EFFECTS OF *YERSINIA ENTEROCOLITICA* INFECTION ON THE DEVELOPMENT OF THE SMALL INTESTINE IN NEWBORN PIGLETS

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ABSTRACT

A model of bacterial gastroenteritis has been developed in which the effects of *Yersinia enterocolitica* infection on the structural and biochemical development of the small intestine have been examined in neonatal piglets both during the infection period (3 and 5 days postinfection) and during the subsequent recovery period after antibiotic therapy (at 14 days). The potential of oral bovine lactoferrin and another bovine milk protein for preventing or reducing the effects of *Y. enterocolitica* gastroenteritis have been evaluated in these piglets.

Newborn, colostrum-deprived piglets were inoculated orogastrically with a high dose (about 3 x 10^{10} colony forming units/ml) of *Y. enterocolitica* serotype 0:3, biotype 4. Diarrhoea began between 40 hours and 4 days after inoculation in 18 of the 19 animals and microabscesses, the typical lesions of Yersiniosis, were present in the mucosa of the small intestine in all infected piglets. At 5 days postinfection, microabscesses also were present in the liver of 7 of 8 piglets, and in the mucosa of the stomach in 2 animals. The mucosal damage and resulting malabsorption were reflected in the lower plasma glucose, Na^+ and Cl^- concentrations.

Yersinia enterocolitica infection reduced the body weight but not body length, but did not significantly affect the gastrointestinal tract length or weight or the growth of non-intestinal organs except the liver. There were markedly lower lactase and sucrase, but not maltase and Na^+-K^+-ATPase, activities in the small intestine. The mucosal protein and DNA contents and the ratio of RNA to DNA in the small intestine were not significantly different in infected animals. Rapid proliferation of crypt cells resulted in crypt enlargement in the entire small intestine, but reduced vacuolation of the epithelium of the distal small intestine.

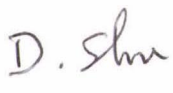
Following institution of effective antibiotic therapy, gastrointestinal lesions were absent. Compared with controls, the piglets gained body weight at the same rate, although remaining lighter in weight, and organ weights and concentrations of plasma Na^+ and Cl^-, but not glucose, were no different. Previously-infected piglets retained an altered profile of disaccharidase activity with a lower lactase activity, higher maltase and sucrase activities and early appearance of sucrase activity in the ileum. There were fewer vacuoles in the epithelium of the distal ileum.

A bovine milk fraction, but not bovine lactoferrin, appeared to reduce the severity of the infection due to *Y. enterocolitica*, there being shorter crypts, fewer proliferating crypt cells and higher lactase activity. The group means for the lesion number were also much lower although not significantly different. Oral supplementation with bovine lactoferrin in the milk formula did not have any beneficial effects in the infected piglets. In non-infected piglets, lactoferrin appeared to have trophic effects on the kidney and the small intestinal crypts, increased the lactase activity and caused an unexplained reduction in plasma glucose concentration and liver weight.

Yersinia enterocolitica enteritis in newborn, colostrum-deprived piglets accelerated the maturation of the epithelium of the small intestine, indicated by reduced enterocyte vacuolation and an altered disaccharidase profile.

STATEMENT

This is to certify that the work on which this thesis is based was carried out by the undersigned, and has not been accepted in whole or in part for any other degree or diploma. Assistance received is specifically recorded in the Acknowledgements section bound with this thesis.



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LIST OF ABBREVIATIONS

A/AG	acid/acid with gas
AB/PAS	Alcian blue/periodic acid-Schiff
ANOVA	Analysis of Variance
BMF	bovine milk fraction
BrDU	5-Bromo-2'-deoxy-uridine
BSA	bovine serum albumin
BSS	biocil surgical scrub
CFU	colony forming units
CIN	cefsulodin-irgasan-novobiocin
CRL	crown rump length
C_{RNA}	RNA concentration
DAB	3,3 diaminobenzidine tetrahydrochloride
DF	degree of freedom
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetate
EGF	epidermal growth factor
FITC-D	fluorescein-isothiocyanate-labelled dextran
HE	haematoxylin and eosin
Ig	immunoglobulin(s)
K/A	alkali/acid
Lf	lactoferrin
LIA	lysine iron agar
M	molar
mM	millimolar
mmol	millimole
mOsm	milliosmole
MS	mean square
N	normal
Na^+-K^+-ATPase	sodium-potassium adenosine triphosphatase
NRL	nose rump length
NS	not significant
OD	optical density
PBS	phosphate buffered saline
P_i	inorganic phosphate
rpm	revolutions per minute
SE	standard error of the mean
TGE	transmissible gastroenteritis
TPN	total parenteral nutrition
TSB	tryptone soy broth
TSI	triple sugar iron
TW	tryptone water
U	unit
µmol	micromole
V. cholera	*Vibrio cholera*
VP	Voges-Proskauer
Y. enterocolitica	*Yersinia enterocolitica*

INTRODUCTION

Neonatal life is a time of rapid development of the gastrointestinal tract and one of vulnerability to infectious diseases resulting in diarrhoea. Diarrhoea is more dangerous in the young than in the adult because the young lose more body fluid relative to body weight than the adult during the diarrhoea (Black *et al.*, 1984) and also because the young need a longer time to rebuild the villi after damage by enteropathogens (Moon, 1978). Diarrhoeal diseases are the leading cause of childhood death, and in some parts of the world they are responsible for more years of potential life lost than all other causes combined (Guerrant and McAuliffe, 1986). It has been estimated that each year 4.6 to 6 million children (more than 12,600 a day) die from diarrhoeal illness in Asia, Africa, and Latin America (Snyder and Merson 1982; Warren 1990; Guerrant *et al.*, 1990). Infectious diarrhoea not only causes childhood death but also growth retardation (Martorell *et al.*, 1975; Rowland *et al.*, 1977). The impact of enteritis in the newborn on gut development and maturation, however, is poorly documented, nor are the long term effects after clinical recovery well known.

It is important that the effects of infectious enteritis in the neonate on the growth of the internal organs, particularly the small intestine, and on the development of the small intestinal enzymes are evaluated. Further, more information is needed to determine whether organ growth and development and digestive functions are impaired after recovery from the infection. In the present study, the pig was chosen for the animal model because of the similarities of its intestinal anatomy, physiology and nutritional requirements to those of humans (Pond and Houpt, 1978). Neonatal pigs have been successfully used to study the effects of rotavirus and TGE virus on gastrointestinal structure and function (Kelly *et al.*, 1972; Davidson *et al.*, 1977; Kerzner *et al.* 1977; Shepherd *et al.*, 1979; Graham *et al.*, 1984). *Yersinia enterocolitica* was chosen as the pathogen because in humans *Y. enterocolitica* infection causes diarrhoea but is rarely fatal (Mair and Fox, 1986), in contrast to other common enteropathogens e.g. *Escherichia coli*, rotavirus and TGE virus. Robins-Browne *et al.* (1985) and Schiemann (1988) have demonstrated that *Y. enterocolitica* infection caused diarrhoea in caesarean-delivered piglets which were deprived of colostrum. This suggests that a model of bacterial gastroenteritis in naturally-delivered, colostrum-deprived piglets could be used

for the study of the development of the neonate at different times during a period of diarrhoea.

Breast feeding confers protection from intestinal infections on the human infant. Components of colostrum and mature milk - specific antibodies, leucocytes, lactoferrin and lysozyme - are known to have antibacterial properties. *In vitro* studies demonstrated that colostrum and mature milk prevented enterobacterial growth (Nagy, 1975; Brock *et al.*, 1983). Although bacterial gastroenteritis can be successfully treated with antibiotics, it would be better if the disease could be prevented, especially in the very young. Some components of bovine milk would appear to be potential antibacterial agents which could easily be administrated by oral supplementation to the neonate to increase its ability to resist gastroenteritis.

