

The Modulating Effects of Dietary Fiber and Short-Chain Fatty Acids on
Enterocyte Differentiation, Maturation,
and Turkey Coronavirus Infection

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Abstract

In a number of mammalian species, susceptibility to enteric coronavirus infection has been shown to be age-related. This is thought to be associated with enterocyte maturation and receptor protein expression. One of the factors that can influence differentiation and maturation of enterocytes is the availability of short-chain fatty acids (SCFA) in the intestinal lumen. These compounds are by-products of the bacterial fermentation of dietary fiber and serve as the primary energy source for enterocyte metabolism.

The overall objective of this dissertation was to evaluate the effects of dietary fiber and short-chain fatty acids on enterocyte differentiation, maturation, and susceptibility to coronavirus infection in turkeys.

Initial work involved the development of an indirect immunoperoxidase assay (IPA) for the identification and localization of turkey coronavirus (TCV) in paraffin-embedded, acid-ethanol fixed tissue. IPA was found to be superior to indirect immunofluorescent antibody test (IFA) for this and other diagnostic purposes.

To evaluate cellular differentiation and maturation, an SDS-PAGE/immunoblot technique was developed to determine relative levels of villin expression in turkey embryos. Villin is an actin-bound cytoskeletal protein known to be expressed in increasing quantities at the apical surfaces of maturing enterocytes. Villin expression level was found to increase linearly as a function of embryo age. Villin localization was performed by IPA on paraffin-embedded, acid-alcohol fixed tissue. As enterocytes (embryos) matured, villin was found to concentrate at the apical surfaces and eventually at the basolateral membranes. Experiments were also conducted to see what effect *in ovo* butyrate administration would have on developing embryonic enterocytes. Butyrate has been shown to enhance differentiation of

non-neoplastic and neoplastic cells in culture as well as promote healing of damaged intestinal epithelium in human. Villin expression was significantly enhanced in embryos receiving 0.2 and 0.3 M butyrate 36 hours post-administration. Butyrate appeared to enhance villin expression and therefore enterocyte maturation in a dose-dependent manner.

Susceptibility of turkey embryos to TCV infection as a function of age and butyrate treatment was investigated as well as epithelial localization of TCV infection in poult. The level of TCV infection of epithelium was found to increase with embryo age between 17 and 23 days. Poults showed higher levels of infection on the distal 2/3 of villi and no evidence of infection in the intestinal crypts. Butyrate administration in 21-day-old embryos followed by TCV inoculation caused a significant increase of the number of infected cells per villus. This data suggested that butyrate might be used as a means to manipulate enterocyte susceptibility to TCV infection.

In the final set of experiments, the effects of fiber-fortified poult diets containing 5% cellulose or 5% guar gum on luminal SCFA levels, enterocyte maturation, and TCV infection were investigated. SCFA levels in cecal contents were determined by gas chromatography. Enterocyte maturation was assessed by the determination of villin expression on immunoblot and the severity of TCV infection was determined by IPA and lesion score. Fiber-fortified diets enhanced SCFA production and villin expression, but contrary to embryo studies, TCV infection appeared to be reduced. In general, poults performed better on the diet containing cellulose.

Mechanisms regarding the roles of dietary fiber and SCFA in enterocyte differentiation, maturation, and TCV susceptibility are proposed as well as future directions for research. The *in ovo* and poults system used in this research may serve as models for further investigation of the influences of host and dietary factors on enteric viral infection and recovery.

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CHAPTER 1.

Literature Review

1.1 Characteristics and Classification of Coronaviruses

Morphologically, coronaviruses are pleiomorphic but roughly spherical particles of 60 to 200-nm-diameter with a characteristic fringe of 20-nm-long, petal-shaped surface projections that create a solar corona-like appearance in negatively stained electron micrographs, which originates the name “coronavirus”.

Coronaviruses are positive, single, stranded, RNA viruses with a very large non-segmented genome of approximately 32 Kb (Lai and Cavanagh, 1997). RNA is polyadenelated at the 3' end and capped at the 5' end. Arranged in the 5' → 3' direction, about two third of the genome encodes a very large RNA polymerase and is followed by genes encoding structural proteins, which include surface (S), integral membrane (M), and nucleocapsid (N) proteins. Hemagglutinin-esterase (HE) is another structural protein that forms a short-spike surface projection, and is identified in group-II coronaviruses.

All coronaviruses are classified in the genus coronavirus within the family coronaviridae. Based on antigenic relationship, coronaviruses have been serologically classified into three major groups (Lai and Cavanagh, 1997). Group I includes human coronavirus (HCV) 229E, transmissible gastroenteritis virus (TGEV), porcine endemic diarrhea virus (PEDV), canine coronavirus (CCV), and feline infectious peritonitis virus (FIPV). Group II includes human coronavirus (HCV) OC43, mouse hepatitis virus (MHV), bovine coronavirus (BCV), porcine hemagglutinating encephalomyelitis virus (HEV), and turkey coronavirus (TCV). Group III includes avian infectious bronchitis virus (IBV).

1.2 Mechanism of Coronavirus Infection

The spike or S glycoprotein, a surface structure that is anchored non-covalently to M protein, is responsible for the initiation of cellular infection by binding to receptors expressed on target cells (Hansen et al., 1998). It is comprised of two subunits, S1 and S2, which can be separated by trypsin cleavage. S1 is an N-terminal subunit that forms the spike with a

globular head, and S2, a carboxy-terminal subunit, comprises the stalk, transmembrane, and intracytoplasmic domains. S1 expresses a receptor-binding activity (Kubo et al., 1994); S2 does not (Taguchi, 1995). Hemagglutinin esterase (HE) which is present in some coronaviruses (Dea et al., 1986; Spaan et al., 1988; Vlasak et al., 1988) is believed to help virus-receptor binding and facilitate the infection (Krempl et al., 2000).

1.2.1 Receptor Proteins as Determinants of Coronavirus Infection

The essential role of receptor proteins as determinants of coronavirus infection has been extensively researched. These proteins can be categorized broadly into three groups: 1) aminopeptidase N, a receptor for group I coronaviruses, 2) sialic acid, a receptor for HE positive (group II) coronaviruses, and 3) mouse hepatitis virus receptor, a receptor for mouse hepatitis virus (MHV).

Aminopeptidase N (APN) is considered to be the functional receptor for group I coronaviruses. As a type II membrane glycoprotein with zinc-dependent catalytic activity, it is an enzyme which preferentially releases neutral amino acids from the N-terminus of oligopeptides. It is expressed in several tissues and is most abundant in the enterocytes of the small intestine and in the epithelium of renal proximal tubules (Sjostrom et al., 2000). APN mediates the infection of TGEV in swine (Delmas et al., 1994; Delmas et al., 1992), HCV-229E in humans (Yeager et al., 1992), CCV in dogs (Kolb et al., 1998), and FIPV in cats (Tresnan et al., 1996). It has also been shown that feline APN can serve as a receptor for the entire group I coronaviruses (Tresnan and Holmes, 1998). Determinants mediating HCV-229E infection are located within the N-terminal domain of human and feline APN. The C-terminal domain of feline, porcine, and canine APN is involved in the infection of the other group I coronaviruses in a species-specific manner (Kolb et al., 1998). According to Delmas et al., (1994) the virus-binding site of porcine APN is different from the catalytic site of the enzyme.

9-O-acetylated sialic acid has also been reported as a binding receptor for BCV (Schultze and Herrler, 1992; Vlasak et al., 1988), TGEV (Krempl et al., 2000; Schultze et al., 1995) and HCV-OC43 (Krempl et al., 1995; Vlasak et al., 1988). Binding activity of this acetylated sialic acid involves a hemagglutination reaction. HE had been considered to be responsible for hemagglutination, but Schultze et al. (1991) found that the reaction between S

protein and 9-O-acetylated sialic acid exhibited a more efficient hemagglutination, and proposed that S protein might play a potential role in the primary attachment of the virus to cell surface receptors. The loss of sialic acid binding ability due to a single point mutation of S protein markedly reduced the enteropathogenicity of TGEV (Krempl et al., 1998). In addition, this point mutation renders TGEV susceptible to the detergent octylglucoside (Krempl et al., 2000). Therefore, it has been proposed that sialic acid binding activity helps the virus to survive contact with detergent-like substances present in the gastrointestinal tract (Krempl et al., 2000).

Mouse hepatitis viral receptors (MHVR) have been identified as glycoproteins in the carcinoembryonic antigen family (CEA) (Williams et al., 1991). These CEA-related proteins are recognized in a broad range of tissues, including renal proximal tubules, hepatocytes, and respiratory, intestinal, and endocrine epithelial cells (Godfraind et al., 1995). More than a single type of CEA can be utilized as a receptor for MHV (Compton, 1994; Yokomori and Lai, 1994).

The importance of these receptors to coronavirus infection has been demonstrated. MHV-A59 infectivity was eliminated by the presence of a monoclonal antibody recognizing the 110 kDa receptor and a 58 kDa fragment later identified as MHV receptor 1 (MHVR1) (Dveksler et al., 1993; Williams et al., 1990). The absence of viral binding proteins in the intestinal brush border membrane of adult SJL/J mice was suggested as a determinant of their resistance to MHV-A59 infection (Boyle et al., 1987). Upon finding a MHVR1 homologous protein that exhibited negative MHV-A59-binding activity, Williams et al., (1990) suggested that the resistance of SJL/J mice was due to the mutation of receptor proteins. Ohtsuka and Taguchi (1997) later reported the presence of a viral receptor protein, MHVR2, in resistant SJL/J mice; however, the activity of MHVR2 was markedly less than the activity of MHVR1 in susceptible BALB/c mice. Therefore, it seems to be that a strong virus-receptor binding is needed to initiate the infection.

Transfection with receptor-encoding genes has been found to induce susceptibility to coronavirus infection in many resistant cell lines (Delmas et al., 1993). Human embryonic kidney cells were found to be susceptible to FIPV, HCV-229E, and TGEV after being transfected with cDNA of feline APN (Kolb et al., 1997). Transfection with recombinant

deletion clones of receptor genes rendered formerly resistant, receptor-negative, hamster cells susceptible to MHV-A59 infection (Dveksler et al., 1993). By examining different recombinant deletion clones, the recognition site for MHV-A59 was located within the N-terminal domain of MHV-receptor protein (Dveksler et al., 1993).

1.2.2 Virus Entry

The mechanism of coronavirus entry, either by plasma membrane fusion or endocytosis, is not yet known. The route of entry for TGEV (Rossen et al., 1994), MHV-A59 (Rossen et al., 1995), FIPV (Pedersen et al., 1981), and HCV-229E (Wang et al., 2000a) is confined to the apical surface of polarized epithelial cells. BCV appears to infect Madin-Darby canine kidney (MDCK) cells via the apical membrane only (Schultze and Herrler, 1995; Schultze et al., 1996). Lin et al. (1997) found infection at the basolateral membrane of human rectal tumor (HRT-18G) cells although the efficiency was low. Different results from these studies were obtained from the examinations in different cell types; therefore, these studies suggest that different cells mediate BCV infection differently.

Due to the fact that they are enveloped, internalization of coronaviruses requires membrane fusion. Whether this takes place at the lysosomal membrane (endocytic pathway) or at the plasma membrane remains to be determined. To date, evidence suggests that both mechanisms are possible. Delayed replication of MHV as a result of a sodium chloride treatment indicates that virus internalization might involve lysosomal membrane fusion (Krzystyniak and Dupuy, 1984; Mizzen et al., 1985). Based on other virus systems, acidic conditions in the lysosome are required to trigger the uncoating of viral nucleocapsids and the fusion between virus envelope and lysosomal membrane (Helenius and Marsh, 1982; Kielian et al., 1986). Therefore, replication would be impaired in viruses dependent on this pathway when intracellular pH increases (Mauracher et al., 1991; Shibata et al., 1983). Evidence indicating viral entry via an endocytic pathway was seen using electron microscopy to track the process of TGEV internalization (Hansen et al., 1998). This evidence included virus-receptor binding, receptor recruiting, and formation of virion containing endosomes.

In contrast, it has been shown by colloidal gold-mediated immunoelectron microscopy that BCV gains entry by direct fusion with the plasma membrane (Payne et al., 1990), and does not require acidic intracellular conditions. A similar mechanism has been

reported for MHV (Gallagher et al., 1991; Kooi et al., 1991). In fact, MHV has been shown to utilize either of these pathways depending on the nature of the target cells and the strain of virus (Nash and Buchmeier, 1997).

1.3 Pathogenesis

Coronaviruses cause serious diseases in a variety of species. Different forms of pathology and clinical signs are produced depending on the type and strain of coronavirus and target organs of infection. For example, infection with TGEV, TCV, BCV, and CCV causes enteritis. The clinical manifestation of infection with MHV and HCV-229E range from paralysis to encephalitis, and in the case of IBV, tracheitis, bronchitis and nephritis may be seen. Different host species and strains also respond to infections differently. Age is another factor that affects severity of infections.

1.3.1 Cytopathogenesis, and Spreading of Progeny

S protein plays a pivotal role in the induction of cell fusion which leads to cell lysis. Protease activity is required for this to take place (Mizzen et al., 1987). Trypsin or chymotrysin cleaves the spike protein of MHV into two subunits of almost equal size (Sturman and Holmes, 1984). One of the subunits is acylated and transported to the plasma membrane during the maturation of virions, and it is thought that its assimilation at the plasma membrane may activate cell fusion (Sturman et al., 1985). A single point mutation of S protein by substitution of aspartic acid for histidine in spike cleavage signal are known to suppress virus-induced cell fusion and are thought to be a cause of persistent MHV infection (Gombold et al., 1993a; Gombold et al., 1993b). Trypsin cleavage is also required for BCV-induced cell fusion (St Cyr-Coats et al., 1988), which is mediated by S2, transmembrane subunits (Yoo et al., 1991).

Virus entry and cell fusion may require other factors in addition to the major receptor-binding proteins. Virus binding to both sialic acid and APN is considered important to the stabilization of viral attachment and the development of entero-pathogenicity with TGEV (Krempl et al., 1998). In the case of MHV, HE and S protein activity are both required (Gagneten et al., 1995). Resistance of certain receptor-positive murine cells to internalization of MHV indicated that the availability of receptors might not be the only

restriction factor for virus entry (Yokomori and Lai, 1994). Asanaka and Lai (1993) demonstrated that resistance to MHV infection could also be the result of a defect in a cellular factor required for viral uncoating. Delmas et al. (1993) reported that some cell lines failed to produce progeny even though TGEV infection was successfully introduced by transfection with porcine APN.

The level of receptors expressed on target cells seems to be related to infection and cytopathological induction by coronaviruses. Murine 17 Cl 1 cells expressing a low MHVR level were shown to be less susceptible to MHV-A59 and to serve persistent infection (Sawicki et al., 1995). After 600 passages, viruses began to exhibit differences from the original MHV-A59. Changes included slower rates of RNA synthesis and N protein development, and a broader range of target cells (Schickli et al., 1997). It was therefore proposed that chronic coronavirus infection in tissues expressing a low receptor level might select for viruses with altered receptor recognition and extended host range. Using HeLa cell lines with varying MHVR density, Rao and Gallagher (1998b) demonstrated that a low density of MHVR was conducive to infection but not the development of cytopathology, thus suggesting that a higher receptor level was required for MHV to induce syncytium formation. This study also revealed that cell lysis may not be a consequence of virus-induced syncytia alone. The accumulation of S protein-receptor complex observed by these researchers was hypothesized as a possible cause of acute cell death (Rao and Gallagher, 1998a).

Overexpression of receptor proteins has been found to impair production of viral particles (Delmas et al., 1995). When testis cell clones which overexpressed porcine APN were infected with TGEV, lower numbers of viral particles were produced and smaller plaque diameters were formed. The addition of anti-porcine APN antibody helped to restore virion production, so interference with viral production was thought to be associated with a late maturation step and the release of infectious virions from cells. In addition, this study demonstrated that if there was an interaction between S and receptor proteins inside the endoplasmic reticulum or Golgi apparatus, it did not affect the spike maturation. The inhibition of virus production due to excessive receptor proteins has also been reported with MHV-A59 (Gallagher, 1995). However, contrary to the report of Delmas et al. (1995), it was

suggested that the interaction between excessive receptors and spike proteins blocked the transport of S protein to the Golgi apparatus. The actual mechanism remains to be explored.

In addition to cell fusion and lysis, coronaviruses trigger infected cells to undergo apoptosis. The occurrence of apoptosis depends on cell types. MHV induced apoptosis in DBT cells when E protein was expressed in the cells (An et al., 1999). TGEV-induced apoptosis in swine testes cell cultures was believed to aggravate the infection (Sirinarumitr et al., 1998). MHV-3 infection was compared in two different cell types, and apoptosis was induced in cells that showed no syncytium formation but was not induced in those that formed syncytia (Belyavskiy et al., 1998). This might suggest that apoptosis is a host defense mechanism designed to limit infection. In support of this, Eleouet et al. (2000) found that during TGEV-induced apoptosis, viral nucleocapsid protein was digested by activated caspases. Apoptosis following retinal infection with MHV was found concurrently with the upregulation of genes associated with cytotoxic T cells and the clearance of infectious virions (Wang et al., 2000b).

Rossen et al. (1995) found that, in contrast to TGEV in which virions escaped infected cells through the apical surface, MHV progeny were released from the basolateral membrane. They suggested that these different routes of virus spread might be the explanation for the nature of infections i.e., systemic infection for MHV and local infection for TGEV. In a later study, the release of MHV virions was found to be dependent on cell type, suggesting that different cell types might target viral vesicles differently (Rossen et al., 1997). In addition to TGEV, BCV and TCV virions also preferentially escape from host cells through the apical surface (Lin et al., 1997). Viral progeny might be able to spread from infected cells to neighboring cells by way of syncytia formed in the course of infection. This is supported by the observation that receptor proteins are not required for MHV spread after initial infection (Gallagher et al., 1992). Apparently, syncytium formation can be induced by S protein without receptor protein mediation (Gallagher et al., 1993).

1.3.2 Species Specificity, Tissue Tropism, and Age-Dependent Resistance

Data has suggested that characteristics, including species specificity, tissue tropism and age resistance are associated with receptors specifically recognized by coronaviruses. Benbaccer et al. (1997) found that resistant cells became susceptible to CCV, FIPV, and

TGEV infection after transfection with a chimeric APN containing a canine domain. These cells were also susceptible to TGEV infection after transfection with APN containing a porcine domain, but were still resistant to CCV and FIPV infection. Interestingly, they also found that TGEV, even though it cannot infect bovine cells, could use chimeric human/bovine APN as a receptor. Similar evidence has been reported in which FIPV, despite being unable to recognize human or porcine APN, can infect cells expressing a human/porcine APN chimera (Hegyí and Kolb, 1998). Delmas et al. (1994) showed that human APN could not substitute for porcine APN in serving as a receptor for TGEV despite sharing 80% amino acid sequence homology. HCV-229E, which infects only human cells or cells expressing human APN was found to exhibit a binding activity to cells derived from dogs, cats, pigs, and humans (Levis et al., 1995). This suggested that the species specificity of HCV-229E might not be determined by the virus-receptor binding step alone, but may also involve subsequent steps during viral internalization.

Tissue tropism exhibited by coronaviruses is thought to be a function of the presence of specific receptors. Principal target tissues for replication of MHV-A59 in BALB/c mice, including colon, small intestine, and liver have the highest amount of MHVR glycoproteins (Williams et al., 1991). Also, the expression of human APN at the cellular surface corresponds to HCV-229E infection of various human neuronal and glial cell lines (Lachance et al., 1998).

Factors influencing age-dependent resistance to coronavirus infections have been investigated. Comparatively higher levels of interferon and macrophage activity, corresponding with a reduction of clinical disease were observed in weanling vs suckling mice infected with MHV (Lucchiari and Pereira, 1990; Taguchi et al., 1979). From this study, it was concluded that the stage of immune system development seems to be responsible for age-dependent resistance. However, administration of anti-viral antibody and purified T cells failed to enhance resistance of young SJL mice to the development of acute central nervous system disease (Stohlman et al., 1980). This suggested that age-dependent resistance might be due to maturation of a specialized adherent cell population. In contrast, Pickel et al. (1981) reported that susceptible young mice could be protected by maternal immunity, pre-immunized spleen cell transfer, or inactivated JHM virus administered prior to

infection. This study suggested that differences in resistance to coronaviral infection due to age were influenced by immunological events but not by the different stages of immunocompetence. Weingartl and Derbyshire (1993) suggested that saturable binding sites on the plasma membrane of villous enterocytes in newborn piglets might be associated with the fact that newborn piglets are more susceptible to TGEV infection than weaned piglets. In addition, saturable binding sites of susceptible villous enterocytes might explain tissue tropism when compared to unsaturable binding sites of resistant cryptal cells. A later study found that the binding sites on the enterocyte plasma membrane did not correspond with the distribution of aminopeptidase N (Weingartl and Derbyshire, 1994). A 200-kDa protein isolated from enterocyte plasma membrane was identified and suggested as an additional receptor required for TGEV infection because monoclonal anti-200-kDa protein could block TGEV infection. The distribution of this protein was proportional to the saturable binding sites (Weingartl and Derbyshire, 1995)

1.4 Turkey Coronavirus Associated Diseases

1.4.1 History

In 1951, a disease of turkeys known as “mud fever” was first described in the literature (Peterson and Hymas, 1951). A similar outbreak affecting major production areas in Minnesota was described by Pomeroy and Seiburth (1953) who referred to the disease as “bluecomb”. It was clinically characterized by acute onset of depression, inappetance, diarrhea, and weight loss. Turkeys of all ages were affected and the condition was considered highly contagious.

