Models to Study Colonisation and Colonisation Resistance

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This review describes various *in vivo* animal models (humans; conventional animals administered antimicrobial agents and animals species used; gnotobiotic and germ-free animals), *in vitro* models (luminal and mucosal), and *in silico* and mathematical models which have been developed to study colonisation and colonisation resistance and effects of gut flora on hosts. Where applicable, the advantages and disadvantages of each model are discussed. *Key words*: colonisation, colonisation resistance, animal models, *in vitro* colonisation models, *in silico* and mathematical colonisation models.

INTRODUCTION

The gastrointestinal (GI) tract contains a complex, dynamic, and spatially diversified microbial ecosystem which is established and maintained during life in humans and animals. The factors which allow microbial components of this open ecosystem to establish and maintain their regional habitats, each contributing to the 'homeostasis' inside the GI tract, are largely unknown. The microflora interacts with its host, man, both locally, due to its intimate contact with the intestinal mucosa, and systemically, influencing diverse responses and functions: immunological, physiological, anatomical, metabolic, nutritional and toxicological. Therefore it is important to have methods for studying the gut microflora, particularly its composition, metabolic activities, products that may influence the host, and abiotic factors that govern the gut ecosystem.

The study of the composition and metabolism of the colonic flora presents considerable methodological problems. Attempts to circumvent these problems have led to the development of numerous *in vivo* and *in vitro* models to simulate the human colon and its microbial population.

There appears to be no single ideal method for studying the ecology and metabolic activities of the human colonic flora, and the problem must be tackled by a variety of means, each of which has intrinsic advantages and disadvantages of complexity, convenience and suitability, but which together provide a more accurate view of the ecosystem.

In this review, we describe *in vivo* animal models, *in vitro* models and *in silico* and mathematical models which have been developed to study colonisation and colonisation resistance and effects of gut flora on hosts.

I ANIMAL MODELS TO STUDY COLONISATION AND COLONISATION RESISTANCE

Several different *in vivo* experimental models have been used by investigators to show that the indigenous intestinal biota functions to maintain homeostasis in the intestinal tract and thus prevents colonisation by pathogenic microorganisms. In order to study the relationships between the host and various components of its microflora it is essential to use germ-free animals. Comparing these animals to animals associated with a complex flora (holoxenic) or with some known bacteria (gnotobiotic) allows us to define the role played by the microbial flora as a whole or by some of its components in the physiology of the host or in colonisation resistance.

Once a functional stability (as opposed to absolute numerical or compositional stability) is established, it also mediates protective effects against intestinal colonisation by pathogenic microorganisms. The best evidence for this colonisation resistance of the intestinal flora stems from the observation that germ-free animals are more susceptible to disease than corresponding conventional animals. For example, whereas a germ-free mouse can be killed with 10 cells of *Salmonella enteritidis*, 10^6 cells are required

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to kill a conventional mouse (1). The presence of the intestinal flora is the important factor for this difference because the LD_{50} for germ-free and conventional mice is the same regardless of whether the animals are challenged intravenously or intraperitoneally. Support for this claim comes from the experience of clinicians with antibiotics given orally. This practice often induces intestinal infections resulting in enteritis and diarrhoea. In chickens the inclusion of subtherapeutic levels of antimicrobial growth promoters in the feed frequently prolongs the excretion of *Salmonella* in the faeces (2) and similar effect is obtained with various pathogens in mice dosed experimentally with antibiotics (3–6). In all cases the antimicrobials suppress the protective flora and allow the pathogen to survive. Another source of supporting evidence comes from experiments in which dosing with faecal suspensions has been shown to prevent infection.

In vivo models of colonisation resistance have involved three types of experiments:

- those comparing infant and adult animals;
- those comparing germ-free or gnotobiotic animals with their conventional counterparts;
- those comparing animals whose resident microorganisms have been suppressed by an antimicrobial agent with animals possessing an undisturbed normal biota.

Conventional animals

Conventional animals have many limitations, but they have often been used to compare infant and adult animal (e.g. succession of colonisation) and in studies administering selected drugs to animals (7, 8). In addition, conventional animals are irreplaceable controls when gnotobiotic and/or genetically engineered animal models are used.

Advantages

- Full realism in the case of farm animals
- High degree of realism in other species: data apply to a fair degree to related species
- Fewer ethical restrictions on experiments than with the human model
- Good control over environment (diet, stress, etc.)
- Good control over genetics of subject population

Disadvantages

- Access to intestinal microflora limited
- Ethical restrictions still apply
- Complexity of the model makes interpretation of results difficult

Infant versus adult animals. The dramatic quantitative and qualitative fluctuations in the bacterial populations of the normal intestinal biota that occur immediately after birth up until the time the animal begins to sample solid food indicate that the normal biota is not well balanced. Differences between the bowel biota of infants and that of adults appear to be sufficient to influence colonisation by several species of Clostridia, including *C. botulinum*, *C. difficile*, *C. spiroforme and C. perfringens* type A.

