Bioregulation of the digestive tract microflora

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Summary: Colonisation resistance (CR) is the resistance encountered by ingested micro-organisms when they try to colonise the digestive tract. This review discusses various aspects of the CR, including factors involving the cooperation of host and indigenous microflora. Factors which affect the CR and may decrease it significantly include antibiotic treatment, severe illness, stress and possibly diet. Involvement of the immune system in the mucosa-associated intestinal microflora from birth is outlined, along with its influence on the final composition of the intestinal flora of a subject.

Finally, a new, rapid and very promising technique for determining the CR is discussed. By computer analysis of the morphology of (fresh) faecal bacteria on a microscopic slide and subsequent statistical evaluation of the data, the protective value of the CR can be estimated. This computerised micromorphology analysis can also be accomplished with UV-microscopy. If the faecal flora is preincubated with the host's serum antibodies and sandwiched with fluorescent anti-human isotype antibodies, additional information can be obtained about an individual's humoral immune reactivity to indigenous flora components.

KEYWORDS: Bacteria - Colonisation - Defence mechanisms - Farm animals - Intestinal flora - Regulation - Techniques.

INTRODUCTION

In veterinary medicine, prevention of infection is of great practical and economical importance. Early weaning of animals may be followed by a high incidence of enteric infections, and certain herds or flocks suffer more from infections than others. A critical analysis of the defence mechanisms that exist in the digestive tract may therefore clarify several puzzling observations concerning infections.

The present review will discuss the variety of mechanisms which together form the first line of defence to intestinal infection, called colonisation resistance (CR). We will then consider the implications of abnormal intestinal colonisation for infection, and provide a description of the sequence in which CR develops after birth. The circumstances required for the development of an optimal CR after birth, as well as the requirements for maintenance and improvement of the CR in adult animals, may be easier to understand on the basis of the information presented.

As used in this review, the term indigenous microflora refers to the individual animal's own persistent intestinal microflora. All bacteria that stay for some time (perhaps several weeks) after ingestion, or which simply pass through the digestive tract, are regarded as transient to the animal.

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THE MICROBIAL ECOSYSTEM OF THE DIGESTIVE TRACT

In all animal species, an important microbial ecosystem develops in the digestive tract after birth. Depending on the animal species, this process may take several weeks to months. As will be discussed later, the principle source of this microflora should be the dam. Once the gut has adopted its numerous inhabitants, which are largely anaerobic bacteria, the bacterial community apparently forms a strong ecosystem which, in conjunction with the host individual, keeps its environment (the gut) free of newly ingested bacterial species. The composition of the indigenous bacterial society in the digestive tract adopted by the host is largely determined by the following factors:

1. Chance: Bacteria which are ingested shortly after birth have a better chance than bacteria which enter the digestive tract later on. The time at which the system "closes" varies with animal species from several weeks to months.

2. Enzymes: To settle in the intestines, bacteria must have, either themselves or in conjunction with others, a set of proper enzymes to digest the nutrients available in their specific niche. Many ingested bacteria cannot persist because they lack the capacity to utilise the nutrients available in the intestines of a certain animal. Only those bacteria which live within the mucus layer covering the mucous membrane are protected from the flushing effect of peristaltic movement. Consequently, to become indigenous, bacteria must be able to utilise intestinal mucus as a major source of nutrients.

3. Immunoregulation: In order to live in close proximity with the gut mucosa, bacteria must be immunologically accepted by the host organism.

4. Adhesion: In the oropharynx, the capacity to adhere to the mucosal cells is a prerequisite for bacteria to colonise the area. In contrast, adhesion to the intestinal mucosa is not a strict requirement for colonisation. Bacteria need only to be able to live in the mucus and compete adequately with other bacteria for nutrients.

The selection of bacteria which will finally form the indigenous intestinal flora occurs mainly at the site of primary colonisation, i.e. on oropharyngeal epithelial cells and in the intestinal mucus.

COLONISATION RESISTANCE OF THE DIGESTIVE TRACT

The term "colonisation resistance" (CR) was introduced in 1971 by Van der Waaij et al. (27). The degree of CR of the digestive tract of a particular individual can be estimated in several ways, for example:

a) In a group of animals, by oral inoculation of a representative number of individuals of the group with the same number of (marked) bacteria. The concentration of the orally inoculated strain in the faeces along with the duration of its presence form a measure of the CR of the animal population investigated (27). The CR to a certain bacterium was originally defined as the logarithm of the number of bacteria that results in colonisation for 14 days or longer in 50% of a group of animals, following oral inoculation of all members of the group with the same inoculum size.

b) In conventionally living individual animals (i.e. not bacteriologically isolated, and specific pathogen free (SPF)) a good correlation has been found between the
concentration of a certain oral bacterial infection in the faeces and the average number of different biotypes of Enterobacteriaceae in the faeces (30).

c) Faecal enzyme patterns appear to reflect the intestinal population pattern (21, 37).