In 1973, pleiomorphic particles of 55-220 nm in diameter with characteristic surface projections were detected in the intestine of bluecomb-affected poult by transmission electron microscopy. These were classified as coronaviruses (Panigrahy et al., 1973). During the 1980’s, an outbreak of coronaviral enteritis was reported in Quebec (Dea et al., 1986; Dea and Tijssen, 1988). The Quebec isolate was propagated in human rectal adenocarcinoma cells (HRT-18) which were treated with trypsin to induce cytopathic effect (Dea et al., 1989). This cell-culture-adapted Quebec isolate remained infective to chicken and turkey embryos, and produced clinical signs in challenged poult (Dea et al., 1991). The

Quebec isolate has since been further characterized. Besides the large surface projections (S protein) characteristic of all coronaviruses, TCV possesses smaller projections (Dea et al., 1989), which have been identified as HE protein. These proteins have also been found on BCV, HCV OC43, and MHV. Due to the presence of HE, as well as other antigenic and genomic similarities to BCV (Dea et al., 1990; Verbeek et al., 1991), it has been suggested that TCV be classified as a group II coronavirus (Siddell, 1995). In contrast to this, Guy et al. (1997) have reported a lack antigenic relatedness between BCV and the North Carolina strain of TCV. The North Carolina isolate appears to be more closely related to IBV, a group III coronavirus (Guy et al., 1997). The proper classification of TCV has yet to be resolved.

Over the course of five years (1991-1996), it was estimated that \$136 million were lost by the turkey industry due to Poult Enteritis Mortality Syndrome (PEMS), a condition believe to be primarily associated with TCV infection (Barnes et al., 2000). PEMS is an infectious, transmissible disease that affects turkeys 1-4 weeks of age. It is characterized by diarrhea, poor feed efficiency, stunting, and mortality (Barnes et al., 2000). Researchers have attempted to identify the etiologies, pathogenesis and modes of transmission, in order to develop treatment and prevention programs. In addition to TCV, various other agents, including bacteria (*Salmonella spp.*, *Eschericia coli*, *Clostridium spp.*, *Campylobacter spp.*, *Bacteroides spp.*, and *Arizona spp.*), viruses (reovirus, rotavirus type A and D, turkey enteroviruses, turkey adenoviruses, alphaviruses, infectious bursal disease virus type 2, and astrovirus), and protozoa (*Cochlosoma spp.* and *Cryptosporidium spp.*), have been isolated from field outbreaks of PEMS (Yu et al., 2000; Guy, 1996).

1.4.2 Pathogenesis

TCV infects epithelial cells of the intestine and bursa of Fabricius in turkeys, the only naturally susceptible host. Infected poult initially exhibit shrill chirping, restlessness, anorexia, and diarrhea, followed by depression and huddling. The infection produces high morbidity but low mortality. During the course of disease, reduction in intestinal motility, feed utilization and intestinal transport have been reported (Duke et al., 1969a; Duke et al., 1970a; Duke et al., 1970b; Duke et al., 1969b). Co-infection with enteropathogenic *E. coli* aggravates TCV infection causing high mortality and slow growth (Guy et al., 2000). Fecal-

oral transmission is a major route of horizontal spreading. No vertical transmission has been reported.

Gross necropsy findings include poor body condition, ruffled feathers, and dehydration as indicated by dark colored pectoral muscle and shanks. Intestine are typically distended with fluid, thin wall, and pale in color. Ceca are likewise filled with brown-colored fluid and gas (Deshmukh et al., 1976). None of these lesions are considered to be pathognomonic.

Histopathological findings include inflammation and cellular degeneration, resulting in villus atrophy and bursal lymphoid depletion. Absorptive cells appear swollen with degenerative microvilli. A high number of goblet cells are seen at the villus tip (Adams et al., 1970). The lamina propria is infiltrated with lymphoid cells and is separated from epithelium (Adams et al., 1970; Deshmukh et al., 1976). Villi are shortened and the villus-crypt ratio is reduced (Gonder et al., 1976). The severity of lesions decreases over the length of the intestine from jejunum to ileum and cecum (Deshmukh et al., 1976). Infected epithelial cells are almost completely gone 90 hours post-infection, and the first signs of re-epithelialization such as an increase of mitotic figures is observed at 120 hours post-infection (Deshmukh et al., 1976). The epithelium regains normal cellular populations in two to three weeks (Adams et al., 1970). In embryos inoculated with TCV, similar lesions have been noted (Deshmukh et al., 1976). Ultrastructural changes in intestinal epithelial cells include destruction of microvilli and mitochondria and disruption of mucin production. Marked dilation of the endoplasmic reticulum and cellular vacuolation are also seen (Adams et al., 1972a).

TCV is sensitive to chloroform inactivation (Hofstad et al., 1969). The Minnesota isolate resists 50 °C for up to 60 minutes whereas the Iowa isolate is inactivated after only 15 minutes (Hofstad et al., 1969). The Iowa and Minnesota isolates appear to be stable at pH 5 and 7 but are inactivated at pH 3. TCV survives for a longer period of time under conditions resembling the intestinal environment i.e., a pH of 5.5 and the presence of micro-organisms, than in culture medium, pH 7.0 (Adams et al., 1972b).

TCV agglutinates rabbit and guinea pig erythrocytes (Dea et al., 1986). *In vitro* propagation in several cell lines originating from chickens and turkeys has been attempted

without success (Deshmukh et al., 1973). However, it has been found that the Quebec isolate induces syncytia formation in primary chicken embryo kidney cell culture (Dea et al., 1986). Only the Quebec isolate has been successfully propagated in HRT-18 cells (Guy et al., 1997).

Convalescent birds develop a lifelong immunity to TCV infection (Pomeroy et al., 1975). It has been suggested that this is a function of local secretory antibodies in the intestine (Nagaraja and Pomeroy, 1980). Circulating immunity against TCV is undetectable within the first week after challenge as determined by both IFA and IPA (Guy et al., 1997). However, at two weeks post-challenge, serum antibody levels are significantly elevated.

1.4.3 Diagnosis

Clinical signs and lesions are not definitive for diagnosis of TCV or PEMS. Differentiation must be made from other enteric viral, bacterial, or protozoal infections. TCV can be identified by transmission electron microscopy (TEM) after concentration of intestinal suspensions by sucrose gradient centrifugation (Panigrahy et al., 1973). TEM is not very sensitive; therefore, negative samples have to be re-examined by direct immunofluorescent antibody (FA) (Patel et al., 1975) or indirect immunofluorescent antibody (IFA) tests (Patel et al., 1976). Both immunohistochemistry techniques are based on the reaction between polyclonal anti-TCV sera and the antigen in intestinal cryo-sections. The polyclonal antibody for antigen detection is prepared by multiple challenges in turkey poults while the antigen preparation for serological examination is performed in turkey embryos (aged 22-26 days) as described by Adams and Hofstad (1971). It has been suggested that FA be used for antigen detection during the acute stages of infection, but IFA is actually more commonly used. IFA is also commonly used as a means for serodiagnosis in convalescent flocks. Interpretation requires well-trained personnel and is tedious for large numbers of samples. To facilitate a batch test of field sera for antibody against TCV, IBV enzyme-linked immunosorbent assay (ELISA) has been used (Loa et al., 2000). IBV-ELISA does however have problems with false-positives reactions when serum samples from turkey breeder flocks are tested (Hooper, personal communication). A reverse transcriptase-polymerase chain reaction (RT-PCR) assay has been developed for TCV detection (Breslin et al., 2000).

1.4.4 Treatment and Control

Treatment of birds experiencing TCV and PEMS is difficult. Milk replacer, electrolytes and glucose do not appear to be effective as supportive therapies (Dziuk et al., 1970). Antibiotics such as neomycin, aureomycin (Hofstad et al., 1969) and fluoroquinolones have been found to be occasionally useful in reducing the severity and duration of clinical disease.

The 1950-1970s Minnesota outbreaks were eventually controlled by the implementation of biosecurity programs. Biosecurity still appears to be the best method for disease prevention and control.

1.5 Intestinal Epithelium Development and Enterocyte Maturity Identification

Understanding the determinants of enterocytes susceptibility to TCV infection seems to be of great benefit for planning of disease prevention and control. Evidence indicates that enterocyte maturity plays a role in enteric coronavirus infectivity in other species. This enterocyte maturity might be a determinant of TCV infection as well.

The intestinal epithelium is histologically characterized as simple columnar. It plays a crucial role in the modification of large complex nutrients so that they can be absorbed and transported through blood or the lymphatic system and used by cells throughout the body. The folding of the epithelium to form finger-like structures called villi increases the functional surface area thereby maximizing the efficiency of food digestion and absorption. Intestinal epithelial cells possess a selectively permeable cell membrane consisting of specific protein channels and membrane transporting systems.

Proliferation of epithelial cells occurs in the lower crypt region (Hodges and Michael, 1975; Potten et al., 1992; Takeuchi et al., 1998). Newly generated undifferentiated cells ascend the villus to replace old cells that are exfoliated at the tip (Wice and Gordon, 1992). While these cells migrate along the crypt-villus axis, they undergo differentiation and maturation into particular cell types, such as argentaffin cells or endocrine cells, goblet cells or mucus secreting cells, and absorptive cells. Intestinal epithelium is well organized in a single layer in which each cell is bound to its neighboring cells by junctional complex. Tight

junctions, which separates the cell membrane into two regions i.e., apical and basolateral are essential for the regulation of para-cellular transport between intestinal lumen and intercellular space (Gasbarrini and Montalto, 1999; Madara and Marcial, 1984; Madara et al., 1990). A number of membrane proteins expressed during the differentiation process is targeted unevenly between two membrane compartments creating a polarity in differentiated cells.

A group of cytoskeletal proteins—calmodulin complex, villin, fimbrin, myosin, fodrin, zonula adherens bundle, calpactin I, 200 kDa/140 kDa protein, and intermediate filament—plays an essential role in organizing the two different structural regions, microvilli and terminal web of the intestinal brush border membrane (Fath and Burgess, 1995; Maroux et al., 1988; Peterson and Mooseker, 1993). The microvillus structure is supported by a microvillar core, a group of actin microfilaments bundled by fimbrin in a hexagonal array. These actin microfilaments are anchored to the microvillus plasma membrane by a lateral bridge consisting of 110 kDa polypeptide and calmodulin.

Epithelial cells undergoing differentiation express specific protein-encoding genes. Some proteins expressed at a certain stage of cellular development such as enterocyte maturation can be identified by means of specific protein expression determination. Enterocyte maturity can be determined physically by the expression of cytoskeletal protein, including villin, fimbrin, actin and 110 kDa protein (Carboni et al., 1987; Peterson et al., 1993; Shibayama et al., 1987). It can also be determined functionally by the presence of brush border enzyme activities, including sucrase-isomaltase (Oates et al., 1997) and alkaline phosphatase (Sanderson et al., 1996; Schroder et al., 1999).

Villin is a calcium dependent, actin-bound cytoskeletal protein that is expressed specifically in epithelial cells of the intestine and proximal renal tubule. The expression level of villin is associated with cellular differentiation and the organization of the brush border membrane, including microvilli, which develop in late stages of enterocyte differentiation (Maunoury et al., 1988; Robine et al., 1985). Villin gene expression is induced in Caco-2 cells during post-confluence differentiation and also in HT-29 cells undergoing butyrate-induced differentiation (Hodin et al., 1997). Increase and relocation as well as concentration of villin at the apical surface have been correlated with enterocyte maturation (Shibayama et

al., 1987). Therefore, it seems that villin expression may be a possible means by which the relationship between cellular maturity and susceptibility to TCV infection could be assessed.

1.6 Effect of Short-Chain Fatty Acids on Enterocyte Metabolism

Luminal SCFA, by-products of bacterial fermentation, are essential for metabolism of the intestinal epithelial cells. Atrophy of the epithelium has been observed in situations in which bacterial fermentation and SCFA production have been negatively affected i.e., the absence or decrease of dietary fiber (Lund et al., 1993; Sakata, 1987; Sakata, 1997) or germ-free conditions (Furuse et al., 1991; Rolls et al., 1978). Inhibition of butyrate utilization in colonocytes, due to blocking of β -oxidation, causes ulcerative colitis (Roediger and Nance, 1986). Studies performed on patients with colitis detected normal glucose and glutamate but impaired butyrate utilization in ileal and colonic mucosa, suggesting that butyrate is a major source of energy for enterocytes and colonocytes (Ahmad et al., 2000; Chapman et al., 1994; Chapman et al., 1995). Feces from patients with active ulcerative colitis were found to contain inhibitor(s), which could block the oxidation of butyrate in colonocytes (Jorgensen and Mortensen, 1999). Such inhibition of butyrate metabolism causes enterocytes to starve to death (Roediger, 1980; Roediger, 1990).

1.6.1 Uptake of Short-Chain Fatty Acids

The SCFA absorption rates vary along the intestinal tract (Rechkemmer and von Engelhardt, 1988; von Engelhardt and Rechkemmer, 1992). This variation may be due to differences in membrane permeability of epithelial cells and luminal environments in different parts of the intestine (Rechkemmer and von Engelhardt, 1988). Because they have a very short carbon chain structure, SCFA are water soluble and can be readily absorbed by epithelial cells. They do not require bile acids for micelle formation as do longer fat molecule (Westergaard and Dietschy, 1976). It is still questionable whether they are absorbed in an ionized or non-ionized form via active or passive transport. Generally, the ionized forms are unable to pass the lipid-bilayer membrane; therefore, a simple diffusion of non-ionized or protonated forms has been postulated. However, considering the average pKa of SCFA, which is around 4.8, it seems that they are most likely in ionized forms in the gut lumen (von Engelhardt et al., 1998).

Substantial evidence regarding the relationship between HCO_3^- and SCFA absorption suggests that SCFA can be absorbed in ionized forms via a carrier-dependent, ion-exchange transport (Genz et al., 1999). High concentrations of bicarbonic anhydrase have been detected in the small intestine, cecum, and colon where SCFA are produced and absorbed. This enzyme hydrolyzes CO_2 (end-product of metabolism) to HCO_3^- that is released from epithelial cells in exchange with SCFA. Both diffusion and carrier-mediated SCFA- HCO_3^- exchange are used for SCFA transport across basolateral membrane in colonocytes (Reynolds et al., 1993).

Another mechanism of SCFA absorption involving HCO_3^- has been proposed for the diffusion of protonated forms (Ruppin et al., 1980). This mechanism involves luminal HCO_3^- and H^+ which are generated by the hydration of CO_2 , a by-product of bacterial fermentation. This in turn may result in protonation of SCFA and thereby facilitate diffusion. It has also been suggested that luminal SCFA protonation may be a function of H^+ release from cells in response to a Na^+ influx. This Na^+ influx also drives water absorption (Bowling et al., 1993; Ruppin et al., 1980; Scharrer and Lutz, 1990).

SCFA-driven water absorption has been suggested as a compensation mechanism in adult pigs to prevent fluid loss due to TGEV infection (Argenzio et al., 1984). In human patients with acute watery diarrhea, dysfunction and restoration of rectal water and Na^+ absorption was detected along with very low levels of luminal SCFA during the onset of clinical signs and levels ten times higher during recovery (Ramakrishna and Mathan, 1993). Ample evidence supporting the effect of SCFA on water and electrolyte absorption in patients with diarrhea has been found (Alam et al., 2000; Bowling et al., 1993; Clausen et al., 1991; Homann et al., 1994; Roediger, 1994).

1.6.2 Short-Chain Fatty Acid Utilization

Numerous studies suggest that enterocytes use SCFA, particularly butyrate, as a major source of energy (Krishnan, 1998; Jorgensen, 1997; Velazquez, 1997; Basson, 1996; Marsman, 1995; Firmansyah, 1989; Cummings, 1987; Roediger, 1982). The carbon structure of acetic acid can be incorporated into lipids of the intestinal mucosa (Gangl, 1975), while propionic acid can be used in the gluconeogenesis pathway (Grohn, 1985). Some of the SCFA are transported out of the intestine into the blood (Cummings, 1987). Uptake and

metabolism of these SCFA by the liver give rise to ketone bodies, including acetoacetate and β -hydroxybutyrate, which can be used by intestinal cells (Roediger, 1982). Furthermore, butyrate has been shown to affect the proliferation and differentiation of enterocytes (Vaziri, 1998; Hodin, 1997, 1996; Petit, 1993).

SCFA is actually one of the preferred substrates for colonocyte metabolism. Besides the CO_2 end-product, oxidation of butyrate in rat colonocytes gives rise to intermediates, including lactate and 3-hydroxy-butyrates *in vivo* (Fitch and Fleming, 1999), and 3-hydroxybutyrate and acetoacetate *in vitro* (Ardawi and Newsholme, 1985; Krishnan and Ramakrishna, 1998). Like adult colonocytes, neonatal colonocytes prefer butyrate as a metabolic substrate (Krishnan and Ramakrishna, 1998). The relative contribution of substrates to the net adenosine triphosphate (ATP) production in rat colonocytes was calculated to be 45-50% from butyrate, 20-25% from glucose, and 30-35% from other sources, including acetate, propionate, palmitate, and glutamine (Krishnan and Ramakrishna, 1998). Another study using the same cell type reported that about 86% of total oxygen consumption measured came from SCFA utilization and this oxidation was not affected by the presence of acetoacetate, L-glutamine, or D-glucose (Roediger, 1982). In a study comparing colonocyte oxidation of C2-C8 fatty acids, valerate, hexanoate, and octanoate seem to be metabolized at a rate equivalent to that of butyrate but higher than propionate and acetate (Jorgensen et al., 1997). Measurements of $^{14}\text{CO}_2$ production in isolated pig colonocytes suggested that ammonia, another bacterial metabolite, reduced β -oxidation of butyrate but had no effect on acetate oxidation in the TCA cycle. In contrast, ammonia increased glycolytic oxidation of glucose, which was suggested as a possible effect on phosphofructokinase activity in glycolysis (Darcy-Vrillon et al., 1996). It was suggested that butyrate utilization by proliferating colonocytes spares pyruvate for anabolism (Butler et al., 1990), and this effect was augmented by propionate.

1.7 Effect of Short-Chain Fatty Acids on Cytokinetics

Intensive research has shown that SCFA, especially butyrate has a perturbing effect on the cell cycle of various cell types originating from the intestine, liver or kidney. Butyrate is interesting because it can enhance differentiation and apoptosis while inhibiting

proliferation of many transformed cell lines. Tsao et al. (1982) reported the stimulating effect of butyrate on the differentiation of HRT-18 cell line based on the induction of morphological changes (cell enlargement, flattening, and increased membranous process formation) and increase in membrane-associated enzyme activities (up to 10-fold for AP, 3-fold for γ -glutamyl transpeptidase, and 2-fold for sucrase activity). This effect of butyrate was also detected on human colon adenocarcinoma cell lines, including Caco-2, SW620, and HT-29 as determined by increased dipeptidyl peptidase, lactase, sucrase and IAP activities and villin expression (Barnard and Warwick, 1993; Basson et al., 1998; Hodin et al., 1996; Mariadason et al., 2000; Sanderson et al., 1996). This effect was later demonstrated to occur in a time- and dose-dependent fashion (Hodin et al., 1996). Butyrate-induced polyploidization, another evidence of cell-cycle perturbation, was also shown to vary depending on cell types (Yamada et al., 1992).

The mechanism of butyrate-induced modification of epithelial cytogenetics has been intensively explored on a molecular level. Butyrate inhibits proliferation by arresting the cell cycle in various cell types at a certain stage such as at G_1 in HT-29 cells (Barnard and Warwick, 1993), at $G_{0/1}$ - S phase transition in human urothelial cell lines (Larsen et al., 1995), and at G_1 and G_2 in peritoneal carcinoma cells (Boisteau et al., 1996). The mechanism of butyrate-induced growth arrest and apoptosis is thought to be associated with polyacetylation and dephosphorylation of histone proteins and/or the replication of proto-oncogene, c-myc (Bernhard et al., 1999; Buckley et al., 1996; D'Anna et al., 1980). Butyrate-induced apoptosis was found to correlate with histone acetylase inhibition, resulting in polyacetylation and conformational change of histone $H_{1(0)}$. This conformational change exposes the chromosome to surrounding chemicals including enzymes that could cause cells to undergo apoptosis. In contrast, Chabanas et al. (1985) suggested that the butyrate-enhanced production and accumulation of $H_{1(0)}$ is unlikely the mechanism determining G_1 arrest because cell proliferation resumed without $H_{1(0)}$ reduction after withdrawal of butyrate. Butyrate-induced growth arrest and apoptosis of rat and human colonic carcinoma cells also correlated with the increase of glucose consumption and mitochondrial activity, suggesting the alteration of metabolic activity (Boisteau et al., 1996; Heerdt et al., 1997). An *in vivo* study reported a linear relationship between the levels of butyrate production and histone

acetylation in wheat bran-fed rats, but no terminal differentiation induction was detected (Boffa et al., 1992). These influences on epithelial cytokinetics have been postulated as a potential means of cancer prevention and control of butyrate.