There is experimental evidence in animals which suggests that variations between bowel biota of infants and adults may account for differences in their susceptibility to botulism. Using infant mice as a model, Sugiyama and Mills (9) experimentally reproduced the limited age susceptibility to *C. botulinum* intestinal overgrowth.

Infant hamsters closely parallel infant humans in their susceptibility to asymptomatic intestinal colonisation by toxigenic *C. difficile*. *C. difficile* colonizes the intestinal tracts of non antibiotic treated hamsters between 4 and 11 days of age (10). Hamsters younger and older than 4 to 11 days are resistant to *C. difficile* intestinal colonisation unless first treated with an antimicrobial agent. The development of resistance to *C. difficile* intestinal colonisation correlates with the time at which the hamsters begin to sample solid food.

Conventional animals administered antimicrobial agents. Treatment of conventional animals and humans with antibiotics often causes an increase in susceptibility to intestinal colonisation with pathogens (6, 11).

A number of studies have been undertaken in hamsters, guinea pigs, rats and mice to test the hypothesis that intestinal biota components that normally suppress *C. difficile* are eliminated by antibiotic administration, allowing the pathogen to attain unusually high population levels (12–14).

There have been many studies where attempts have been made to reconstitute colonisation resistance to different infections in animals using faecal or caecal homogenates from healthy normal animals of the same species.

Animal species used to study colonisation and colonisation resistance. Many animal species have been used, the most common ones being the mouse model, followed by rat, guinea pig, pig, chicken, Japanese monkey, Mongolian gerbil, ferret and quail.

Mouse. Pazzaglia et al. (15) used an animal model for studying *Aeromonas*-associated diarrhoea pathogenesis. Protein-malnourished mice were challenged orally with *Aeromonas* strains to determine if diminished levels of resistance would allow the induction of a diarrhoeal response. Although mice consumed 10^8 cfu per day for a minimum of 4 days, none became ill due to *Aeromonas spp.* ingestion. *Aeromonas spp.* were isolated from 75% of faecal cultures obtained 7 days after initial challenge, indicating bowel colonisation had occurred.

Chiang et al. (16) studied mutations in the toxin-coregulated pilus of *Vibrio cholerae* and the result of this mutation for colonisation in the infant mouse model. All four mutant strains demonstrated autoagglutination defects,

and all were highly defective for colonisation in the infant mouse model. These results support the previously proposed correlation between autoagglutination and colonisation.

Whitman et al. (17) studied factors promoting colonisation and the efficacy of decontamination therapy with antimicrobial agents. They developed a model of gastrointestinal colonisation with vancomycin-resistant *Enterococcus faecium* in CF1 mice. They demonstrated the importance of antibiotics in predisposing to gastrointestinal colonisation with vancomycin-resistant *Enterococcus spp.* Although treatment with ramoplanin temporarily suppressed the organism, recurrence of colonisation due to relapse or reinfection occurred.

Lim et al. (18) used a murine model to study the role for type 1 fimbriae expressed by most *E. coli* in colonisation. The results from experimental infections and cases of cystitis in women suggested that type 1 fimbrial genes were transcribed both in the bladder and in the kidney. However, those bacteria found in the urine and not attached to the uroepithelium were not transcriptionally active for type 1 fimbrial genes.

Pei et al. (19) worked on *Campylobacter jejuni* colonisation in mice. They found that mutation in the *peb1A* locus of *C. jejuni* reduced the rate and duration of intestinal colonisation (*peb1A* encodes an adhesin mediating cell adherence). So adherence plays in this case an important role in intestinal colonisation.

Rat. Heidt et al. (20) established colonisation resistance in SPF rats using a rat-derived microflora. They concluded that culturing of intestinal donor-microflora contents of selectively decontaminated animals could be a useful way to obtain a species-specific donor-microflora which could be used to start new SPF units.

Bovee-Oudenhoven et al. (21) studied the effects of calcium and fermentation by yoghurt bacteria on the resistance of rats to *Salmonella* infection. They concluded that in addition to fermentation by yoghurt bacteria, calcium in milk products strongly enhanced the resistance to *Salmonella* infection by lowering luminal cytolytic activity or diminishing the availability of iron for pathogen growth, or both. The same authors (22) later studied in the protective effect of dietary lactulose and calcium phosphate against *Salmonella* infection SPF rats. The lactulose-fed rats had a better colonisation resistance, translocation was reduced by dietary calcium, whereas lactulose was ineffective. Their conclusions were that the combination of dietary lactulose and calcium phosphate was protective against *Salmonella* infection.

