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# Parameters and Techniques to Determine Intestinal Health of Poultry as Constituted by Immunity, Integrity, and Functionality

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## Abstract

The intestinal microflora lives in intimate contact with its surrounding intestinal wall and the bacteria can exert beneficial or deleterious effects on the host, depending on whether they are classified as probiotics or as pathogens. The interaction is determined on one hand by characteristics of the microorganisms, and on the other hand by characteristics of the intestinal wall. Together they determine the health status of the intestine. This review describes parameters and techniques (with advantages and disadvantages) available for poultry to identify the characteristics of the intestinal health, as constituted by three components: immunity, integrity, and functionality. To investigate intestinal immunity, *in situ* detection of various cell populations of the immune system with specific monoclonal antibodies using immunocytochemical staining is a reliable, semi-quantitative method. *In vitro* assays to measure functional aspects of T lymphocytes, B lymphocytes, plasma cells, natural killer cells, macrophages, and phagocytes are applicable to intestinal wall tissue. For investigation

## Abbreviations

EGF: epidermal growth factor  
ELISA: enzyme-linked immunosorbent assay  
FACS: fluorescein-activated cell sorter  
HOX: homeobox genes  
IFN- $\gamma$ : interferon-gamma  
IGF: insulin-like growth factor  
IL-2: interleukin-2  
ITF: intestinal trefoil factor  
PCR: polymerase chain reaction  
TGF- $\beta$ : transforming growth factor-beta

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of intestinal integrity, *in situ* detection of villous height and crypt depth and their ratio, and villus arrangement is still an easy, routine histological method. In addition, expression levels of specific molecules, such as E-cadherin, different growth factors, and trefoil factor, seem promising parameters. To investigate functionality of the intestine, the permeability can be measured as the rate of transport of tracer molecules across the epithelial surface. Furthermore, determination of the level of mucus secretion and its composition are a valuable tool. These parameters for immunity, integrity and functionality, or a combination thereof, are indispensable to investigate the influence of intestinal microorganisms on intestinal health.

## Introduction

The intestinal microflora represents a complex and concentrated group of microorganisms. The exact composition of the intestinal microflora is hard to define, but it is assumed that hundreds of species colonize the intestine, of which the strictly anaerobic bacteria far outnumber other types (Zhu *et al.*, 2002). Recently several reviews have been published on the intestinal microflora and the newest techniques for analysis (O'Sullivan, 2000; Vaughan *et al.*, 2000). In very general terms, we can categorize the various components of the intestinal microflora into potentially pathogenic or health-promoting groups. From the viewpoint of harmful properties, intestinal bacteria may be involved in the onset of localized or systemic infections, intestinal putrefaction, toxin formation, and production of mutagenic and carcinogenic substances. Alternatively, some intestinal organisms, for example lactobacilli and bifidobacteria, may confer general health-promoting benefits such as vitamin production, stimulation of the immune system through non-pathogenic means, triglyceride lowering, and inhibition of the growth and establishment of harmful microbial species.

During the last few years, much attention has been focussed on pathogenic bacteria on one hand and health-promoting bacteria on the other hand, not only in humans, but also in farm animals. Human health is regularly endangered by pathogenic microorganisms of animal origin, of which infectious particles of transmissible spongiform encephalitis, *Salmonella enteritidis*, and *Campylobacter jejuni* receive much public attention. The potential ban on the preventive use of antibiotics in feed even increases the need to apply other techniques to lower the burden of pathogenic bacteria in meat and eggs. Hence even in farm animals, like poultry, pigs and cattle, probiotics and competitive exclusion microflora are applied in the field

(Mulder *et al.*, 1997; Huber, 1997).

The human infections by farm animal products are often due to insufficient function of the defence in the intestinal wall of the farm animals. *Campylobacter jejuni*, for example, is a highly infectious organism in humans causing gastrointestinal disease in most cases and in some cases also Guillain-Barré syndrome, an inflammatory demyelinating neuropathy, develops. In contrast in chickens, *C.jejuni* is part of the intestinal microflora and causes no disease. The intestinal defence against *C.jejuni* is limited, develops slowly, and does not lead to exclusion of the bacteria (Cawthraw *et al.*, 1994). This also occurs with *S. enteritidis*, another important cause of food-poisoning, that can survive in the intestine of poultry in a state of disease-free carriage (Barrow, 1996). It is generally assumed that improvement of the innate and specific defence in the intestinal wall will lead to lower numbers of pathogenic bacteria in the intestinal tract of farm animals and thus to less human infections.

Both the pathogenic and health-promoting microflora can be divided into at least two sub-ecosystems: the luminal microflora and the mucosal microflora. The composition of the luminal microflora is mainly determined by the nutrients available, the rate of passage, and the effects of antimicrobial substances. In contrast, the composition of the mucosal microflora is mainly determined by the host's expression of specific adhesion sites on the enterocyte membrane, the rate of mucus production, the production of secretory immune globulins and the extrusion of cellular material from the membrane into the mucus. Therefore, the mucosal microflora will interact intimately with the intestinal wall of the host. As a result, the mucosal microflora and the intestinal wall will mutually influence each other and together they determine the health status of the intestine.

The successful application of probiotics and exclusion microflora, as well as the improvement of the innate and specific defence to prevent zoonotic infections, require detailed knowledge of microorganisms on one hand and the intestinal wall on the other hand. Here, a comprehensive overview will be given of parameters and related techniques to examine the intestinal wall. Three aspects of the intestinal wall are discriminated: immunity, integrity, and functionality. Immunity is defined as the cells and products belonging to the immune system in the gut. Integrity is defined as the cells and products constituting the barrier against leakage or translocation of feed components, microbial toxins, and microorganisms from the lumen to the body. Functionality is defined as those aspects involved in feed degradation and absorption. Obviously, these three aspects are intermingled and hard to separate *in vivo*. Nevertheless, improvement of intestinal health can occur at all three aspects and thus they may require individual investigation.

## Immunity

Immunity of the intestinal wall can be divided into innate and specific (adaptive) defences that have to cooperate to reach a level of immunity that protects against dietary antigens and infections with pathogens (Figure 1). The innate defence is mainly formed by natural killer cells,

granulocytes and macrophages and their secreted products, such as nitric oxide and various cytokines. These cells constitute the first line of defence operating immediately after infection thereby limiting the growth and spread of a pathogen and thus limiting the acute phase response. In addition, macrophages and granulocytes also function as effector cells during the late phase of the specific immune response to eliminate the pathogen. In addition, cells of the innate defence system produce important cytokines that contribute to the specific immunity. Specific immune responses, can be divided into humoral immunity (antibodies produced by B lymphocytes and plasma cells) and cellular immunity (helper and cytotoxic T lymphocytes). Whether merely humoral immunity or cellular immunity, or a combination of both, is induced, is an intrinsic property of a pathogen and is often related to the cell type that is infected. Both types of immunity are characterized by high specificity for the pathogen and by memory formation. The specificity for the pathogen is determined by T-cell receptors located on the cell surface of T lymphocytes and of immunoglobulins on the cell surface of B lymphocytes. Only upon binding of (sub-) molecular structures of the pathogen to these receptors in combination with various co-stimulatory signals from other cell types, such as cytokines, can a primary immune response to a pathogen start. During the immune response, the pathogen is generally eliminated from the animal and then the immune response will fade out. At the same time, however, memory T and B lymphocytes are formed and are disseminated throughout the body. Upon renewed contact with the pathogen, these cells will exert a faster and more effective immune response, the so-called secondary or memory response.