The experiments reported here had four main aims. The first was to establish that vaginally-delivered, colostrum-deprived piglets could be used for a repeatable animal model of *Y. enterocolitica* enteritis. The second aim was to investigate the effects of *Y. enterocolitica* infection on gut development during neonatal life. The third was to determine whether or not the development of the piglets was compromised in comparison with control animals after they had recovered from severe diarrhoea. The fourth was to assess the effects of bovine lactoferrin and another bovine milk protein on gut development in neonatal piglets infected with *Y. enterocolitica.*

Chapter 1

GENERAL LITERATURE REVIEW

The gastrointestinal tract develops very early in the foetal life of mammals. Most of its structures are present before birth and continue to mature during early postnatal life. Development of the small intestine follows a proximal-distal pattern in both the foetus and the neonate. Villi, microvilli, crypts, intestinal glands and other structures first appear and subsequently show maturational changes in the duodenum and their development proceeds distally. Enzymes are first present in the small intestine in different mammalian species at different times during development e.g. all disaccharidases are present before birth in humans but in pigs, rats, and lambs, only lactase and cellobiase are well-developed at birth, and trehalase, sucrase, maltase and isomaltase develop during neonatal life.

Being in continuity with the external environment, the gastrointestinal tract is exposed to food and may also come in contact with enteropathogens, which may alter its digestive and absorptive processes. Enterotoxin from some enteropathogens e.g. *Salmonella* or *Escherichia coli* stimulate secretion of Na^+ or Cl^- or inhibit electrolyte absorption by the mucosa of the small intestine. Invasion by pathogens destroys intestinal epithelial cells on the villi which desquamate into the intestinal lumen, resulting in the loss of membrane-bound enzymes associated with the microvilli, such as lactase, sucrase and maltase. Decreased activities of these enzymes may cause maldigestion and malabsorption resulting in diarrhoea.

In this Chapter, the development of the structures and enzymes of the small intestine, both before birth and during the neonatal period, the pathophysiology of enteritis, especially yersiniosis, and the role of milk and colostrum in the newborn will be reviewed.

1.1 MORPHOLOGICAL DEVELOPMENT OF THE GASTROINTESTINAL TRACT

The gastrointestinal tract develops embryologically from a simple gut tube formed by

the folding of a single layer of endoderm and an associated outer mesenchymal component. The endoderm gives rise to the epithelial lining of glandular epithelium while the muscle, blood vessels, connective tissue and serosa are derived from the mesoderm. The gut tube consists of three parts: the foregut, from which the stomach, pancreas and descending duodenum develop; the midgut, from which the rest of the small intestine develops and the hind gut which gives rise to the large intestine (Arey, 1974).

1.1.1 Development of the Small Intestine in the Foetus

The primitive gut is at first a fairly straight tube extending throughout the length of the body (Patten, 1959). The intestine rapidly elongates, at a greater rate than the embryo as a whole, and begins to form a loop which undergoes torsion, followed by protrusion into the umbilical cord, then re-entry into the abdominal cavity when this enlarges (Arey, 1974). During its formation, the small intestine is a simple tube lined by a single layer of epithelial cells surrounded by mesenchyme but, after rapid elongation, the intestinal tube thickens and the epithelium becomes stratified (Bryden *et al.*, 1972; Grand *et al.*, 1976).

The earliest indication of mucosa is the appearance of subepithelial aggregates of mesenchymal cells which cause projections into the central lumen of the overlying stratified epithelium of two to six layers in thickness (Johnson, 1910; Grand *et al.*, 1976; Moxey and Trier, 1978). The stratified cells are characterised by large nuclei with prominent nucleoli (Moxey and Trier, 1978). The stratified epithelium is separated from the surrounding mesenchymal cells by a continuous basal lamina (Grand *et al.*, 1976). Smooth muscle cells and blood vessels appear within these mesenchymal invaginations as their development progresses.

1.1.1.1 Villus formation

In the small intestine, the rudimentary villi begin as a stratified epithelium with a central core of mesenchyme (Toofanian, 1976). The degree of stratification of the epithelial lining of these protrusions then gradually decreases to a simple layer of columnar epithelium which initially lines only the apex of developing folds or villi, but as the villi mature the columnar epithelium appears along the sides of the villus (Moxey and Trier, 1978). The columnar epithelium rapidly proliferates. Once villi have formed in the

duodenum, the lumen soon becomes completely filled by columnar epithelium. Similarly, rapid proliferation occurs in a caudal direction (Johnson, 1910; Grant *et al.*, 1976; Moxey and Trier, 1978). Later, only the epithelium between villi remains stratified, and the number and length of the villi increase (Moxey and Trier, 1978). Subsequently, while villus maturation and lengthening occur proximally, villi begin to appear in more distal parts of the small intestine (Grand *et al.*, 1976).

1.1.1.2 Crypts

The crypts always form later than the villi (Johnson, 1910; Grand *et al.*, 1976; Moxey and Trier, 1978). The crypt is a progenitive zone (Trahair and Robinson, 1986). Cells divide in the crypts and migrate up the villi to be shed from the tip (Eastwood, 1977). Crypt enterocytes differ from villus enterocytes not only in their structure but also in their function. In the crypt, the epithelium is composed of poorly differentiated cells rich in thymidine kinase activity. As these cells migrate to the villus tip, many proteins are synthesized, e.g. disaccharidases and Na^+-K^+-ATPase (Gall *et al.*, 1977). The proliferation of epithelial cells in the small intestine is usually limited to the crypts in the foetus, neonate and adult, except in foetal rats, in which studies with thymidine-H^3 have shown that proliferation of the intestinal epithelium is not limited to the crypts or extreme base of the villi, but occurs along the entire length of the villi in the duodenum as late as one day before birth (Hermos *et al.*, 1971).

1.1.1.3 Epithelial vacuolation

Soon after villus formation, vacuoles first occur in the cytoplasm of some villus absorptive cells and are subsequently very evident in the small intestine in foetal humans (Johnson, 1910; Moxey and Trier, 1979), sheep (Trahair and Robinson, 1986) and pigs (Hardy *et al.*, 1971). Vacuolated cells become fewer in later gestation in human foetuses (Johnson, 1910; Moxey and Trier, 1979). In pigs, in which the gestation period is about 114 days, vacuolated cells are very extensive in the small intestine of foetuses between 93 and 101 days (Hardy *et al.*, 1971). Vacuolated cells remained in the distal ileum until 4 weeks after birth in pigs (described in 1.1.2.1). The vacuoles usually occur in the apical cytoplasm and progressively displace the nucleus towards the base of the cell. Occasionally, a vacuole is lateral to the nucleus, or there are two vacuoles, one above and the other below the nucleus. The vacuoles which lie below the nucleus are usually more irregular in shape and often smaller (Hardy *et al.*, 1971). These vacuolated

cells remain in the distal small intestine in the lamb until 48 hours after birth, but disappear by 6 days after birth (Trahair and Robinson, 1983).

1.1.1.4 Goblet cells

Undifferentiated and differentiated goblet cells are present in both the stratified and simple columnar epithelium during development of the small intestine (Moxey and Trier, 1978). In the stratified epithelium of the foetal small intestine, there are goblet cells ranging from undifferentiated cells containing only a few secretory granules, to cells containing some granules which generally are not sufficiently abundant to distend these cells into a typical "goblet" shape, to differentiated goblet cells which are indistinguishable from those found on the villi of the normal adult small intestine (Moxey and Trier, 1978). The nucleus of goblet cells is located in the basal portion of the cell and the supranuclear region is filled with many large secretory granules. Goblet cells seem to be more prevalent in the proximal than in the distal small intestine in early gestation but this apparent difference decreases with increasing gestational age. The number of goblet cells in the small intestine increases with advancing gestational age (Moxey and Trier, 1978).