In other experiments (23), they concluded that extra calcium phosphate added to a lactulose diet improved the resistance to colonisation and translocation of *Salmonella enteritidis*. This was probably mediated by a calcium-induced stimulation of lactulose fermentation by the intestinal microflora and reversion of the lactulose-mediated increased luminal cytotoxicity, which reduced damage inflicted on the intestinal mucosa.

Caplan et al. (24) developed a neonatal rat model of necrotising enterocolitis (NEC), a common gastrointestinal disorder affecting premature infants. The interaction of the main purported risk factors of NEC such as formula feeding, asphyxia, bacteria, and prematurity can be studied in this test system.

Pig. Berends et al. (25) discussed the main elements of a descriptive epidemiological model for *Salmonella spp.* in the pre-harvest stages of pork production, and the subsequent quantification of risk factors. Under the current circumstances, the lack of farm hygiene, contaminated feed, the use of broad spectrum antibiotics, a positive *Salmonella*-status of animals before transport, the lack of transport hygiene and transport stress are the most important risk factors regarding infections with *Salmonella spp.* They concluded that better prevention and control can be achieved by (i) very strict and consistent farm hygiene in combination with promotion of the colonisation resistance of animals kept together with a prudent use of broad spectrum antibiotics; (ii) simultaneous execution of control programmes at breeding farms, multiplying farms and finishing farms; (iii) separate transport and slaughter of the animals thus produced.

Nagy et al. (26, 27) studied the colonisation capacity of enterotoxigenic *E. coli* (ETEC) strains that lack adhesins in weaned pigs. The authors also studied the pilus-mediated adhesion of ETEC strains in pigs and suggested that adhesion and colonisation by these ETEC isolates is dependent on receptors that develop progressively with age during the first 3 weeks after birth.

Chicken. Van der Waaij D and BD (28) compared the colonisation resistance of the digestive tract in different animal species and in man. They attempted to determine the colonisation resistance of the digestive tract by biotyping *Enterobacteriaceae* in samples of different animal species: rodents, chickens, dogs and monkeys. Man did not differ from monkeys; however, both differed from the rodent species studied and from dogs. The same year, Berchieri et al. (29) studied the inhibition of colonisation of the chicken alimentary tract with *S. typhimurium* by pre-colonisation with an avirulent mutant. Strains of *S. infantis* and *S. heidelberg*, chosen because they colonized the chicken alimentary tract, produced inhibition of a wider range of serotypes.

Ferret. Andrutis et al. (30) studied the infection of the ferret stomach by isogenic flagellar mutant strains of *Helicobacter mustelae*. They concluded that flagellar motility is an important virulence factor for colonisation and pathogenesis in the *H. mustelae* ferret model.

Japanese monkey. Kubota et al. (31) used Japanese monkey and Mongolian gerbils because it is the animal model that can sustain persistent colonisation with *Helicobacter pylori*.

Quail. Catala et al. (32) studied the contribution of oligofructose to the protective role of bifidobacteria in experimental necrotising enterocolitis in quails. It was demonstrated that, irrespective of the environmental conditions, the use of oligofructose helped to prevent the overgrowth of bacteria implicated in necrotising enterocolitis in preterm neonates.

Gnotobiotic and germ-free animals

Advantages

- Good control over flora parameters (if present)
- Reduced complexity of flora facilitates interpretation of the data

Disadvantages

- Reduced realism (fewer interspecies and host microflora interactions)
- The ethical restrictions still apply
- The complexity of host makes interpretation of results complex

The advantage of gnotobiotics lies in the ability to control the composition of the environment in which a multicellular organism develops and functions. To study, e.g., the cross-talks that occur between microorganisms and their hosts, it is necessary to first define cellular function under germ-free conditions and then to evaluate the effects of adding a single or defined population of microbes.

The combined use of genetically manipulatable model organisms (genetically engineered microbes and transgenic mammals) and gnotobiotics has the potential to provide new and important information about how bacteria affect normal development, establishment and maintenance of the mucosa-associated immune system, and epithelial-cell functions.

The importance of the indigenous microbial biota in protecting against intestinal colonisation by exogenous bacteria has also been demonstrated in gnotobiotic animals. Many bacteria are able to colonize the intestinal tracts of germfree animals, whereas the colonisation of conventional animals with the same microorganism is difficult (33, 34).