An opposite effect of SCFA, including butyrate, has been reported *in vivo*, in which growth of the intestinal epithelium is promoted (Gibson et al., 1998; Sakata, 1987). This suggests that SCFA may have a systemic effect on the intestinal epithelial cytokinetics and this effect is strong enough to counteract the local effect found in *in vitro* studies. Evidence supporting a possible systemic effect of butyrate on the intestinal epithelial cells was reported by Sakata (1989). Another reason for different results observed between *in vitro* and *in vivo* studies may be because the *in vitro* studies primarily used carcinoma cells. Normal cells appear to respond in the opposite manner (Gibson et al., 1992; Luciano et al., 1996). Gibson et al. (1999) found that the paradoxical effect of butyrate in neoplastic vs non-neoplastic cells seemed to be related to the stages but not to the origins of the cells.

1.8 Short-Chain Fatty Acids and Enterocyte Susceptibility to Viral Infection

Butyrate increases cellular susceptibility to cytomegalovirus (Radsak et al., 1989; Tanaka et al., 1991; Wu et al., 1994) and herpes simplex virus infections *in vitro* (Ash, 1986). The mechanism is not yet known, but it has been suggested that butyrate might mediate cell development in a way that increases the number of susceptible cells or the cellular proteins required for the infection. No information concerning the effect of butyrate or SCFA on susceptibility to coronavirus infection has been published.

1.9 Short-Chain Fatty Acids and Treatment of Enteritis

SCFA have been researched as a treatment for patients in need of intestinal epithelial restoration such as those who have undergone colon resection or those with ulcerative colitis. Mice which underwent colon resection and surgical anastomosis showed faster recovery rates after receiving colonic lavage with a SCFA solution (acetate : propionate : butyrate = 6 : 3 : 1) twice a day for three days when compared to those receiving 10% hypertonic glucose, 10% providone-iodine, or saline lavage (Aguilar-Nascimento et al., 1995; Aguilar-Nascimento et al., 1997). SCFA have been used as an effective, economical treatment for

patients with ulcerative colitis (Kim, 1998). A daily butyrate enema effectively treated diarrhea and enteritis experimentally induced by acid enema in humans (Butzner et al., 1996). This healing effect is thought to be the result of increased cell proliferation (Aguilar-Nascimento et al., 1999), which is contradictory to what is observed in carcinoma cell lines. In addition to cell-cycle mediation, SCFA might facilitate the healing process by promoting intestinal epithelial cell migration (Wilson and Gibson, 1997; Wilson and Gibson, 2000). Furthermore, SCFA-enhanced water absorption is beneficial for patients with diarrhea (Alam et al., 2000; Homann et al., 1994).

In birds, the effects of SCFA on stimulation of intestinal epithelial growth have been documented (Furuse et al., 1991; Rolls et al., 1978). However, their use as treatment in cases of enteritis has not been reported. Individual administration of SCFA enemas, as performed in humans (Cummings, 1997) is an impractical technique for flock treatment. Purified SCFA cannot be delivered to the intestine via oral administration because most will be absorbed at the foregut (Hume et al., 1993). However, regular retrograde movement of luminal contents from the distal gut e.g., ceca, does occur (Clemens et al., 1975; Lai and Duke, 1978). Therefore, manipulation of bacterial fermentation, along this retro-peristaltic movement, may be a mechanism by which SCFA could be supplied to the small intestine and colorectum of the bird.

1.10 Dietary Fiber

Dietary fiber can be broadly defined as an indigestible carbohydrate component based on the activities of mammalian digestive enzymes. Carbohydrate, a polyhydroxyaldehyde or polyhydroxyketone structure, naturally exists in various forms. Starch consists of glucose subunits that are linked by α -1,4 glycosidic bonds to form a polysaccharide backbone. Branches formed by α -1,6 glycosidic linkages at various positions along the backbone create different complex carbohydrates. Amylose has no branches, while glycogen, a storage form of carbohydrate found in animal liver and muscle, has branches at approximately every 10 glycosidic bonds and amylopectin has branches at approximately every 30 glycosidic bonds. Dextrose, a monosaccharide, is the most available form for utilization and is derived from polysaccharide through enzymatic digestion in the gastrointestinal tract. α -Amylase, an

enzyme produced by salivary glands and pancreas in mammalian species, can digest α -1,4 glycosidic linkages, to produce smaller units e.g., oligosaccharides, di- and monosaccharides. Dextrinase, a brush border enzyme can digest α -1,6 glycosidic linkages. Other forms of polysaccharide such as cellulose (β -1,4-glucan) and hemicellulose (β -1,4-D-glucan substituted with xylose, galactosylxylosyl and fucosylgalactosylxylosyl units) are indigestible and therefore are considered to be fiber. Cellulose and hemicellulose are stable in hot water, but there are other forms of fiber which resist enzyme digestion and are soluble in hot water. These soluble fibers are mainly found in pectin and gum.

In avian and monogastric farm animals, fiber is a non-utilizable feed component, and therefore, to maximize feed efficiency, its incorporation in feed is generally limited. However, recent research conducted mostly in humans has suggested an important role of dietary fiber as associated with health and diseases. Fiber delays food digestion and absorption, resulting in the reduction of postprandial glyceemic peak and hypercholesterolemia with benefits to patients with some common health problems such as obesity, diabetes, and coronary heart disease (Anderson et al., 1999; Anderson et al., 2000; Fernandez, 2001; Groop et al., 1993; MacMahon, 1999; Sreedevi and Chaturvedi, 1993).

The effect of dietary fiber on proliferation and differentiation of intestinal epithelial cells has been of great interest. Evidence suggests that low-fiber diet intake is conducive to the development of colon cancer; however, the mechanism is not understood (Erhardt et al., 1997; Hill, 1999). Fiber might bind to carcinogenic and toxic substances; as a result, exposure of the epithelial cells to these substances is diminished. In contrast, a synergistic effect of dietary fiber and carcinogens in the induction of colon cancer has been reported (Jacobs, 1983b; Jacobs, 1984). In rats administered with a carcinogen, 1,2-dimethylhydrazine, wheat bran diet induced mucosal cell hyperplasia in the distal colon, but had no effect on cell mass and had a reducing effect on RNA content in the proximal colon (Jacobs and Lupton, 1984). This study also showed that wheat bran diet reduced the exfoliation and migration rates of cecal epithelial cells, but facilitated the migration rate of epithelial cells at the proximal colon. Soluble fiber induced hyperplasia of small intestinal mucosal cells and increased brush border enzyme activities (Chun et al., 1989). The effect on epithelial cytokinetics is varied depending on types of fiber (Jacobs, 1983a; Langhout et

al., 1999). Different types of fiber also induce mucosal cell hyperplasia at different preferential sites (Konishi et al., 1984; Lee et al., 1993). Lee et al. (1993) also reported that the stimulation of intestinal cell proliferation was a result of an interactive function between fiber and fat.

Fiber is thought to mediate intestinal epithelial cytokinetics via SCFA, a by-product of bacterial fermentation (Goodlad et al., 1989). These SCFA, including acetic, propionic, and butyric acids are produced mainly in the ceca and to a lesser extent in the crop in avian species. Indigestible components such as fiber or non-starch polysaccharides make up most of ingesta that passes through the intestinal tract and supplies bacteria with a substrate of fermentation. The proportion of acetic, propionic, and butyric acid varies depending on type of substrate, individual variation and the type of fermentative micro-organisms present (Bourquin et al., 1992). Soluble fiber, such as pectin and guar gum promotes bacterial fermentation and SCFA production especially butyrate (Edwards et al., 1992; Titgemeyer et al., 1991). Elevated luminal SCFA has been found to mediate the proliferation and differentiation of intestinal epithelial cells (Edwards et al., 1992; Jacobs, 1983a; Langhout et al., 1999; Lee et al., 1993; Lupton, 1995).

1.11 Statement of hypotheses

According to the literature regarding coronaviruses in other species, the infectivity of coronaviruses seems to be markedly influenced by the presence of host cell receptors which can be specifically recognized by viral attachment proteins. The expression level of receptors is directly proportional to cellular maturity. For enterocytes which are a target of TCV infection, SCFA are a major energy source of metabolism and butyrate in particular has an effect on cell cycle mediation. SCFA in the intestine are produced by bacterial fermentation for which dietary fiber serves as a main substrate.

Therefore, to explore the host and dietary factors which influence TCV infection the following hypotheses have been proposed:

1. Host susceptibility to TCV infection is a function of enterocyte maturity.
2. SCFA, especially butyrate, can stimulate enterocyte maturation, and thereby influence host susceptibility to TCV infection.

3. Dietary fiber as a substrate for microbial fermentation can result in increased SCFA production and therefore influence host susceptibility to TCV infection.

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CHAPTER 2.

An Indirect Immunoperoxidase Assay for the Detection of Turkey Coronavirus in Fixed Paraffin-Embedded Tissue

2.1 Abstract

An indirect immunoperoxidase assay (IPA) was developed to identify turkey coronavirus (TCV) in fixed paraffin-embedded intestinal tissue. Fixation with 1% acetic acid in 95% ethanol was found to be superior to 10% neutral buffered formalin or periodate lysine 1% paraformaldehyde. The sensitivity of IPA on fixed tissue was also determined to be greater than that of indirect immunofluorescence assay on frozen tissue, which is the method most commonly employed for sero-diagnosis of TCV infection. The described assay has the advantages of simplifying sample shipment and storage as well as enabling more precise cellular localization of TCV.

2.2 Introduction

Turkey coronavirus (TCV) is one of the primary agents thought to be associated with Poultry Enteritis Mortality Syndrome (PEMS), an enteric condition of young turkeys characterized by diarrhea, elevated mortality, poor feed efficiency, and sub-optimal growth (Guy et al., 1997). A direct immunofluorescent antibody (FA) test for detection of TCV in cryo-sections, using polyclonal anti-TCV serum, was described by Patel et al (1975). The difficulty with antigen detection in frozen sections is that it requires shipping and handling of frozen infectious material as well as using equipment such as cryostat which is not commonly found in most veterinary diagnostic laboratories. Also it is for the most part a qualitative assay and provides little information regarding tissue structure and viral localization. Later in 1976, an indirect FA (IFA) technique for the detection of serum antibody against the virus was reported (Patel et al., 1976). In IFA, TCV-positive cryo-sections used to detect anti-TCV antibody also carry IgG, which reacts to the secondary antibody, fluorescein-conjugated anti-turkey IgG, causing non-specific background fluorescence. This non-specific background especially that is increased in the examination of

undiluted field sera as well as tissue autofluorescence diminishes the contrast in specific reactions causing interpretation difficulties. At present, IFA is used for both antigen and antibody detection.

Immunohistochemical assays, using fixed paraffin-embedded tissue have been developed for coronavirus identification in other species (Dar et al., 1998; Gerna et al., 1982; Shoup et al., 1996; Tammer et al., 1995; To and Bernard, 1992; Zhang et al., 1997). It was felt that the development of an indirect immunoperoxidase assay (IPA) for TCV would alleviate some of the problems noted with IFA and provide a more practical tool for TCV surveillance.

2.3 Materials and Methods

Viruses. Twenty-four-day-old turkey embryos were injected intra-amniotically with 0.2 mL supernatant derived from homogenized embryonic turkey intestine infected with Indiana isolate TCV (37th passage). After 24 hours of incubation, the small intestine was collected, ground, and suspended in phosphate buffered saline (PBS), pH 7.4 to make a 10% w/v suspension. The suspension was clarified by centrifugation at 1000 x g for 10 min after which the supernatant was collected and frozen at -70 °C. Two additional serial passages were performed as described and the supernatants were pooled, diluted 1:10 v/v with PBS (pH 7.4), frozen (-70 °C), and retained as stock. The Embryo Infective Dose 50 (EID₅₀) of the stock was determined according to Reed et al. (1938) by IFA and later verified by IPA on both frozen and fixed samples. The concentration of TCV in the stock was found to be 5.0 x 10⁴ EID₅₀/ml.

Animals. Fertilized turkey eggs and 1-day-old poults were obtained from primary turkey breeder stock (British United Turkey of America (BUTA), Lewisburg, WV) known to be coronavirus free. The eggs were incubated to the desired age for each experiment and poults were held in isolation.

Tissues. For the purposes of assay development, ten 24-day-old embryos were inoculated intra-amniotically with 1 x 10³ EID₅₀ and ten 3-day-old poults were orally inoculated with 5 x 10³ EID₅₀ of TCV. After 24 hours of incubation, intestinal segments from the middle of the duodenal loop and the area around the Meckel's diverticulum were

obtained and split for IFA and IPA. For IFA, the samples were immediately frozen in embedding medium (Sakura Finetek USA, Inc, CA). Cryo-sections (5 μm) were cut, mounted on charged glass slides (Fisher Scientific, PA), fixed in absolute acetone for 10 min and dried at room temperature. Slides were placed in boxes, vacuum-sealed to prevent moisture accumulation, and frozen at $-70\text{ }^{\circ}\text{C}$. For IPA, samples were fixed in 1% acetic acid in 95% ethanol (AE) or 10% neutral buffered formalin (NBF) or freshly prepared periodate lysine 1% paraformaldehyde (PLP) (McLean and Nakane, 1974). After 24 to 48-hour fixation, the samples were processed, embedded in paraffin and sectioned at 5 μm . The sections were mounted on charged glass slides and stored at room temperature for future testing. Negative controls for IFA and IPA studies were prepared similarly using intestinal sections from uninfected embryos and poults.

Antibodies. Polyclonal turkey anti-TCV serum was prepared by orally challenging 2-week-old turkey poults with 5×10^3 EID₅₀ of TCV stock. Sera were collected at 2 weeks post-challenge and pooled. The optimum working dilution for positive serum was determined by IFA, using frozen coronavirus-positive tissue sections. Negative control serum was obtained from uninfected poults of the same hatch.

Immunohistochemistry. IFA was performed, using polyclonal turkey anti-TCV as the primary antibody and fluorescein isothiocyanate (FITC) (Kirkegaard & Perry Laboratories, MD) and Texas Red (Kirkegaard & Perry Laboratories, MD) conjugated goat anti-turkey IgG as the secondary antibodies. Processed tissues were mounted with 50% glycerol in PBS and examined using a fluorescent microscope. The conditions for IPA, including antibody and conjugate concentration, and incubation times were optimized. Endogenous peroxidase was quenched by incubating the sections in 3% H₂O₂ for 10 min, then washing once in PBS for 10 min. Sections were overlain with 0.5% 3,3'-diaminobenzidine-4HCl (DAB) containing 0.1% H₂O₂ in Tris buffer saline for 5 min then washed in PBS for 10 min. The slides were counterstained with hematoxylin for 1 min, rinsed in tap water, dehydrated in graded ethanol (30 sec in each 80, 95, 100, 100, and 100%) and finally clarified in absolute xylene. After clarification, slides were permanently mounted (Alban Scientific, MO), coverslipped and examined, using a regular light microscope. Photomicrographs were taken with a digital camera.

Experimental challenge and assay verification. Experiment 1: The relative sensitivities of IFA and IPA for detection of seroconversion were determined by performing an endpoint dilution with pooled positive serum. TCV positive embryonic tissues were prepared as previously described. IFA and IPA based methods for serological testing were then compared as follows. Thirty-five, 2-week-old poults were inoculated with 5×10^3 EID₅₀ of TCV. Sera were then collected at 0, 7, 14, and 21 days post-challenge and used as the primary antibody in both the IFA and IPA procedures. Experiment 2: The relative sensitivities of IFA and IPA were determined for antigen detection. Ninety, 24-day-old embryos were inoculated as previously described for antigen preparation with the exception that post-inoculation incubation time was varied. Fifteen embryos each were sacrificed at 6, 12, 16, 20, 24, and 28 hours post-inoculation. Intestinal samples were collected and either frozen for IFA or fixed in AE for IPA. NBF and PLP were not used for fixation in this segment of the study because they were earlier found to be inferior to AE. Experiment 3: IFA and IPA were compared in the diagnosis of field serum samples.

2.4 Results

The TCV challenge dose of 1×10^3 EID₅₀ was adequate to produce 100% infection in 24-day-old turkey embryos. Likewise, 5×10^3 EID₅₀ produced 100% infection in 2-week-old poults.

The fluorescence associated with both FITC and Texas Red was adequate for visualization of TCV infected embryonic intestine. However, a low level of background fluorescence due to auto-fluorescent or non-specific binding was noted. Texas Red appeared to generate better contrast and demonstrated less quenching than FITC.

All three fixatives allowed recognition of the viral antigen by polyclonal anti-TCV serum. However, IPA visualization in tissues fixed with AE was considerably better due to enhanced reaction intensity. The general structure of fixed paraffin-embedded tissue was well preserved. Viral antigen could be clearly identified within the cytoplasm of individual enterocytes. Figure 2.1a shows IPA performed on AE-fixed embryonic intestine 24 hours after inoculation with TCV. Nuclear counterstaining with hematoxylin created a clear contrast with the brown, cytoplasmic staining of DAB associated with TCV antigen. TC

infected enterocytes could be seen at the base, lateral wall and tip of the villi, with a few noted in the crypt. Degenerated TCV-infected enterocytes could be seen in the intestinal lumen. Figure 2.1b shows the adjacent tissue section similarly processed except for the use of negative control serum as a primary antibody. Little non-specific binding was encountered as indicated by the absence of brown staining. A higher magnification of embryonic jejunum shows large accumulations of TCV antigen in the cytoplasm of infected cells (Fig. 2.1c). In turkey poult, the distal two-third of the villus showed evidence of TCV infection. No infected cells were noted in the crypt cells and only minimal numbers in the proximal third of the villus (Fig. 2.2).

A comparison of antigen detection by IFA and IPA infected embryos over time is summarized in Table 2.1. Of 15 samples, 6 (40%) and 13 (87%) were found to be TCV-positive at 12 hours post-inoculation by IFA and IPA respectively. The number of detectable positives increased to 9 (60%) and 14 (93%) at 16 hours, and to 15 (100%) and 15 (100%) at 20, 24, and 28 hours post-inoculation.

Table 2.2 shows the relative sensitivities of IFA and IPA in detection of anti-TCV antibodies in serum. IPA was capable of detecting positives at a higher dilution (1:2500) than IFA (1:1280).

In experimentally infected poult, both IFA and IPA were unable to detect seroconversion at 1 week post-infection, but by 2 and 3 weeks post-infection both assays detected seroconversion in (35/35) 100% of the poult.

2.5 Discussion

IPA as reported herein can simplify and improve detection of TCV by allowing the use of fixed paraffin-embedded tissue. Samples can be processed by equipment generally available in most diagnostic laboratories and can be examined using a regular light microscope. This technique also solves some of the problems associated with using frozen samples, including inconvenience and risk of transporting infectious samples, as well as sample deterioration. Likewise, it eliminates the need for maintaining large numbers of pre-prepared TCV-positive frozen sections for IFA examination of field sera. Fixed paraffin-embedded sections can be stored at room temperature.

As reported by other investigators (Gerna et al., 1982; To and Bernard, 1992), our studies showed that ethanol was superior to aldehyde as a fixative for immunohistochemical detection of coronavirus. When aldehydes are used, partial loss of antigen detection typically occurs unless an enzymatic treatment of tissue sections is done prior to application of the primary antibody (Dar et al., 1998; Gerna et al., 1982; Shoup et al., 1996; Tammer et al., 1995; To and Bernard, 1992; Zhang et al., 1997). This required selecting and optimizing of types and concentrations of the enzymes and timing of digestion.

Although Texas Red was found to provide a higher level of color contrast when compared to FITC, background fluorescence was still present. This could be a problem in the case of weak positives as well as the explanation for lower sensitivity of IFA versus IPA. It has been our experience that undiluted field sera often produce a higher level of background, especially when hemolysis has occurred; making interpretation difficult.

IFA slides are difficult to archive due to the instability of fluorescence and the intolerance of the frozen sections to permanent mounting. Permanently mounted IPA slides can be stored for longer periods of time with only minimal loss in quality.

The use of fixed, paraffin-embedded tissue sections of IPA technique permits histopathological evaluation as well as viral localization studies because cellular architecture is well preserved. Positive reactions can easily be differentiated from non-specific background by reconfirming the intra-cytoplasmic localization of viral antigen. This also enhances diagnostic efficiency especially in the case of field serum testing when undiluted field sera are being tested and background staining becomes a problem. This could also be part of the explanation to the higher sensitivity of the IPA in the detection of either early infection or low-level serum antibody as demonstrated in this study.