Levels of CR

In conventional mice with a stable microflora, there is usually a strong suppressive effect on orally inoculated organisms. This means that conventional mice may have a high CR. For example, in the case of most potentially pathogenic bacteria, $10^8$ to $10^9$ bacteria (for which no mucosal immunity exists) are required to establish colonisation for several (at least two) weeks in 50% of the group investigated. However, SPF mice may also have a CR lower than in conventional counterparts (16).

A lowered CR may also be due to substantial quantitative and possibly also qualitative changes in the indigenous microflora, hampering secretion of mucus and perhaps also decreased mucosal cell desquamation. This may occur during periods of stress, during aging, severe illness and most obviously during chemotherapy for cancer or for some time after irradiation. Recovery of the host organism mostly implies recovery of its indigenous gut flora and thus the CR (33).

Extremely low CR values are to be expected in animals under treatment with broad-spectrum antibiotics, particularly if the antibiotic is applied orally (23, 35, 39). Antibiotic concentrations in the intestines may become sufficiently high to suppress growth of many, if not all CR-associated indigenous bacteria. During broad-spectrum antibiotic treatment, one should therefore be aware of the enhanced susceptibility of the patient to bacteria or yeasts that are resistant to the concentration of the antibiotic established in the gut. Sensitive bacteria, even if they are pathogenic, will die along with the indigenous species due to antibiotic activity in the intestinal contents.

To summarise, the CR of the digestive tract can be lowered by incompleteness of the flora, by poor condition of the mucous membranes (nutrient depletion) and by antibiotics.

STABILITY OF COLONISATION RESISTANCE

To become a member of an existing gut ecosystem, in other words to become indigenous, three sequential barriers must be crossed by a newly ingested bacterium.

1. The bacterium must be able to survive among numerous indigenous bacteria that are continuously freed from the mucus layer and mixed with intestinal contents. Indigenous bacteria often produce substances like volatile fatty acids or bactericidic substances which are toxic to other newly ingested microbes. Examples of bacteria that are susceptible to volatile fatty acids are: *Clostridium difficile*, *Campylobacter* species, *Salmonella* species and perhaps also staphylococci.

2. The bacterium must be able to compete for nutrients in the mucus layer.

3. The bacterium should not be rejected immunologically.

In practice, these barriers are crossed only by bacteria which fulfil the requisite criteria early in the life of the host organism. The more complete the flora of an animal,
the more it utilises all available nutrients, leaving nothing for the relatively small number of newly ingested and otherwise suitable strains. The average number of bacteria ingested each day may vary from one animal species to another. Only in animals which consume carcasses of other animals will the ingested number of bacteria approximate numerically to those present in their intestines. In other animal species, the daily intake is only a fraction of the normal number of indigenous bacteria in the intestines ($10^{11}$ per gram of contents). As a result of the barriers to colonisation formed by the indigenous flora in close cooperation with the host organism, ingested bacteria rarely manage to colonise the gut permanently. This may occur in carnivorous animals which have ingested another animal's complete gut contents (huge numbers of bacteria), and is less likely to occur in herbivorous animals.

Unfortunately, no information is available about the stability of composition of the indigenous intestinal flora in carnivorous animals as opposed to herbivores. It may not differ much, because in man (for example) it is impossible to implant mixtures of intestinal bacteria indigenous to other humans for longer than a few days (9, 18, 32). This is presumably due to differences in antigenic composition of intestinal bacteria (serological difference) (40), regardless of the fact that they belong to the same genera and species.

Once established, the indigenous intestinal flora thus appears in general to be qualitatively stable.