Experiments in gnotobiotic animals also support the importance of the intestinal biota in protecting the host against *C. difficile* associated intestinal disease. When introduced into germfree mice, rats or hares, *C. difficile* rapidly establishes a stable population (35, 36). On the other hand, these same species of animals with a conventional microbiota are resistant to *C. difficile* intestinal colonisation (37, 38).

Pecquet et al. (39) used gnotobiotic mice to study the impact of *Saccharomyces cerevisiae* on colonisation of potentially enteropathogenic microorganisms.

Bacteroides thetaiotaomicron and fucosylation. A simplified gnotobiotic animal model such as that used by Bry et al. (40) should aid the study of open microbial ecosystems (see also Hooper et al. 41). Comparison of conventional and germ-free NMRI mice revealed that production of fucosylated glycoconjugates and an (1, 2)-fucosyltransferase messenger RNA in the epithelium of the small intestine requires the normal microflora. Colonisation of germ-free mice with *Bacteroides thetaiotaomicron*, a component of this flora with the capacity to utilize L-fucose, restored the fucosylation programme, whereas an isogenic strain carrying a transposon insertion that disrupts its ability to use L-fucose as a carbon source did not. The induction was dependent on density and did not appear to require direct binding of bacteria to the host epithelium. Therefore, *B. thetaiotaomicron* must be able to metabolize fucose in order for it to signal the epithelium to synthesize these fucosylated glycoconjugates.

It is known that fucosylated glycoconjugates mediate attachment of pathogens to epithelial surfaces (42) and also provide a source of nutrients for members of the indigenous flora (43). Thus, the ability of *B. thetaiotaomicron* to regulate fucosylation programmes in the distal intestine may affect both the ability of other components of the normal flora to establish a stable niche and the vulnerability of the intestine to colonisation by pathogens. In this context it is interesting to note that the colonisation of the ileum of NMRI mice by the fucosidase-producing *Bifidobacterium infantis* is enhanced when it is introduced together with *B. thetaiotaomicron* (44). *B. thetaiotaomicron* is able to modify host epithelial differentiation in a way that supports its own growth and permits colonisation by a second microbe.

Nurmi concept or competitive exclusion (CE) explaining barrier effect. Often quoted types of mechanisms concerning the inhibition of growth of pathogenic bacteria are competition for limiting nutrients and mucosal attachment sites, and production of antibacterial substances (see a separate review in this supplement by Fons et al.). In this context it has been shown in chickens that their susceptibility to *Salmonella* colonisation is probably due to the delayed establishment of normal intestinal microflora when reared by mass production. *Salmonella* colonisation could be prevented by introduction of intestinal (e.g. caecal or faecal) microflora from adult bird into newly hatched chickens. This so-called Nurmi concept (45) or competitive exclusion (CE) has been further developed (46, 47) and it is currently still applied for the control of *Salmonella* spp. in poultry. Their is a growing interest in the use of CE products for both other pathogens than *Salmonella* spp. and other domestic animals (48, 49).

Raibaud et al. (38) demonstrated a marked barrier effect against *Salmonella typhimurium* by the bacteria present in the digestive tract of conventional chickens maintained in an isolator. In gnotobiotic adult mice, the caecal flora

from these chickens also exerted a marked barrier effect towards *S. typhimurium*, but the elimination of the pathogen from the faeces was slower. After maintenance in gnotobiotic mice for 60 days, this flora was still effective when reinoculated into 4-day-old gnotobiotic chickens.

WHO consultations on vaccination and competitive exclusion against *Salmonella* infections in animals take place regularly (the last meeting in October 1998 in Jena/Germany was organized by the FAO/WHO Collaborating Centre for Food Hygiene and Zoonoses at the Federal Institute for Health Protection of Consumers and Veterinary Medicine in Berlin). Since the broad range of different competitive exclusion flora products cannot be defined as either a vaccine or a medical product, the WHO recommends a special product category called 'normal gut flora', which is a preparation of live obligate and facultative anaerobic bacteria originated from normal healthy individuals (specified pathogen-free and quality controlled) of a domestic animal species. The CE bacterial preparations are often undefined cultures (e.g. taken from chicken caeca). A defined culture with the potency and stability equivalent to that of undefined cultures has not been developed. In chickens mixed cultures containing a lower number of strains (< 50) are generally less protective, and cultures containing only one genus (e.g. *Lactobacillus* or *Bifidobacterium*) are generally not effective (50, 51). In contrast, the omission of Lactobacilli from the protective microflora results in decreased resistance (52). Since the mechanism of protection is not clear, there are no reliable criteria for selecting potentially protective strains.