2.6 References

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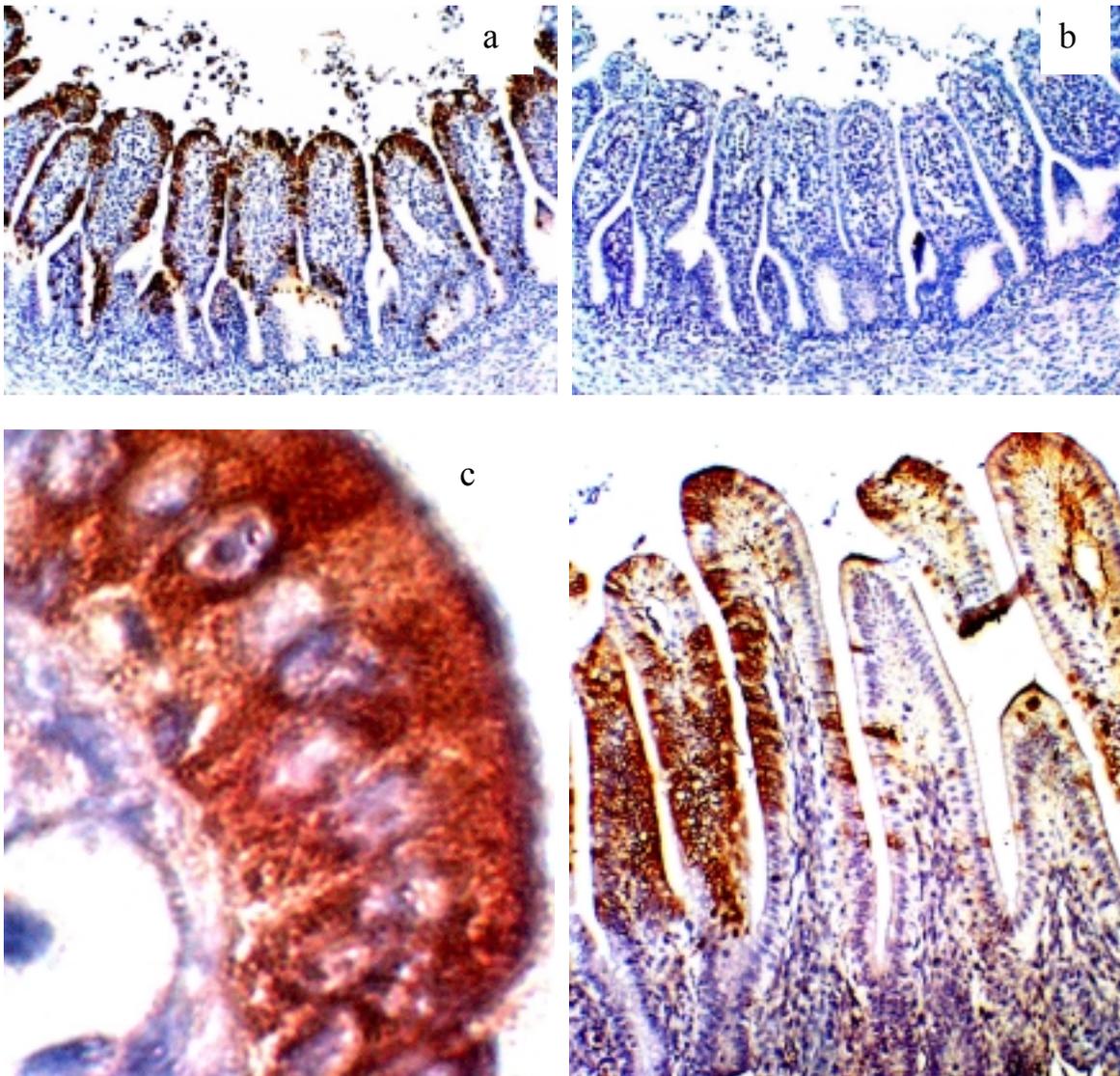


Figure 2.1 IPA performed on AE-fixed embryonic intestine 24 hours after intra-amniotic inoculation with TCV.

a) Infected duodenal enterocytes demonstrated by dark brown cytoplasmic staining. b) IPA using TCV negative control serum as primary antibody, counter stained with hematoxylin, prepared from section adjacent to that shown in “a”. c) Intracytoplasmic localization of TCV in individual jejunal enterocytes.

Figure 2.2 IPA performed on jejunum from a 3-day-old poult orally inoculated with TCV.

Table 2.1 TCV identification as a function of time post-challenge.

Methods	Incubation time (hr)					
	6	12	16	20	24	28
IFA (no. pos./total)	0/15	6/15	9/15	15/15	15/15	15/15
IPA (no. pos./total)	0/15	13/15	14/15	15/15	15/15	15/15

Table 2.2 Comparison of IFA and IPA on the basis of endpoint dilution with known positive anti-TCV serum.

Methods	Serum dilution						
	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
IFA	++++	+++	++	++	+	neg	neg*
IPA	++++	+++	++	++	+	+	neg

* +++++ to + = decreasing reaction intensity, neg = visually negative.

CHAPTER 3.

The Effect of Butyrate on Villin Expression and Enterocyte Maturation in Turkey Embryos

3.1 Abstract

Villin expression was investigated as an indicator of enterocyte maturity in developing turkey embryos and later used to determine the effect of butyrate on enterocyte maturation. Villin was detected by indirect immunoperoxidase assay on fixed paraffin-embedded tissue. Relative levels of villin expression in crude intestinal extracts were determined based on the optical density of immunoreactive bands on Western blot. Villin was expressed in the cytoplasm and on the apical membranes of enterocytes in 17 and 19-day-old embryos. By 21 days, villin expression dramatically increased on the apical membrane. This was even more pronounced in 23-day-old embryos with some lateral membrane localization being noted. It was found that the expression level of villin increased linearly in association with embryo age indicating a positive correlation between villin expression and enterocyte maturity. Twenty-one-day-old embryos were injected intra-amniotically with butyrate at concentration ranging from 0.1 to 0.5 M. Villin expression was significantly enhanced in embryos receiving 0.2 and 0.3 M butyrate at 36 hours post-administration. It appears that butyrate enhances villin expression and enterocyte maturation in a dose-dependent manner.

3.2 Introduction

Detection and quantification of brush border cytoskeletal proteins is one of the strategies historically used to assess differentiation and maturation of enterocytes. These proteins, which include actin, fimbrin, myosin I, and villin are essential elements in the structural development of the brush border (Carboni et al., 1987; Heintzelman and Mooseker, 1990; Maroux et al., 1988). Villin and fimbrin are required for the bundling of actin filaments that form the structural core of the microvillus (Arpin et al., 1988; Bretscher and

Weber, 1980; Friederich et al., 1990; Grone et al., 1986; Pringault et al., 1986; West et al., 1988). The actin filament bundle is anchored to the plasma membrane by myosin I.

Villin is considered essential to microvillus assembly because it is only expressed in those cells which develop brush borders i.e., epithelial cells of the intestine and renal proximal tubule (Bretscher and Weber, 1979; Bretscher and Weber, 1980). It is responsible for the control of actin filament elongation, a process which is Ca^{++} concentration gradient dependent. In humans, rats, and chickens, villin expression and cellular localization in intestinal epithelium appears to correlate well with the formation of microvilli during the terminal stages of enterocyte maturation (Fath and Burgess, 1995).

Enterocyte maturation is energy dependent. One of the major energy sources utilized by these cells is butyrate, a short-chain fatty acid (SCFA) (Chapman et al., 1995; Fitch and Fleming, 1999; Krishnan and Ramakrishna, 1998). In avian species, butyrate along with several other SCFA's is produced as a result of bacterial fermentation in the ceca and to a lesser extent, the crop. Although there is some uncertainty with regard to the exact effect, it is apparent that butyrate availability may directly influence the rate at which enterocytes differentiate (Basson et al., 1998; Morita et al., 1982; Sanderson et al., 1996; Tanaka et al., 1989) and proliferate (Boffa et al., 1992; Mathers et al., 1993; Sakata, 1987). To date, most of this research has been done *in vitro* using transformed cell lines of human origin. The effect of butyrate on turkey enterocyte development has not been previously investigated.

The objectives of this research were to investigate the relationship between villin expression and enterocyte maturation and then to determine the effect of butyrate administration in turkeys.

3.3 Materials and Methods

Experiment 1.

Animals. A randomized complete block design was used to study the relationship between enterocyte maturation and villin expression. The experiment was performed in three blocks, with 32 turkey embryos per block. Initially, in anticipation of a 20% infertile / embryo mortality rate, a total of 40 eggs were set for each block. Four sets of 10 eggs each were

staggered 2 days apart. This permitted simultaneous harvesting of 17-, 19-, 21-, and 23-day-old embryos.

Villin localization. For villin localization, 2 of 8 embryos from each age group were euthanized by cervical dislocation. Intestinal segments (1 cm) were collected from two locations, the middle of the duodenal loop and just anterior to Meckel's diverticulum. These were immediately fixed in 1% acetic acid in 95% ethanol. After 24 hours, samples were embedded in paraffin, sectioned at 5 μ m, and mounted on charged slides (Fisher Scientific, PA). These were dried in a hot air oven at 45 °C for 3-4 hours, and then stored at room temperature.

Immunoperoxidase staining was performed using the following protocol. Sections were first deparaffinized in absolute xylene. Hydrogen peroxide (3.0%) was applied for 10 min to quench endogenous peroxidase activity. Slides were incubated at room temperature (RT) for 2 hours with primary antibody i.e., monoclonal mouse-anti-chicken villin (Immunotech, France), diluted 1:50 in PBS, pH 7.4 in a moist chamber. Cross-reactivity of this antibody with turkey villin was previously determined (data not shown). Slides were washed by immersion in PBS for 10 minutes. Incubation with secondary antibody i.e., peroxidase labeled goat-anti-mouse IgG (Kirkegaard & Perry Laboratories, MD), diluted 1:200 in PBS, pH 7.4 was performed for 30 minutes at RT followed by a second PBS wash. Finally, visualization was achieved by applying 3-3' diaminobenzidine-4HCl (DAB) substrate for 5 min. Slides were evaluated under regular light (daylight filter) using an Olympus BH-2 microscope.

Villin expression level. For the study of villin expression, 6 of 8 embryos from each age group were euthanized by cervical dislocation. Full-length intestinal samples extending from the ventriculo-duodenal junction to the ileum at the level of the cecal tips were collected and frozen in liquid nitrogen. These were later homogenized with 1.0 mL of distilled water in an Omni Mixer 17105 homogenizer (Omni International, CT), lyophilized, and stored at -40 °C for further testing.

Lyophilized crude intestinal extract (2 mg) was re-suspended in 190 μ L of Laemmli sample loading buffer (Laemmli, 1970) and boiled in a water bath for 3 min. β -mercaptoethanol was added to bring the final concentration to 1:100 w/v. Samples were

reheated at 95 °C in a heating block for 5 min then clarified by centrifugation at 15,000 rpm for 4 min. Each sample was loaded at 7 µL onto a 7.5% sodium dodecyl sulfate resolving gel and subjected to electrophoresis (SDS-PAGE) at 200 V for 45 min using a mini-gel apparatus (Hoefer Scientific Instruments, LA). Pooled intestine from 25-day-old embryos was also run as a reference standard. Proteins separated by SDS-PAGE were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked at RT with 1.5% bovine serum albumin (BSA) and 1.5% skim milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST). After 1 hour, the blocking solution was discarded and the membrane was washed once in TBST for 5 min and then in TBS for 5 min. The membrane was incubated with monoclonal mouse-anti-chicken villin, diluted 1:200 in TBST for 4 hours at RT. The antibody solution was discarded and the membrane was washed twice in TBST and twice in TBS, 5 min per wash. Incubation with peroxidase-labeled goat-anti-mouse IgG (Kirkegaard & Perry Laboratories, MD), diluted 1:1000 in TBS was performed for 1 hour at RT, followed by two TBST and TBS washes as previously described. DAB substrate was applied for 10-15 sec and a final 5 min distilled water rinse was performed.

Immunoblots were scanned using a flatbed scanner. Protein band images were analyzed for optical density (OD) using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). To account for variation between blots, OD's were corrected based on the intensity of the reference standard. The results were reported as means ± SEM. Differences among means were analyzed using a one-way ANOVA (SAS, Version 8.1, SAS Institute, Cary, NC) with significance being assigned at a level of $P < 0.05$. Block effect was considered to be negligible ($ss < 5\%$). Post-ANOVA analysis between age groups was performed using Tukey's range procedure and regression analysis was done to determine the relationship between embryo age and villin expression level.

Experiment 2.

A randomized complete block design was used to study the effect of butyrate on villin expression. The experiment was performed in three blocks, with twenty four 21-day-old turkey embryos per block. Within blocks, embryos were divided into 6 treatment groups i.e.,

0.1, 0.2, 0.3, 0.4, and 0.5 M butyrate in PBS, pH 7.4 plus a negative (PBS) control. Treatments were administered by intra-amniotic injection, 0.2 mL per embryo. At 48 hours post-treatment, embryos were euthanized by cervical dislocation and the intestinal samples were collected for SDS-PAGE and immunoblot analysis as previously described.

Results were reported as means \pm SEM. Differences among means were analyzed using a one-way ANOVA (SAS, Version 8.1, SAS Institute, Cary, NC) with significance being assigned at a level of $P < 0.05$. Block effect was considered to be negligible ($ss < 5\%$). Post-ANOVA analysis comparing butyrate treatments with the control was performed using Dunnett's t test and regression analysis was done to determine the relationship between butyrate concentration and villin expression.

3.4 Results

Experiment 1.

Fixed paraffin imbedded tissues revealed the presence of villin at the apical surface and in the cytoplasm of intestinal epithelial cells of 17- and 19-day-old embryos (fig. 3.1a and 3.1b). In 21-day-old embryos, a significant increase in the density of villin at the apical surface was observed (fig. 3.1c). The intestinal epithelium of 23-day-old embryos appeared to be well differentiated based on the presence of specific cell types like goblet cells (fig. 3.1d). Villin was found to be concentrated on the apical membrane and lateral portion of the basolateral membrane producing a clearly demarcated boundary between cells (fig. 3.1d). The density was greatly increased. Goblet cells appeared completely devoid of villin.

Immunoblots revealed a single protein band of approximately 95 kDa (fig. 3.2). Relative villin concentration increased significantly with age in a linear fashion ($y = 0.898x - 12.381, r^2 = 0.89$) (fig. 3.3).

Experiment 2.

Villin was expressed at higher levels in all butyrate treated embryos when compared with controls (fig. 3.4). However, significant elevations were only noted with the 0.2 M and 0.3 M treatments. The relationship between butyrate concentration and villin expression was curvilinear with peak expression being observed at 0.3 M.

3.5 Discussion

Actin-binding proteins including villin, are highly conserved among various species (Markus et al., 1994; Schleicher et al., 1988); (Bazari et al., 1988; Pringault et al., 1986; Way and Weeds, 1988). The presence of a protein in intestinal epithelium of turkey embryos that reacts with monoclonal anti-chicken villin on immunoperoxidase stained sections as well as the detection of a 95 kDa protein derived from intestinal homogenates corroborates this fact and at the same time validates the use of monoclonal anti-chicken villin as a reagent for detection of turkey villin.

Fixed paraffin-embedded tissue sections permit the study of villin localization and enable the identification of different epithelial cell types. From serial examination of developing embryos, it was determined that villin appears initially in the cytoplasm of absorptive epithelial cells and then relocates to the apical and basolateral membranes. This logically coincides with age dependent differentiation and maturation of these cells in the embryo. Similar findings have been reported in developing chicken embryos (Shibayama et al., 1987) and human adenocarcinoma cells (Peterson et al., 1993). It has been found that villin expression and localization play a key role in the assembly of brush border microvilli (Friederich et al., 1990; Heintzelman and Mooseker, 1990; Robine et al., 1985; Shibayama et al., 1987). Our observations regarding villin localization are consistent with these previous findings. On the other hand, basolateral membrane deposition as observed in mature cells supports the notion that villin may serve additional functions. In chickens, it has been proposed that the presence of villin at this site aids in the formation of plicae, which increase surface area available for transport of nutrients between cell and the interstitium (Heintzelman and Mooseker, 1990).

A lower concentration of villin staining was observed in young enterocytes of turkey embryos. This could either be a reflection of lower villin expression levels in these immature cells or a decrease in the sensitivity of IPA detection in the case of diffuse cytoplasmic villin. Based on antigen-capture ELISA, some investigators have suggested that villin is present at its final concentration even in undifferentiated crypt cells (Fath et al., 1990). To address the sensitivity issue, we used SDS-PAGE and western blotting techniques which concentrate apical and cytoplasmic villin into a single, tight, immunoreactive band. Therefore it is

believed that our data truly demonstrate that villin expression is lower in immature enterocytes of turkey embryos and that it can be used as a measure of enterocyte maturity in turkeys.

In turkey embryos, butyrate administered by intra-amniotic injection, appears to stimulate turkey enterocyte differentiation and maturation as indicated by increased villin expression. Butyrate has been found to enhance differentiation and apoptosis while inhibiting proliferation of many transformed cell lines. Tsao et al. (1982) reported that butyrate stimulates differentiation of HRT-18 cells based on induction of morphological changes (cell enlargement, flattening, and increased membranous process formation) and increase in membrane-associated enzyme activity. Butyrate is known to specifically enhance dipeptidyl peptidase, lactase, and sucrase activities as well as villin expression in several human colon adenocarcinoma cell lines, including Caco-2, SW620, and HT-29 (Barnard and Warwick, 1993; Basson et al., 1998; Hodin et al., 1996; Mariadason et al., 2000; Sanderson et al., 1996). This effect appears to be time and dose dependent in HT-29 cells (Hodin et al., 1996).

The mechanisms through which butyrate enhances enterocyte differentiation have yet to be fully resolved. Butyrate has been shown to inhibit proliferation by arresting the cell cycle at the G₁ phase in HT-29 cells (Barnard and Warwick, 1993), at the G_{0/1}- S phase transition in human urothelial adenocarcinoma cells (Larsen et al., 1995), and at G₁ and G₂ in peritoneal carcinoma cells (Boisteau et al., 1996). Butyrate is also thought to influence growth and apoptosis by inhibiting histone acetylase and thereby promoting polyacetylation and dephosphorylation of histone proteins (Bernhard et al., 1999; Buckley et al., 1996; D'Anna et al., 1980). In rat and human colonic carcinoma cells, this appears to correlate with increased glucose consumption and mitochondrial activity, suggesting an alteration in metabolic activity as well (Boisteau et al., 1996; Heerdt et al., 1997).

In non-neoplastic cells, various effects have been observed. Butyrate reduces the expression of phenotypic markers and has no effect on proliferation of colonic epithelial cells grown *in vitro* (Gibson et al., 1992). However, butyrate has been reported to promote the growth of the intestinal epithelium *in vivo* (Gibson et al., 1998; Sakata, 1987).

There may be several explanations for the reported variation in the response to butyrate administration. First it is logical to think that there are inherent morphological and physiological differences between neoplastic and non-neoplastic cells (Gibson et al., 1992; Luciano et al., 1996). Second, since the effect of butyrate appears to be dose dependent (Hodin et al., 1997; Sakata, 1987), *in vivo* studies may be complicated by the presence of fermentative bacteria in the gastrointestinal tract which produce SCFA. The *in ovo* system used in our study appears to be a hybrid between the *in vivo* and *in vitro* systems. It capitalizes on the use of normal cells in a physiologically normal environment and due to its sterility, does not have the confounding effect of endogenously produced SCFA.

