoetendal *et al.* (2002) also reported that the predominant mucosa-associated bacterial communities in the human GIT were host specific. This was in agreement with Simpson *et al.* (2000), who found, by use of 16S rRNA gene-targeted PCR and denaturing gradient gel electrophoresis fingerprinting in pigs, that each individual maintained a unique faecal bacterial community which was stable over time, suggesting a strong host influence.

From a functional point of view, Macfarlane *et al.* (1992) investigated human colon contents (including the ascending, transverse, and descending areas) of sudden-death victims, and showed that lumen conditions such as pH and concentration of fermentation products in these parts differed both between individuals, and between different areas of the GIT. Differences in microbial activity between individuals were also shown by comparing the *in vitro* fermentation capacity of microbial inocula from the large intestine (caecum, colon and rectum) of different pigs (Bauer *et al.* 2004). Using specific substrates, it was shown that there were significant differences in microbial activity between individuals both in terms of the rate and endproducts of fermentation.

To allow for studies of structure, function and dynamics of complex microbial ecosystems at sufficient resolution, novel culture-independent high-throughput approaches, including microarray techniques, are being developed (Zhou, 2003; Zoetendal *et al.* 2004).

Development of the intestinal microbiota from birth

After birth, the intestinal microbiota takes some time before developing a stable community (Gaskins, 2001). Colonisation is a complex process of natural selection and ecological succession. It depends on various factors, some of which are of host origin, such as the genome and physiology of the animal, while others are of microbial origin, such as bacterial interactions.

During the first few weeks of life, microbial succession in the GIT of human infants, pigs (Moughan *et al.* 1992),

chickens (Barrow, 1992) and calves (Smith, 1965) is remarkably similar, even though other species are exposed to greater numbers of bacteria from faecal and environmental sources, compared with man. After birth, the germfree GIT is rapidly colonised by anaerobic and facultative anaerobic bacteria. Culture studies have indicated that in general, human infants are initially colonised by species showing a high reductive potential (for example, *Enterobacter*), which metabolise O_2, thus indirectly encouraging the growth of anaerobic bacteria including lactobacilli and bifidobacteria, *Bacteroides* and clostridia (Mackie *et al.* 1999; Teitelbaum & Walker, 2002). However, in human infants, it has also been shown that the composition of the intestinal microbiota varies according to the infants' diet. Several studies have shown that the microbiota of breast-fed infants is often dominated by bifidobacteria and lactobacilli, while the microbiota of formula-fed infants contains more *Bacteroides*, *Clostridium* and *Enterobacteriaceae* (Balmer & Wharton, 1989). According to a study of Stark & Lee (1982), breast-fed and formula-fed infants in the first week of life were colonised by enterobacteria and enterococci, followed by bifidobacteria, *Bacteroides* spp., clostridia and anaerobic streptococci. Until solid food was introduced at weaning, breast-fed babies had a simple microbiota consisting of bifidobacteria and relatively few enterobacteria and enterococci. Formula-fed babies during the corresponding period were more often colonised by other anaerobes in addition to bifidobacteria, and had higher counts of facultatively anaerobic bacteria. It would seem that breast-feeding created an intestinal environment that favours a simple microbiota of bifidobacteria and few other micro-organisms (Bullen *et al.* 1976; Kleessen *et al.* 1995), although not all studies could confirm this result (Lundequist *et al.* 1985). The intestinal microbiota of the breast-fed infant may be composed of a relatively narrow spectrum of Gram-positive bacterial species, due to the presence of only a few dominant species in breast milk (Martín *et al.* 2003). More recently, such differences in microbial colonisation have been confirmed by molecular techniques (Harmsen *et al.* 2000). Favier *et al.* (2002) investigated the succession of bacterial communities in human neonates, by monitoring 16S rRNA gene diversity in faecal samples using PCR–denaturing gradient gel electrophoresis. The first colonisers belonged to *E. coli* or *Clostridium*, followed after a few days by *Bifidobacterium*, which then remained predominant throughout breast-feeding. After weaning, *Clostridium*, *Ruminococcus*, *Enterococcus* and *Enterobacter* spp. appeared, with microbial denaturing gradient gel electrophoresis profiles becoming more complex and also more stable with increasing age.

Differences between the microbiota of breast-fed and formula-fed infants have also been shown with regard to differences in fermentative activity. According to Edwards & Parrett (2002), the ability of faecal microbiota from breast-fed infants to ferment complex carbohydrates seems to develop more slowly than that of formula-fed infants. *In vitro* fermentation of soyabean polysaccharide and guar gum was shown to increase progressively, but not to be significantly developed until late weaning (Parrett *et al.* 1997). Differences in fermentative activities of formula-fed infants at different stages of weaning were not significant, which might indicate a faster maturation of their colonic microbiota (Parrett & Edwards, 1997). However, in another study of Parrett *et al.* (1997), no differences in fermentation of several carbohydrates (soyabean polysaccharide and a fructo-oligosaccharide (FOS)) were found between faecal cultures of breast-fed and formula-fed infants, as measured by SCFA production. They showed that soyabean polysaccharides were poorly fermentable compared with faecal cultures from adults. Parrett & Edwards (1997) suggested that one reason for this might be that before weaning, the intestinal microbiota of infants is primarily adapted to fermentation of lactose, hexoses and oligosaccharides from milk, with the consequence that enzymes needed to ferment other carbohydrates may be absent or inactive. The authors further suggested that ingestion of carbohydrates in the diet could stimulate the microbiota, and thereby improve the fermentation of such substrates (Parrett *et al.* 1997). However, McVeagh & Miller (1997) suggested that the oligosaccharides present in human breast milk may act as 'soluble fibre' in the large intestine of human babies, providing a substrate which could stimulate microbial fermentation. This might lead to an adaptation of the infant's microbiota to fermentation of more complex carbohydrates, without the need for addition of other carbohydrates before weaning.