1.1.2 Development of the Small Intestine in the Neonate

The degree of maturation of the gastrointestinal tract at birth varies from species to species. On this basis, mammals can be classified into two groups: altrical species in which the small intestine is immature at birth and must undergo postnatal maturation, and precocious species in which the young are comparatively mature at birth.

In altrical species, e.g. the rat, mouse and rabbit, there is a postnatal period of maturation up to the time of spontaneous weaning, about 3 weeks in the rat. At birth, the small intestine of the newborn rat is immature and must undergo a 3 week period of postnatal maturation. Neither cells resembling adult absorptive cells nor well-defined crypts are present until birth. At birth, only a few immature crypts are present in the duodenum. The first crypts are identified in the ileum 4 days after birth (Calvert and Pothier, 1990). Crypt depth steadily increases until 15 days in both the proximal and the distal regions (Trahair, 1989). The epithelial cells do not become vacuolated until 6 hours after birth. Epithelial cells grow taller and thinner after 4 days (Dunn, 1967). Epithelial growth results in increased mucosal thickness. In the proximal small intestine,

the mucosal thickness increases steadily to day 10-12 and then reaches a plateau. In distal regions, the mucosal thickness begins to decline at about day 17, declining to about 25% of the maximum thickness (at 13-14 days) by day 20 (Trahair, 1989; Calvert and Pothier, 1990).

In contrast to the newborn rat, in species in which the young are comparatively mature and independent at birth (precocious), the gastrointestinal tract is well prepared for enteral nutrition having undergone the major phases of its development *in utero*. The horse and human are such species and their young exhibit little postnatal maturation of the gastrointestinal tract during the early neonatal period (Weaver, 1986). The intestine in newborn piglets and lambs is not as mature as that in human infants. The villi in the distal small intestine are taller than those in the proximal region in newborn lambs (Trahair and Robinson, 1986). In piglets, immature epithelial cells at birth have a strikingly different morphology and function from those in the adult.

1.1.2.1 Maturation of the mucosa of the small intestine in neonatal piglets

At birth, the characteristics of the epithelial cells of the jejunum and ileum of piglets are poor development of the glycocalyx over the microvilli, apical tubules, the apical position of the nucleus and the subnuclear location of the Golgi apparatus. Before the animals have been suckled, vacuoles are absent in the duodenum and jejunum whereas in the ileum, the absorptive cells contain empty vacuoles of moderate size and have variable nuclear positions (Staley *et al.*, 1969; Clarke and Hardy, 1971; Hardy *et al.*, 1971; Moon, 1972). Epithelial cells absorb intact colostral protein and macromolecules and become vacuolated cells during the neonatal period. The colostral protein is absorbed into the epithelial cell by pinocytosis through invaginations (Clark, 1959), then into apical tubules to form apical vacuoles. The vacuoles never occur in the epithelium of the crypts in any part of the small intestine (Moon, 1972). The epithelial cells assume the mature non-vacuolated appearance at different times in different regions of the small intestine.

The epithelial cells of the duodenum and jejunum change dramatically from 1 to 36 hours following the onset of suckling (Staley *et al.*, 1969; Clarke and Hardy, 1971). The apical tubules are most numerous in the cells on the upper part of the villus and there are fewer tubules in the cells adjacent to the crypts. The most striking change is the presence of colostric vacuoles. Only 1 hour after piglets are first suckled, some cells

on the upper two-thirds of the villus contain vacuoles. The small vacuoles are distributed throughout the cytoplasm while the large ones are located in the apical end of the cell. Most epithelial cells contain colostric vacuoles by 6 hours (Staley *et al.*, 1969). Numerous vacuoles are present both in the apical and in the basal cytoplasm and all villus cells have apical nuclei (Sibalin and Björkman, 1966). Vacuoles are prominent in the absorptive cells lining the upper two-thirds of the villus. Generally, they are smaller when in the supranuclear position and larger below the nucleus. On the upper part of the villus, the vacuoles are full of acidophilic protein, but the vacuoles on the lower part of the villus appear to be empty.

Villus cells containing colostric vacuoles become fewer in the duodenum at 36 hours of age in suckled piglets. At this time, the apical tubules are still beneath the terminal web region but the colostric vacuoles are small and very few in number. The Golgi apparatus has shifted slightly from a subnuclear position toward the apical surface. At 48 hours, colostric vacuoles are scarce. The hirsute layer is well developed and is especially prominent on the tips of the microvilli. The nuclei are located in the base of the cell and the Golgi apparatus is supranuclear. At this time, the epithelial cells of the piglet duodenum cannot be distinguished from those on mature epithelium (Clarke and Hardy, 1971) and the ability to absorb macromolecular protein has been lost by the duodenal absorptive cells. In the jejunum, the number of cells with vacuoles becomes progressively reduced from 3 days (Clarke and Hardy, 1971). The nuclei are in the base of the epithelial cells at 4-5 days. At 10 and 11 days of age, vacuoles are no longer present in the jejunal epithelium (Clarke and Hardy, 1971; Moon, 1972). If newborn piglets are prevented from sucking, no vacuoles are observed in the epithelial cells of the jejunum at 77 hours of age (Clarke and Hardy, 1971).

In the ileum, particularly the terminal ileum, the features of the epithelial cells at birth are different from those in the duodenum and jejunum. The terminal ileum of unsuckled piglets (3- or 23-hour-old) contains villi bearing variable numbers of cells with empty vacuoles of moderate size and with variable nuclear positions (Clarke and Hardy, 1971; Hardy *et al.*, 1971; Moon, 1972). The nuclei are basal within 24 hours after suckling (Xu *et al.*, 1992a). The density of vacuolation does not decrease until 14 days, after which time the vacuolated cells tend to be restricted to the upper part of the villus and they disappear by 21 days (Clarke and Hardy, 1971; Moon, 1972).

The amount of lymphoid tissue in the terminal ileum is variable and increases with age.

In 3- or 4-day-old, suckled piglets, there is little lymphoid tissue underlying the mucosa and there is little difference in the degree of vacuolation of those villi which overly and those which do not overly lymphoid tissue. Beyond this age, as lymphoid tissue increases both in its depth and in the proportion of the intestinal circumference involved, vacuolation becomes less extensive or absent on villi overlying lymphoid tissue. Neighbouring villi which do not overly lymphoid tissue are still heavily vacuolated (Clarke and Hardy, 1971; Moon, 1972).

Closure, or cessation of absorption, is the time required for immature neonatal intestinal epithelium to mature to a point where it can no longer absorb macromolecules (Lecce, 1966). Closure occurs at 36-48 hours after birth in naturally suckled pigs (Brambell, 1958; Broughton and Lecce, 1970). The intestinal transmission of macromolecules in the preclosure piglet is governed by the amount of protein and proteinase inhibitors in the intestine (Weström *et al.*, 1985). These authors demonstrated that feeding colostrum (bovine and porcine) markedly enhanced the transmission of both bovine serum albumin (BSA) and fluorescein-isothiocyanate labelled dextran 70,000 (FITC-D). Furthermore, increasing amounts (50-200 mg/ml) of ingested BSA and bovine immunoglobulin G (IgG), significantly increased the transfer of the labelled protein FITC-D compared with 10 mg/ml of ingested BSA or IgG. Proteinase inhibitors obtained from sow colostrum or soy bean also enhanced the transmission of both BSA and FITC-D. Closure is delayed if newborn piglets are prevented from sucking. When piglets were prevented from sucking for 70 hours (Lecce and Morgan, 1962) or 106 hours (Payne and Marsh, 1962) after birth, antibody absorption still occurred during this time.