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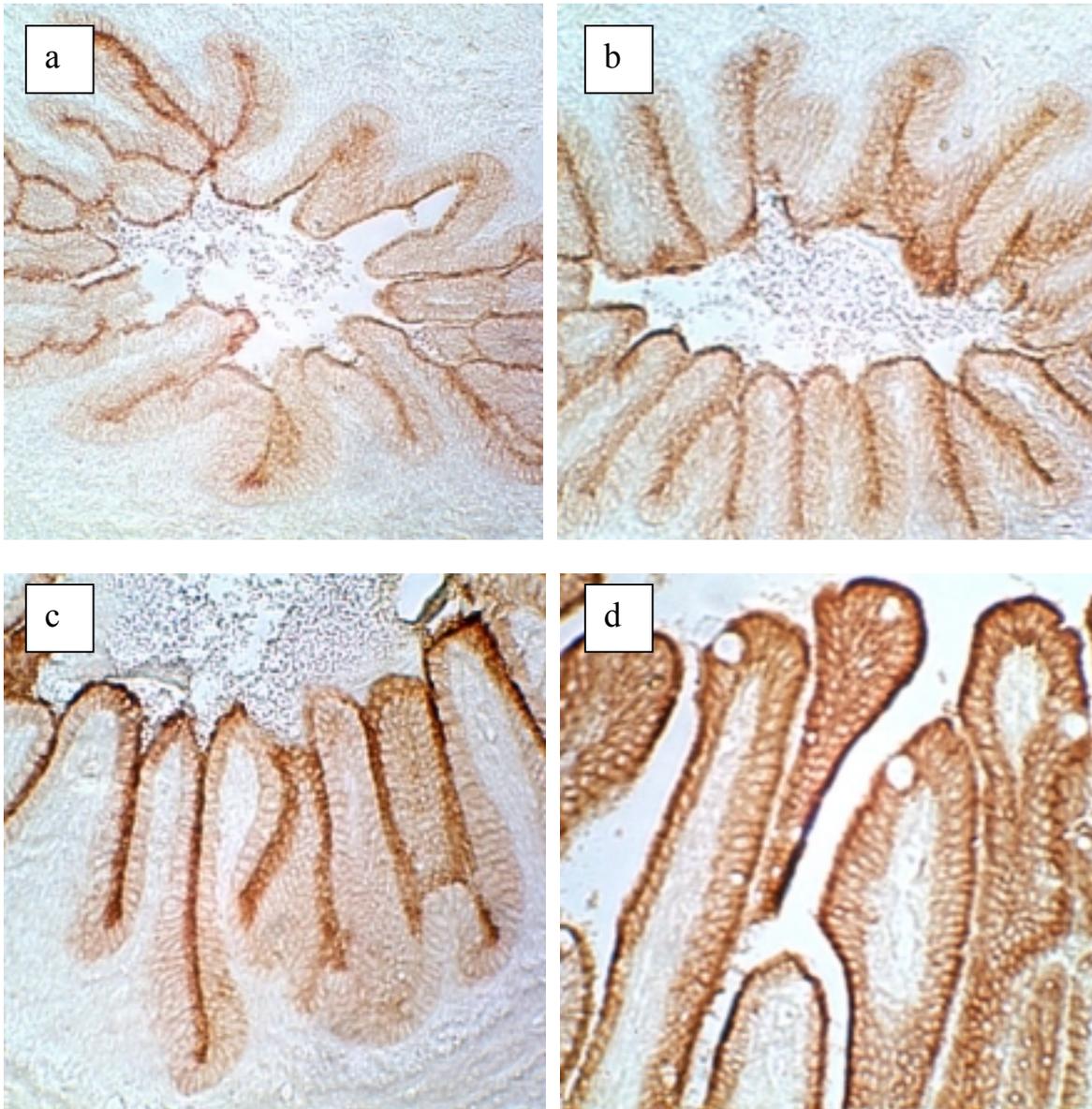


Figure 3.1 Villin localization in intestinal epithelial cells of developing embryos.

a) of 17 and b) of 19 days old: apical membrane localization of villin with diffused cytoplasmic localization. c) of 21 days old: increased concentration of villin at the apical surface. d) of 23 days old: very high concentration at the apical membrane and basolateral membrane. No villin is detected in goblet cells.

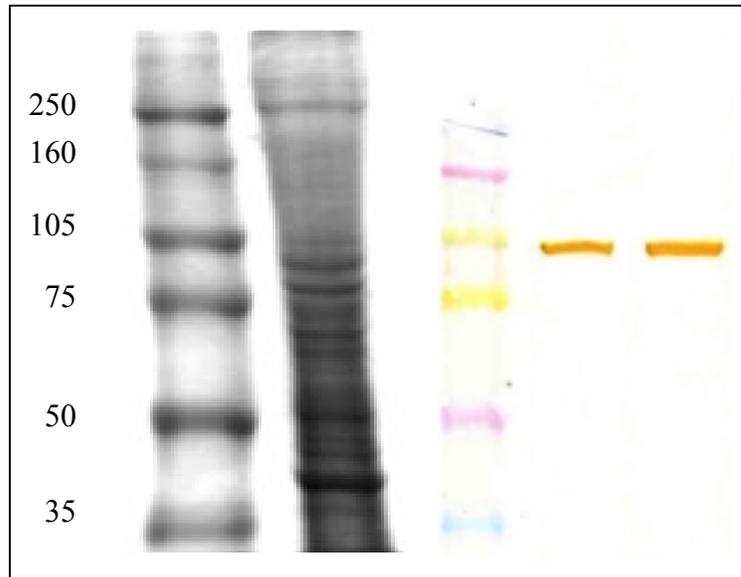


Figure 3.2 Villin detection on immunoblot.

Lane 2 shows protein separation from crude intestinal extract of 23-day-old embryos by SDS-PAGE. Lane 4 and 5 show immunoblots of intestinal samples from 21- and 23-day-old embryos. A single band of approximately 95 kDa was identified by monoclonal mouse anti-chicken villin. Lanes 1 and 3 are standard protein markers.

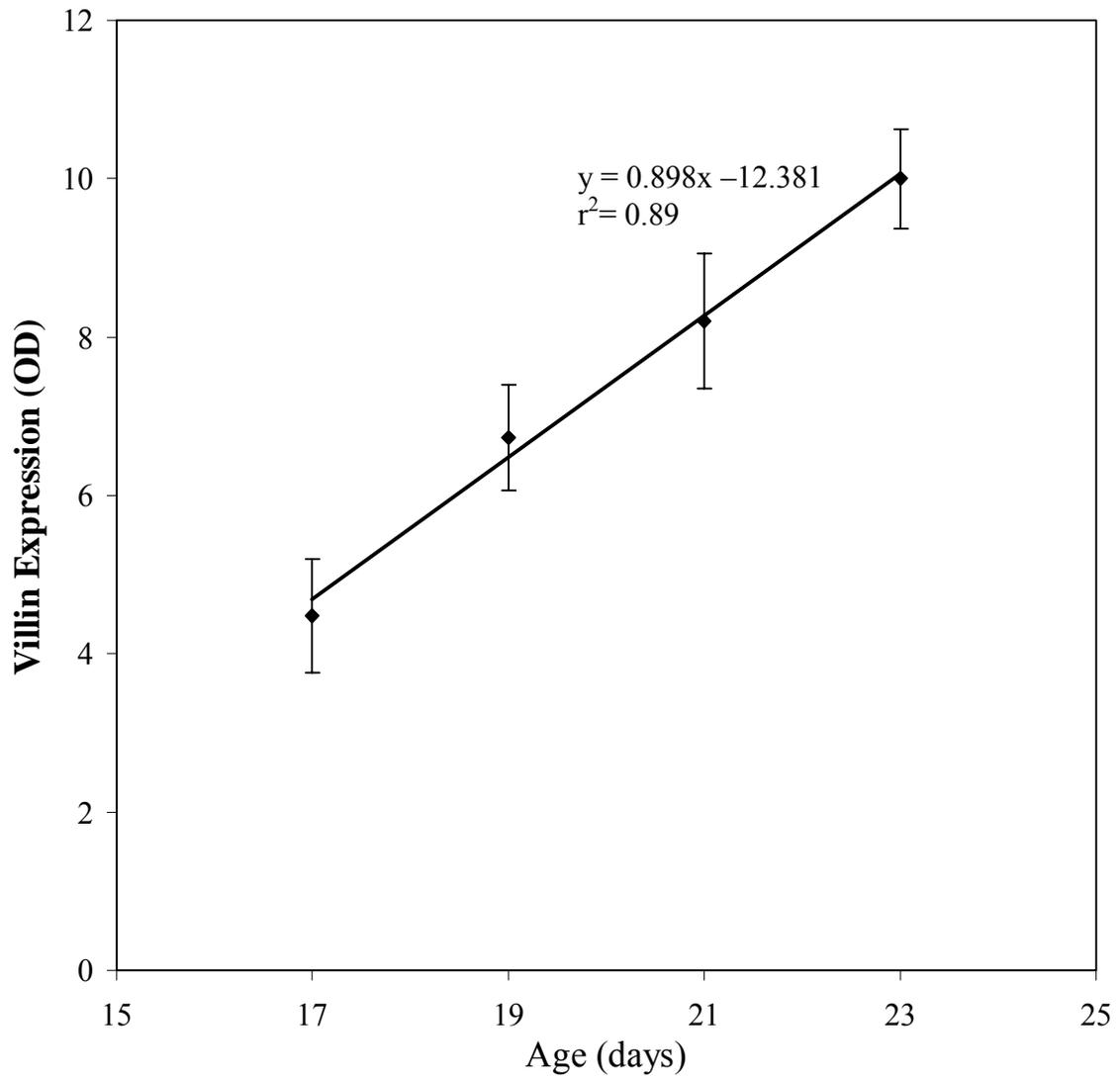


Figure 3.3 Linear relationship between embryo ages and villin expression levels. Values are expressed as means \pm SEM of relative villin expression levels in crude intestinal extract determined by OD on immunoblots.

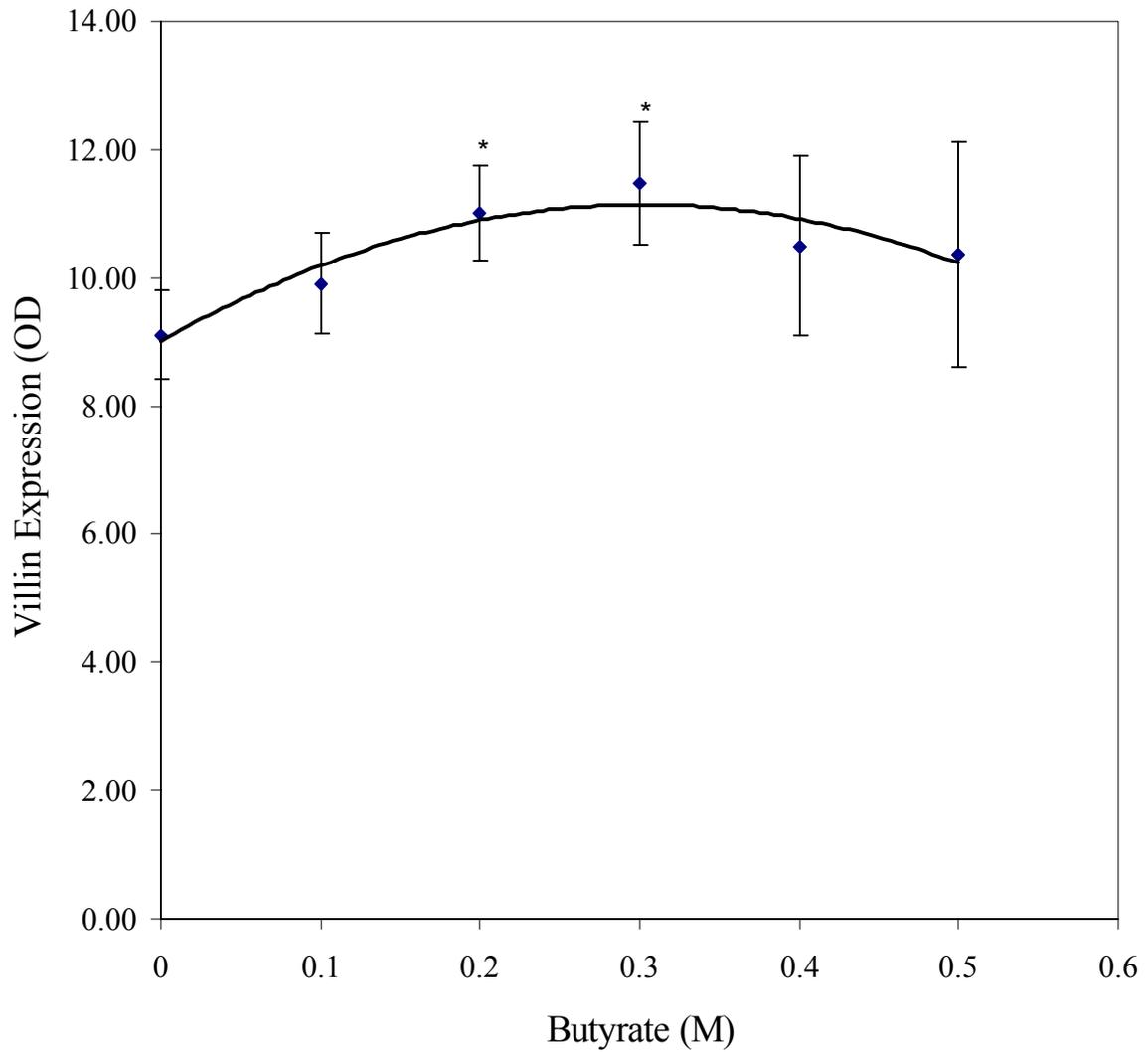


Figure 3.4 Effect of butyrate on villin expression.

Values are expressed as means \pm SEM of relative villin expression levels in the intestine of embryos treated with butyrate at different concentrations. Significant differences of treatment means compared to control (0 M butyrate) assessed by Dunnett's *t* test are indicated by *.

CHAPTER 4.

The Effect of Butyrate on Enterocyte susceptibility to TCV infection

4.1 Abstract

Susceptibility of turkey embryos to turkey coronavirus (TCV) infection as a function of age and butyrate treatment was investigated. Epithelial localization of TCV infection was also studied in poults. Infection, based on the appearance of intracellular antigen in intestinal epithelial cells, was determined 24 hours post-inoculation in 17-, 19-, 21-, and 23-day-old embryos as well as 3- and 18-day-old poults by indirect immunoperoxidase assay. Embryos appeared refractory to infection at 17- and 19-days-of-age. Evidence of limited infection was noted in 21-day-old embryos and 23-day-old embryos were found to be highly positive for intracellular TCV antigen. Poults also showed a high number of antigen-positive cells on the distal two thirds of the villi. No infected cells were seen in the intestinal crypts. Further examination of 19 day-old embryos by electron microscopy revealed a lack of cellular internalization despite the presence of virus in the intestinal lumen. Butyrate (0.2 M) administered by intra-amniotic injection in 21-day-old embryos followed by TCV inoculation 36 hours later, caused a significant increase in the number of infected cells per villus. It is believed that the effect of butyrate is related to its ability to enhance enterocyte differentiation and maturation. These data suggest that butyrate may be used as a means to manipulate enterocyte susceptibility to TCV infection.

4.2 Introduction

Turkey Coronavirus (TCV) is a positive, single stranded RNA virus with a “crown-like” envelope, characteristic of the family Coronaviridae. The envelope and its associated proteins play a major role in the process of cellular infection. Spike (S) protein, a large, club-like protein protruding from the envelope surface has been shown to be necessary for cellular internalization of virus (Ballesteros et al., 1997; Delmas et al., 1994; Godet et al., 1994; Hansen et al., 1998). Host receptor proteins which bind to the viral S protein are therefore

believed to be important determinants of such things as species specificity (Benbaccer et al., 1997; Holmes et al., 1997; Kolb et al., 1997), tissue tropism (Compton, 1994; Compton, 1998; Godfraind et al., 1995; Yokomori and Lai, 1994; Zelus et al., 1998) and age susceptibility (Weingartl and Derbyshire, 1993; Weingartl and Derbyshire, 1994).

As epithelial cells differentiate, proteins necessary for nutrient absorption and the maintenance of structural integrity begin to appear on or near the cell surface (Freeman, 1995). One such protein, aminopeptidase-N has been proposed as the receptor for coronavirus group I, which includes transmissible gastroenteritis virus (TGEV) (Kolb et al., 1998). Age related susceptibility to TGEV infection is believed to be directly related to the appearance of aminopeptidase-N on the cell surface (Weingartl and Derbyshire, 1993).

TCV infects the epithelium of the intestine and bursa of Fabricius in turkeys (Guy et al., 1997). As with other Coronaviridae, the species specificity and tissue tropism exhibited by TCV are anticipated to be a result of the viral recognition of specific receptor proteins expressed on epithelial cells. Even though a receptor protein for TCV has not yet been identified, it is likely that it follows an age related pattern of emergence similar to that of aminopeptidase-N.

The purpose of this study was to explore the relationship between enterocyte maturity and susceptibility to TCV infection in turkey embryos. Also, since our previous work has suggested that butyrate administration may stimulate enterocyte maturation, the effect of butyrate on enterocyte susceptibility to TCV infection was investigated.

4.3 Materials and Methods

Experiment 1.

Animals. A randomized complete block design was used to study the relationship between enterocyte maturation and TCV infection. The experiment was performed in three blocks, with 40 turkey embryos per block. Initially, in anticipation of a 20% infertile / embryo mortality rate, a total of 48 eggs were incubated for each block under normal conditions (38 °C with 60 % humidity). Four groups of 12 eggs were set at 2 day intervals in a standard drum incubator. This permitted simultaneous inoculation of 17-, 19-, 21-, and 23-day-old embryos with TCV. Following this experiment, an additional 5 embryos were set for

electron microscopic study. This was done to investigate the physical interaction between TCV and target enterocytes in the event that resistance to infection was found.

Twenty, day-old turkey poults obtained from TCV negative breeders were raised in a battery and provided with feed and water *ad libitum*. At 3 days-of-age, 10 poults were randomly selected and orally inoculated with TCV. Those remaining were inoculated at 18 days-of-age.

TCV Inoculation. Ten embryos of each age were inoculated by intra-amniotic injection with 1×10^3 EID₅₀ of TCV prepared as previously described (chap. 2). Each of the 20 poults was orally inoculated with 5×10^3 EID₅₀ of the same preparation.

Sample preparation and evaluation. Susceptibility of embryos to TCV infection was determined at 24 hours post-challenge based on the identification of intracellular TCV antigen by indirect immunoperoxidase assay (IPA). Briefly, two intestinal segments (1 cm) were collected, one from the middle of the duodenal loop and the other from the area located just anterior to Meckel's diverticulum and immediately fixed in 1% acetic acid in 95% ethanol. After 24 hours, samples were embedded in paraffin and sectioned (5 μ m). Tissue sections were mounted on charged slides (Fisher Scientific, PA), and dried in a hot air oven at 45 °C for 3-4 hours. Slides were deparaffinized in absolute xylene, then endogenous peroxidase activities were quenched by incubation with 3% H₂O₂ for 10 min followed by a 10-min wash by immersion in PBS, pH 7.4. Slides were subsequently incubated with polyclonal turkey anti-TCV antibody for 30 min at room temperature (RT) in a moist chamber. After a 10-min wash as previously described, they were incubated with secondary antibody i.e., peroxidase-conjugated goat anti-turkey IgG (Kirkegaard & Perry Laboratories, MD), diluted 1: 200 in PBS, pH 7.4 at RT for 30 min. Following another wash, substrate solution, 3,3'-diaminobenzidine-4HCl (DAB) containing 1% H₂O₂ in TBS, was applied for 5 min. Slides were washed, dehydrated in graded ethanol and permanently mounted. Tissue sections were examined under a regular light (daylight filter) for the presence of intracellular antigen, using an Olympus BH2 microscope.

For electron microscopy, 1 cm intestinal segments were collected from 5, 19-day-old embryos and placed in an ice-cold fixative containing 1.5% formalin, 2% paraformaldehyde in cacodylate buffer. After chilling, the segments were trimmed to 2 mm, placed in fresh

fixative and stored at 4 °C for 2 hours. Samples were then dehydrated through graded ethanol and embedded in Lowicryl K4M[®] at -35 °C in an UV chamber. Ultra-thin (100 nm) sections were placed on nickel grids and post-fixed using 2% osmium tetroxide for 15 min. Sections were stained with uranyl acetate and lead citrate and examined using a Joel 100 CXII transmission electron microscope.

Experiment 2.

Experimental Designs. A randomized complete block design with two blocks of twenty 21-day-old turkey embryos, 10 for butyrate treatment and 10 as negative controls were used to investigate the effect of butyrate on enterocyte susceptibility to TCV infection. Embryos were injected intra-amniotically with either 0.2 mL of 0.2 M butyrate in PBS, pH 7.4 or 0.2 mL of PBS, pH 7.4. At 36 hours post-treatment, each embryo was inoculated with 1×10^3 EID₅₀ TCV via the same route. Intestinal samples were collected, slides prepared, and IPA's performed as described for Experiment 1.

Tissue sections were examined under regular light (daylight filter) for the presence of intracellular antigen, using an Olympus BH2 microscope. To quantify the level of TCV infection, 100x digital images were analyzed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). Based on the condition of each intestinal cross-section, as few as three and as many as twelve longitudinal villus sections were evaluated per embryo. Only complete villi (base to tip) were included in measurements, hence the variation in number of villi counted per section. Density plots corresponding to IPA intensity were generated. Infected cells were enumerated based on the number of density peaks per villus and treatment means calculated.

Statistical Analysis: Differences between treatment and control means were analyzed using a non-parametric, Wilcoxon Scores (Rank Sums) procedure (SAS, Version 8.1, SAS Institute, Cary, NC), and significance was established at $P < 0.05$. The block effect was considered to be negligible with $ss < 5\%$.

4.4 Results

Experiment 1.