Differences in microbial composition may also occur when different milk formulae are fed. Hoey *et al.* (2004) used fluorescent *in situ* hybridisation with rRNA-targeted oligonucleotide probes to investigate the influence of a soya-based infant formula on faecal microbial composition in infants compared with cows' milk-fed controls, between 4 and 12 months of age. It was found that faecal bacterial numbers for bifidobacteria, bacteroides and clostridia (and total bacteria counts) were significantly lower for the soya group compared with the cows' milk group.

In suckling piglets, on the other hand, the population of faecal bifidobacteria seems to be numerically low (Mikkelsen *et al.* 2003), or even absent (Konstantinov *et al.* 2004*a*). Lactobacilli, however, establish early in the piglet's intestine, and, although succession does occur throughout the pig's lifetime, they remain a predominant member of the intestinal microbiota (Tannock *et al.* 1990; Naito *et al.* 1995; Stewart, 1997). At weaning, which generally occurs early, the transition from milk to a solid diet leads to dramatic changes in the composition of the microbial population during the 7–14 d after weaning (Hillman, 2001). According to Ewing & Cole (1994), numbers of lactobacilli and other beneficial bacteria decrease in times of stress, as do their beneficial effects, allowing potential pathogens such as coliforms to increase. Franklin *et al.* (2002) found that lactobacilli populations in different GIT sections (jejunum, ileum, caecum) declined to lower levels in early-weaned pigs (17 d), compared with pigs weaned at 24 d. Konstantinov *et al.* (2004*b*) have reviewed recent studies investigating the GIT microbial diversity during weaning in piglets.

Offering creep feed to suckling piglets might alter the GI microbiota, thereby preparing it for the dietary challenges

occurring after weaning (King & Pluske, 2003). However, pre-weaning feed consumption is highly variable both between and within litters (Bruininx *et al.* 2002), and the amounts of creep feed ingested are often very small. Thus, mere provision of creep feed may not necessarily lead to any statistically significant changes in the GI microbiota (Jonsson & Hemmingsson, 1991).

Influence of maternal milk components on neonatal gastrointestinal tract microbial composition

As for human infants, porcine milk contains a large variety of anti-inflammatory, antimicrobial and immunomodulatory agents, which may help to compensate for delays in development of the neonatal immune system or to support the establishment of a beneficial commensal microbiota. This is of particular importance in the pig, as their multi-layered placenta prevents the transfer of maternal antibodies to the fetus. This contrasts with man and rodents, which benefit from a trans-placental passage of maternal serum antibodies during embryonic development (Butler, 1998; Salmon, 1999). Therefore, maternal antibodies, bioactive peptides such as growth factors and cytokines, as well as maternal cells such as phagocytes, lymphocytes and epithelial cells in mammary secretions, are of substantial importance in the early postnatal period in piglets (Salmon, 1999; Wagstrom *et al.* 2000). Immuno-active components present in mammary secretions of pigs have been reviewed by Wagstrom *et al.* (2000), and for human subjects by Labbok *et al.* (2004).

Carbohydrates

Milk can also contain components such as glycoproteins, glycolipids, mucins and oligosaccharides (Newburg, 1999), some of which exhibit antimicrobial activity, but which may also act as growth promoters for bifidobacteria (Kunz & Rudloff, 1993). Mostly, these substances have been found in human milk, though some are also found in porcine milk, for example, lactoferrin (Masson & Heremans, 1971). Compared with other host species, human milk is considered to be unique in terms of its complex oligosaccharide content (Rudloff & Kunz, 1997).

Some oligosaccharides are known to be potent inhibitors of bacterial adhesion to epithelial cells by acting as receptor analogues to mucosal adhesion molecules (Kunz & Rudloff, 1993; Kunz, 1998; Peterson *et al.* 1998). Such oligosaccharides have been shown to protect infants from many infectious agents (Carlson, 1985). Colonisation of epithelial surfaces is often the first step in the process of infection by a pathogen. Host–pathogen interactions are often mediated by the attachment of proteins (lectins) present on the microbial surface to oligosaccharide chains of glycoproteins and glycolipids on the eukaryotic cell (Karlsson *et al.* 1992). The pathogen protein-receptor sites have strict requirements for their oligosaccharide ligands, usually consisting of three to five monomers. This specificity is probably one of the main factors determining host species and site of initial colonisation. For example, lacto-*N*-tetraose and lacto-*N*-neotetraose act as cell surface receptors for *Streptococcus pneumoniae*, while fucosylated oligosaccharides are receptors for *E. coli*. Kunz *et al.* (2000) summarised some of the oligosaccharides found in human milk, which are known to act as receptor analogues for bacterial pathogens.

Human milk oligosaccharides may act also as specific bifidogenic factors, supporting the survival of these bacteria (Beerens *et al.* 1980). Such natural prebiotics (3–6 g/l in milk; Kunz & Rudloff, 1993) consist mainly of a lactose core substituted with *N*-acetyl glucosamine, galactose, fucose and sialic acid, resulting in over 100 different compounds (Kunz & Rudloff, 1993). Oligosaccharides in human milk have been shown to be resistant to enzymic hydrolysis in the upper GIT (Engfer *et al.* 2000; Gnoth *et al.* 2000).