The cells that constitute the innate and specific defence are not randomly located in the intestinal wall, but they are found in three distinct areas. Most prominent are the organized lymphoid tissues, such as caecal tonsils, Meckel's diverticulum, and Peyer's patches, although the latter do not always develop (for review see Jeurissen *et al.*, 1994). The second discrete area is the lamina propria of the intestine. In this loose connective tissue, most if not all cell populations associated with defence are localized in large numbers. The third area is formed by the epithelium. In the epithelium, only specific cell populations can be detected depending on the part of the intestine: T and B lymphocytes in the small intestine, and macrophages in the esophagus (Vervelde and Jeurissen, 1993). Because of the structure of the intestine with its many villi, the total surface of the intestinal epithelium is enormous; in humans, it is estimated to be 300 m<sup>2</sup>. For the chicken, accurate calculations are not available. The importance of the leukocytes in the epithelium (intra-epithelial) and in the lamina propria to defence must therefore not be overlooked and may in fact be more substantial than that of the organised lymphoid tissues. The presence, the size and the structure of the organized lymphoid tissues are dependent on the type of chicken, their age, and the antigenic pressure (Jeurissen *et al.*, 1989). The numbers of leukocytes in the epithelium also increase with age in specific pathogen free layer-type chickens (Befus *et al.*, 1980; Lillehoj *et al.*, 1992; Vervelde and Jeurissen, 1993) and in broiler type chickens (Songserm 2001). In inbred

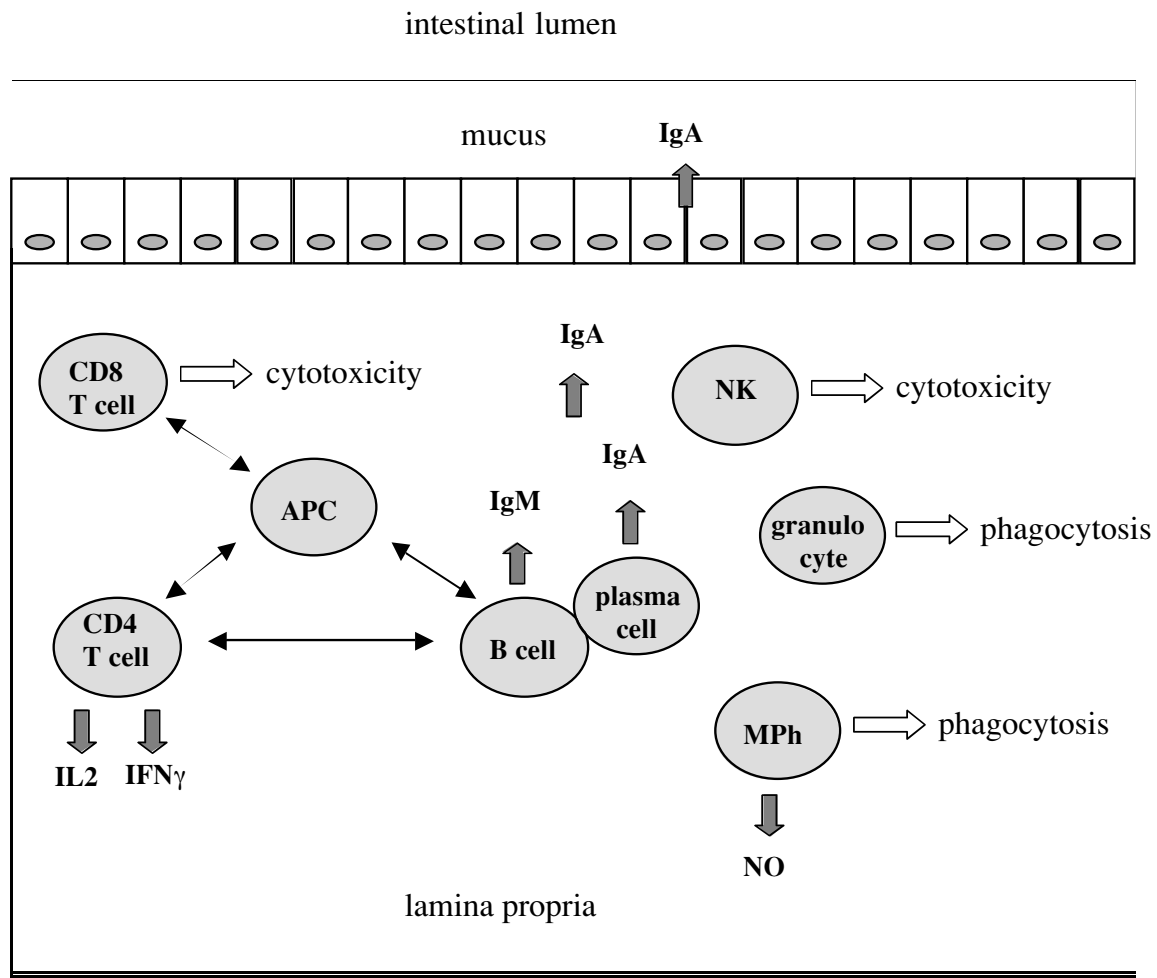


Figure 1. A schematic representation of the innate and specific immunity in the intestine. APC: antigen-presenting cell; CD4: helper phenotype; CD8: cytotoxic phenotype; IFN $\gamma$ : interferon gamma; IgA: immunoglobulin A; IgM: immunoglobulin M; IL2: interleukin 2; MPh: macrophage; NK: natural killer cell; NO: nitric oxide.

strains of broilers, differences in postnatal development of T-lymphocyte subpopulations (Lillehoj *et al.*, 1992) and of T-lymphocyte ontogeny (Goidl and Thesis, 1985) were observed.

The capacity of immunity, as part of the total defence, can be deduced from the presence and function of the individual cells of the immune system by recognition of their cellular determinants. Within cell populations, the function can be further discriminated by the presence of cellular determinants, such as CD4 and CD8 associated with helper and cytotoxic functions respectively, by the production of cellular proteins associated with their function such as antibodies, cytokines, free radicals, or enzymes, or by their specific function, such as cytotoxicity, antibody-production, or phagocytosis.