**INFLUENCE OF DIET ON THE INDIGENOUS FLORA**

The diet may provide an additional source of nutrients to intestinal bacteria, influencing the composition of the flora (6, 12). Only if the food consists of pure intestinal mucus will the luminal flora of the intestines be quantitatively and qualitatively identical to the indigenous flora in the mucus secreted by the host organism. Since food never consists of pure undigested mucus of the same animal species, the luminal flora always differs quantitatively in composition from the mucosal flora. On some diets certain indigenous bacterial species may be enhanced in growth, whereas on other diets other indigenous species may grow better. In this way, diet can modulate the composition of the flora, and may influence the CR positively, enhancing the barrier to newly ingested bacteria by stimulating growth of the bacteria which are competitive, or producing toxins for the newcomers. Diet can have a negative effect by lowering the barrier to newcomers, giving growth preference to less competitive and toxic indigenous species.

Diet may also influence the CR negatively in a different manner. If newborn animals are maintained for several weeks on a low-protein diet, this may affect the immunoregulation of the composition of indigenous microflora. Their T-cell system may develop poorly under such circumstances, with impaired capacity to develop oral tolerance to their indigenous flora (17, 19). If the protein deprivation lasts too long, the subject may be unable to develop the well-balanced immune response required for normal immunoregulation of flora composition, even when the amount of dietary protein is increased.

The direct influence of diet on the luminal intestinal flora, as opposed to the mucosal flora, is summarised in Figure 1. Protein-deficient diets lower T-cell activity. This is particularly important in young subjects which are in the process of selecting a proper (protective) indigenous microflora.
TRANSLOCATION OF (POTENTIALLY) PATHOGENIC BACTERIA

Another event associated with the quality of the CR which is of practical importance is translocation of bacteria from the luminal side of the intestinal mucosa to the mesenteric lymph nodes, liver and spleen (3, 28). Translocation occurs more readily and massively when a large number of certain bacteria (in particular, potentially pathogenic species) is colonising the mucous membrane (3, 28). Massive colonisation by potentially pathogenic bacteria or yeasts is generally reflected by relatively high concentrations of these microbes in the faeces (Fig. 2).

Normally, the translocation of potentially pathogenic bacteria is not noticeable clinically. The translocating bacteria become phagocytosed soon after passing through the mucosa, before an infectious process can develop. Phagocytosis may occur in the submucosal tissues and may play a role in the transportation by macrophages (38) of these bacteria into the mesenteric lymph nodes, liver or spleen. In these lymphoid organs, translocating bacteria may induce humoral immunity. Potentially pathogenic bacteria such as *Escherichia coli* and other not essentially pathogenic Gram-negative enterobacilli apparently induce only a low titre of circulating antibodies. Most of the antibodies, being of the IgA isotype, are excreted with mucus into the intestinal tract (14). Essentially pathogenic bacteria such as *Salmonella* and *Shigella*
Diagram showing the correlation between oral inoculation dose, subsequent intestinal colonisation and the occurrence of positive cultures of lymphoid organs. High intra-intestinal numbers of E. coli are associated with translocation into the lymph nodes and spleen.

species may induce the production of much higher titres of circulating antibodies of a different (IgG) isotype. In this case, the term "translocation" is inappropriate, as invasion is clinically apparent and may cause severe illness.

To summarise, not only pathogenic but also potentially pathogenic bacteria may translocate from the mucosal mucus layer into the submucosal tissues and the regional lymphatic organs if they colonise the mucus in sufficiently high numbers.

ROLE OF THE IMMUNE SYSTEM IN THE DETERMINATION AND CONTROL OF THE COMPOSITION OF THE INDIGENOUS MICROFLORA

The immune system is in continuous contact with intestinal contents through its gateways, the tonsils and Peyer's patches. Epithelial cells overlaying the Peyer's patches (called M cells) are specialised in absorbing large molecules, including phagocytosis of larger particles or even intact bacteria from the intestinal lumen. After uptake from intestinal contents, this antigenic information is transported through the M cells to the underlying tissues of the Peyer's patches, which contain macrophages, B cells and T cells. Much of the antigenic information is digested and destroyed by the macrophages to become non-immunogenic. The extent to which this occurs seems to be related to the amount of adjuvant (polyclonal stimulating) activity produced by endotoxin and other substances in the bacterial cell wall, such as peptidoglycan.
(7) and one of its derivatives, muramyl dipeptide (24). There is strong evidence that at least some indigenous bacteria of the intestinal flora are non-immunogenic (2, 8). This might be due partly to cross-antigenicity of antigenic determinants of (indigenous) bacteria involved with host tissue antigens (5).