1.1.2.2 Growth of the small intestine

There is a rapid increase in the weight of the small intestine in suckled piglets (Widdowson *et al.*, 1976; Xu *et al.*, 1992a) and puppies (Heird *et al.*, 1984) during the first 24 hours after birth, the entire small intestine growing more rapidly than the body as a whole (Widdowson *et al.*, 1976). The gain in weight in the small intestine during the first day was 78% compared with an 8% gain in body weight during the same period (Xu *et al.*, 1992a). The duodenum increased 42% in weight during the first 24 hours, while the jejunum and ileum grew even more rapidly, by 70-77%, in suckled piglets (Widdowson *et al.*, 1976; Xu *et al.*, 1992a) and suckled puppies (Heird *et al.*, 1984). The small intestine increased 30% in weight in suckled rabbits (Hall and Widdowson, 1979). After 24 hours, the weight increase of the small intestine in suckled piglets

slowed to 17% for each day from day 2 to day 10 (Widdowson *et al.*, 1976). The fastest growth was in the jejunum, particularly the jejunal mucosa, which doubled in weight during the first 24 hours (Widdowson *et al.*, 1976; Xu *et al.*, 1992a), but changed little over the next 2 days (Xu *et al.*, 1992a) and 9 days (Widdowson *et al.*, 1976), whereas the muscular layers gained less weight per day during the first 24 hours than they did over the next 2 or 9 days. Associated with the tissue weight gain there was a 23-24% increase in small intestinal length during the first day (Widdowson *et al.*, 1976; Xu *et al.*, 1992a). In the following 2 days, the increase in the length of the small intestine each day was 2% of that measured at 24 hours (Xu *et al.*, 1992a), but increased by 6% each day from days 2 to 10 (Widdowson *et al.*, 1976).

The small intestine increases not only in weight and length but also in the overall diameter and in the number and length of the villi. The diameter of the small intestine increased by 15% during the first 24 hours (Xu *et al.*, 1992a) and by 30% by day 10 (Smith and Jarvis, 1978). The absorptive area doubled by 10 days (Smith and Jarvis, 1978). Most of the villi in the intestine of the newborn pig appear to be similar in length although there are a small number of shorter ones present. Those from 9-day-old animals appeared to be divided into two populations, one of very long and the other of very short villi. The long villi were about twice the length of those from the newborn intestine. It has been suggested that the short villi had formed during postnatal development whereas the long villi were elongations of villi which were present at birth (Smith and Jarvis, 1978).

The cellular population in the small intestinal mucosa, indicated both by the number of cells from the base of the crypt to the tip of the villus and by the mucosal DNA content, increased progressively with age during the first 3 and 6 days (Widdowson *et al.*, 1976; Smith and Jarvis, 1978; Xu *et al.*, 1992a). The DNA content increased 84-154% by 3 days after birth (Xu *et al.*, 1992a). In suckled piglets, the total protein content of the small intestinal mucosa increased dramatically during the first 24 hours (Widdowson *et al.*, 1976; Xu *et al.*, 1992a) then decreased during the next 3 days (Xu *et al.*, 1992a) or 7 days, but became stable by 2 weeks (Aumaitre and Corring, 1978). It has been demonstrated that feeding of colostrum stimulated intestinal protein synthesis more actively than milk feeding in newborn lambs (Patureau Mirand *et al.*, 1990) and piglets (Burrin *et al.*, 1992). The transient increase in mucosal protein might have been due partly to the retention of unchanged colostral proteins in the enterocytes of the villi during the first 24-36 hours (Widdowson *et al.*, 1976; Xu *et al.*, 1992a). However, there

may be species differences, because newborn rabbits, which do not absorb large quantities of protein after birth, also show an increased protein content of the small intestine during the first 24 hours and a slight decrease on the following day (Hall and Widdowson, 1979).

1.1.2.3 Factors associated with gut growth in the neonate

After birth, the newborn mammal must adapt quickly from parenteral to enteral nutrition, the regulated intravenous delivery of nutrients via the placenta being replaced by intermittent delivery via the gastrointestinal tract (Weaver, 1986). The growth of the gastrointestinal tract is very fast during the neonatal period, particularly in the first 24 hours (1.1.2.2). Rapid growth of the gastrointestinal tract is associated both with the rich nutrient supply and the presence of trophic factors in colostrum and mature milk.

Colostrum-suckled piglets had a greater small intestinal weight than piglets fed either mature milk or 5% lactose for 24 hours (Simmen *et al.*, 1990). At 24 hours of age, the small intestinal weight in colostrum-suckled piglets was 1.9-fold that of piglets fed water (Widdowson *et al.*, 1976). In pigs, feeding of either colostrum or mature milk was effective in promoting neonatal gut development, as reflected by RNA content: the total RNA content in the small intestine and in the intestinal mucosa was similar by 24 hours for colostrum- and milk-fed piglets, but less for piglets fed 5% lactose (Simmen *et al.*, 1990). Colostrum-suckling increased the mucosal DNA content of the small intestine in beagle puppies during the first 24 hours compared with artificial feeding (Heird *et al.*, 1984) but in contrast, over the same time, no difference in DNA content was seen in suckled and artificially-fed newborn piglets (Simmen *et al.*, 1990).

One contributing factor to the rapid increase in weight of the intestine in suckled piglets, puppies and rabbits during the first 24 hours after birth may be the high protein content of colostrum compared with that in mature milk (12.0, 14.2 and 17.8 g/ml in colostrum and 1.1, 2.2 and 5.8 g/ml in milk in woman, cow and sow respectively) (Widdowson, 1985). Burrin *et al.* (1992) have demonstrated that both sow colostrum and mature milk stimulate intestinal protein synthesis in newborn piglets and that colostrum-fed piglets have greater protein synthesis than milk-fed piglets.

Colostrum and mature milk contain, in addition to nutrients, hormones and factors which specifically stimulate gut growth. Beagle puppies fed artificial bitch milk gained as

much body weight as those that were suckled, but there was no appreciable increase in the weight of the intestinal mucosa or in mucosal protein and DNA contents (Heird *et al.*, 1984). Newborn rats fed for 40 hours on an artificial formula isocaloric with expressed rat milk had significantly lighter intestines, which contained less DNA and RNA than those fed rat milk (Berseth, 1987b). Insulin, present in human and pig colostrum and mature milk, is believed to influence small intestinal growth and development. The concentrations of insulin in human and pig colostrum are 3- to 30-fold those in serum (Kulski and Hartmann, 1983; Slebodzinski *et al.*, 1986; Weström *et al.*, 1987). High doses of oral insulin increase the small intestinal mucosal weight and its protein and RNA contents in newborn miniature pigs (Shulman, 1990). Epidermal growth factor (EGF) is a major growth-promoting agent in human milk (Carpenter, 1980). EGF stimulates cellular proliferation in the epidermis (Cohen and Elliott, 1963) and intestinal mucosa (Klein and Mckenzie, 1980; Al-Nafussi and Wright, 1982). EGF-fed animals have heavier and longer intestines which contain more DNA and RNA than animals fed no EGF (Berseth, 1987a). EGF also influences the maturation and the proliferation of the enterocytes in the suckled mouse (Malo and Menard, 1982). Insulin-like growth factors-1 and -2, found in higher concentrations in human colostrum than in human mature milk (Donovan *et al.*, 1991), may also stimulate gut growth.

Enteral nutrition is very important for growth and function of the gastrointestinal tract, particularly of the small intestine. Given an equal caloric intake, there was no difference in body weight gain between pigs receiving total parenteral nutrition (TPN) and pigs fed an artificial milk formula for 3 weeks both at 3 days of age (Morgan *et al.*, 1987) and at 6 weeks of age (Goldstein *et al.*, 1985). In contrast, there were significant differences in anatomical measurements throughout the gastrointestinal tract, which was lighter in weight and shorter in length in TPN pigs. These differences were most marked in the proximal small intestine. The stomach and pancreas were also lighter, and villi were shorter in the proximal small intestine in TPN pigs.