#### ***In Situ* and *ex Vivo* Detection of Specific Cells and Proteins**

Several techniques are available to examine the contribution of various cell populations to the innate and

specific defence. They can roughly be divided into *in situ* *ex vivo* techniques and *in vitro* techniques. Both types of techniques have their advantages and disadvantages. *In situ* techniques, such as immunocytochemistry, are based on the use of small parts of tissue, for example the intestine, that are treated to keep the structure well preserved. The main advantage is that the interaction between various cell populations is not disrupted and can be visualized. To visualize the interaction, a panel of monoclonal antibodies against leukocytes, B lymphocytes, plasma cells, IgM, IgG, IgA, T lymphocytes of helper and cytotoxic function, monocytes, macrophages, antigen-presenting dendritic cells, follicular dendritic cells, epithelial cells, endothelial cells and other cell populations is often applied on serial sections. From the specific recognition of cell phenotypes by monoclonal antibodies, the putative function can be deduced. This concept has proven to be very powerful in dissecting the contribution of various cell populations to defence in the intestinal wall (Vervelde *et al.*, 1996; Jeurissen *et al.*, 2000). An elaborate overview of monoclonal antibodies that is available to investigate the

various cell populations in the chicken intestine is given elsewhere (Jeurissen and Janse, 1998; Jeurissen *et al.*, 1998). During immune responses, large encapsulated structures develop in the organized lymphoid tissues that consist of B lymphocytes only, the so-called germinal centers. These germinal centres reflect the maturation of the immune system, either by age or by antigenic pressure and is suitable for use in young broilers (Jeurissen *et al.*, 1989; Jeurissen and Janse, 1994). Immunocytochemistry is rather laborious, but the slides with sections can be retained for years. A disadvantage is that the technique is merely qualitative and it can only be semi-quantitative with much effort.

*Ex vivo* and *in vitro* techniques, such as analysis in the Fluorescence Activated Cell Sorter (FACS) and bioassays, are based on isolated single cell populations and for this purpose the structure has to be disrupted. For the isolation of intestinal wall cells, techniques have been described for mice and successfully applied on chicken tissues that are based on the use of digestive enzymes (Van der Heijden and Stok, 1987). Representative single cell populations can easily be obtained from the organized lymphoid tissues. It is nearly impossible, however, to obtain a single cell population selectively from the epithelium or the lamina propria, although it has been claimed in some studies (Chai and Lillehoj, 1988; Myers and Schat, 1990). Furthermore, it is known from other species that certain cell populations, e.g. plasma cells, are very sensitive to disruption and thus the obtained single cell population may not be representative of the *in vivo* situation. Most *in vitro* techniques are developed and applied in layer-type birds. Broiler chickens are genetically distinct from layer-type chickens and therefore it is to be anticipated that some techniques have to be adjusted for broilers. A comparison of 4-week-old layer-type chickens and broilers for their cellular and humoral response to a protein antigen demonstrated significant differences between these two types of chickens. We have also found that titres against Newcastle disease virus were much higher after vaccination in layer-type chickens than in broilers. Nevertheless, FACS analysis will result in accurate numbers of cells within the single cell suspension. In combination with immunocytochemistry for rough comparison, FACS analysis is a powerful tool to investigate changes in cell populations. The numbers of CD4 and CD8 cells, for example, can be used to calculate the CD4:CD8 ratio and this parameter has been used as an indicator of intestinal health.

A relatively new method to quantify amounts of all sorts of proteins is quantitative PCR. This technique is applicable for those molecules of which the gene has been cloned. In the chicken, expression of cytokines is performed in different studies as a method to investigate infection (Kaiser *et al.*, 2000; Yun *et al.*, 2000). To investigate further which cells contribute to the expression of a certain molecule an *in situ* hybridization can be done.

#### Functional Assays

In addition to the mere qualification by immunocytochemistry and quantification by FACS analysis of intestinal wall cells, several functional assays are available

to further examine the role of various cell populations in intestinal health and disease. To investigate the function of T lymphocytes, that form the backbone of both the adaptive cellular and humoral immunity, several techniques have been described. They comprise both *in vivo* assays (adoptive transfer, delayed-type hypersensitivity skin reaction) and *in vitro* assays (lymphocyte proliferation, major histocompatibility complex-restricted cytotoxicity, cytokine production), but few of these have been proven applicable to intestinal wall cells. To measure lymphocyte stimulation, cell suspensions of spleen, the best source for these cells, are stimulated with antigen *in vitro* and the resulting cell proliferation is measured on the basis of the incorporation of radiolabelled thymidine into the DNA. Lymphocyte stimulation assays are applied routinely for various viruses, bacteria, parasites and protein antigens. CD4 positive helper cells divide in general to a greater extent upon antigen stimulation than other lymphocytes. Upon activation, helper T-lymphocytes secrete cytokines like interleukin 2 (IL-2) and interferon- $\gamma$  (IFN- $\gamma$ ). Therefore IL-2 and IFN- $\gamma$  levels in culture supernatants reflect T-cell activation. IFN- $\gamma$  produced *in vitro* by stimulated T-lymphocytes is measured in a bioassay based on stimulation of the virally transformed macrophage cell line HD11 with culture supernatant to produce nitric oxide, that is subsequently determined with Griess reagent. IL-2 produced *in vitro* by stimulated T lymphocytes can be measured in a bioassay whereby the culture supernatant is used to induce proliferation of Concanavalin A prestimulated spleen cells, whilst having no effect on unstimulated lymphocytes (Schauenstein *et al.*, 1982). This assay is time consuming, but even more hampered by the lack of reproducibility.

Natural killer (NK) cell activity is characterized as the cytotoxic activity against tumor cells and virally infected cells, irrespective of their genetic background. These cells represent a major proportion of lymphocytes infiltrating infected tissues in early stages of infection. Studies on the nature of NK cells are hampered by the lack of specific cell markers, but these cells seem to be related to T lymphocytes with respect to function and cell markers. NK cells have been identified in the chicken spleen (Schat *et al.*, 1986) and the intestinal wall (Chai and Lillehoj, 1988). Assays to measure NK activity have been reviewed by Fahey and York (1987) and Sharma and Schat (1990), but NK activity is mostly measured using the avian leukosis virus-transformed cell line RP9 (Sharma and Coulson, 1979). In this assay, the RP9 target cells are labeled with radioactive chromium and cultured with serial dilutions of splenic lymphocytes of control chickens and experimental chickens that have undergone certain treatment (infection, vaccination, feed additives, housing system).