**Oral tolerance**

Much antigenic material in the intestines appears to become immunologically neutral in the Peyer’s patches by the induction of immune tolerance. This rendering of an individual immunologically unresponsive to a certain antigen (ovalbumin) (4) or group of antigens like sheep erythrocytes (1) by repeated massive oral inoculation, is called “oral tolerance”. Oral tolerance is due to induction of specific activity in T-suppressor cells (38). In certain animal species, oral tolerance is easier to induce early in life than at an adult age (15).

**Intestinal antibodies**

In adult animals, most ingested bacteria and nutritional antigens sampled by the Peyer’s patches result in antibody (IgA) and/or cellular immunity. The IgA-isotype of antibodies may, after excretion with the mucus, interfere with adhesion of bacteria to the mucous membrane, or with absorption of larger (immunogenic) molecules (13). The latter would otherwise induce IgE production and thus cause allergy (2). IgA is also an agglutinating antibody, capable of agglutinating bacteria to form large aggregates. By agglutinating bacteria in the mucus layer that have just induced IgA production or have previously done so, their clearance by intestinal flow is promoted and colonisation is thus terminated or prevented (10, 25).

The cellular immunity provided by T cells also appears to be important in controlling the CR. T cells may exert both positive and negative influences on the intestinal mucosa itself, affecting the indigenous flora (22). The mitotic activity of cells in the crypts of Lieberkühn can be favoured by release of lymphokines from T cells. On the other hand, enterocytes affected by pathogens are actively killed by T cells and desquamated, resulting in shortening of villi, which can be regarded as a negative influence (26).

To summarise, the gut-associated immune system can respond either negatively (by immune tolerance) or positively by producing IgA antibodies which are secreted with the mucus into the intestinal tract.

**DEVELOPMENT AND INSTRUCTION OF THE GUT-ASSOCIATED LYMPHOID TISSUES AFTER BIRTH**

The intestinal ecosystem is established soon after birth. The first encounter of bacteria and antigens of other sources with lymphoid cells occurs in the Peyer’s patches. In this period, the lymphoid organs and particularly the gut-associated lymphoid tissues (GALT) “determine” their type of response to the bacterial flora of the developing mucosa. A moderate (normal) mitotic activity in the crypts may represent optimal circumstances for both the host organism and indigenous flora to establish and exert its protective function, the CR. The less vigorous a cellular immune response is to the mucosa-associated bacteria that should compose the indigenous intestinal flora, the less energy is wasted. Enhanced crypt cell proliferation as well as rejection of affected cells along the villi strongly impose on nutritional sources
of the host organism. In contrast, a short crypt and a long villus imply rest and maximal goblet cell (mucus) formation, so that the indigenous flora can flourish.

Because there are clear differences between individuals in the CR of the digestive tract, both in adult humans (20) and in animals (34), it seems unlikely that the difference in flora composition between individuals is determined merely by the immune system, either through oral tolerance permitting the microbes involved to stay, or by limiting colonisation through IgA secretion and/or cell desquamation associated with increased crypt activity. This is the more unlikely because significant and persistent individual differences in the CR have been found in mice of the same inbred strain. Although the animals were taken from six different litters they were genetically identical, born in the same week and maintained in the same animal room (36).

At least in mice, the dam is presumably the first and most important source of bacteria for the indigenous flora of her offspring. The litters of different genetically identical dams acquire during 3-4 weeks of age (before weaning) a CR which is similar in quality to that of the dam. Enhanced crypt activity by specific (bacterial) T-cell stimulation due to an intestinal pathogen in these first three weeks of life is undesirable. This would interfere with a successful "take" of bacteria from the dam's indigenous flora, or appropriate bacteria from other sources. Enhanced crypt activity upon contact with a pathogen is not selectively harmful to the pathogen. After a decrease of the CR due to the immune disruption of the developing flora, new bacteria, possibly the same as when the dam is still available, may begin recolonising the infant animal. The new colonising bacteria may have difficulty in inducing oral tolerance, even though they possess the proper enzymes.

To summarise, before weaning and soon after birth, bacteria which properly fit the intestinal niches of the subject may induce immunological unresponsiveness more readily than after weaning and during adulthood.