Intestinal samples collected from 17-day-old turkey embryos were negative for TCV by IPA (fig. 4.1a) as were those from 19-day-old embryos (fig.4.1b). Examination of embryos from these same age groups using transmission electron microscopy revealed the presence of extracellular, corona-like particles near the apical epithelial cell surface (fig 4.3). In 21-day-old embryos, TCV antigen was detected in the cytoplasm of epithelial cells located at the tips of villi by IPA (fig. 4.1c). The intensity of the IPA reaction was even more pronounced at 23-day-of-age where the cytoplasm appeared completely filled with TCV antigen (fig. 4.1d and 4.1e). As would be expected for Coronaviridae, no antigen was detected in the nuclei of infected cells or in goblet cells (white arrow). The concentration of TCV positive cells was higher in the distal half of villi than in the proximal (basal) half. Some of the degenerating IPA positive cells at the villus tips appeared ready to exfoliate as evidenced by detachment of the basal membrane from the lamina propria (red arrow). In some jejunal samples, infection of epithelial cells from the base to the tip of villi was noted (fig. 4.1f). However, TCV antigen was not found in the deepest regions of the crypts. In poults, TCV antigen was detected mostly in the cells located at distal two-thirds of the villus and none was in the crypts (fig. 4.2a and 4.2b).

Experiment 2.

Figure 4.4 shows the number of infected cells per villus in butyrate treated and control embryos. A significant increase in number of infected cells in the duodenum (19.65 ± 6.65 , 210%) and in the jejunum (15.35 ± 5.51 , 240%) was noted in those embryos receiving intra-amniotic injections of butyrate versus those receiving injections of sterile PBS (9.35 ± 6.97 and 6.40 ± 5.80 respectively).

4.5 Discussion

Results from this study indicate an age-related susceptibility of turkey embryos to TCV infection. Increased cellular susceptibility along the crypt-villus axis seems to be associated with the maturation process of intestinal epithelium. Arising from stem cells,

newly proliferated cells differentiate and mature while ascending the villus to replenish old, exfoliated cells. In our previous study, it was determined that epithelial cell differentiation and maturation were related to the cellular localization and expression level of villin. The proximity to fully differentiated goblet cells was also considered an indicator of maturity. In the current study, 17 and 19 day-old embryos were refractory to infection with TCV. In keeping with our previous results, we would suggest that this is most likely due to a lack of receptor expression in epithelial cells which are poorly differentiated at this stage of embryonic development. As embryos mature (21, 23 day-old), the number of infected cells increases, first at the villus tip (21 day-old) and then over the entire length of the villus from tip to base (23 day-old). This phenomenon may be the result of a gradual accumulation of well-differentiated, receptor bearing cells on the villus. Cells in the bottom area of the crypts remain undifferentiated even in 23-day-old embryos, hence they cannot, and do not become infected. In poults, the distal two-thirds of the villus appears to contain differentiated cells which are susceptible to infection. Cells in the crypts and the proximal one-third of the villus i.e., that portion closest to the base, are not infected. In both cases, the accumulation or lack of accumulation of susceptible cells on the villus may be related to the absence or presence of physical stimulation by ingesta that would normally trigger epithelial cell turnover. Similar findings have been reported in swine infected with TGEV (Shoup et al., 1996). In fact, the low turnover rate of enterocytes in suckling piglets is thought to greatly influence the severity of infection with TGEV (Moon et al., 1973).

In our previous study (chap. 3), enterocyte maturation and differentiation in turkey embryos was shown to be enhanced by intra-amniotic butyrate administration. The current data would suggest that this is the mechanism by which butyrate enhances susceptibility to TCV infection. Butyrate has been reported to increase susceptibility to cytomegalovirus infection in human epithelial thyroid papillary carcinoma cells (Tanaka et al., 1991) and in human melanoma cells (Wu et al., 1994). It also has been shown to increase susceptibility to herpes simplex virus in mouse neuroblastoma cells (Ash, 1986). It is believed that species specificity and tissue tropism with herpes viruses is associated with the presence of receptor(s) that are expressed during certain stages of cellular development (Lafemina and

Hayward, 1988; Smith, 1986). Our data would suggest that this holds true for TCV infection in turkeys as well.

4.6 References

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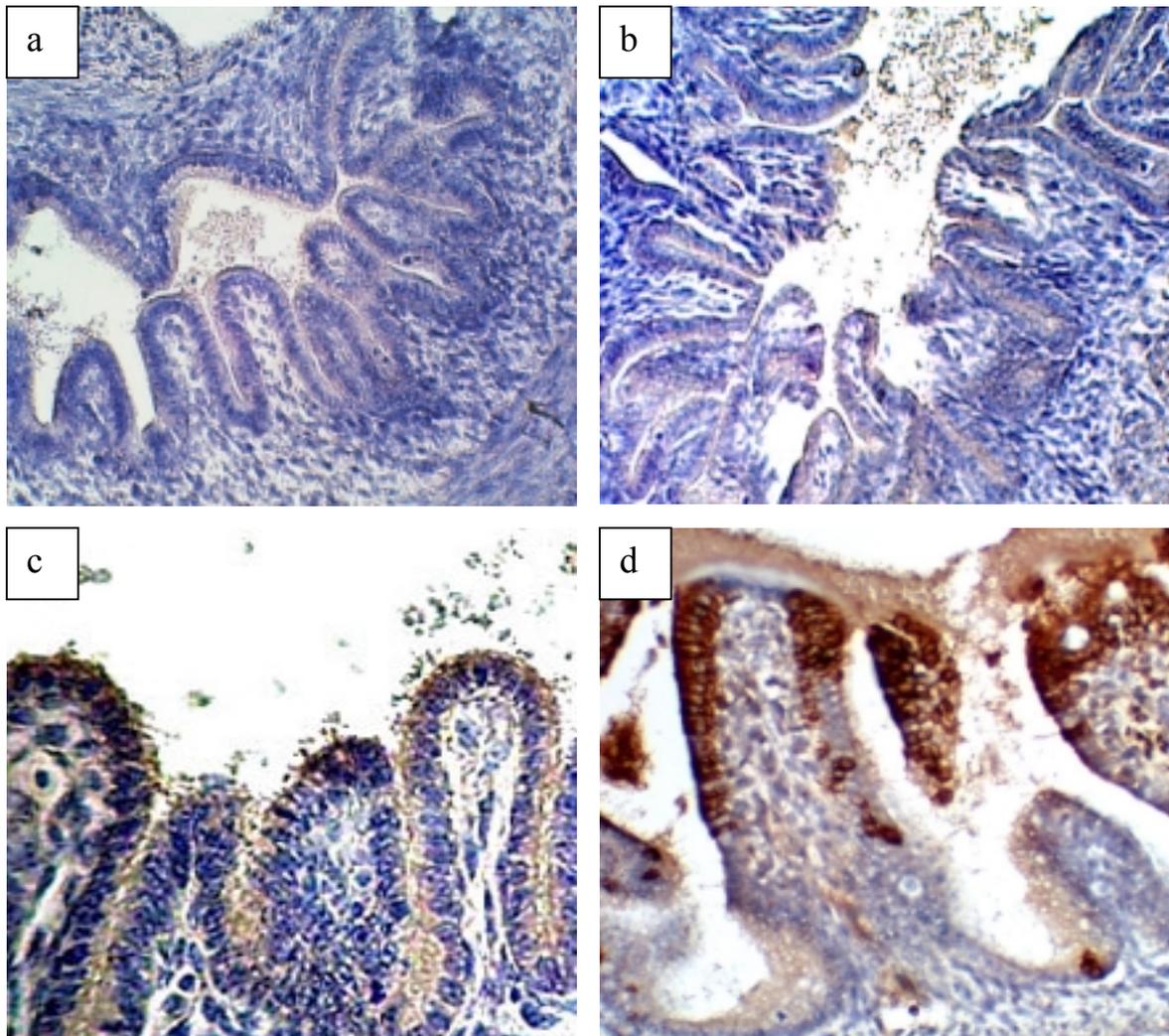


Figure 4.1 TCV infection in developing turkey embryos.

a) 17-day-old embryos show negative infection. b) 19-day-old embryos show negative infection. c) 21-day-old embryos show slight brown IP staining in the cytoplasm. d) 23-day-old embryos show a high concentration of intracellular antigen. Infection was occasionally found in the crypts.

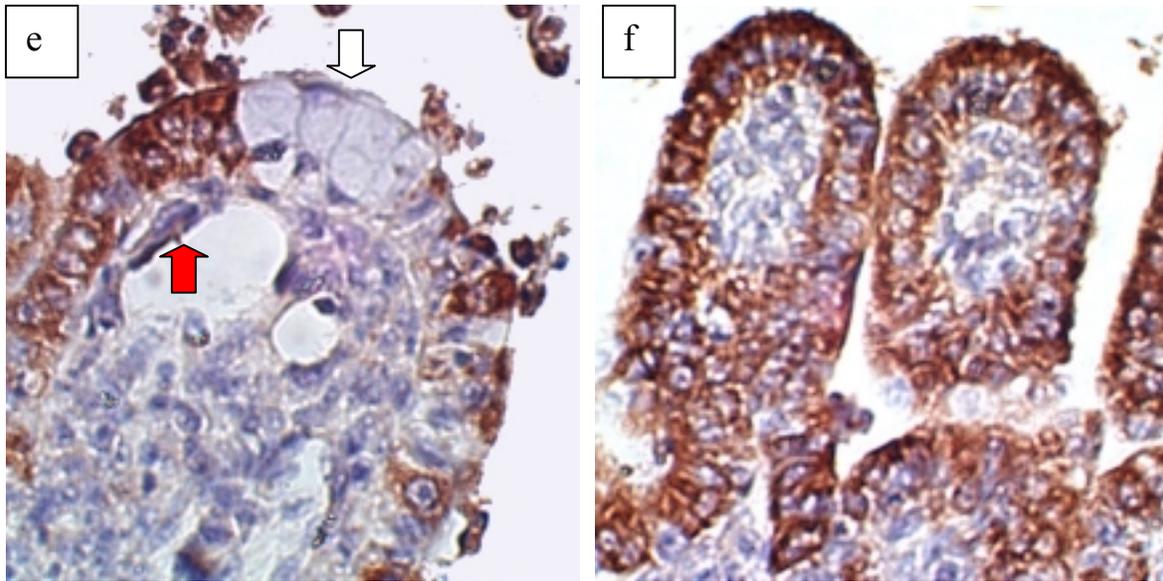


Figure 4.1. e) 23-day-old embryos show negative infection in goblet cells (white arrow) and a separation of infected epithelial cells from lamina propria (red arrow). f) 23-day-old embryos show massive infection in the jejunum from tips to bases of the villi.

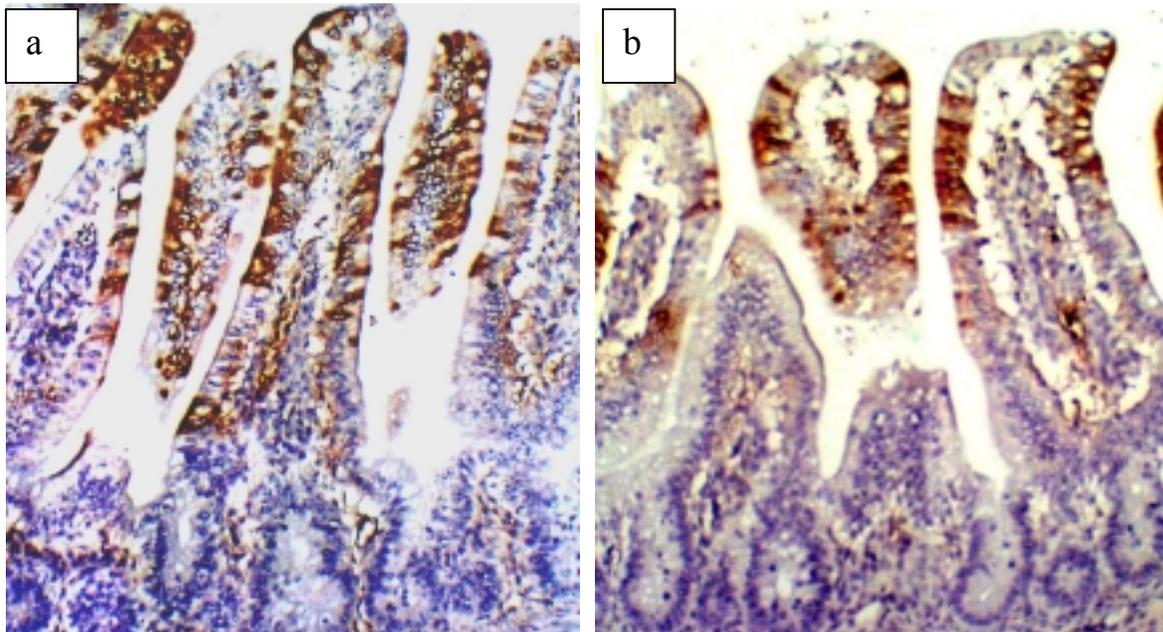


Figure 4.2 TCV infection in turkey poults.

a) 3-day-old poults and b) 18-day-old poults show infection concentrated in two thirds of the villi. No infection was detected in the crypts.

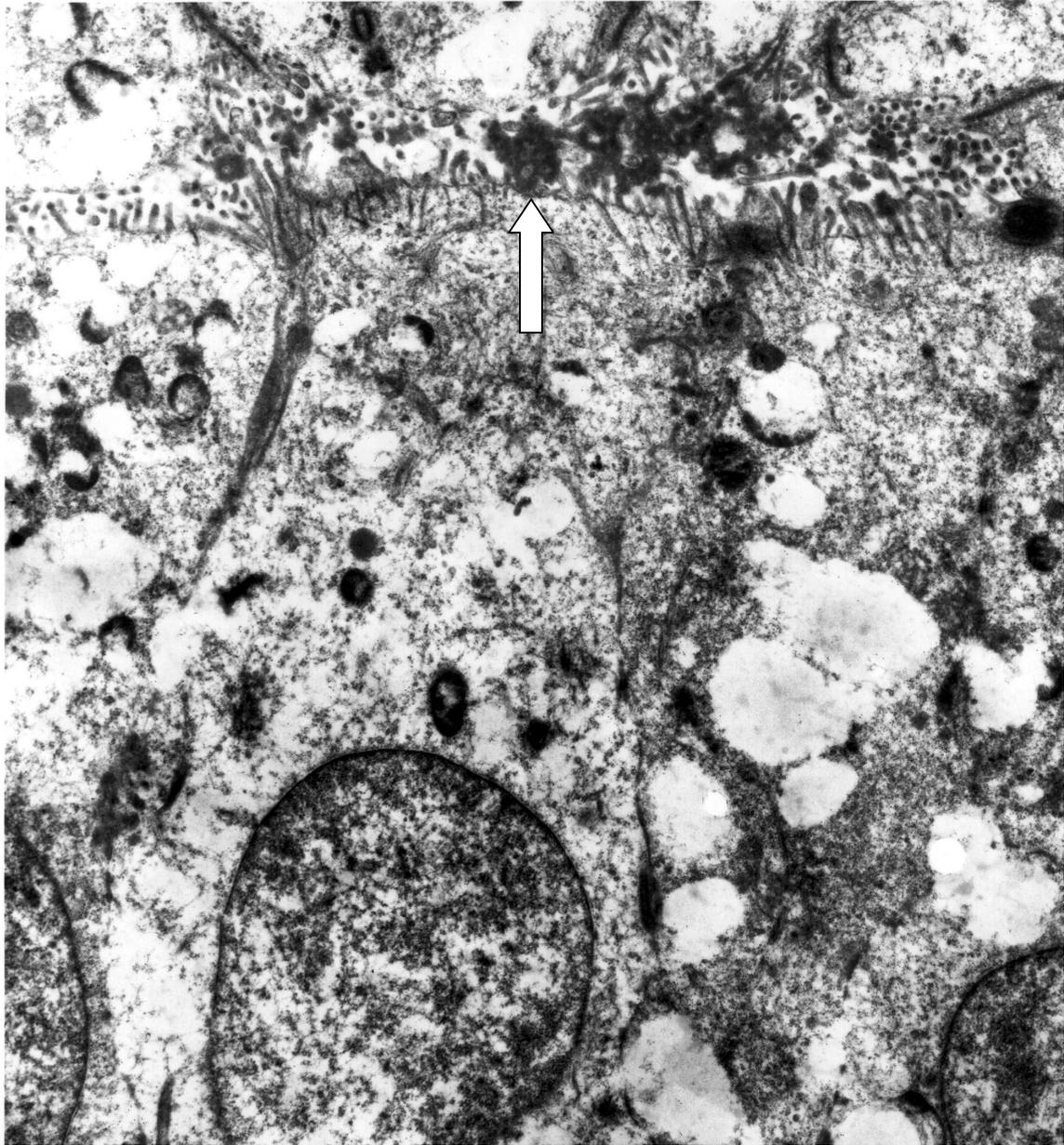


Figure 4.3 Transmission electron micrograph of intestinal epithelium of 19-day-old embryos inoculated with TCV.

At 24 hours post-inoculation, TCV is found in intestinal lumen (arrow).

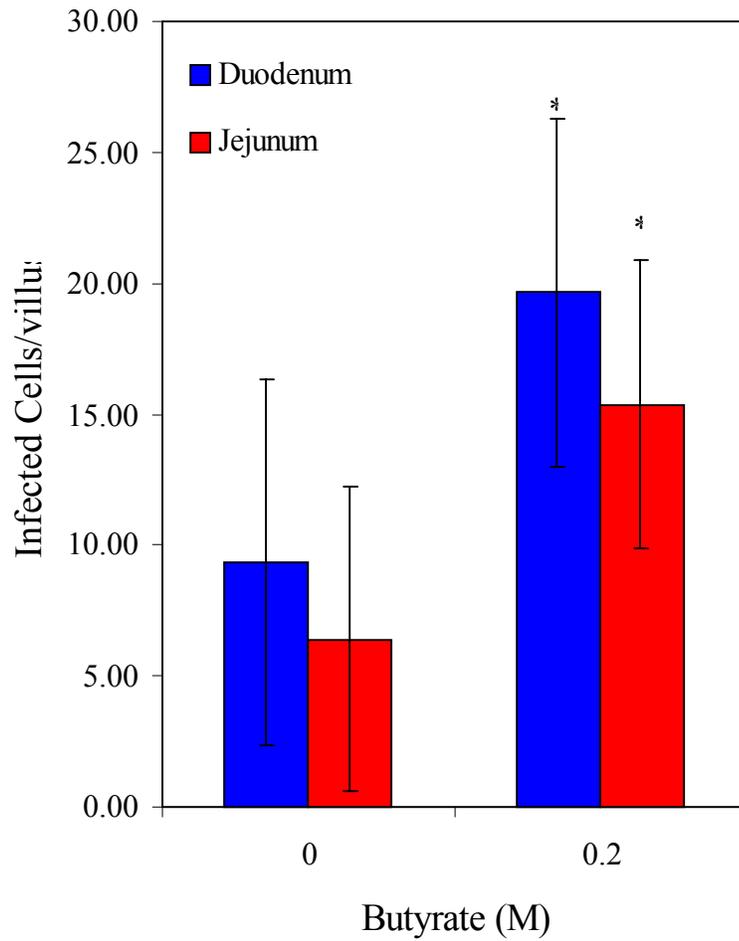


Figure 4.4 Effect of butyrate treatment on TCV infection in turkey embryos. Values are expressed as means \pm SEM. Significant difference compared to control is denoted by *.

CHAPTER 5.

Effect of Fermentable Diets on Enterocyte Maturation and Turkey Coronavirus Infection

5.1 Abstract

The effects of cellulose (C) and guar gum (G) fortified and basal (B) diets on intraluminal fermentation, enterocyte maturation, and turkey coronavirus (TCV) infection were investigated in turkey poults. Levels of the short-chain fatty acids (SCFA), acetate, propionate, butyrate, iso-butyrate, valerate, and iso-valerate in cecal contents were determined. Cellular differentiation and maturation of enterocytes in the small intestine were assessed by measuring the relative levels of villin expression as indicated by immunoblot analysis. TCV infection was evaluated in fixed paraffin-embedded intestinal tissue by indirect immunoperoxidase assay. Body weight, rate of gain, and feed conversion ratio (FCR) were determined to evaluate the effect of diet and TCV infection on performance. Production of acetate and butyrate was significantly higher in diet C compared to B. Acetate, butyrate, iso-butyrate, and iso-valerate were significantly higher in diet G compared to B. But only acetate, iso-butyrate, and iso-valerate were significantly higher in diet G compared to C. Villin expression was found to be significantly higher in diet G compared to C and B. The extent of TCV infection i.e., lesion score, was highest in diet B and lowest in G. There were no significant differences in body weight or rate of gain between birds fed diets B and C. However, TCV infection reduced their performance. The best FCR was obtained with diet C in both infected and un-infected groups. The poorest body weights, rates of gain, and FCR were seen with diet G, despite the fact that this group has the lowest lesion score. These data suggest that diets C and G promote luminal SCFA production, which in turn stimulates enterocyte differentiation and maturation. However, these same diets seem to reduce the level of TCV infection. This appears to be contrary to observations in embryos, but may reflect the additional effect of these diets on cellular turnover/replacement rate or other factors such as mucus production.