Antibodies form an essential component for protection against potentially pathogenic micro-organisms with important functions in the clearance and neutralisation of pathogens and their products. In the chicken, three classes of immunoglobulins exist, IgM, IgG and IgA, which are secreted by plasmablasts and plasma cells. Presently, the Enzyme-Linked ImmunoSorbent Assay (ELISA) is the most commonly used assay to determine specific antibodies in serum. For poultry, ELISA's have been developed that are

commercially available from, among others, Idexx, Kirkegaard and Perry Laboratories, Svanova, and BioChek. These ELISA's are based on coating the plate with pathogen-derived antigens. After incubation with chicken sera, the bound pathogen-specific antibodies are recognized by conjugates specific for chicken IgM and IgG. At present, specific ELISA's are available for the following pathogens: avian encephalomyelitis virus, avian influenza virus, avian leukosis virus, avian reovirus, avian rhinotracheitis virus / pneumovirus, chicken anaemia virus, haemorrhagic enteritis virus, infectious bronchitis virus, infectious bursal disease virus, infectious laryngotracheitis virus, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, Newcastle disease virus, *Ornithobacterium rhinotracheale*, reticuloendotheliosis virus, *S. enteritidis* and *Salmonella typhimurium*. In the field, ELISA's are mainly used to check the vaccination status of a flock. None of these ELISA's has been used with fluids that are more representative for the defence that occurs in the intestinal tract, such as bile or intestinal washings, and in addition the IgA isotype is not separately determined. It may be worthwhile and feasible, however, to investigate whether and how bile, tears, intestinal washings, or faeces can be applied in these commercial ELISA's, especially because vaccination with most of the pathogens mentioned above is a routine procedure in layers and broilers.

In addition, The ELISA technique can be used to measure the concentration of total IgM, IgG, and IgA, irrespective of the specificity. Especially in young animals, this can be a valuable tool to determine the maturation of the immune system. This ELISA is based on a catching antibody on the plate, specific for one of the isotypes. After incubation with the test fluid (serum, bile, faeces, or intestinal washing), the bound immunoglobulins are detected with an antibody specific for the light chain in subsequent steps.

The ELISPOT assay is essentially based on the same principles as the ELISA, except that single cell suspensions are incubated for several hours in antigen-coated plastic wells and that subsequently the antibodies secreted by these cells form spots that can be visualised with monoclonal antibodies against chicken IgM, IgG or IgA. This technique has been developed for the chicken using single cell suspensions of spleen and small intestine and both for antigen-specific antibody responses and total amount of immunoglobulin isotype (Koch and Jongenelen, 1989). This quantitative technique is rather laborious and highly influenced by the procedure used to produce single-cell suspensions.

The functional activity of macrophages and granulocytes can be determined *in vitro* using an assay measuring phagocytosis and killing of bacteria, Congo red stained yeast cells, or sheep red blood cells. In this assay when using bacteria, single cell suspensions from the spleen, the intestinal wall or abdominal cavity are incubated for a short period with bacteria and then treated with a suitable antibiotic to remove non-phagocytosed bacteria from the surface and medium. Then cells are lysed and the numbers of internalised bacteria are counted with routine bacteriological techniques. The outcome is a measure of the phagocytosis capacity of the macrophages

and granulocytes. To examine the killing capacity of these cells, they are cultured for some time and then treated as above to determine the decrease of intracellular bacterial count. This assay is applicable as a general measure of defence against bacteria in all broilers. In chickens that were infected with *S. enteritidis*, the phagocytosis and killing of bacteria was increased (Kramer *et al.*, 1999). For the killing of intracellular pathogens, nitric oxide is supposed to be very important in chickens, as in other animals. In a bioassay, single cell suspensions of macrophages are cultured *in vitro* with or without stimulation and subsequently the nitric oxide secreted in the supernatant is measured with Griess reagent. After application of this technique to broiler chickens, we found that macrophages in control animals could not be stimulated to produce nitric oxide, whereas macrophages of *Salmonella*-infected chickens did produce nitric oxide (Kramer *et al.*, 2001). This was probably a result of the previous *in vivo* stimulation by *Salmonella*. Nitric oxide can also be measured in body fluids, such as blood serum and plasma, and urine. NOx in plasma can be analysed calorimetrically using Griess reagents after reducing the NO<sub>3</sub> to NO<sub>2</sub> with nitrate reductase according to Verdon *et al.* (1994). Increased NOx (NO<sub>2</sub> + NO<sub>3</sub>) concentrations were detected in blood plasma of chickens infected with *Eimeria acervulina* and *Eimeria tenella* six days postinfection (Allen, 1999). Based on their results a positive relationship between NOx concentrations and the severity of coccidiosis lesions was suggested. Although also others demonstrated increased NOx in urine using mammals, these results need further validation in chickens.

### Integrity

Integrity of the intestinal wall is of major importance to protect the individual against leakage of unwanted substances from the intestinal lumen into the submucosal tissue (Figure 2). These unwanted substances can consist of large feed components, microorganisms, or microbial toxins. Integrity of the intestinal wall is primarily formed by the continuous layer of epithelial cells, that form a strong barrier. In addition, the mucus layer on top of the epithelium also contributes to the barrier function. The mucus layer is produced by goblet cells, that are located in between the epithelial cells. It is generally accepted that integrity of the mucus and the epithelium is crucial for the resistance to enteric disease (Mantle and Allen, 1989). The intestinal epithelium is formed in the crypts, where the cells proliferate. From there, epithelial cells migrate along the basement membrane towards the tip of the villi and during this process, they mature. At the tip of the villi, epithelial cells undergo apoptosis and they are discarded into the intestinal lumen. The mean lifespan of epithelial cells is 48-72 hours. Obviously, intestinal health requires a balance in proliferation, maturation, and apoptosis of the epithelial cells, and in this process many different factors are involved.

### Differentiation and Development

The differentiation of intestinal epithelial cells can be measured by the ratio between apoptotic and proliferating