Proteins

It has been assumed that significant bifidogenic activity may also be associated with milk protein (Bezkorovainy & Topouzian, 1981; Petschow & Talbott, 1990), either by direct stimulation of growth, or by antimicrobial effects. Liepke *et al.* (2002) showed that proteolytic fragments of major human milk proteins are effective growth factors for bifidobacteria. Secretory Ig is a highly protective agent, which can prevent colonisation and invasion by pathogens (for a review, see Brandtzaeg, 2003). The results of Liepke *et al.* (2002) showed that a proportion of secretory Ig may actually support growth of bifidobacteria. Lactoferrin, a glycoprotein which has been reported to promote the growth of bifidobacteria (Petschow & Talbott, 1991; Hentges *et al.* 1992), has already been extensively discussed in terms of its antimicrobial activity (Sanchez *et al.* 1992). Both proteins may therefore be important in influencing an infant's intestinal microbiota, by exerting both antimicrobial effects and selective growth stimulation of bifidobacteria.

Other compounds

Human milk also contains nucleotides (Gil *et al.* 1986; Balmer *et al.* 1994) and gangliosides (Rueda *et al.* 1998), which, when added to formula milk have been shown to increase colonisation by bifidobacteria, although Balmer *et al.* (1994) could not confirm this finding. Bifidobacterial growth was also stimulated by the glycomacropeptide of bovine κ-casein (Poch & Bezkorovainy, 1991) due to its glycan side chain. However, bovine glycomacropeptides also inhibit bacterial and viral adhesion (for a review, see Brody, 2000). Furthermore, antimicrobial action has been shown for a fragment of bovine casein-α_{s2} which is not present in human milk, and which inhibits growth of *E. coli* and *Staphylococcus* strains (Zucht *et al.* 1995). Bovine milk proteins such as α-lactalbumin (Pellegrini *et al.* 1999) and β-lactoglobulin (Ouwehand *et al.* 1997) may also exert antimicrobial activity. Table 1 summarises some examples of components of human milk which exert antimicrobial or microbial growth-promoting activity, while Table 2 gives an overview of components of porcine milk and their putative influence on intestinal microbiota.

Table 1. Components of human milk with possible influence on intestinal microbiota

	Mode of action	Reference
Antimicrobial agents		
Lysozyme	Bacteriolytic enzyme, cleaves peptidoglycans in cell walls of bacterial pathogens	Chipman & Sharon (1969)
Lactoferrin	Fe-binding glycoprotein, inhibits growth of certain pathogens by competing with bacteria for the ferric ion	Bullen (1975) Brock (1980) Sanchez *et al.* (1992) Lönnerdal & Iyer (1995)
Lactoferricin	Aminoterminal peptide of lactoferrin, exerts antibacterial activity against *Streptococcus mutans* and *Vibrio cholerae* and inhibits attachment of enteropathogenic *Escherichia coli* to intestinal cells	Tomita *et al.* (1991) Yamauchi *et al.* (1993) Edde *et al.* (2001)
Oligosaccharides	May function as receptor analogues that inhibit the binding of enteric or respiratory bacterial pathogens, or their toxins, to epithelial cells	Kunz & Rudloff (1993)
Mucins	Heavily glycosylated milk proteins, interfere with bacterial or viral adherence	Schroten (2001)
Milk lipids	NEFA and monoacylglycerols created by the digestion of neutral fats in milk may exert antiviral, antibacterial and antiprotozoal activity	Isaacs & Thormar (1991) Issacs (2001)
	Milk fatty acids can damage bacteria by disrupting their cell membranes	Hamosh (1998)
Growth promoters of protective enteric bacteria		
Lactoferrin or its proteolytic generated fragments	Growth-promoting effect on bifidobacteria?	Petschow & Talbott (1991) Hentges *et al.* (1992) Nuijens *et al.* (1996) Liepke *et al.* (2002) Griffiths *et al.* (2003)
Oligosaccharides	Growth-promoting effect on bifidobacteria ('natural prebiotics')	György *et al.* (1974) Kunz & Rudloff (1993) Newburg (1999)
Casein	Glycoprotein, enhances proliferation of *Bifidobacterium bifidum*	Bezkorovainy & Topouzian (1981)

Influence of milk-associated bacteria

Human breast milk provides a continuous source of micro-organisms to the infant gut. It has been estimated that an infant consuming 800 ml milk per d will ingest about $1 \times 10^5 - 1 \times 10^7$ bacteria while suckling (Heikkilä & Saris, 2003). Bacteria commonly isolated from breast milk of healthy women have included staphylococci, streptococci, lactobacilli and enterococci (Gavin & Ostovar, 1977; West *et al.* 1979). These bacteria should be considered as components of the milk, rather than as contaminants (Martín *et al.* 2004). Heikkilä & Saris (2003) investigated bacterial diversity in expressed breast milk from healthy women. In this study, the predominant species were commensal staphylococci (64%) and oral streptococci (30%), with *Staphylococcus epidermis*, *Strep. salivarius* and *Strep. mitis* as the most common isolates. This agreed with earlier work which indicated that commensal staphylococci and streptococci were the predominant bacterial species in breast milk (Carroll *et al.* 1979; Eidelman & Szilagyi, 1979; West *et al.* 1979), although they may have originated from the maternal skin during breast-feeding (West *et al.* 1979). Staphylococci and streptococci, especially *Staph. epidermis* and *Strep. salivarius* have also been identified from stool samples of breast-fed infants (for example, Kirjavainen *et al.* 2001; Favier *et al.* 2002), suggesting that infant faecal microbiota might reflect the bacterial composition of breast milk. Such commensal bacteria originating from breast milk may exert inhibitory effects against pathogens such as *Staph. aureus* (Heikkilä & Saris, 2003). In a more recent study of Beasley & Saris (2004), molecular methods were used to screen human milk for bacteria to reveal antibacterial activity caused by production of nisin, a bacteriocin produced by *Lactococcus lactis*. It has been suggested that nisin-producing *L. lactis* may protect mothers and infants from pathogenic skin bacteria, such as *Staph. aureus* (Pittard *et al.* 1991).