The composition of feeds affects the gastrointestinal tract and function in newborns. Newborn guinea pigs, which were fed an artificial formula isocaloric with natural guinea pig milk for 8 days, had a similar body weight, length of the intestine, weight of small intestinal mucosa, and contents of mucosal protein and mucosal DNA to those in suckled animals (Weaver *et al.*, 1991). Dowling (1988) reviewed the effects of enteral nutrition on gut structure and function. Maintenance of normal gut structure and function seems to depend on the stimulus provided by regular food intake, which may

also partly explain the diminishing proximal-to-distal gradient in mucosal mass. When the intestine is deprived of its ingested nutrients, this proximal-to-distal gradient tends to disappear. A deficiency of luminal nutrients leads to hypoplasia. Long term malnutrition causes slow intestinal growth, including a lower mucosal weight, lower concentration of protein and DNA in the mucosa and shorter villi in neonatal rats (Winick and Noble, 1966) and rabbits (Butzner and Gall, 1988a).

1.2 DEVELOPMENT OF ENZYMES IN THE GASTROINTESTINAL TRACT

Many enzymes, such as pepsin in the stomach and amylase, disaccharidase, peptidase, lipase and Na^+-K^+-ATPase in the small intestine, appear during foetal life and their activity subsequently increases during the neonatal period. Pepsinogen secretion increases during neonatal life in humans (Grand *et al.*, 1976), and in pigs is low at birth and markedly increases from 2 to 6 weeks after birth (Lewis *et al.*, 1957). Lipase and alkaline and acid phosphatase are present during intrauterine life and after birth the total activities of these enzymes rise with age (Rokos *et al.*, 1963; Sprague *et al.*, 1963; Widdowson *et al.*, 1976). Peptidases, a large group of enzymes, are present in the foetus of humans (Lindberg, 1966) and pigs (Lindberg and Karlsson, 1970) early in gestation and in the rat foetus in later gestation (Lindberg and Owman, 1966). The highest activity of peptidase is in newborn animals which are not colostrum-fed (Lindberg and Owman, 1966; Lindberg and Karlsson, 1970). In suckled newborns, within a few hours of birth, the activity of these enzymes decreases to adult values (Lindberg and Owman, 1966; Lindberg and Karlsson, 1970).

One of the major groups of brush border enzymes, the disaccharidases, is unique to the small intestine in the adult (Alpers, 1987). Disaccharidase activity is highest in the jejunum and the proximal ileum and less in both the duodenum and the distal ileum in the adult (Lebenthal, 1982). Under physiological conditions, the disaccharidases hydrolyse oligosaccharides and disaccharides into monosaccharides - glucose, galactose or fructose - which are absorbed into the portal blood.

1.2.1 Disaccharidases

Disaccharidases, a group of hydrolytic enzymes located in the brush border membrane of the enterocytes of the small intestinal mucosa, include β-galactosidase (lactase), and α-glucosidases, including sucrase, isomaltase, maltase and trehalase (Rubino *et al.*, 1964;

Kidder and Manners, 1980; Dahlqvist, 1984). Lactase activity develops during intrauterine life in all mammals, whereas α-glucosidases develop in either the intrauterine or neonatal periods, the pattern of development and the time required to reach the adult level being species specific (Galand, 1989). Disaccharidase activity is usually described as a specific activity which is expressed as μM of substrate hydrolysed per g of protein or per g of wet tissue. The lactase activity of the brush border of the small intestinal mucosa hydrolyses dietary lactose. On the other hand, α-glucosidase hydrolysis to disaccharides is complicated. With the exception of trehalase, which has absolute substrate specificity, other α-glucosidases have quite complicated cross-specificity for different substrates, especially for maltose, which is hydrolysed at a considerable rate by all the enzymes (Dahlqvist, 1984).

1.2.1.1 Development of lactase

Lactase activity is detectable early in gestation, remains low for some time, then increases before term and declines during the neonatal period. In the human foetal jejunum, this enzyme is detectable, but the activity is very low until the 24th week of gestation when it begins to increase (Antonowicz *et al.*, 1974). At birth, lactase activity in the mucosa of the small intestine is high in humans (Antonowicz *et al.*, 1974), most pig breeds (Bailey *et al.*, 1956; Walker, 1959a; Hartman *et al.*, 1961; Manners and Stevens, 1972; Widdowson *et al.*, 1976; James *et al.*, 1987), rats (Rubino *et al.*, 1964; Koldovský *et al.*, 1966a; Lebenthal *et al.*, 1972) and lambs (Walker, 1959b). Lactase activity is usually only present in the small intestine, however, it is detectable also in the colon of rats in the early postnatal period, with a peak at 4 days after birth (Freund *et al.*, 1990), and in neonatal piglets, although the enzyme activity is very low (1.2-1.4 U/mg protein) (Murray *et al.*, 1991).

Lactase activity decreases gradually in neonatal life, the rate of decrease depending on the species. In normal human infants between 2 and 11 months of age, the level of lactase activity decreases to between half and a quarter that at term (Grand *et al.*, 1976). In most piglets, the activity remains high during the first week of neonatal life and falls at 6 weeks of age to minimum levels, similar to those in mature pigs (Bailey *et al.*, 1956; Walker, 1959a; Hartman *et al.*, 1961; Manners and Stevens, 1972; Widdowson *et al.*, 1976; James *et al.*, 1987), however, in some breeds, e.g. Hampshire and Chester White, the lactase activity at birth is lower and rises to a maximum by 15 days (Ekstrom *et al.*, 1975). In rats, lactase activity is high at birth and gradually falls to low levels

at 20 days of age (Rubino *et al.*, 1964). In the lamb, lactase activity is fairly constant throughout the first 5 weeks of life (Walker, 1959b).

1.2.1.2 Development of α-glucosidases

Of all the α-glucosidases, maltase is the only one which is always present at birth in all mammals which have these enzymes (Galand, 1989). In humans, all α-glucosidases are detectable at 10 weeks of gestation, increase gradually over the second trimester (Dahlqvist and Lindberg, 1966; Jirsova *et al.*, 1968; Antonowicz *et al.*, 1974), and then dramatically rise by term (Newcomer and McGill, 1966). In other mammals, the time for development of α-glucosidases is different from that in humans. A low level of maltase activity and near absence of sucrase activity are seen at birth in rats (Rubino *et al.*, 1964; Lebenthal *et al.*, 1972), mice (Arthur, 1968; Moog *et al.*, 1973), rabbits (Galand, 1986), pigs (Bailey *et al.*, 1956; Walker, 1959a; Hartman *et al.*, 1961; Manners and Stevens, 1972; Aumaitre and Corring, 1978), dogs (Welsh and Walker, 1965) and cats (Hore and Messer, 1968). The activities of these enzymes gradually increase during the neonatal period and reach adult levels at different times depending on the species and the feeding regime. In the rat, mouse and rabbit, there are low levels of α-glucosidases during the suckling period, up to 15 days, and a dramatic (10-30 fold) increase during the third week (weaning period). Adult levels are attained by 25 days in the rat and mouse and by 40 days in the rabbit (Galand, 1989). In sheep (Walker, 1959b) and cattle (Siddons, 1968), sucrase activity is absent during the whole of life, whereas maltase and trehalase are low at birth and do not increase further throughout life.