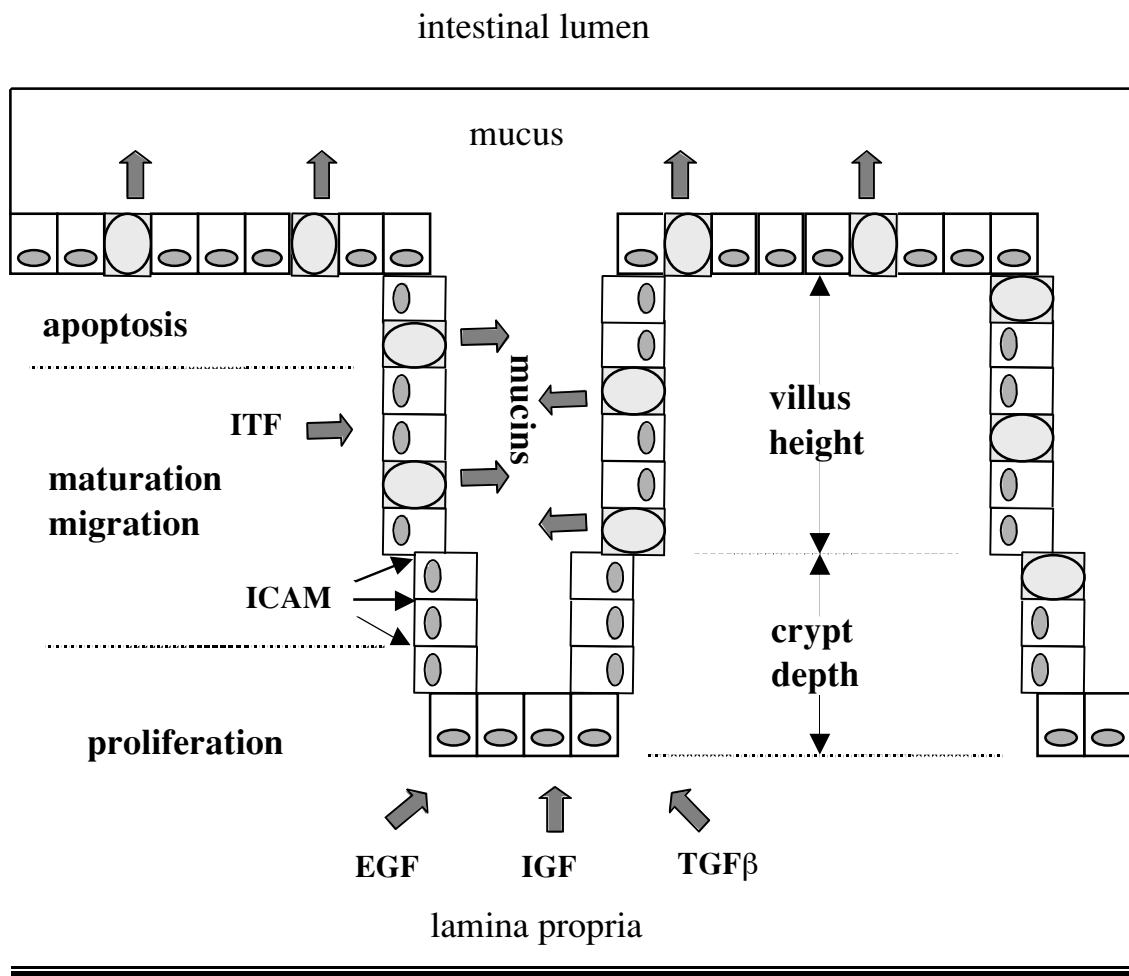


Figure 2. A schematic representation of integrity of the intestine. In the continuous layer of epithelial cells (white), single goblet cells (grey) are located. EGF: epidermal growth factor; ICAM: intercellular adhesion molecule; IGF: insulin-like growth factor; ITF: intestinal trefoil factor; TGFβ: transforming growth factor beta.

cells. Proliferation of cells is mostly found in the crypts. After proliferation the cells migrate and differentiate towards the top of the villus. Then the cells become apoptotic and leave the mucosal layer. A difference in ratio between apoptosis and proliferation might indicate whether the intestinal mucosa has a balanced differentiation. Apoptotic and proliferating cells can be stained with routine histological procedures and counted (Uni *et al.*, 1998b). In addition, DNA:RNA ratio, DNA:protein ratio, and RNA:protein ratio are considered to be reliable parameters for the pattern of intestinal development. The determination of DNA, RNA and protein concentrations in 1 gram of intestinal tissue, together with morphological measurements, has provided new knowledge about the pattern of intestinal development (Uni and Sklan, 1999). The DNA concentration in a tissue reflects its rate of mitosis in a cell population, with the protein to DNA ratio indicating the cell size (Jin *et al.*, 1998). The ratio of RNA to protein was proposed by Waterlow *et al.* (1978) as an index for ribosomal capacity. The concentrations of DNA, RNA and

their ratios to protein content, undergo natural variation during the development of the gastrointestinal tract with the pattern of changes described by Jin *et al.* (1998), Uni *et al.* (1998a), and Iji (1999). However, the patterns reported have not always been consistent, and differences between species, and broilers and layers have been seen. Iji (1999) reported lower DNA concentrations (cell population) and an increased RNA concentration and protein:DNA (cell size) throughout the small intestine between hatching and 21 days of age. Uni *et al.* (1998a), using the slightly different timescale of day 14 incubation to day 7 post-hatching, reported a consistent increase in DNA concentration in the duodenum of both broilers and layers.

The villus orientation and shape can be classified in classes of villi including tongue-shaped, finger-shaped, leaf-shaped, ridged-shaped and convoluted villi. In young birds, the villi appear as zigzag ridges (Lim and Low, 1977), with a control corn and soy flour based diet resulting in mainly ridge- and leaf-shaped villi arranged in a zigzag pattern (Langhout, 1998). It is thought that a regular pattern of

blunted shortened villi is more functional and efficacious than a mixture of long villi and blunt villi in an irregular pattern. Caspary (1992) suggests that the presence of shorter and more tongue-shaped instead of finger-shaped villi may reduce the effective surface area for nutrient absorption, particularly at the villus tips.

In addition to villus arrangement, crypt depth and villus height are studied by several investigators (Ferrer *et al.*, 1995, Uni *et al.*, 1995, 1996, 1998a,b,c; Yamauchi *et al.*, 1996). In general, a high villus height and villus/crypt ratio are associated with a well-differentiated intestinal mucosa. Schneeman (1982) suggested that shorter villi (relative to crypt depth) had fewer absorptive cells and more secretory cells. Interestingly, broiler strains selected for rapid weight gain, show additional villus volume after hatching, although the rate of change thereafter is not different from slow growing strains (Uni *et al.*, 1995). Counting the amount of villi (per cm<sup>2</sup>), and measuring the height of villi and depth of crypt is another well-known way to investigate the intestinal morphology of the chicken.

In between the epithelial cells, goblet cells are located, secreting mucin that is used in the mucinous lining of the intestinal epithelium (Schneeman, 1982). Increased crypt cell proliferation may alter the number of goblet cells and may change the mucin composition and physicochemical properties of the mucus layer (Smits, 1996). The determination of goblet cell number is an established technique using intestinal segments stained with Alcian Blue-Periodic Acid Schiff (Smits, 1996). Differences in numbers of goblet cells in the small intestine have been observed when comparing broiler lines which are more or less susceptible to malabsorption syndrome. Although a higher density in goblet cells may result in an increase in mucin secretion, the number of goblet cells cannot be used to quantify this mucin secretion. Currently, we have demonstrated that MUC-2 content, being the most important intestinal mucin in poultry, was increased in 3-week-old broilers, when the intraluminal microbial activity was stimulated via dietary pectins.