Influence of dietary components on the gastrointestinal microbiota

Fats

While significant knowledge is available on the toxic effect of dietary fat on ruminal micro-organisms (Jouany, 1994), only little is known concerning the influence of dietary fat on the GI bacteria of single-stomached animals. It has been suggested that dietary fat, if it escapes pre-caecal digestion, might reduce the number of micro-organisms in rats. This was supported by data showing a reduced methane production in pigs receiving fat in their diets (Christensen & Thorbek, 1987). However, in a human study, Cummings *et al.* (1978) found no effect of diets with high or low animal fat (62 or 152 g/d) on the relative numbers of faecal bacterial groups, including *Enterobacteriaceae*, *Enterococcus*,

Table 2. Components of porcine milk with possible influence on intestinal microbiota

Agent	Putative mode of action	Occurrence and reference
Lysozyme	Bactericidal activity	Hill & Porter (1974) Nagy *et al.* (1976) Schulze & Muller (1980)
Transferrin	Antimicrobial	Thoren-Tolling & Martinsson (1974)
Lactoferrin	Antimicrobial Growth-promoting effect on beneficial bacteria?	Elliot *et al.* (1984) Masson & Heremans (1971)
Milk lipids	Antimicrobial effect?	Decuypere & Dierick (2003) (Low quantity of medium-chain fatty acids)
Oligosaccharides	Receptor analogue? Growth-promoting effect on beneficial bacteria?	Staples *et al.* (2002) (Low quantity)
'Bifidus factor'	Growth-promoting effect on beneficial bacteria?	György *et al.* (1954)
Casein	Growth-promoting effect on beneficial bacteria?	Holt & Jenness (1984) Kauf & Kensinger (2002)
Peptide produced by casein digestion, for example, casomorphin	Biologically (immunomodulatory) active?	Meisel (1986) Meisel & Frister (1989)

Bacteroides, lactobacilli and clostridia. Kuda *et al.* (2000), on the other hand, showed a decrease in faecal *Bacteroidaceae* after feeding mice with fish oil, compared with mice fed beef tallow. Interestingly, the number of faecal bifidobacteria was greater for the fish oil-fed animals.

According to Dänicke *et al.* (1997), significantly higher pH values were measured in most intestinal segments of broiler chickens, when beef tallow was used as a dietary fat instead of soyabean oil. Dänicke *et al.* (1999) then investigated the effect of the same dietary fat types on selected bacterial groups adhering to the intestinal epithelium in broiler chickens. The beef tallow-fed animals showed increased numbers of cocci in the jejunum and ileum, but decreased total anaerobic bacteria and enterobacteria. However, from the study of Dänicke *et al.* (1999), it was not clear whether the growth of cocci was actively stimulated by beef tallow, or whether it occurred as a result of an inhibition of other species. For example, the secretion of bile acids may have been stimulated by the presence of beef tallow.

As detergents, bile acids possess potent antimicrobial activity. However, some members of the intestinal microbiota have developed mechanisms to resist their action (Gunn, 2000). Some cocci are distinguishable by their growth in the presence of bile salts and, thus, these bacteria, such as enterococci (Gunn, 2000; Strompfová *et al.* 2004), may become dominant when bile acid concentrations are increased. However, some lactobacilli can deconjugate conjugated bile acids (Tannock *et al.* 1994) though it is unknown whether such activity is sufficient to affect bile salt metabolism. The type of dietary fat may also influence the intestinal microbiota indirectly, through its impact on digesta viscosity, intestinal transit time, and digestion in the small intestine (Dänicke *et al.* 1999).

The antimicrobial activity of SCFA and medium-chain fatty acids (MCFA; fatty acids consisting of 6 to 12 C), as well as their derivatives, was first demonstrated by Kabara *et al.* (1972). Since then, antimicrobial activity of MCFA has been shown for group B *Streptococcus* and *Haemophilus influenzae* (Isaacs *et al.* 1995) and for *Listeria monocytogenes* (Wang & Johnson, 1992), amongst others. Using both *in vitro* and *in vivo* models in mice, Petschow *et al.* (1998) showed antimicrobial activities of medium-chain monoacylglycerols on bacterial enteropathogens (for example, *Vibrio cholerae* or enterotoxigenic *E. coli*). The bactericidal effect of digestion products of bovine milk triacylglycerols was examined *in vitro* by Sprong *et al.* (2001). For all pathogenic bacteria tested (*E. coli*, *Salmonella enteridis*, *Campylobacter jejuni*, *L. monocytogenes* and *Clostridium perfringens*), C10 : 0 and C12 : 0 fatty acids were found to be toxic. Sphingosin, which is formed in the intestine from dietary sphingolipids (Schmelz *et al.* 1994), had the greatest bactericidal effect. Tsuchido *et al.* (1985), investigating the effect of SCFA and MCFA on *Bacillus subtilis*, found that the antimicrobial effect of MCFA could be mediated by the induction of an autolytic enzyme causing cellular lysis. Another mechanism proposed for the antimicrobial action of fatty acids is the destabilisation or disintegration of the cell membrane (Thormar *et al.* 1987; Isaacs, 2001). This latter mechanism was investigated by Bergsson *et al.* (2001) using MCFA and their monoacylglycerols on Gram-positive cocci. Monocaprin was shown to be the most effective agent against *Staph. aureus*.