STUDY OF COLONISATION RESISTANCE-RELATED FLORA TO ESTIMATE ITS PROTECTIVE VALUE

As described above, the CR of the digestive tract is determined by oral inoculation of a sufficient dose of an identifiable (by a certain antibiotic resistance pattern) potentially pathogenic bacterium (27, 30). Determination of the concentration of this bacterium in the faeces during the days following contamination, provides a measure of the CR for the bacterium tested in the animal(s) tested. This method is as laborious as the alternative test used to measure the CR of an individual directly, namely, by comprehensive biotyping of Enterobacteriaceae species in a number of consecutive faecal samples of an individual (29, 30, 31). The "microflora-associated characteristic" (MAC) technique of Welling (37) and Midtvedt (21) is easier, but provides only an indirect insight into the population pattern of the gut.

Because of the high costs of comprehensive biotyping of Enterobacteriaceae and the indirectness of the tests available, a new, more direct approach for measuring the CR has been developed by Meijer in our laboratory. A software programme for micromorphology analysis measures eight different parameters which determine the microscopic morphology of each bacterium. For each sample this is carried out on between 700 and 1,000 bacteria in a washed faecal suspension on a microscopic slide. A statistical evaluation, performed by the same software, provides detailed
information about the population pattern in the faeces of an individual (Figs. 3 and 4). Data provided in both Figures make it possible to calculate a “centre of gravity” for the population. This population pattern may correlate well with CR. Direct micromorphological analysis of faecal bacteria may also make it possible to investigate the occurrence, titre and isotype of antibodies produced by a host organism to components of its indigenous faecal flora. By mixing washed bacteria with different dilutions of serum from an individual, antibodies to bacteria on the slide can be revealed by using fluorescent anti-human (isotype) globulin antibodies in a sandwich technique. The same software has been adapted to this type of investigation. By making UV-microscopic and phase-contrast observations on the same series of microscopic views, the morphology of the bacteria against which circulating antibodies are active can be studied. In this way, the isotype of the anti-bacterial antibodies and the titre can be determined.

![Figure 3](image)

**Figure 3**

Scatter plot of bacterial morphologies measured in a microscopic preparation of human faeces

For clarity the shapes of the actual bacteria measured have been plotted. The morphology of each bacterium in the figure is characterised by two factors, containing both size and shape information.

This new micromorphology analysis rapidly provides accurate information about bacterial numbers per gram of faeces, as well as the qualitative aspects of their morphology. In contrast to the equipment used by Goldstein *et al.* (11), the costs of the hardware (an ordinary AT computer with an extension for image acquisition and analysis, plus a CCD-camera of high resolution) and the software are within the financial reach of practically every research and clinical laboratory that is interested in studying changes of the faecal flora.

In summary, the composition of the intestinal (faecal) flora and serological evidence of its immunologic interaction with the host organism can be studied under
In Figures 4a and b, two opposite views are shown of the number distribution to provide a complete presentation.
the following circumstances: development since birth, changes during antimicrobial treatment, modulations due to different diets, and the pattern in patients with a particular disease that might correlate with the pattern of the flora (such as intestinal disease, systemic autoimmunity, malignancies and severe trauma).

**BIORREGULACIÓN DE LA MICROFLORA DEL TRACTO DIGESTIVO. – D. Van der Waaij.**

Resumen: La resistencia a la colonización (RC) es la resistencia que encuentran los microorganismos ingeridos cuando tratan de colonizar el tracto digestivo. Este artículo presenta los diferentes aspectos de la RC, incluidos los factores que suponen una cooperación entre el huésped y la flora indígena. Los tratamientos antibióticos, las enfermedades graves, el estrés y, tal vez, el régimen alimenticio se cuentan entre los factores que afectan la RC y que pueden disminuirla significativamente. Se describe la función del sistema inmune, desde el nacimiento de un individuo, en la microflora asociada a la mucosa intestinal, así como su influencia posterior en la composición final de esta última.

Finalmente, se considera una nueva técnica, rápida y promisoria, para determinar la RC. El valor protector de la RC puede evaluarse analizando con una computadora la morfología de las bacterias fecales (en heces frescas), colocadas en el portaobjetos de un microscopio, y efectuando luego una evaluación estadística de los resultados. Este análisis micromorfológico asistido por computadora también puede utilizar la microscopía UV. Se pueden obtener informaciones suplementarias acerca de la respuesta inmune humoral de un individuo contra los componentes de la flora indígena efectuando una preincubación de la flora fecal con los anticuerpos del suero del huésped y colocándola en «sandwich» entre anticuerpos fluorescentes dirigidos contra los isotipos humanos.

PALABRAS CLAVE: Animales de granja - Bacterias - Colonización - Flora intestinal - Mecanismos de defensa - Regulación - Técnicas.

**REFERENCES**