### Endogenous Factors Regulating Gut Development and Growth

One of the growth factors that is important in gut development and growth is insulin-like growth factor (IGF). This growth factor promotes, besides growth, also collagen synthesis and is reported to be important in chicken embryo development. Levels of IGF differ between layers and broilers and the growth rate of chickens depends on the amount of IGF. The serum levels of chicken IGF can be detected in a heterologous radioimmuno-assay. In chickens, the IGF receptor is expressed in most tissues. Some of the IGF-binding proteins in chicken are known. These binding proteins are used to deliver the IGF protein at the right place IGF I mRNA is expressed in the sub-mucosal regions and crypt cells and the IGF receptor amount is decreased by age. The amount of growth factor is not correlated with the expression of IGF. The IGF I serum levels are decreased in chickens with spiking mortality syndrome (Davis *et al.*, 1997). Some research is done to investigate the effect of IGF on the integrity of the intestine. Most studies were done to investigate the effect of IGF on

wound healing in the intestine. Damage was accomplished in different ways. In all cases the IGF addition had a positive healing effect. The route of administration of IGF and the time frame seems to be important for an optimal result (Howarth *et al.*, 1998; Shimoda *et al.*, 1997). IGF I reduces cell atrophy in the intestine and stimulates intestinal growth in neonates. It has also been described that IGF I in rats increases the villus height and crypt depth (Zang *et al.*, 1995; Mantell *et al.*, 1995; Steeb *et al.*, 1994).

For intestinal mucosal defence, epidermal growth factor (EGF) is important. This growth factor can reduce infections and can positively effect wound healing in the intestine. Many studies have been performed in human, mice, rabbit and other species. It is a factor that increases regeneration and maturation of the epithelium of the intestine (McAndrew *et al.*, 2000; Elliott *et al.*, 2000). Another growth factor that is important for chicken intestinal development is transforming growth factor beta (TGF- $\beta$ ). This growth factor is not only important for embryo development but also acts as a defence factor. After infection with coccidia, such as *Eimeria acervulina*, the expression of TGF- $\beta$  increases in the intestine of the chickens (Jakowlew *et al.*, 1997).

The homeobox (*HOX*) genes are important in the development of the embryo. It is known that this group of genes is involved in the development of the gut (Beck *et al.*, 2000). In the chick embryo for example, gastric glands expressing embryonic pepsinogen are formed in the glandular stomach and the epithelial cells of the gizzard differentiate into mucus secreting cells (reviewed by Yasugi and Mizuno, 1990). The organ specific differentiation of the epithelium occurs along the embryonic axis in the anterior-posterior direction and is induced by regional specific signals released by the visceral mesoderm. *HOX*-genes might be involved in this timed and regional morphogenesis of the digestive tract. Yokouchi *et al.* (1995) showed that the *Abd-b* subfamily genes of the *HoxA* cluster are expressed in restricted domains of the endoderm along the anterior-posterior axis of the chick embryo. Difference in *HOX* gene expression might be an indication of the balance in development and susceptibility to infection of these chickens.

### Migration

Integrity of the intestinal wall demands an optimal migration of epithelial cells from the crypt towards the tip of the villus. This migration is dependent on the adhesion molecules between the epithelial cells, like E-cadherin (L-cam in birds) and the complex that is formed between E-cadherin and the catenin family and the actin filaments (Efsthathiou *et al.*, 1998; Wong *et al.*, 1998). Other proteins involved in the migration process are the tight-junction proteins like occludin and zonula occludens-1. The apical intercellular adhesion molecule 1 (ICAM-1) is up-regulated after bacterial invasion, thereby increasing the adhesion of neutrophils to the epithelial cells (Huang *et al.*, 1996).

The ability of epithelial cells to migrate across the basement membrane to cover defects is the initial step in the repair process. A protein that is involved in wound healing is the intestinal trefoil factor (ITF). This protein stimulates migration of epithelial cells and does not promote

proliferation and growth. This protein is excreted by goblet cells and stimulates epithelial migration (Wright, 1998). ITF is not the only protein known in this family; also trefoil factor 1 and 2 (TF1 and TF2) are important members. All three factors are expressed in the intestine in special compartments of the gut and all play a role in migration. Immunohistochemical staining with antibodies specific for ITF demonstrate this protein in goblet cells, enterocytes and colonocytes. *In situ* hybridisation showed RNA expression in goblet cells only. This means that ITF is secreted by goblet cells and is absorbed by the other cells. Resurfacing of the intestine by epithelial cells is an effect of the ITF production by the goblet cells (Itoh *et al.*, 1999). ITF is a small stable secretory protein that is protease resistant and therefore very useful in external administration of the product for re-epithelisation of the intestinal villi (Wright, 1998; Taupin *et al.*, 2000). The trefoil factor family plays an important role in the development of the intestine. After injury of the epithelial cells in mice, the expression of ITF is increased, and the number of goblet cells is decreased. This means that ITF is responsible for protection of the mucosal layer (Itoh *et al.*, 1999). After infection of micro-organisms in the intestine, the expression of trefoil factors increased as well, resulting in an increased

protection of the mucosa. In chickens, only one member of the trefoil factor family is known (Tabata and Yasugi, 1998). It is unknown whether antibodies used for other species will crossreact with chicken molecules.

**Functionality**

A schematic representation of intestinal functionality is given in Figure 3.

**Physicochemical Parameters**

Important physicochemical parameters that affect microbial activity in the intestinal tract are the viscosity and the pH of the gut contents. Digestion and absorption of nutrients within the gut are affected by the physicochemical conditions, with several factors such as viscosity, pH and osmolality of the chyme implicated as important within the bird. An increase in intraluminal viscosity is thought to reduce the rate of nutrient absorption, by increasing the thickness of the unstirred water layer covering the mucosa cells (Johnson and Gee, 1981). An increase in digesta viscosity may limit the mixing of nutrients with pancreatic enzymes and bile acids within the gastrointestinal tract (Edwards *et al.*, 1988) and also the movement of nutrients