In suckled pigs, sucrase activity remains low in the mid-jejunum and is undetectable in other parts of the small intestine at 7 days after birth. This enzyme continues to increase with age for at least 4.5 years (Bailey *et al.*, 1956; Hartman *et al.*, 1961; Kidder and Manners, 1980; James *et al.*, 1987). Sucrase levels in the small intestine are very variable between individual piglets, developing more rapidly in some pigs than in others (Manners and Stevens, 1972). Low maltase activity is present in the pig foetus at 105 days gestation and at birth (Aumaitre and Corring, 1978). In the duodenum, maltase activity does not change between birth and adulthood (Galand, 1989), but in the jejunum and ileum the activity increases with age and is high by 6 and 8 weeks of age (Bailey *et al.*, 1956; Hartman *et al.*, 1961; Aumaitre and Corring, 1978). Maltase activity continues to increase with age for 30 weeks, after which time there is no further rise

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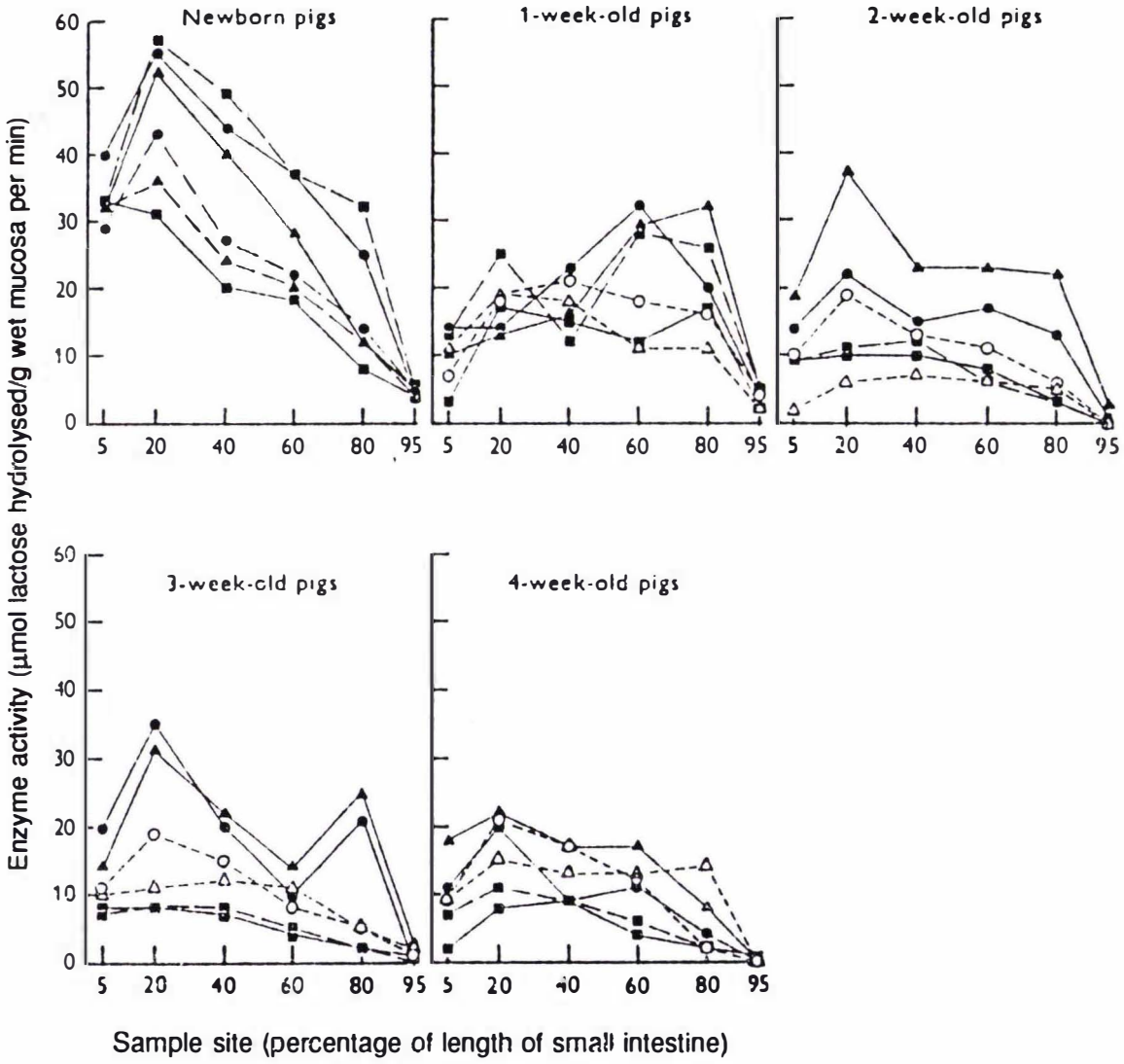

Figure 1.1 Lactase activity in the mucosa at six standardized sites along the small intestine of individual members of pairs of pigs from three litters of Large White x Wessex pigs killed at weekly intervals from birth to 4 weeks of age. Litter I, sow-reared, ■———■, ■— —■. Litter 2, sow-reared, ▲———▲, ▲— —▲; artificially-reared, △- - -△. Litter 3, sow-reared, ●———●, ●— —●; artificially-reared, ○- - -○. (From Manners and Stevens, 1972)

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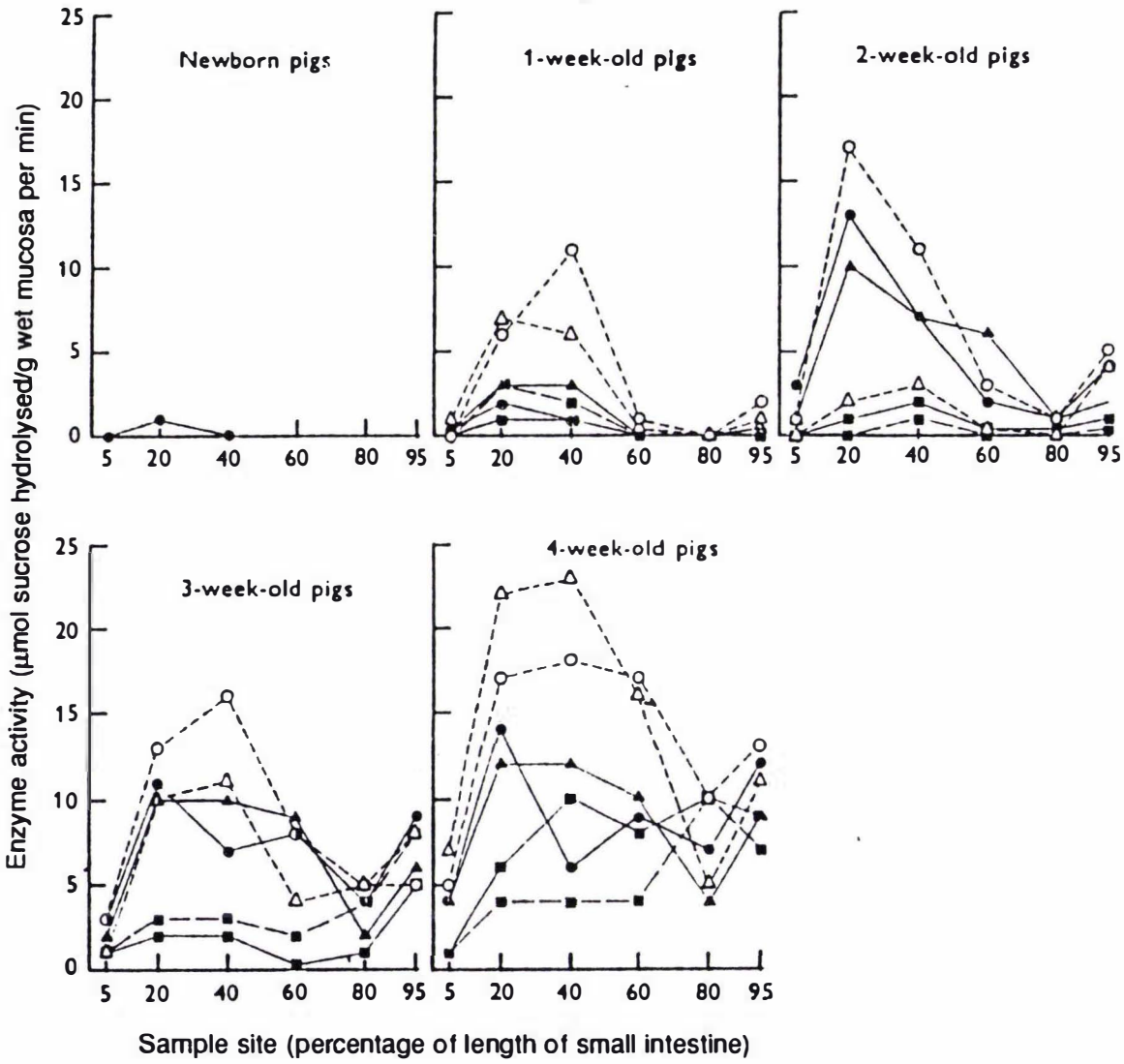