However, the repellent odour and taste of such fatty acids makes their use as feed additives for young animals problematic, as a decreased feed intake would strongly compromise their efficacy. A further reduction in efficacy may result from the direct absorption of these acids in the stomach or proximal small intestine (Clark *et al.* 1969; Dierick *et al.* 2002*b*; Decuypere & Dierick, 2003). It has been suggested that the *in situ* generation of MCFA originating from triacylglycerols containing MCFA might overcome this problem. The MCFA could then be released by exogenously supplied lipases, as well as endogenous preduodenal lipases which are present in several host species. Using *in vitro* and *in vivo* methods, Dierick *et al.* (2002*a*,*b*) investigated the effect of such a combination of triacylglycerols containing MCFA and exogenous lipolytic enzymes as feed supplements in piglets. Microbial counts showed a strong suppressive effect of the MCFA released by the exogenous enzymes on the intestinal microbiota in piglets, suggesting their use as an alternative for antimicrobial growth promoters. The possibilities and limitations of combining triacylglycerols containing

MCFA and exogenous lipolytic enzymes as an alternative to in-feed antibiotics have been reviewed by Decuypere & Dierick (2003).

Proteins

Proteins are known to be important substrates for some intestinal bacteria (Macfarlane *et al.* 1986). However, it has also been shown that several potential pathogens are predominantly protein-fermenters, and would therefore grow more prolifically when proteins are freely available (Macfarlane & Macfarlane, 1995). For example, excessive protein intake was shown to favour the growth of undesirable species such as *C. perfringens*, and to reduce faecal counts of bifidobacteria. Under such conditions, faecal consistency was softer, and excretion of *C. perfringens* enterotoxin and other metabolic endproducts related to microbial protein decomposition was increased (Zentek, 1995*a*,*b*; Van der Steen *et al.* 1997).

Fermentable carbohydrates

The carbohydrate fractions, which escape digestion by mammalian enzymes and are therefore potentially available as substrates for microbial fermentation, include NSP (plant cell wall polysaccharides, pectins, gums), resistant starch and non-digestible oligosaccharides (NDO). The effect of these fractions on the GI microbiota is related to the availability of that carbohydrate to the bacteria as a substrate, i.e. to their fermentability. For example, the microbial breakdown of specific NSP is influenced by the chemical structure of the carbohydrate polymers present (Botham *et al.* 1998), such as the degree of lignification. It is generally assumed that the more soluble carbohydrates are more readily available and therefore fermentable (Stephen & Cummings, 1979), though it has been shown that this is not always necessarily the case (Bauer *et al.* 2001). This study, using faecal inocula of unweaned piglets and cumulative gas production as a measure of fermentation kinetics (Williams *et al.* 2005), showed that insoluble fibre (soya hulls) was better fermented in terms of production of gas and SCFA, compared with a soluble substrate (guar gum).

Non-starch polysaccharides. The NSP (together with lignin) are the principal components of plant cell walls, which differ in their chemical composition and physical properties, both within and between plant sources. The main NSP structures commonly found in feed ingredients of plant origin are all non-α-glucan polymers such as cellulose, β-glucans, arabinoxylans, arabinogalactans, galactomannans, xyloglucans and rhamnogalactouronans (pectins) (Cummings & Englyst, 1987; De Lange, 2000). Pectins, which are usually included in the NSP fraction, are structurally based on a polymer of galacturonic acid residues with rhamnose and arabinose substituents. A variable proportion of the uronic carboxyl groups in pectin are esterified with methanol (Adrian, 1976). Gums, such as gum arabic (a complex arabinogalactan polysaccharide associated with a glycoprotein) and guar gum (a galacto-mannan), have been shown to be well fermented (Bauer *et al.* 2001).

Using anaerobic culture techniques to investigate faecal samples from pigs, Varel *et al.* (1984) showed that diets high in dietary fibre (35 % lucerne meal, compared with a control diet, 0 % lucerne meal) increased the number of cellulolytic bacteria without changing the total number of micro-organisms. Gums, on the other hand, have been shown to exert bifidogenic effects. Gum arabic is completely fermented in the human colon (Ross *et al.* 1983). Guar gum is readily fermented by the human faecal microbiota (Salyers *et al.* 1977), and also showed bifidogenic effects, during enteral feeding (Okubo *et al.* 1994).

β-Glucans are structural components of cereals (wheat and barley), and of fungal cell walls, which cannot be digested by mammalian enzymes, and are therefore potentially useful substrates for GI fermentation (Englyst *et al.* 1989; Edney *et al.* 1991). In poultry, they may exert negative effects on animal performance due to their viscosity, which has been shown to impair digestive and absorptive processes (Choct & Annison, 1992; Fuller *et al.* 1995). However, they may have beneficial effects on microbial composition in terms of increasing the number of lactobacilli; Jonsson & Hemmingsson (1991) showed a correlation between the diet of piglets and the occurrence of cultivable faecal lactobacilli with an ability to degrade β-D-glucans. Dongowski *et al.* (2002) investigated the effect of barley-rich diets on the GIT in young rats, and found decreased counts of coliforms and *Bacteroides* for rats fed the barley-based diets, with concomitant increased *Lactobacillus* counts. Also, SCFA production was higher for the barley-fed animals compared with the control group.