5.2 Introduction

Fermentable diets are those which contain fiber, an indigestible polysaccharide existing primarily in plants. Fiber can be classified as soluble and non-soluble based on its characteristic in water. Although fiber is nutritionally unavailable to humans and animals, deprivation seems to cause problems. Growth and function of intestinal epithelial cells is greatly influenced by the presence of fiber in the diet. Both chemical and physical effects have been postulated. Fiber when fermented by intestinal bacteria is broken down into short-chain fatty acid (SCFA). These by-products, especially butyrate influence the proliferation, differentiation, and maturation of intestinal epithelial cells (McIntyre et al., 1993; Perrin et al., 2001; Van Munster and Nagengast, 1993). Fiber may also reduce the exposure of intestinal epithelial cells to carcinogens by forming complexes or by shortening intestinal transit time (Kestell et al., 1999; Sjodin et al., 1992). Fiber may also enhance production of mucus in the gastro-intestinal tract (Lundin et al., 1993), which serves as a physical barrier to intestinal pathogens. It may also enhance gastro-intestinal epithelial cell turnover/replacement (Jacobs and White, 1983; Wilson and Gibson, 1997; Wilson and Gibson, 2000).

Our previous studies have suggested that butyrate enhances differentiation, maturation, and the susceptibility of enterocytes to TCV infection in turkey embryos. Knowing that butyrate is a by-product of fermentation, it was the objective of this study to determine whether diets containing fiber would have a similar effect when fed to turkey poults.

5.3 Materials and Methods

Experiment 1.

Animals. A randomized complete block design with subsampling was performed in three blocks, with three treatment groups per block. Each treatment group consisted of 25, one-day-old, female turkey poults obtained from a breeder flock known to be coronavirus-free. Upon arrival, poults were sorted by body weight and then randomized so as to create three treatment groups of equal weight distribution. They were brooded in wire batteries under constant light and provided feed and water *ad libitum*. Groups were randomly assigned the

following diets: basal (B), basal fortified with 5% cellulose (C), and basal fortified with 5% guar gum (G).

Feed formulation. A fiber-free, casein-cornstarch basal diet (table 5.1) containing 10% inert material was formulated to meet nutritional requirements as recommended by the National Research Council (1994). This diet was then used in the preparation of treatment diets by replacing the inert component i.e., sand, with cellulose (Solkafloc[®], Fiber Sales & Development Corporation, OH) or guar gum (Multi-Kem Corporation, NJ). To reduce a problem of low palatability, purified diets were then mixed 1 : 1 w/w with a commercial starter feed. Thus the final feeds contained either 5% sand, 5% cellulose, or 5% guar gum. They were then extruded under pressure without heat and pelleted.

SCFA analysis. After 8 days of treatments, 10 poults were randomly selected from each group and euthanized by chloroform inhalation. This method of euthanasia was chosen over cervical dislocation to prevent spastic evacuation of the ceca. Contents from both the left and right ceca were collected into 5 mL polystyrene tubes which were then frozen in ethanol/dry ice, and stored at -40 °C. Sample analysis entailed dilution of cecal contents with distilled water (1:10 w/v). As an internal standard, 2-methyl-butyrate was added to each sample. Oxalic acid was also added to promote conversion of all SCFA to their protonated form. Samples were centrifuged at 17,000 x g for 5 min at 5°C. Supernatants were collected and stored at 4-6°C. Gas chromatographic analysis was performed using a Hewlett Packard, HP 5890A gas chromatograph equipped with Supelco Tight Spec[®] Column (80/120 Carbopack B-Da/ 4% Carbowax 20 M) and flame ionization detector. The level of SCFA was measured in $\mu\text{mole/ gram-wet weight}$ of cecal contents and results reported as mean \pm SEM.

Villin expression. The duodeno-jejunum (from the ventriculo-duodenal junction to Meckel's diverticulum) and ileum (from Meckel's diverticulum to the ileocecal junction) were removed, opened longitudinally, washed with ice-cold PBS (pH 7.4), placed in 5 mL polystyrene tubes and frozen in ethanol/dry ice. Samples were then lyophilized, ground, and stored at -40 °C. SDS-PAGE and immunoblots were performed as previously described (chap. 3) and results reported as mean \pm SEM.

TCV inoculation and evaluation of infection. Remaining poults were orally inoculated with 5×10^3 EID₅₀ of TCV (Indiana isolate). Twenty-four hours post-inoculation, they were

ethanized by chloroform inhalation and two intestinal segments (1 cm) collected, one from the middle of the duodenal loop and the other from the region just anterior to Meckel's diverticulum. These samples were immediately fixed in 1% acetic acid in 95% ethanol. After 24 hours fixation, tissues were embedded in paraffin, sectioned (5 μ m), and mounted on charged slides (Fisher Scientific, PA). Slides were dried at 45 °C for 3-4 hours and stored at room temperature. As previously described (chap. 2), an indirect immunoperoxidase assay (IPA) was used to detect TCV antigen. Unlike the embryos used in previous studies, poult presented a logistical problem in that the number of complete villi per intestinal cross section was low. Therefore, instead of using the number of infected cells/ villus as an indication of infection severity, a classical lesion scoring system was developed (fig. 5.1). Sections having only an occasional infected cell were given a lesion score of 1. Those with infection involving less than 2/3 of a villus were given a score of 2. Those with most of the cells infected on the distal two-thirds of a villus and intact epithelium were given a score of 3. Those showing infection over the entire crypt-villus axis and separation of epithelial layer from lamina propria were given a score of 4.

Statistical analysis. Differences among means were analyzed by one-way ANOVA (SAS, Version 8.1, SAS Institute, Cary, NC). Post-ANOVA analysis was performed using Tukey's studentized range test for SCFA production and villin expression. Duncan's multiple range test was used for the analysis of lesion score. Significant differences were determined at $P < 0.05$.

Experiment 2.

Animals. A randomized complete block design with subsampling was performed in two blocks, with three treatment groups per block. Each treatment group consisted of 25, one-day-old, female turkey poult obtained from a breeder flock known to be coronavirus-free. Feed and water were provided *ad libitum*. Individual body weights were determined every other day for 8 days.

TCV inoculation and evaluation. On day 9, poult in each group were randomly assigned into two groups challenged orally with 5×10^3 EID₅₀ Indiana isolate TCV. Body weight was measured on day 0, 2 and 4 post-inoculation and reported as means \pm SEM. Feed

consumption was also measured during this time period to permit the calculation of feed conversion ratios (FCR).

Statistical analysis. Differences among means were analyzed by one-way ANOVA (SAS, Version 8.1, SAS Institute, Cary, NC) taking into account covariance of initial weights. Post-ANOVA analysis was performed using Tukey's studentized range test. Significant differences were justified at $P < 0.05$.

5.4 Results

The effect of dietary fiber on SCFA production is shown in figure 5.1. SCFA levels were highest in the cecal contents of poult fed diet G and lowest in poult fed B. Production of acetate and iso-butyrate was significantly higher in C compared to B. Acetate, butyrate, iso-butyrate, and iso-valerate were significantly higher in G compared to B. Acetate, iso-butyrate, and iso-valerate were significantly higher in G compared to C.

Figure 5.2 shows the effect of dietary fiber on villin expression in the duodeno-jejunum and ileum. Villin levels were found to be significantly higher in both regions of the small intestine in poult fed diet G when compared to those fed C or B. Poult fed diet C had levels that were higher than those fed B, but this difference was not statistically significant.

The effect of dietary fiber on TCV infection is shown in Table 5.2. Based on infection score, the greatest level of TCV infection was seen in poult fed diet B followed by those fed diet C. The lowest level of infection was seen in poult fed diet G.

Figure 5.3 shows the effect of dietary fiber on body weight over time. There were no significant differences in growth rates between poult fed diets B and C. However, from day 2 onward, poult fed diet G weighed significantly less than those fed C or B. In fact, growth rate was dramatically depressed and these birds experienced severe mucoid diarrhea, depression, emaciation, and a mortality of 5 to 30 % over the course of the study.

The effect of dietary fiber and TCV infection on body weight over time is shown in figure 5.4. Infection with TCV significantly reduced mean body weights in birds fed diets B and C from day 2 onward. Infection clearly reduced the rate of gain, but no effect due to diet was demonstrable. Birds receiving diet G weighed significantly less than those receiving B

or C regardless of infection. Likewise, the rate of gain for those on diet G was dramatically reduced.

Un-infected poults fed diets B, C, and G had FCRs of 1.31, 1.27, and 3.62 respectively. Infected poults fed these same diets had FCRs of 1.82, 1.77, and 15.56 respectively.

5.5 Discussion

In this study, it was demonstrated that the production of SCFA could be enhanced by the addition of fermentable feedstuffs i.e., fiber, to the diet. Two forms of fiber were used, soluble e.g., guar gum (5%) and non-soluble e.g., cellulose (5%). Guar gum, although it resulted in the highest level of SCFA production across the board, had negative side effects which included diarrhea, poor weight gain and FCR.

SCFA are primarily produced in the ceca and colon, it might be assumed therefore, that their effects would be limited to distal intestine. However, it is known that birds exhibit anti-peristaltic movement in their intestine (Dziuk and Duke, 1972) that could possibly extend the effect of luminal SCFA well into the proximal small intestine (Adams et al., 1970). Part of that effect, at least as observed in our studies, is to enhance the differentiation and maturation of absorptive enterocytes. Previous work with embryos indicates that this may correlate with increased susceptibility of enterocytes to TCV infection, presumably because the receptors necessary for TCV attachment and internalization are expressed in greater numbers as cells mature.

In poults fed fermentable (fiber-fortified) diets, the effect on TCV susceptibility does not appear to be the same; at least not at first glance. In contrast to embryos which were administered butyrate by direct injection, poults fed fiber-fortified diets showed less evidence of TCV infection at 24 hours post-inoculation. This was despite the fact that their intestinal epithelium seemed to contain plenty of susceptible, mature cells as indicated by villin expression. An explanation for this phenomenon may be that in embryos, enterocytes are undergoing maturation but cell turnover on the villus is limited because there is no contact with ingesta. In fact, it is possible that the process of cell turnover may not even begin until after hatch when the first food is ingested. Therefore, mature, susceptible enterocytes would

accumulate on the villus. The large number of infected cells seen in 23-day-old embryos (chap. 4) reflects this. In poult, cell turnover likely occurs at a continuous rate due to natural aging and contact with ingesta. The rapid exfoliation and replacement of these mature cells might eliminate the infection before virus can multiply to concentration necessary for detection by IPA. Therefore, the number of infected cells on the villus might be lower in poult. Several *in vivo* studies in other species have shown that butyrate facilitates the proliferation and healing of damaged gut (Boffa et al., 1992; Butzner et al., 1996; Jacobs and Lupton, 1984; Key et al., 1996; Sakata, 1987). It has also been shown to promote the migration of colonocytes (Wilson and Gibson, 1997). If the latter holds true in poult, then there is the potential that fiber-fortified diets may enhance cell turnover / replacement and potentially speed recovery following infection with TCV. In the current set of experiments poult were only followed for 4 days post-TCV infection. During that time, there did not appear to be much difference in performance between birds receiving the basal feed and those receiving the cellulose fortified feed. Additional studies may reveal that beyond 4 days, the beneficial effect of cellulose in the diet with regard to recovery from TCV infection becomes more pronounced.

The diet containing guar gum (soluble fiber) produced peculiar results. Maximal SCFA production was observed, but the overall performance of the birds was extremely poor. Cell differentiation and maturation were enhanced but TCV infection was low. As proposed above, it may have been that cell turnover was increased, therefore, TCV infected cells were rapidly replaced on the villus. Another possibility is that increased mucus secretion associated with this diet may have had a protective effect. This is corroborated by the observation that poult on the guar gum diet experienced a mucoid diarrhea. Increases in the number of goblet cells and mucus secretion have been noted in hamsters fed diets containing fiber (Lundin et al., 1993). Surface mucins are thought to inhibit receptor binding and infection of rotavirus (Chen et al., 1993).

In general, it may be concluded from this study that dietary fiber and its fermentative by-products play an important role in intestinal mucosal cytokinetics and as such may be manipulated to alter the course of TCV infection in poult. Dietary fiber appears to reduce lesion scores and improve performance post-infection. These effects appear to vary with the

type of fiber i.e., soluble versus insoluble. Additional study evaluating higher level of cellulose or lower level of guar gum may be valuable in fine tuning the effect of these purified diets. Evaluating diets that contain more common fiber sources such as oat or wheat bran which have higher soluble and insoluble components respectively, may be of practical value.

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Table 5.1 Composition of casein-cornstarch basal diet.

Ingredients	Amount (%)
Soy oil	1
Corn starch	52.35
Casein ¹	29
Arginine	1
DL-methionine	0.15
NaHCO ₃	0.1
Limestone	0.75
Ca ₂ PO ₄	3.6
Salt	0.4
K ₂ CO ₃	0.95
Choline chloride	0.4
Selenium premix (0.06%)	0.05
Vitamin premix ²	0.05
Trace mineral premix ³	0.2
Inert Substance ⁴	10

¹ Acid-precipitated casein containing 93 ± 1% protein (dry basis); American Casein Company, NJ, USA.

² Containing at the minimum per kilogram of premix: vitamin A, 4500000 IU; vitamin D₃, 2000000 IU; vitamin E, 18000 IU; niacin, 36000 mg; D-pantothenic acid, 8000 mg; riboflavin, 4000 mg; pyridoxine, 2000; thiamine, 1400 mg; folic acid, 1000 mg; menadione, 900 mg; biotin, 90 Mg; and vitamin B₁₂, 7 mg.

³ Containing at the minimum of 3.2% Fe, 9.6% Mn, 11.2% Zn, 1800 ppm Cu, and 850 ppm I.

⁴ Used in B diet: sand; used in C diet: Solka-Floc, purified cellulose, Fiber Sales & Development Corporation, OH, USA; used in G diet: Guar gums, Multi-Kem Corporation, NJ, USA.

Table 5.2 The effect of dietary fiber on TCV infection.

Diet	Lesion score	
	Duodenum	Jejunum
B-diet	2.20 ± 0.27 ^a	1.90 ± 0.16 ^a
C-diet	1.55 ± 0.17 ^b	1.40 ± 0.13 ^b
G-diet	1.30 ± 0.13 ^b	1.10 ± 0.10 ^b

Means in each column followed by different letter are significantly different (P < 0.05).

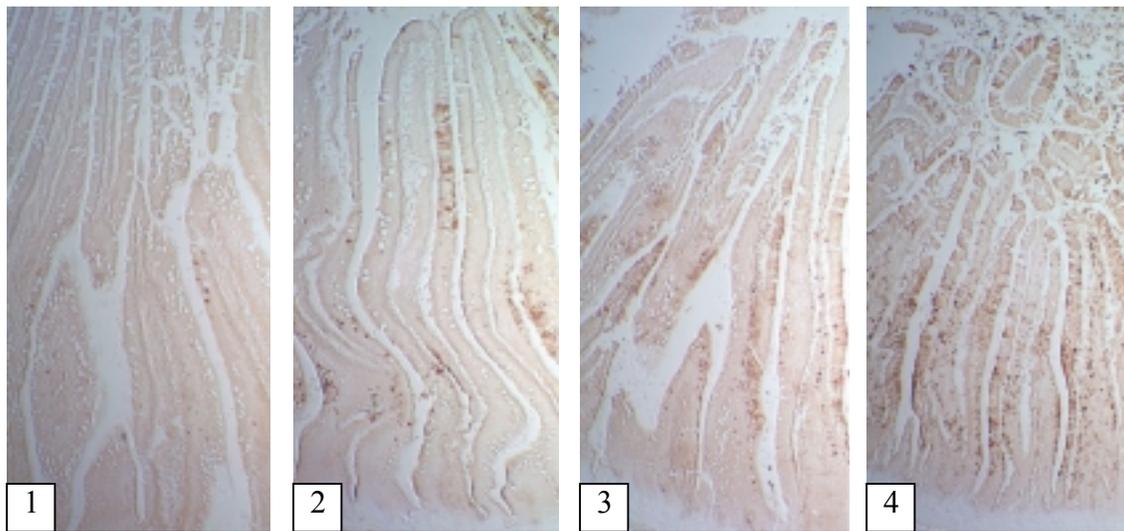


Figure 5.1 Lesion scoring criteria.

Lesion score 1: only an occasional infected cell. Lesion score 2: infection involving less than 2/3 of a villus. Lesion score 3: infection involving approximately two-thirds of a villus with intact epithelium. Lesion score 4: infection over the entire crypt-villus axis with separation of epithelial layer from lamina propria.

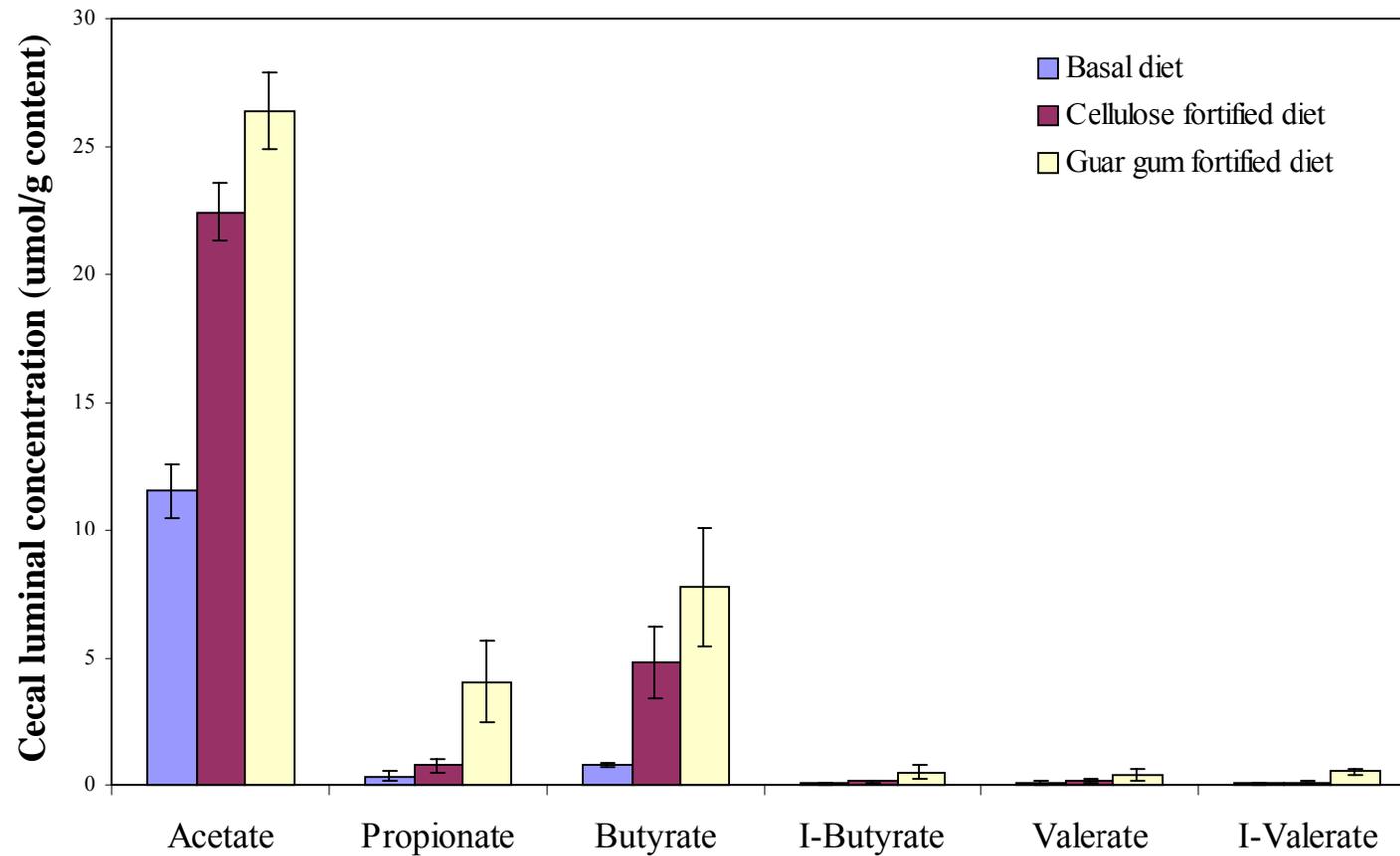


Figure 5.2 The effect of dietary fiber on short-chain fatty acid production.