Figure 1.2 Sucrase activity in the mucosa at six standardized sites along the small intestine of individual members of pairs of pigs from three litters of Large White x Wessex pigs killed at weekly intervals from birth to 4 weeks of age. Litter I, sow-reared, ■———■, ■— —■. Litter 2, sow-reared, ▲———▲, ▲— —▲; artificially-reared, ▵- - -▵. Litter 3, sow-reared, ●———●, ●— —●; artificially-reared, ○- - -○. (From Manners and Stevens, 1972)

(Kidder and Manners, 1980).

The similar, but not identical, patterns of development of sucrase and maltase could be associated with the origin of the activities of the two enzymes. Sucrase activity is wholly derived from the sucrase-isomaltase complex, whereas 80% of maltase activity originates from the sucrase-isomaltase complex and 20% from a maltase-glucoamylase complex (Galand, 1989). The pattern of development of maltase activity is similar to that of isomaltase and sucrase. The maltase activity present at birth has been demonstrated to be associated with maltase-glucoamylase in suckled rats (Galand and Forstner, 1974) and rabbits (Galand, 1986). It is well known that the sucrase-isomaltase complex first appears during the neonatal period in rats (Simon *et al.*, 1979) and rabbits (Sebastio *et al.*, 1986). Simon *et al.* (1979) observed that the sucrase-isomaltase activity in the rat was completely absent until day 17, the stage at which weak activity appeared in the crypt compartment; at day 21, these enzyme activities also appeared in the upper-crypt and the villi. Afterwards, they gradually increased in the upper-crypt and the villi but always remained higher in the crypt.

1.2.1.3 Disaccharidase along the small intestine

In the normal adult, disaccharidase activities are highest in the jejunum and the proximal ileum and are lower in both the duodenum and the distal ileum (Lebenthal, 1982). The jejunum is the site of the first appearance of a specific disaccharidase activity and is also where a high enzyme activity first begins to decline during the maturation process. Figure 1.1 shows the development of lactase activity in both suckled and artificially-reared piglets from birth to 4 weeks (Manners and Stevens, 1972). In newborn piglets, lactase activity was high, with a peak at the 20% site (corresponding to the proximal jejunum) in almost all animals. From the peak, activity fell in both proximal and distal directions with the lowest level at the 95% site of the small intestine (corresponding to the terminal ileum). Between birth and 1 week of age, there was a considerable fall in lactase activity in the proximal half of the small intestine, particularly in the proximal jejunum. From 1-4 weeks of age, lactase activity continuously declined throughout the entire small intestine.

Figure 1.2 shows the development of sucrase activity in both suckled and artificially-reared piglets from birth to 4 weeks (Manners and Stevens, 1972). At birth, sucrase activity was initially very low at the 20% site in the small intestine (corresponding to

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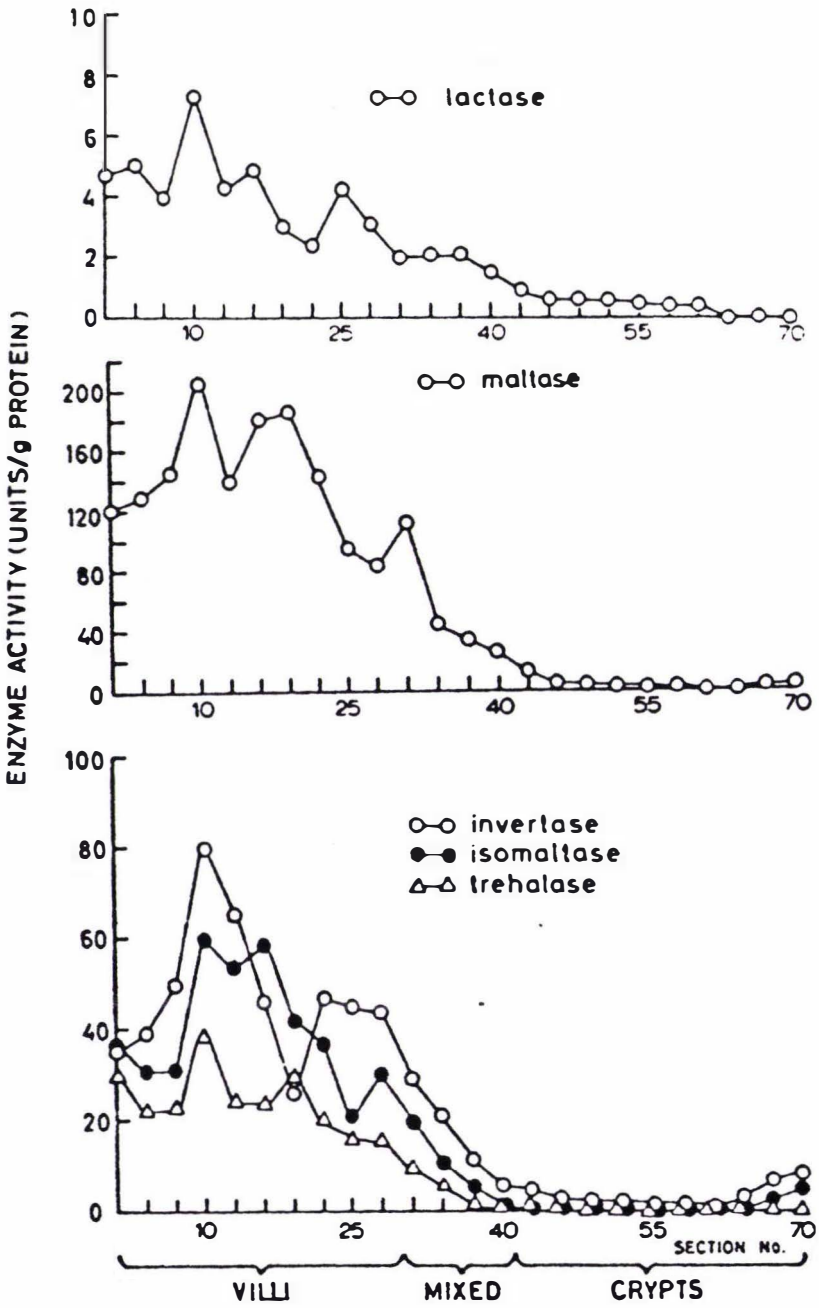

Figure 1.3 Distribution of disaccharidase activities in the villi and crypts of the adult rat jejunum. The enzyme activities have been calculated as "specific activities" (units per gram protein). The highest activity is found in the apical half of the villi, while the crypts do not contain measurable activities. (From Dahlqvist and Nordström, 1966)