Guo (2003) investigated the effects of different polysaccharide fractions from the mushrooms *Tremella fuciformis* and *Lentinus edodes*, and from a herb, *Astragalus membranaceus*, on the caecal bacterial community of chickens *in vitro*. Specific PCR amplification of 16S rRNA gene fragments in combination with denaturing gradient gel electrophoresis was used to analyse the microbial community before and after *in vitro* fermentation, using chicken caecal contents as the original inoculum. The polysaccharide extracts led to significant shifts in the bacterial community when fermented *in vitro*.

Resistant starch. According to Englyst *et al.* (1992), resistant starch is classified into three groups: type I, representing physically inaccessible starch such as whole or partly milled grains; type II, representing starch in granules such as raw potato; type III, representing retrograded starch produced by heat treatment during feed processing. More generally, resistant starch refers to the fraction of starch that escapes enzymic digestion in the human small intestine (for example, McBurney *et al.* 1988). The presence of resistant starch in feeds is related to many factors including the amylose:amylopectin ratio, the granule structure of the starch, the physical form of the feed, the effects of processing, and the presence or absence of NSP, amylase inhibitors, lectins and phytate (Cummings & Englyst, 1987). For a recent review describing the classification of resistant starch, see Champ *et al.* (2003).

Resistant starch provides a carbohydrate source for bacterial growth which has been shown to yield high concentrations of butyric acid (Wang *et al.* 2004). In man, the predominantly amylolytic bacteria belong to the genera *Bifidobacterium*, *Bacteroides*, *Fusobacterium* and *Butyrivibrio* (Cummings & Englyst, 1987). Wang *et al.* (2002) examined four diets containing 40 % amylopectin maize starch, amylomaize starch, carboxymethylated amylomaize starch or acetylated amylomaize starch, for their effect on the composition of colonic bacteria in mice. They found significant increases in the faecal bifidobacteria for mice fed the amylopectin, amylomaize and acetylated amylomaize starch diets. A significant decrease in the faecal population of coliforms was observed for mice fed acetylated amylomaize starch. A promoting effect on indigenous bifidobacteria of diets containing resistant starch has also been demonstrated for rats and pigs (Brown *et al.* 1997; Kleessen *et al.* 1997; Silvi *et al.* 1999).

Non-digestible oligosaccharides. The NDO fraction comprises carbohydrates such as FOS or inulin, and is currently the most popular candidate as a so-called 'prebiotic'. Prebiotics have been defined as specific ingredients which are added to the human diet and which are believed to enhance the beneficial activity of specific members of the microbiota, such as lactobacilli or bifidobacteria in the large intestine (Gibson & Roberfroid, 1995). Inulin is a plant fructan which shows a degree of polymerisation ranging from two to sixty fructose units. Inulin molecules having a degree of polymerisation of less than twenty fructose units are generally defined as FOS, and are a mixture of predominantly tri-, tetra- and pentasaccharides (Gibson & Roberfroid, 1995; Van Loo *et al.* 1995). Other NDO currently used as prebiotics include transgalacto-oligosaccharides (TOS) which are a mixture of tri-, tetra-, penta- and hexasaccharides (Ekhart & Timmermans, 1996), and raffinose, which is widely distributed in plants (Rathbone, 1980). Other oligomers that may have a possible prebiotic effect include lactulose, and oligosaccharides containing xylose, mannose and galactose (Gibson & Roberfroid, 1995). For a detailed listing and description of NDO currently used as prebiotics, see Grizard & Barthomeuf (1999).

In vitro fermentation using human faeces showed that inulin and FOS selectively stimulate the growth of bifidobacteria and may produce an environment (increased SCFA concentrations and/or decreased pH) that is not favourable to the growth of certain pathogenic organisms such as *E. coli* and *C. perfringens* (Wang & Gibson, 1993). Generally, these substrates can be utilised by lactobacilli, *Bacteroides*, streptococci and enterobacteria, but are not utilisable by *E. coli* (Hidaka *et al.* 1986). McDonald (2001), using weaned piglets naturally colonised with haemolytic *E. coli*, reported decreased proliferation of *E. coli* in response to inulin in the diet. Xu *et al.* (2002) showed increased viable counts of *Bifidobacterium* and *Lactobacillus* in the small-intestinal and proximal colonic contents of pigs fed a diet supplemented with FOS (4 and 6 g/kg diet), as compared with the control diet. Concomitantly, they found reduced counts of *Clostridium* and *E. coli.*

Kleessen *et al.* (2003) investigated the effect of fructan-rich Jerusalem artichoke (as 0·5 % syrup in drinking water) on viable counts of selected caecal bacteria in broiler chickens up to 35 d of age. The authors found that Jerusalem artichoke resulted in significantly smaller numbers of total aerobes, *Enterobacteriaceae* and *C. perfringens*, suggesting that the Jerusalem artichoke may have suppressed potential pathogens in broilers' caeca.