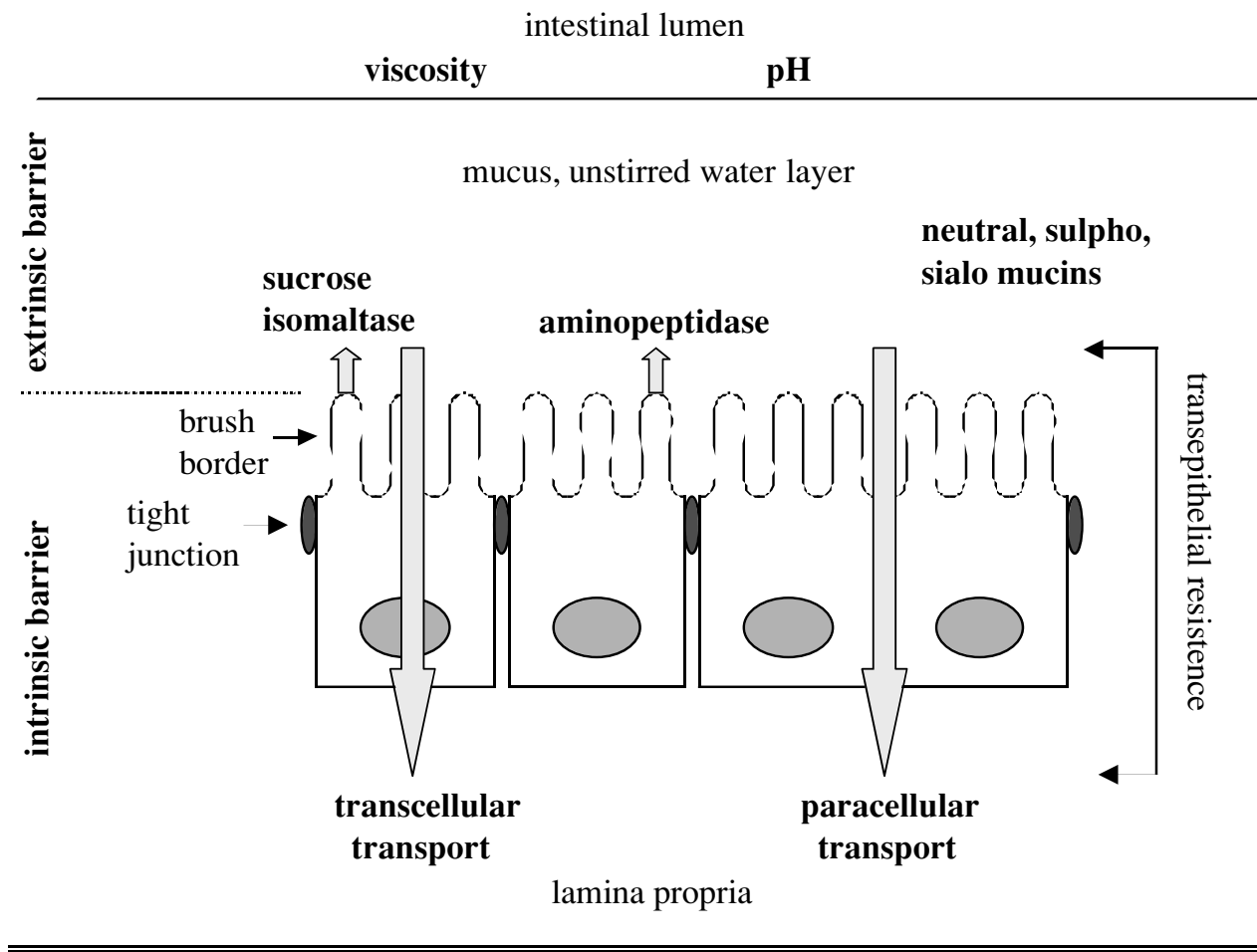


Figure 3. A schematic representation of functionality of the intestine.

from the lumen to the mucosal surface (Fengler and Marquardt, 1988), which will limit digestion and absorption. Since increased viscosity reduces mixing and passage rate, this is thought to decrease luminal oxygenation and allow increased microbial production due to increased residence time (Bedford, 1996). The measurement of digesta viscosity is an established technique (Bedford and Classen, 1993), requiring the collection and centrifugation of the small intestinal contents, with viscosity of the supernatant determined using a viscometer. A potential drawback with viscosity determination is that there must be sufficient digesta to obtain adequate supernatant after centrifugation for the viscosity measurement. Intraluminal viscosity depends upon the diet composition and reported ileal values range from 3.1 cP on corn diets, 2.4 cP on maize diets and 33.5 cP on rye-fed birds (Langhout, 1998).

Whilst digesta viscosity has been seen as important in poultry, it is not the only factor responsible for differences in anti-nutritive effects of non-starch polysaccharides (Langhout, 1998). They reported differences in broiler chick performance at similar digesta viscosity values, showing that an altered retention time of ingested feed in the gizzard caused by nutrition leads to fall in gastric pH. Measurement of pH is straightforward, with the pH of the aqueous fraction determined by inserting a micro pH electrode into the same fraction in which the viscosity was assessed. It is important to measure pH immediately to minimise carbonate buffering. The gastrointestinal pH decreases as the chyme passes from the crop into the proventriculus and gizzard and then becomes progressively less acidic along the length of the small intestine. Hurwitz and Bar (1968) showed that the pH is regulated very efficiently in the ileum of laying hens. However, dietary modification, for example by the inclusion of carboxyl methylcellulose has been shown to result in a linear decrease in ileal pH in broilers (Van der Klis and Voorst, 1993). This may be important in terms of gut barrier functions, and also in relation to mineral absorption since it affects both the solubility of minerals as well as the size of mineral complexes (Van der Klis and Voorst, 1993). More recently, Von Engelhardt *et al.* (1998) reported that the pH in the luminal surface microclimate of the guinea pig colon is rather constant, close to neutrality with an effective buffering capacity in the presence of bicarbonate. Thus the microclimate at the luminal surface of the colonic epithelial cells is largely independent of changes in bulk luminal pH. Indeed, when the pH was altered between 5.4 and 7.4 the short chain fatty acid absorption rates were not affected, and hence, it may be that pH determination, at least in the large intestine, may not be a good parameter of microbial short chain fatty acid production and absorption.

### Intestinal Permeability

Permeability of the intestinal mucosa has been defined as the capacity of the mucosal surface to be penetrated by specific substances through unmediated diffusion (Montalto *et al.*, 1997). There are two theories about permeation routes. The first hypothesis concerns a transcellular route (through small pores), and a paracellular route (through larger channels) and lipophilic pathways, whilst the second hypothesis attributes the key role to paracellular tight

junctions.

In animals from which urine collection is easily achieved, changes in intestinal permeability can be evaluated by simple non-invasive tests, administering probe molecules orally. Fleming *et al.* (1993), Miki *et al.* (1996), and Den Hond *et al.* (1999) orally administered non-metabolised sugars (e.g. L-rhamnose, mannitol, lactulose, 3-O-methylglucose) in rats and humans and measured their appearance in urine, to assess small intestinal absorption pathways and the integrity and permeability of the intestinal mucosa. An increased permeability (lactulose/mannitol ratio) indicated mucosal damage. This non-invasive test to assess gut mucosal damage and sample preparation is simple and fast (sugars resolved within 10 min, with 93.3% mean recovery for all sugars). In poultry, the production of excreta, rather than separate urine and faeces, may reduce the practical application of this approach as birds have to be cannulated prior to such a test, although in other animals, such as the pig, they may have considerable merit.