When defined cultures are tested in gnotobiotic mice, the resistance produced is less than that obtained in the conventional animal (53, 54). In opposition to this, Ducluzeau et al. (55) demonstrated antagonistic effects of simplified fractions of intestinal microflora obtained from conventional mice comparable to that of the total flora. When *Escherichia coli* K-12 and *Clostridium* E were established in gnotobiotic mice before the introduction of *Shigella flexneri* SF2, the latter was reduced to a level below detection. The authors suggest that the bacterial antagonism is related to the production of an antibiotic substance active against *S. flexneri* SF2.

While there is no doubt that intestinal microflora can protect the host against intestinal colonisation by pathogens, there is a lack of good evidence that the organisms currently being used as probiotics are those which are responsible for the beneficial effects of the intestinal microflora. In this context, a great interest exists concerning animal models which are able to show protective effects mediated by probiotics in animals when challenged with pathogens. Up to date, only a few studies using mice have been successful. These include the protective effect of *Lactobacillus casei* and *Lactobacillus acidophilus* against *Shigella sonnei* infection (56), the antagonistic activity exerted by *L. casei* against *S. typhimurium* (57), and the prevention by Lactobacilli against *Helicobacter pylori* infection (58). Rodrigues et al. (59) also showed protection against *S. typhimurium* and *S. flexneri* colonisation obtained in conventional and gnotobiotic mice previously associated with *Saccharomyces boulardii* (this effect was not due to the reduction of the bacterial populations in the intestine). Nevertheless, the underlying mechanisms of the resistance against colonisation by gut pathogens mediated by probiotic cultures remain unknown.

Human flora-associated rodents. In an attempt to circumvent some of the problems associated with the use of conventional flora animals in experimental studies and yet retain their advantages of convenience and of dietary, environmental, and genetic control, the colonisation of germ-free rodents with human faecal organisms has been explored. This method maintains the microbial flora in an *in vivo* environment similar to that of the human alimentary canal by associating germ-free rats or mice with a suspension of freshly collected human faeces. The similarity of the resultant flora to that of human faeces has been investigated at the bacteriological and the metabolic levels.

Hazenberg et al. (60), who inoculated germ-free mice with suspensions of human faeces from four individuals demonstrated that the gross bacterial composition of the experimental flora was similar to that of man and was distinct from the indigenous murine flora. The human flora remained unchanged when associated with the mice for at least 5 weeks.

Mallett et al. (61) have performed complementary studies to those of Hazenberg in which germ-free rats were associated with a human faecal flora. The flora was characterized on the basis of microbial enzyme activities rather than by bacteriological methods and the flora was found to have many metabolic similarities to the native human flora from which it was derived. Thus, the results of these studies indicate that the human faecal flora retains its bacteriological and enzymic characteristics when associated with gnotobiotic rodents. Such a system provides a model for studying human gut microfloral ecology and metabolism. In particular, it facilitates studies of the interaction between dietary components and the human gut flora (since it is easier to control and manipulate animal diets than those of humans), and, furthermore, it provides the opportunity to study the role of the human flora in toxicity of chemicals.

II *IN VITRO* MODELS TO STUDY COLONISATION AND COLONISATION RESISTANCE

In vitro models offer further simplification and a further level of control to study mechanisms of colonisation and colonisation resistance. *In vitro* modelling allows researchers to study the influence of specific prebiotics, probiotics and drugs on the microflora. Like the *in silico*

and mathematical models, experiments can be done in these systems which no ethical committee would allow in any *in vivo* setting.

Advantages

- Ethical restrictions are absent
- Good control over species in model flora
- Reduced complexity of host and flora facilitates interpretation of the data
- Good access to flora in all parts of the model system

Disadvantages

- Reduced realism
- Mucosal and luminal models have not yet been integrated
- No coupling to *in vitro* immune system foreseeable

Luminal models

The simplest, and most widespread *in vitro* model of the *luminal* part of the intestinal microflora is the continuous flow chemostat (62–72). These continuous flow systems have the great advantage that mathematical modelling is straightforward and analytical, steady state solutions can be derived, which allows derivation of kinetic parameters (73–75). However, it is unlikely that a continuous flow system models the situation in the intestine accurately (fortunately!). For this reason, Nuotio and Mead (76) used an intermittent flow system to model the flora of the avian caecum more accurately.

An extension of the single stage continuous flow chamber is the use of multiple stages. Itoh and Freter (77) already use a two-stage simulator (stage meaning compartment of the intestine). Further extensions include the three stage system of Gibson and Wang (78), and the six-stage SHIME (Simulator of the Human Intestinal Microbial Ecosystem) system (79–81). Another multi-compartment model intestinal tract has been developed at TNO, Zeist, the Netherlands (82). Though highly realistic simulations of many features of the gut have been made, the (mucosal) immune system is missing in all systems, and it is hard to see how it could be incorporated.