Studies using combinations of FOS and other NDO have also been performed. For example, Moro *et al.* (2002) analysed faecal samples from newborn infants who had received a formula supplemented with either 4 or 8 g of a mixture of FOS and galacto-oligosaccharides (GOS)/l for 1 month. Compared with the control group receiving a non-supplemented formula, there was a dose-dependent increase in the number of bifidobacteria and lactobacilli in the faecal samples after 28 d. No significant change was observed in other components of the faecal microbiota, particularly *Bacteroides*, *Clostridium*, *E. coli*, *Proteus* and *Klebsiella*. Similarly, Boehm *et al.* (2004) found, by use of culture and fluorescent *in situ* hybridisation, that the addition of an oligosaccharide mixture (FOS and GOS) to an infant formula resulted in an increased number of bifidobacteria, and a reduced number of pathogens, as compared with infants receiving an unsupplemented formula. At a concentration of 0·8 g oligosaccharides/100 ml formula, the amount of bifidobacteria became similar to that typical of breast-fed infants.

TOS can also influence GI microbial composition. They can be utilised by bifidobacteria, lactobacilli, *Bacteroides*, streptococci and enterobacteria (Tanaka *et al.* 1983). A culture study by Smiricky-Tjardes *et al.* (2003) demonstrated the effect of dietary GOS on ileal and faecal bacterial communities in growing pigs. The authors found a significant increase in faecal bifidobacteria and lactobacilli for animals fed diets containing soya solubles and TOS.

Table 3 summarises some recent studies investigating the effects of different fermentable carbohydrates on the composition of the GI microbiota. Results are sometimes conflicting, such as for the use of GOS studied under *in vitro* and *in vivo* conditions. Tzortzis *et al.* (2004) showed that GOS increased bifidobacteria in canine faeces *in vitro*, while *in vivo*, there was no effect on faecal bifidobacteria in a human feeding trial (Satokari *et al.* 2001). Such differences may depend, to some extent, upon the methodology and host species being used. However, it is clear that truly fermentable carbohydrates, chosen as appropriately for the host environment, can have a beneficial effect upon the intestinal microbial community of the young animal. Konstantinov *et al.* (2003, 2004*a*,*b*) introduced pre-tested (*in vitro*) fermentable carbohydrates to newly weaned piglets' diets, which resulted in a greater diversity and more rapid stabilisation of the GI community. It has also been shown that the introduction of fermentable carbohydrates not only resulted in increased beneficial bacteria (bifidobacteria, lactobacilli), but also in decreases in potentially harmful bacteria, such as *C. difficile* (Hopkins & Macfarlane, 2003). Similarly, Konstantinov *et al.* (2004*a*), again using pre-tested fermentable carbohydrates in weaning piglet diets, showed a stimulation of lactobacilli, and a concomitant suppression of *Clostridium*-like species.

Table 3. Studies investigating effects of fermentable carbohydrates on the composition of the gastrointestinal microbiota, by use of molecular techniques

Fermentable carbohydrate	Origin or host species of microbial sample	Influence on microbiota	Reference
In vitro			
Arabinoxylans Starch	Faecal samples of children	Increase in total anaerobe counts and eubacterial rRNA concentrations Degradation of arabinoxylans associated with increased counts of *Bacteroides*	Hopkins *et al.* (2003)
Inulin Levan-type exopolysaccharides FOS Levan	Faecal samples of adult humans	Increase in bifidobacteria No effect	Dal Bello *et al.* (2001)
FOS Galactosyllactose Inulin	Faecal samples of adult humans	Increase in bifidobacteria Concomitant reduction in *Clostridium difficile*	Hopkins & Macfarlane (2003)
Galactosyl-melibiose mixture FOS Melibiose Raffinose	Faecal samples of adult dogs	Increases in bifidobacteria and lactobacilli for all carbohydrates tested Higher increase in bifidobacteria and lactobacilli and higher decrease in clostridia for galactosyl-melibiose mixture compared with FOS, melibiose and raffinose	Tzortzis *et al.* (2004)
In vivo			
Mushroom polysaccharides (*Tremella fuciformis* and *Lentinus edodes*) Herb polysaccharides (*Astragalus membranaceus*)	Caecum, broiler chickens	Increase in bifidobacteria and lactobacilli Decrease in *Bacteroides* spp. and *Escherichia coli* Highest increase in bifidobacteria and lactobacilli for *Lentinus edodes* extract Dose-dependent increase in *E. coli*, bifidobacteria and lactobacilli for all polysaccharides tested	Guo *et al.* (2004)
Sugarbeet pulp and FOS	Faeces, weaning piglets	Increase in *Ruminococcus*-like species Higher bacterial diversity and more rapid stabilisation of bacterial community	Konstantinov *et al.* (2003)
Inulin, lactose, wheat starch and sugarbeet pulp	Ileum and colon, weaning piglets	Higher bacterial diversity in colon *Lactobacillus reuteri* most prevalent in the ileum *L. amylovorus*-like populations most prevalent in ileum and colon	Konstantinov *et al.* (2004*a*,*b*)
Galacto-oligosaccharides Galacto-oligosaccharides + *Bifidobacterium lactis* Bb-12	Faeces, human	No effect on indigenous *Bifidobacterium* population Transient colonisation with *B. lactis*	Satokari *et al.* (2001)

FOS, fructo-oligosaccharides.

Influence of fermentable carbohydrates on nitrogen metabolism in the intestine

In the absence of sufficient energy as carbohydrate, some bacteria may use protein as a source of energy, resulting in the formation of potentially toxic substances such as NH_3, amines and amides (Cummings & Macfarlane, 1991; Macfarlane *et al.* 1992). However, if sufficient fermentable carbohydrate is available, bacteria may utilise NH_3 as an N source for their own growth (Bryant & Robinson, 1962). Accordingly, provision of fermentable carbohydrates can increase NH_3 uptake by GI bacteria. N would then be excreted as microbial protein via the faeces instead of as urea in urine, saving energy to the host and reducing the NH_3 burden to the environment (Mosenthin *et al.* 1992, 1994; Canh *et al.* 1998). For example, the addition of oligofructose to a rat diet (7·5 g per 100 g diet) reduced blood urea and urinary N by 20–30 % (Younes *et al.* 1995). This phenomenon was also shown by Canh *et al.* (1997), who investigated the influence of dietary NSP (sugarbeet pulp) on N partitioning of urine and faeces of fattening pigs. They found that the pigs fed the sugarbeet pulp-based diet excreted 22–37 % less urea in urine than the pigs fed diets with a lower NSP content.