Intestinal integrity can also be assessed *in vitro* with Ussing chambers. This is a useful model for investigating permeability of different intestinal regions having several advantages compared to other *in vitro* techniques, since they are reliable and reproducible in permeability studies in rats and humans (Yang *et al.*, 1999). Indeed, since the gut barrier is made up of many factors (gastric acid, secretory IgA, mucus), one advantage of the Ussing chamber is the ability to isolate the mucosa alone, so that experimental conditions for bacterial movement through the mucosal barrier can be precisely controlled and monitored (Smith *et al.*, 1992).

With  $^{51}\text{Cr}$ -EDTA, tight junction regulated paracellular permeability can be evaluated whilst electro-physiological monitoring of transepithelial resistance and potential difference make it possible to consider the underlying mechanisms. Transepithelial resistance is considered to reflect tissue integrity, with an inverse relationship reported between transepithelial resistance and paracellular permeability (Grubb, 1991; Yang *et al.*, 1999; Amat *et al.*, 1999). Transepithelial potential difference is a voltage measurement that is dependent on the electrogenic ion pumps in the epithelial cell membrane, mainly the Na/K pump, and on the epithelial barrier functions. Transepithelial potential difference is proportional to total ionic flux, an indicator of tissue viability and an indirect measurement of cell metabolism. The structural changes in intestinal morphology (increased crypt depth) as a result of stressors, may affect both permeability and secretory potential and hence also impact on the problems with gastrointestinal disorders as commonly experienced under practical conditions.

### Proteins of the Intestinal Wall

The mucus layer of the gastrointestinal tract is secreted by goblet cells and is the first step in the maintenance of gut integrity and health. Intestinal mucins consist of core peptides (~1,500 to 4,500 amino acids in length) to which are attached hundreds of O-linked oligosaccharide branches. The functional properties of gastrointestinal mucins are: lubrication of epithelial surfaces; diffusion

barrier to nutrients, drugs, ions, toxins, and macromolecules; binding of bacteria, viruses, parasites; protection against proteases; interaction with immune surveillance systems; detoxification by heavy metal binding and interaction of membrane mucins with microfilaments (actins) as outlined by Forstner and Forstner (1994). Several techniques have been developed and modified over the years to measure mucus secretion, and range from being relatively unspecific to the more precise immunoassay methods for mucin glycoproteins. The protein assay method allows direct quantification of the total amount of mucus protein within a specific area of the gut, but without the specificity of the origin of that protein. The insoluble layer can be isolated and within this layer, with use of purification techniques, a particular glycoprotein may be studied and different types of microscopy can determine this. To assess mucus secretion, the use of Periodic Acid Schiff and Alcian Blue histochemical staining are relatively easy techniques that have provided evidence of diet induced changes in mucin composition of goblet cells (Sharma *et al.*, 1997). Such colorimetric techniques are specific ways to measure the glycoproteins that are present in a sample from epithelial surfaces, and whilst time and product consuming, are also a very cheap way to determine mucus quantity. The isolation, characterisation and development of a quantitative ELISA assay for mucin output and flow developed recently for the calf (Montagne *et al.*, 2000), is also thought worth developing for application to the bird. However, the general absence of effective cross-reactivity with mucins between species, means that this would require considerable time to develop the technique.

In many human diseases and pathological conditions, changes in mucin composition have been observed, with the relative proportion of neutral, sulphated and sialylated mucin species altered (Mantle and Allen, 1989). Changes in the composition of the mucus and mucin content may also decrease nutrient absorption or increase the energy requirement for gut maintenance. Whether an increase in mucin secretion is beneficial depends on the thickness of the mucus layer. If this mucus layer is too thin to prevent bacterial invasion, an increase in production can be beneficial. However, it could be detrimental for chickens if the mucus barrier becomes too dense for effective luminal mixing of the digestive enzymes with the ingested feed, and movement to the mucosal surface to occur (Smits, 1996). Increased cell turnover inevitably results in a reduced maturity of goblet cells, which has been reported to result in an increase in the sialo-mucin and a reduction in the sulpho-mucin containing goblet cells (Filipe, 1977; Culling *et al.*, 1981). Thus, the type of mucin produced may be a reflection of the length/maturity of the villus/crypt, and hence a particular mucin profile may be indicative of an altered intestinal morphology. Whether these changes will be sufficiently diagnostic of a particular disease and/or intestinal maturity and/or integrity in the broiler is not yet determined.

Brush border enzymes degrade oligomers and oligopeptides into absorbable components. The activity of brush border enzymes increases with enterocyte differentiation. Changes in small intestinal functioning and hence integrity may be indicated by determining the

activities of brush border enzymes. For example, the activities of the brush border enzymes sucrase-isomaltase and aminopeptidase have been assessed as criteria for typical functioning of the brush border membrane of the small intestine. In chickens and other species some of these enzymes like intestinal aminopeptidase and sucrase-isomaltase, are partially cloned (Uni, 1998, 1999). The activity may be expressed in units per gram of brush border membrane protein and is a well described method (Uni, 1998, 1999; Uni *et al.*, 1998a,b). Intestinal cell lysates are taken and activity is measured. Uni *et al.* (1998a,b) have shown that RNA expression level can also be measured and can be correlated with growth and health.

The intestinal micromicroflora has been shown to deconjugate bile acids (Hylemond, 1985) and as such the determination of deconjugated bile acids has been suggested to be of value in the evaluation of bacterial overgrowth in the small intestine of humans (Masclée *et al.*, 1989). Since conjugated bile acids are vital in the emulsification of fat and fatty acid absorption, their deconjugation may reduce fat digestibility both directly and indirectly. Since reutilization of unconjugated bile acids from portal blood is less effective than of conjugated, a reduced recycling of bile acids may result in a decreased pool size (Juste *et al.*, 1983; Langhout, 1998). As young chicks have a limited capacity to produce bile salts in the first few weeks after hatching (Green and Kellogg, 1987; Inarrea *et al.*, 1989), an increased microbial activity may result in a reduced bile acid concentration in the intestine. Bile acid measurement is well described by Smits (1996) and Langhout (1998) and involves saponification and extraction into the unconjugated and conjugated fractions using Lipidex-DEAP chromatography as described by Tangerman *et al.* (1986). After enzymatic hydrolysis, methylation and solvation, the concentration of conjugated and unconjugated bile acids is determined by gas chromatography (Salemans *et al.*, 1993). The main bile acids present in chicken chyme are chenodeoxycholic acid and cholic acid.

## Conclusions

To investigate the effects of microorganisms on the intestinal health in poultry, several parameters and techniques are available at the levels of immunity, integrity, and functionality. These techniques vary from "old fashioned" routine histology to "modern" *in situ* hybridisation. Using a combination of parameters for immunity, integrity, and functionality, an accurate and detailed insight into the intestinal health of poultry can be obtained.

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