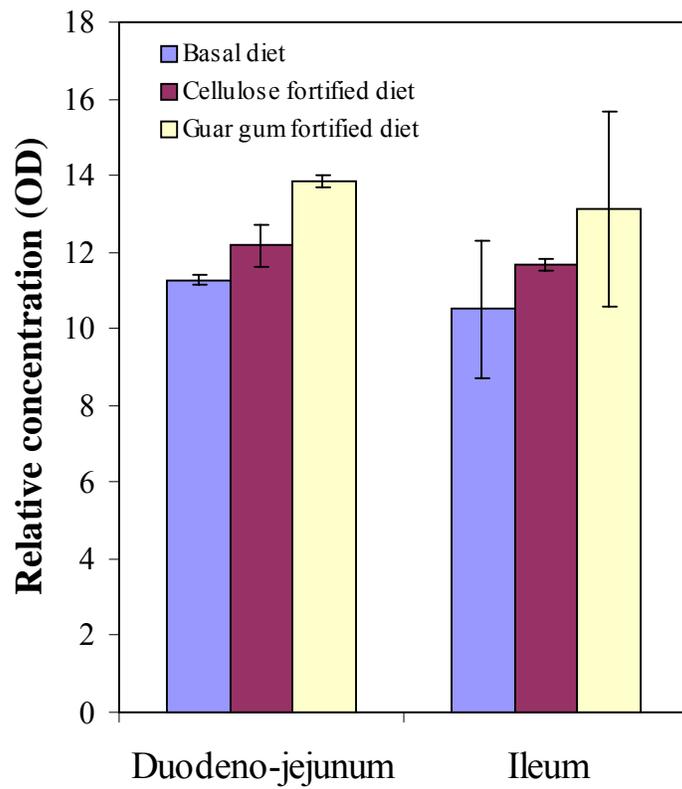


Figure 5.3 The effect of dietary fiber on villin expression in the duodeno-jejunum and ileum.

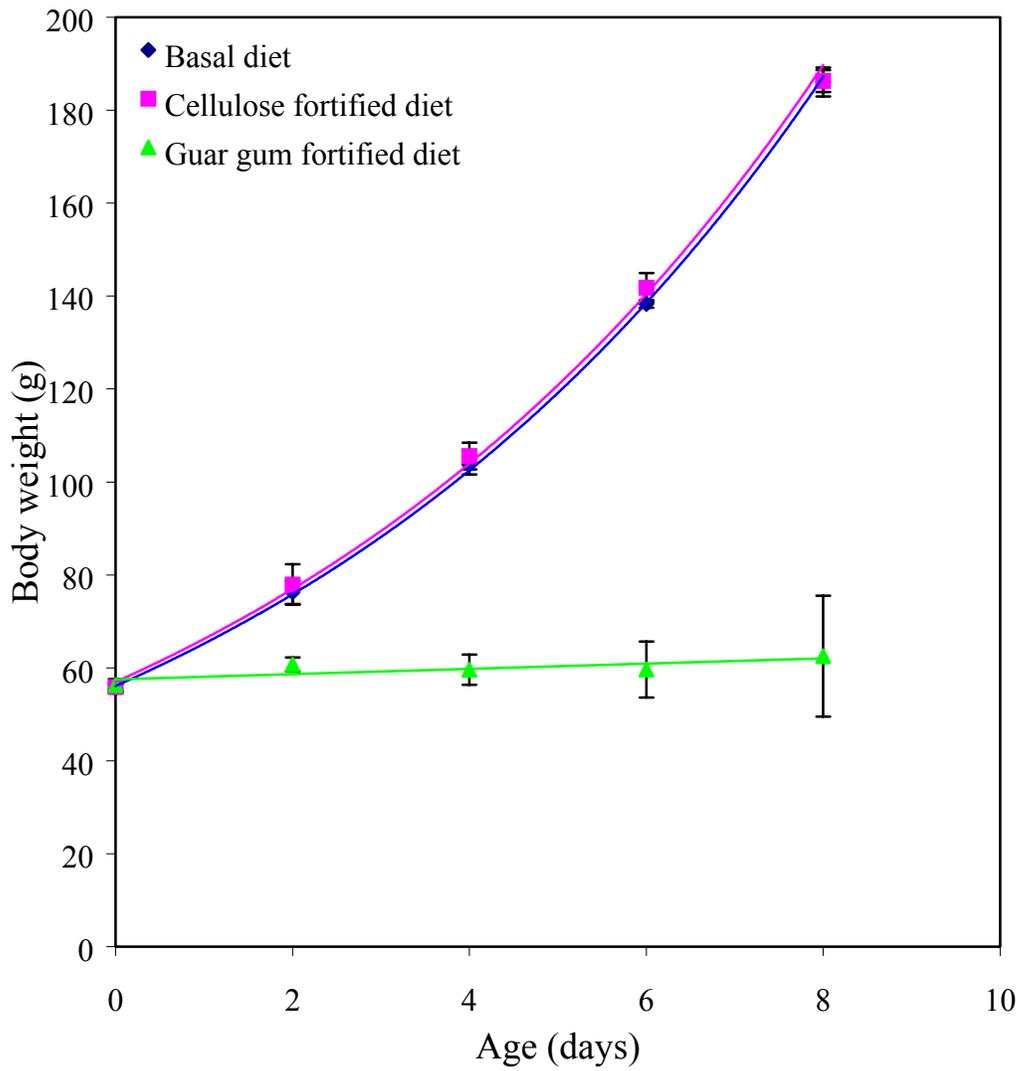


Figure 5.4 The effect of dietary fiber on body weight over time.

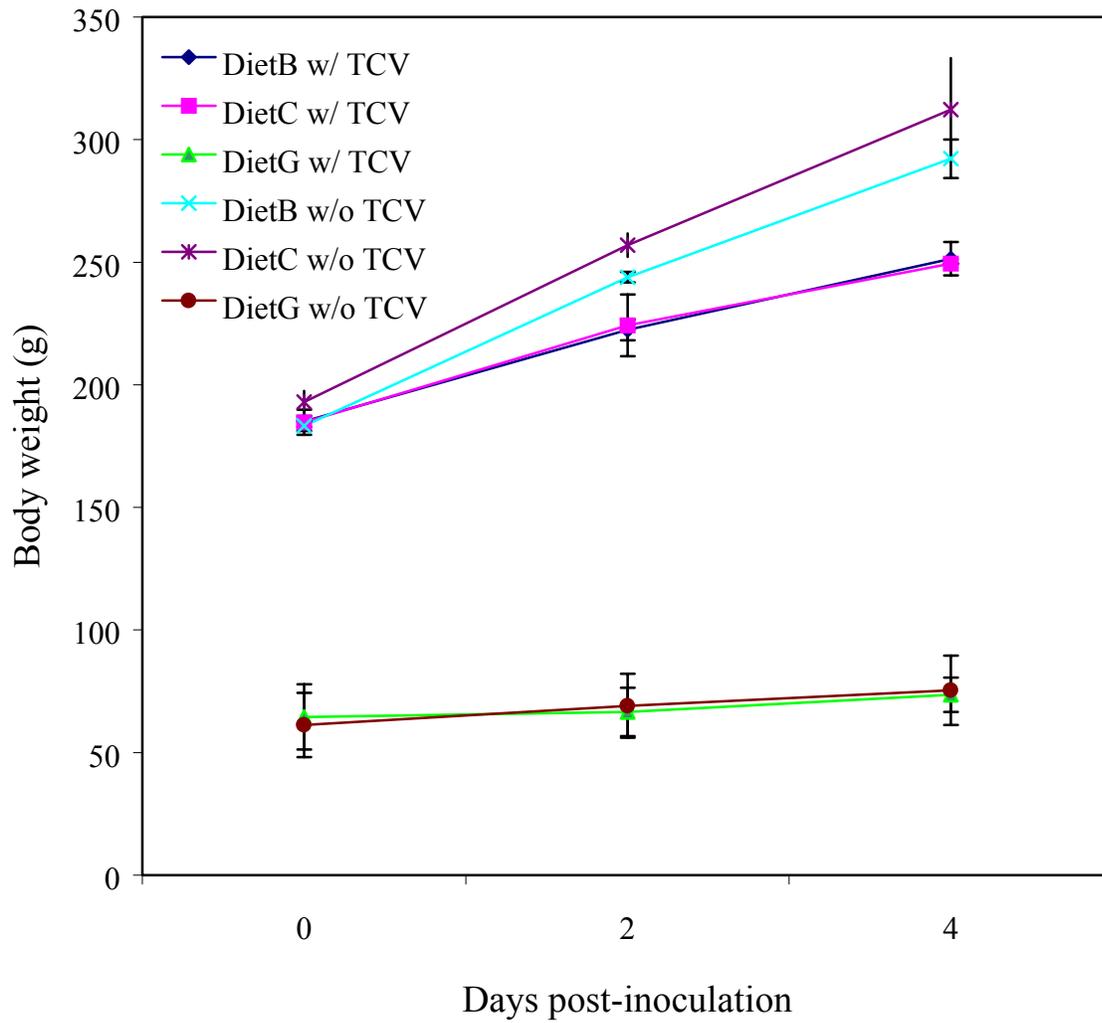


Figure 5.5 The effect of dietary fiber and TCV infection on body weight over time.

CHAPTER 6.

General Conclusions and Discussion

6.1 General Conclusions

The research presented in this dissertation primarily addressed the relationships between enterocyte maturity, susceptibility to TCV infection, and intra-luminal short-chain fatty acids (SCFA). Manipulation of SCFA was performed in both a closed embryo system by way of direct injection and in poults by way of dietary fiber supplementation. Based on developmental age, a positive correlation between villin expression and enterocyte differentiation and maturation was observed in embryos. Villin therefore appears to be a good marker for enterocyte maturity. Butyrate administered by intra-amniotic injection in 21-day-old turkey embryos was found to enhance enterocyte differentiation and maturation in a dose-dependent manner. A positive correlation between TCV infection and villin expression was found in embryos. Mature enterocytes appear to be more susceptible to infection while poorly differentiated epithelial cells are refractory. In keeping with these findings, butyrate was found to increase the severity of TCV infection in embryos.

It was found that dietary fiber increased SCFA levels in the ceca. As found in embryos, SCFA appear to enhance enterocyte maturity as indicated by villin expression but the overall severity of TCV infection was reduced. At first glance, this finding seems contradictory to what had been found in embryo studies previously described, but it is likely that increased proliferation and turnover rate in poults may account for the difference in the number of infected cells present on the villi. In general, birds fed the cellulose-fortified diet performed better than controls even in the face of TCV infection.

6.2 Proposed Mechanisms of SCFA- and Dietary Fiber-Mediated Enterocyte Maturation and TCV Infection

Based on our research and the findings reported by others, the following mechanisms are proposed regarding the roles of dietary fiber and SCFA in enterocyte differentiation, maturation, and TCV infection. Dietary fiber provides a substrate for bacterial fermentation,

primarily in the ceca. The fermentative by-products, SCFA, are flushed from the ceca and travel by retrograde peristaltic motion to the small intestine. SCFA stimulate proliferation, differentiation, and maturation of intestinal epithelial cells. As part of this process, several proteins, including an APN-like receptor protein are expressed on the apical (luminal) surface of the cells. TCV binds to these receptors, is internalized and begins to replicate. Infected cells degenerate and release virus back into the lumen potentially providing a source of virus for re-infection of susceptible enterocytes. Dietary fiber and SCFA continue to stimulate differentiation, maturation, and proliferation leading to rapid replacement of dead and dying cells. Depending on the rate of re-epithelialization, the course of TCV-induced enteritis may be shortened and normal morphological and physiological function more quickly restored.

In the embryo model, SCFA appears to promote differentiation and maturation of enterocytes. Presumably, this coincides with receptor protein expression and is the likely explanation for the observed increase in severity of TCV infection (see figure 6.1, right side). With respect to differentiation and maturation, the effect of SCFA resulting from ingestion of dietary fiber is likely to be the same in poult. However, poult consuming fiber-fortified diets exhibited less severe TCV lesions. This raises a question with regard to differences between the poult and embryo models.

We believe that a plausible answer can be found in the fact that the intestine of the poult is functionally active while that of the embryo is not. Embryos absorb nutrients from the yolk sac, whereas poult must obtain nutrients via ingested feed. To maintain the efficiency of feed digestion, a continuous turnover of intestinal epithelial cells is necessary in poult. This turnover is presumably stimulated by both the physical presence of feed (fiber) in the gut as well as chemical mediators such as SCFA. Therefore, in the course of TCV infection, dietary fiber and SCFA may facilitate not only enterocyte differentiation and maturation but also proliferation and replacement of infected mature cells. In essence the rate of replacement may exceed the rate of infection. Thus, the actual number of cells that contain sufficient virus for IPA detection may be decreased due to exfoliation and replacement even if the number of susceptible cells is increased (see figure 6.1, left side).

The proposed mechanism provides an explanation of the interplay between host, diet, and TCV. It is conceivable however, that similar relationships exist for other enteric viruses such as astroviruses, enteroviruses, and rotaviruses. Thus the embryo and poult models described in this research may be of value in the study of these agents and the numerous other factors that influence the development of clinical enteritis in young turkeys.

6.3 Applications

It seems apparent that dietary fiber and SCFA may be of value in the prevention and treatment of TCV induced enteritis in turkeys. Plausible applications would include: 1) the use of fiber-fortified rations as standard starter feeds in young poults, 2) the use of fiber-fortified rations as therapeutic feeds in the event of TCV or other viral enteritidies, 3) Top dressing standard feeds with high-fiber feedstuffs as a treatment for enteritis, 4) the use of probiotics i.e., bacterial competitive exclusion cultures, in combination with fiber-fortified rations or top-dressed, high-fiber feedstuffs, and 5) the use of stable SCFA as feed additives.

Poults are at greatest risk to infection with enteric pathogens in the first weeks of life. The use of a fiber-fortified starter feed would potentially be of benefit in several ways. It would improve overall gut health by providing a fermentable substrate which would promote SCFA production. This in turn would stimulate differentiation, maturation and continuous turnover of absorptive enterocytes. Dietary fiber would also stimulate mucin production which would provide some protection against invasion by gastrointestinal pathogens. And, should pathogens like TCV be encountered, fiber-fortified feed would essentially help to accelerate the process of infection and recovery by mechanisms already discussed, so that performance is minimally impacted.

Fiber-fortified feed may have therapeutic value if provided at the time enteritis is first detected. As previously indicated, it may help to limit the severity of TCV infection and speed up recovery of intestinal structure and function.

Top dressing with high-fiber feedstuffs like ground alfalfa, oat bran, or wheat bran may be beneficial in the face of enteric viral infections. Poults experiencing enteritis tend to eat litter, presumably because they are seeking a source of fiber. This often produces gizzard impactions and mortality. Providing an alternative to this in the form of a palatable top-

ingredient that is easily passed through the digestive tract would be valuable in itself. The added benefits with regard to SCFA production etc., would be those already described.

The use of probiotics in combination with dietary fiber may be of benefit in two ways. One would be that probiotics, by virtue of the specific bacterial populations they contain, could be used to selectively enhance production of preferred SCFA like butyrate. The other would be that dietary fiber may provide needed substrates that enable beneficial “probiotic” organisms to out-compete other, less desirable intestinal flora. This would in essence enhance their competitive exclusion properties.

The use of stable SCFA i.e., butyric ester, as a feed additive could be a direct means by which luminal butyrate levels could be manipulated. Since the effect of butyrate appears to be dose dependant, this may be a more precise way of controlling the concentration of butyrate and hence its effect on epithelial cytokinetics and TCV infection. It has yet to be determined whether these “stable” SCFA are able to withstand the feed pelleting process or whether they actually reach the mid and distal intestine before being absorbed systemically.

To date, the only truly effective means of preventing of TCV is good biosecurity and the only beneficial therapy appears to be antimicrobials like the fluoroquinolones which are used to treat secondary complications. Most of the areas where turkeys are raised have high concentrations of poultry. This makes biosecurity difficult to maintain. Most of the effective antimicrobials are diminishing in availability due to concern over the development of drug resistance in human foodborne pathogens. This leaves very few alternatives for managing enteritis in turkeys. Therefore, further investigation regarding the benefits of fiber-fortified diets as preventative and therapeutic agents should be pursued.

6.4 Further Research

As a continuation of the research presented in this dissertation, the following areas of investigation should be considered:

1. *Re-examine the effects of cellulose and guar-gum at concentrations other than those used in the current studies.* For guar-gum to be of any value, the concentration must be reduced. Higher levels of cellulose may be of greater

value. Determining the optimal level of performance on these diets would be important.

2. *The effects of dietary fiber (purified) on structural recovery of the intestinal epithelium following TCV infection.* This would involve varying the fiber component of the diet measuring luminal SCFA and radiolabeled uridine incorporation in proliferating enterocytes at selected intervals during the 3 weeks following infection.
3. *The effect of dietary fiber (purified) on functional recovery of the intestinal epithelium following TCV infection.* This would involve varying the fiber component of the diet and measuring luminal SCFA and brush border enzyme activity at selected intervals during the 3 weeks following infection.
4. *The effect of dietary fiber (purified) on mucin production and TCV infection.* This would involve varying the fiber component of the diet and counting the number of goblet cells stained with Periodic Acid Schiff (PAS) and alcian blue, pH2.5. A TCV challenge would be given after 1 week on treatment. The severity of TCV infection would be determined by IPA.
5. *The evaluation of various types of high-fiber feedstuffs on SCFA, enterocyte differentiation, maturation, proliferation, function and recovery following TCV infection.* This would involve the incorporation of feedstuffs like alfalfa meal, oat bran, and wheat bran in the diet combined with the structural and functional assays described above.
6. *The evaluation of various high-fiber feedstuffs as a treatment in field cases of TCV enteritis.* This would involve a case-controlled study where flocks experiencing enteritis on multi-unit farms could be given alfalfa meal, oat bran, and/or wheat bran free choice by top-dressing standard commercial feed. Performance could be evaluated by determining body weights and feed conversion over the three following the onset of enteritis.
7. *Identify the receptor proteins necessary for TCV infection of enterocytes.* Epithelial cell proteins could be separated by SDS-PAGE and virus overlay protein blot assays performed to identify receptor proteins. Monoclonal

antibodies could be prepared against the receptor protein fragments and used for native protein purification and characterization. Preliminary data has already been obtained that indicates the presence of a protein in crude intestinal extracts from turkey embryos that binds TCV.

8. *Study the relationship between the presence of receptor protein and TCV infectivity.* One approach would entail the collection of epithelium from various organs and determining the presence as well as the level of viral binding protein. Another approach would be to determine the presence and the level of viral binding proteins in various tissues from different species including those susceptible and those resistant to TCV infection.

Proposed Mechanisms

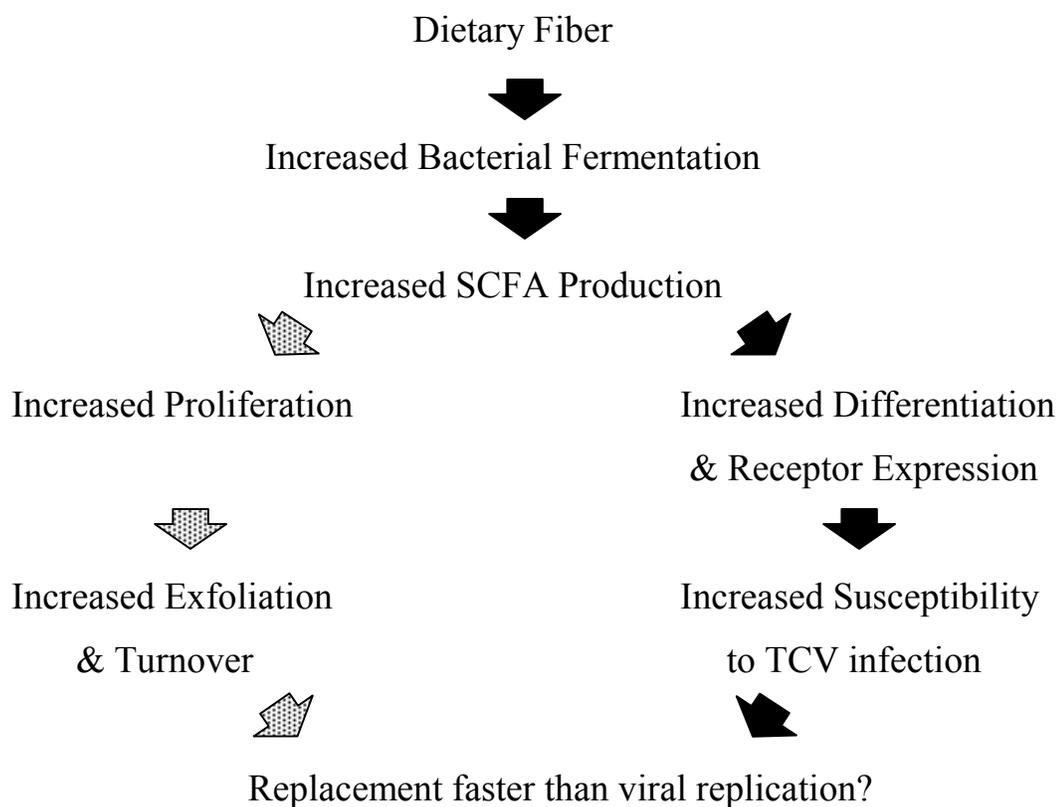


Figure 6.1 Proposed mechanisms for the effect of dietary fiber and SCFA on TCV infection in turkey poults.

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Abstracts:

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