Van Nuenen *et al.* (2003) investigated the effect of inulin on the metabolic activity of the human colonic microbiota with or without the addition of *C. difficile in vitro*. The addition of inulin stimulated the total SCFA production, while suppressing formation of NH_3 and branched-chain fatty acids. While the introduction of *C. difficile* stimulated the production of protein-fermentative metabolites such as NH_3, branched-chain fatty acids and phenolic compounds (for example, indole), this was almost completely avoided by the addition of inulin. It appeared that inulin has the potential to shift the metabolic activity of the human colonic microbiota away from the production of toxic metabolites, both under normal conditions, and under conditions with a disturbed microbiota, i.e. in this case with a higher proportion of *C. difficile*.

Conclusions

Diet appears to be an important factor controlling the composition and metabolic activities of the GI microbiota of single-stomached animals and man. Insufficient work has been done to understand the effect of dietary fat on the single-stomached microbiota. However, any influence is most likely to be direct antimicrobial action, as has been shown for some MCFA and their derivatives, such as some milk lipids, or possibly through interaction with bile acid metabolism. It is generally assumed that fat in the gut of single-stomached species is completely absorbed before it can have any effect on colonic bacteria. This assumption would be invalid, however, for animals or human subjects with small-intestinal disturbances which are associated with poor digestion or fat absorption.

Fermentation of proteins is associated with the growth of potentially pathogenic bacteria and toxic metabolite production, while the fraction of non-digestible (but fermentable) carbohydrates seems to exert beneficial influences on the composition and activity of the GI microbiota. Such carbohydrates may enhance the health-promoting properties of the GI microbiota, such as colonisation resistance against invading pathogens, or production of SCFA and reduction of detrimental substances. Particular focus has centred on the use of prebiotics which selectively stimulate beneficial GI bacteria such as lactobacilli or bifidobacteria. Fermentable carbohydrate supplementation seems to stimulate bacterial diversity in newly weaned piglets, which seems to be essential for rapid stabilisation of the microbial community (Konstantinov *et al.* 2003). Such a mechanism is of special importance for the young animal or infant, in times of rapid change or stress, such as the time of intestinal colonisation after birth or during weaning.

As can be seen from compositional differences in the GI microbiota between breast-fed and formula-fed infants, early diet has a major impact on microbial development. Specific components of maternal milk might support colonisation of the neonate animal or infant with a beneficial microbiota, partly by acting as growth promoters for beneficial bacteria, and partly by exerting antimicrobial activities against potential pathogens. For example, oligosaccharides present in human milk display homology to cell surface pathogen receptors and may therefore inhibit pathogen interactions with host mucosal tissues, so protecting from infection. Prebiotics incorporating such receptor oligosaccharide sequences would then act as 'decoy' molecules for potential pathogenic bacteria (Steer *et al.* 2000).

However, it is not only oligosaccharides which may stimulate beneficial microbiota. It seems that there are more fermentable carbohydrates with prebiotic properties. In terms of health, larger, more slowly fermentable polysaccharides might provide an advantage over the rapidly fermented oligosaccharides currently used as prebiotics, by providing a carbohydrate source for SCFA production and suppression of protein metabolism more distally in the colon. Indeed, there is increasing evidence to suggest that some NDO are completely fermented either by the terminal ileum (FOS) or within the proximal large intestine (TOS), and are therefore unavailable for bacteria in the distal colon (Houdijk, 1998). *In vitro* fermentation studies using faecal inocula have shown that arabinoxylan may be used by *Bifidobacterium longum* as a carbon source (Crittenden *et al.* 2002). Attention should be given, not only to manufactured prebiotics, but also to more 'natural prebiotics', i.e. specific feedstuffs containing fermentable carbohydrates that might enhance microbial activity in a positive way and therefore improve GI health. However, degradation of polysaccharides in the GIT is a process involving consortia of several bacterial species. This means that various factors have to be taken into account, such as substrate availability, nutrient competition, population dynamics and host factors. The possible immunostimulatory action of some polysaccharides (for example, β-glucans) might also be of interest, raising the possibility of an immune-system-related influence on intestinal microbiota, exerted by dietary components.

One should also consider that other bacterial species, apart from the 'classic' easily cultivated bacteria, such as lactobacilli and bifidobacteria, may exert beneficial

influences and be stimulated by specific dietary components. However, the metabolic properties of these bacteria will have to be defined so as to determine their role in colonic metabolism and GI health.

Nutrition of livestock must always include economic considerations, so the potential of dietary modulation of the GI microbiota must be related to improvements in animal health, rather than any change in feed efficiency. Nevertheless, it is essential to understand the colonisation process and the interactions between the host diet and its microbiota. This may aid in predicting the effects of specific dietary components on the development of a beneficial microbiota, and the long-term effects on microbial composition, in order to improve host health, particularly in young animals at the time of weaning, or at other stressful moments in animals' lives. This will be of particular interest as the use of antibiotics as growth promoters is completely banned from animal feeds within the European Union.

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