Itoh and Freter (77) studied the inhibitory effect of *Clostridia* and *Lactobacilli* on *E. coli* populations, comparing results in gnotobiotic mice with those obtained with their two-stage chemostat. Using an intermittent flow system, Nuotio and Mead (76) have shown the inhibitory effect of the avian caecal flora to both entero-haemorrhagic *E. coli* and *S. infantis*. Gibson and Wang (78) used a three-stage continuous flow system to study the effect of a prebiotic (oligofructose) on the intestinal microflora. Asplund et al. (83) studied inhibition of *Yersinia enterocolitica* in an *in vitro* model porcine intestine. Nollet et al. (79) used the six-compartment SHIME system to study the effect of addition of *Peptostreptococcus productus* to the microflora of the ascending colon. They concluded that *P. productus* could be maintained in the microflora if administered regularly and could compete with indigenous methanogens. In the same simulator, Kontula et al. (80) studied the colonisation of their model intestine by *Lactobacillus* GG and its effect on a number of other bacterial species, as well as their metabolite production. Here stable colonisation was observed. Alander et al. (81), also using the SHIME system, studied five potential probiotic strains of lactobacilli, in part to assess the possibilities of using the reactor as a screening method for probiotics.

Bernhardt et al. (71, 72) have studied mycological aspects of the intestinal ecology using a continuous flow system.

Marteau et al. (84) used the TNO system to model survival of lactic acid bacteria in stomach and small intestine and particularly the effect of bile. The TNO model intestine has elaborate, computer-controlled systems to simulate gastric juices, bile, and other secretions and even uptake through the gut wall (82). This study was aimed to validate the simulator. Ganzle et al. (85) studied the effect of bacteriocin-producing *lactobacilli* on *E. coli* and *Listeria innocua* in the system. Apart from studying the survival of the lactobacilli, the degradation of the bacteriocin (curvacin A), and its toxin effect could also be studied.

Mucosal models

Since mucosal adhesion of bacteria is considered to be an important factor in colonisation, several studies of *in vitro* mucosal modelling have been made. Most of these methods use cultures of enterocytes or enterocyte-like cells or mucosal explants. Both host and bacterial factors influencing adhesion or invasion can be studied by these assays.

Knutton et al. (86) used isolated human enterocytes from duodenal biopsies to determine the adhesion of different strains of enterotoxigenic *E. coli*. Bertschinger et al. (87) found a good correlation between *in vivo* and *in vitro* binding of two strains of *E. coli* to porcine enterocyte brush borders. Favennec et al. (88) and Magne et al. (89) studied the mechanisms of adherence of *Giardia intestinalis* and *Giardia duodenalis* to enterocyte-like Caco-2 cells (human colon carcinoma cells). Coconnier et al. (90) found that heat killed *L. acidophilus* could adhere strongly to Caco-2 cells and could inhibit both adhesion and invasion of these cells by four enteric pathogens. Crociani et al. (91) compared the *in vitro* adhesion of bifidobacteria to Caco-2 cells to previous *in vivo* work. Hudault et al. (57) used cultures of Caco-2 cells, which could be protected from invasion by *S. typhimurium* C5, by contact between the invader and either a *Lactobacillus* GG culture, or its supernatant. Similar effects were observed in mice. A similar study by the same group (92) was done to demonstrate the protective effect against invasion of Caco-2 cells by *S. typhimurium* of spent culture supernatants of *L.*

acidophilus strain LA1. Ouwehand et al. (93) used immobilized mucus to study the influence of the normal faecal flora on the adhesion of a probiotic to the mucosa.

A number of studies focus on bacterial translocation mechanisms and in particular, the effect of epithelial damage on the translocation process. Wells et al. (94) used cultures of HT-29 enterocytes to study the effect of calcium on the epithelial junction integrity, and its effect on bacterial translocation. Low calcium availability was found to increase endocytosis and loosened the junctions between the cells. Wells et al. (95) also showed that bacteria can survive intracellularly, after endocytosis by either Caco-2 or HT-29 cells. The same group (96) also studied the effects of hypoxia on bacteria-enterocyte interactions. They concluded that hypoxia causes increased endocytosis, which may in part explain the increased frequency of translocation in tissue ischaemia. Xu et al. (97) found similar results in a slightly different experimental set-up. They also concluded that the tight junction integrity was impaired by hypoxia. Xu et al. (98) have also studied the effect of heat shock and endotoxin stress on enterocyte apoptosis and bacterial translocation.

Inheritance of host factors influencing the numbers of intestinal receptors for six strains of *E. coli* were studied by Hu et al. (99) in enterocyte preparations from 368 pigs. They found evidence for two different receptor types, one low and one high affinity. The low affinity receptor was no longer expressed after 16 weeks of age, whereas the high affinity persisted throughout life. Some pigs showed a mixed phenotype, expressing both receptors. Vogeli et al. (100) used an *in vitro* adhesion assay, using small intestinal enterocyte preparations and an *E. coli* strain, in a study to determine which host genes were involved in mucosal adhesion of the pathogens in pigs.

Binding of bacterial toxins (*C. difficile* toxin A, and cholera toxin) to intestinal brush border membranes from axenic and conventional mice *in vitro* was studied by Lucas et al. (101). The presence of a conventional microflora did not appear to increase the affinity of toxin A to the brush border receptors, but only the number of receptors. They found a good agreement with the *in vivo* results, where conventional mice were more susceptible to toxin A than the axenic mice. No difference was observed for cholera toxin.

III *IN SILICO* AND MATHEMATICAL MODELS TO STUDY COLONISATION AND COLONISATION RESISTANCE

Advantages

- Ethical restrictions absent
- Full control all model parameters, including complexity of the model: from highly simplistic to very complex
- Full access to all data in all parts of the model system, at any time
- Mucosal and luminal models can be combined
- Work on *in silico* immune systems can be integrated
- Cheap and fast

Disadvantages

- Further reduction of realism
- Methodology still in its infancy

Mathematical models and computer simulation (or *in silico*) models are comparative newcomers to the study of the intestinal microflora in general and colonisation resistance in particular. Though mathematical modelling and computer simulation are often considered as one and the same thing, a few important distinctions must be made. A mathematical model consist of a system of equations or mathematically posed rules to describe the dynamics of the (eco)system being modelled. A computer simulation model consists of one or more computer programmes in which a mathematical model is translated into machine instructions, along with code to solve the equations. Therefore, computer simulations require a mathematical model of the system under investigation, but the reverse is not true. The simplest mathematical models can be solved analytically, without the need for computer simulations. In such cases, an *exact* solution of the system of equations is available. When we have to resort to computer simulations, only an approximation of the solution to system of equations can be obtained. This is often not a problem, since the set of equations was only an approximation of the real system anyway.

Mathematical and *in silico* models allow researchers to study the principles of interactions within a model microflora, rather than the effects of specific prebiotics, probiotics, or drugs. Mathematical and *in silico* models also serve as a platform for 'thought experiments,' allowing researchers to test the impact of parameters in a system in which their theory holds. Ecosystems are renowned for their 'counter-intuitive' behaviour, and the predictions of a theory can easily be radically different from what is expected.

Another difference with *in vitro* and *in vivo* work is that the time scale of the experiment can be compressed. Simulation of a bout of toxigenic diarrhoea from onset to complete recovery (15 days) takes just a few minutes on a Cray J932 supercomputer (Wilkinson, unpublished data). Even on modern personal computers, such a simulation might take less than an hour.

A few attempts at mathematical modelling have been made, notably by Freter et al. (102), and more recently by Coleman et al. (74) and the Model Intestinal Microflora In Computer Simulation (MIMICS) system (103). The models have fairly large conceptual differences. The model of Freter and co-workers has no spatial extent, and bacterial

interactions are based on single substrate competition using the Monod equation for growth, and competition for epithelial binding sites. Though various alternatives to the Monod equation, which is essentially a single step, Michaelis-Menten kinetic uptake equation, exist (104, 105), its use in microbial ecological modelling is ubiquitous (73, 106–108). Coleman et al. (74) also omit spatial scale from the model, and allow competition for multiple substrates through multiple substrate uptake through Blackman's bilinear equation (109) and a generalized logistic growth equation which allows for intra and interspecies competition for space. Because the models do not have spatial extent, they can be considered as equivalents of single-chamber, continuous flow chemostats. Indeed, such mathematical models are frequently used to understand the properties of *in vitro* chemostat models (73, 74).

By contrast, the MIMICS system (103) includes spatial scale explicitly, and models bulk transport and diffusive and turbulent mixing. It models the intestine as an axis-symmetric tube of varying diameter, through which there is an influx of bacteria, nutrients and other chemicals, and an efflux of 'faeces.' The model for bacterial growth, competition and the mutualistic interactions are through uptake of oxygen and a single carbon source modelled through interactive Michaelis-Menten terms (73). As such, it resembles the *in vitro* multi-compartment models more closely.

Applications

Substrate competition. In all of the aforementioned models substrate competition is the main interaction between species. Apart from the few models of the gut ecosystem, this type of interaction has been studied extensively in many other ecosystems, and a vast literature on this topic exists (75, 108, 110, 111).

In most studies, both dilution rate and the nutrient composition of the inflowing medium is held constant. One of the main reasons for the use of this approach is that under these assumptions analytical solutions can be derived readily (112). This also allows kinetic parameters to be derived. It can be shown that at given a dilution rate and nutrient concentration, only one species can survive in a stable equilibrium. If multiple substrates are available, *at most* as many species as there are *limiting* nutrients can survive in a stable equilibrium (108). Note that stability in this context means absolute, mathematical stability. The order in which species invade has no influence on the final outcome of the competition.

The effect of temporal variation in the nutrient supply on the competition between species has been studied by a number of authors (106–108). All studies seem to agree that two species may coexist in a stable limit cycle on a single, limiting substrate, in contrast to the situation in the steady state flow rate, where the number of coexisting species cannot be larger than the number of limiting substrates.

The effect of inhibitory substances. Models of growth inhibition generally follow those of inhibition of enzymes, just as growth models are analogous to enzyme kinetic models (113). Presser et al. (114) have produced a mathematical model of the growth rate of *E. coli* as a function of lactic acid concentration and pH. The model includes water activity and temperature. Though the model was not in complete agreement with the data at high lactic acid concentrations, it was satisfactory at lower levels.

Tan et al. (115) provide a mode for substrate inhibition, which can occur at very high substrate concentrations.

Secretion of inhibitory substances. Frank (116) has presented a mathematical model of the interaction between two species of bacteria, which are identical in substrate uptake (using a logistic growth equation), but differ in that one secretes a substance toxic to the other. The production of substrate costs the toxin producer a certain amount of biomass, so at a given level of limiting substrate, its growth will be slower than that of the susceptible species (in the absence of toxin). Frank has shown that this situation leads to a so-called bi-stable equilibrium, which either only susceptibles, or only producers in the ecosystem. If there are sufficient numbers susceptibles, they decrease the available substrate levels to such an extent that a few invading toxin producers cannot grow sufficiently fast to increase their numbers. Besides, the amount of toxin produced by a small number of invaders is too small to have an impact on the susceptibles. If the ecosystem consists of only producers, the susceptibles cannot grow fast enough to outcompete the producers, due to the high levels of toxin. In this situation, the species arriving first usually has the advantage.

Removal of inhibitory substances. A case used as a pilot study for the MIMICS system is an example of removal of an inhibitory (oxygen) by bacteria (aerobes), which must take place before another (anaerobic) species can establish itself (103). In this case, there was only one stable equilibrium, with two co-existing species, but the order in which they could invade the ecosystem was fixed: first the aerobes, and only later the anaerobes. This principle can readily be extended to other types of inhibitory substances.

Models of epithelial binding. Freter et al. (102) modelled the binding of bacteria to different receptors in the gut, though, strictly speaking, he did not model competition for binding sites, since only the invading species could bind to the receptors. A similar model, which includes embedding in mucus, and interaction with the host, has been proposed for the dynamics of *Helicobacter pylori* on the epithelium of the stomach (117). Again, there was no competition between different species, but the general form of the model can be used in studies of competition in the gut. It is unclear whether binding site competition yields a 'first come, first serve' situation, as in the case of toxin production, or whether, as in the case of substrate competition, the order is largely irrelevant.

At a higher level of detail, mathematical models of the spatio-temporal patterns created by bacteria growing on a surface have also been developed (118).

Quorum sensing. Quorum sensing (119), or density dependent behaviour in bacteria may have important implications for colonisation in the gut ecosystem (see a separate review in this supplement by Swift et al.). To date, no mathematical models have been put forward which might be used in the context of colonisation resistance, but it should be straightforward to do so.

Including an immune system. With the advent of computer models of the immune system (120), it should also be possible to link microflora and immune models, for even greater freedom in *in silico* experimentation.

CONCLUSIONS

Animal models provide insight into the accuracy of theoretical predictions in a controlled, but complex system.

In vitro models provide essential data on 'atomic' processes, by a reductionist approach, along with a measure of synthesis in the more complex models.

Mathematical and *in silico* models provide means to create a synthesis, integrating findings from other models into a coherent, quantitative theory.

Human models form the final, integrating step in the research, proving a theory in practice. These models consist in dietary, clinical studies in human and offer full realism. However, several factors have to be controlled such as environment of subjects (diet, stress etc. and genetic characteristics of subject population). Ethical grounds do not allow to perform any kind of toxicological studies. Finally, complexity of models makes interpretation of results hardier. As the realism of the models increases, experimental flexibility is reduced due to ethical questions and limited access to all parts of the models.

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