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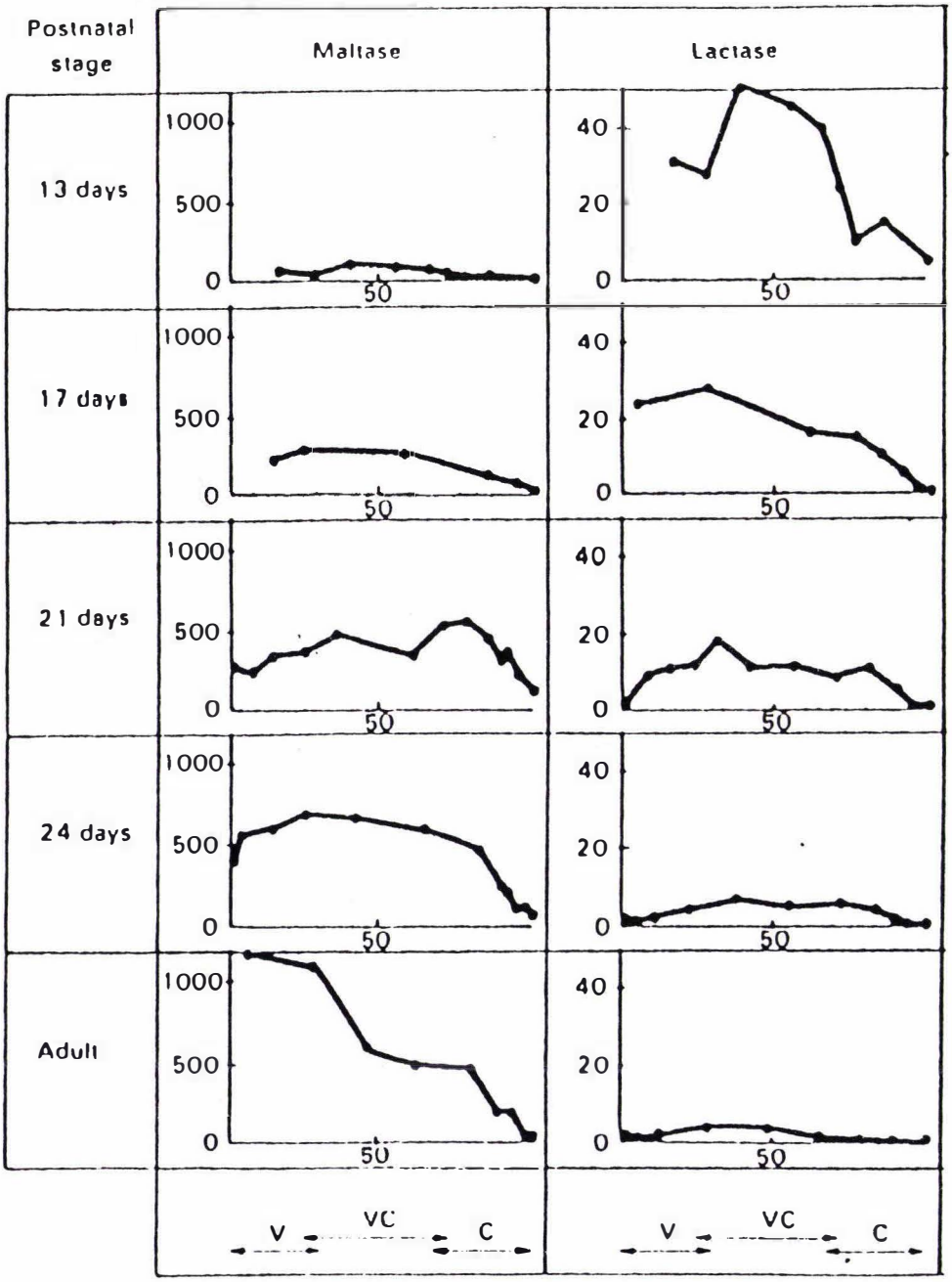

Figure 1.4 Representative gradients of maltase and lactase activities in intestinal cells isolated along the villus-crypt axis at different postnatal stages. Enzymic activities were determined in homogenates of successive cell fractions. The ordinate shows specific activities expressed as units/g protein. (One unit of enzymic activity hydrolyses 1 µmol of substrate/min.) According to the enzymic gradients obtained, isolated cells could be pooled into three cell compartments: V (villus cells), VC (lower villus and upper-crypt cells) and C (crypt cells). (From Simon *et al.*, 1979)

the proximal jejunum) and absent elsewhere, and extended in a proximal to distal direction along the small intestine with age. Sucrase activity was present at the 40% and 60% sites (corresponding to the mid- and distal jejunum) in 1- and 2-week-old pigs, and by 3 weeks of age the enzyme was present in the whole small intestine with the highest activity in the jejunum (Manners and Stevens, 1972).

1.2.1.4 Disaccharidase along the crypt-villus axis

The activities of all disaccharidases are not uniform along the crypt and villus. The localization of these enzymes along the crypt-villus axis has been established in rats from horizontal sections in the adult (Dahlqvist and Nordström, 1966; Fortin-Maoana *et al.*, 1970) and on sequentially isolated cells in the neonate (Simon *et al.*, 1979). The distribution of the specific activity of some disaccharidases along the villi and the crypts in the jejunum of adult rats is illustrated by the study of Dahlqvist and Nordström (1966), in which measurements were carried out on frozen sections cut from the tips of the villi to the base of the crypts (Figure 1.3). The activity was highest in the apical half of the villi, less close to the tips and in the basal part of the villi and essentially zero in the crypts.

During postnatal development, the location of disaccharidase activity along the crypt-villus axis is different when the activity is high from when it is low. Simon *et al.* (1979) demonstrated the evolution of lactase activity, from being high at birth to low at weaning, and of maltase activity, from low to high, along the crypt-villus axis during postnatal development in rats (Figure 1.4). Between 13 and 24 days of postnatal life, maltase had maximal activity in the intermediate cell fractions and the lowest values in the crypt zone, but this enzymic pattern changed by adulthood, when maltase activity becomes highest in the upper-villus cells and decreased progressively towards the base of the crypts. In contrast, lactase activity was always maximal in the middle of the villus and low in the crypts. Up to 17 days, high lactase activity was present along the whole crypt-villus axis with a lower activity in the lower crypt than in the villi and upper crypt. From 21 days of age onwards, lactase activity progressively declined in the villi and upper crypt and completely disappeared in the lower crypts. In a histochemical study using an α-glucosidase-specific stain, Collins *et al.* (1990) found a similar pattern of development of these enzymes in neonatal mice.

1.2.1.5 Factors influencing disaccharidase activity during neonatal life

Several factors can modulate the levels of disaccharidases during development: trophic factors, weaning time and the composition of the diet. It is well known that corticosteroids reduce lactase activity and increase maltase and sucrase activity in neonatal rats (Lebenthal *et al.*, 1972; Henning and Leeper, 1982). However, Yeh *et al.* (1991) demonstrated that cortisone enhanced lactase activity in rats younger than 9 days of age but depressed lactase activity in 19- and 22-day-old rats. In the older rats, cortisone plus thyroxine further depressed lactase activity but thyroxine alone did not. Oral insulin increased lactase activity in newborn, miniature pigs (Shulman, 1990). Both oral and injected EGF increased lactase, maltase and sucrase activities in preweaning and weaned pigs (James *et al.*, 1987; Jaeger *et al.*, 1990).

Early weaning and diets high in sucrose both promote the early development of sucrase and isomaltase activities. Early weaning accelerated the decline in lactase activity, whereas prolonged nursing maintained lactase activity in neonatal rats (Lebenthal *et al.*, 1973; Yeh and Holt, 1985). In piglets weaned at 2 days and reared artificially on diets containing 5% sucrose, sucrase and maltase activities were higher than in those nursed by their dam (Manners and Stevens, 1972). Similar evidence was obtained in the rat (Lebenthal *et al.*, 1972). Rats which were fed a diet containing 2% sucrose from 9 days of age had markedly greater activities of intestinal sucrase and isomaltase compared with those animals receiving a carbohydrate-free diet. The activity of maltase doubled, but lactase activity was unchanged. In piglets fed a greater amount of creep diet (a supplementary diet of high nutrient value for preweaning animals) from day 8 to day 14, the total sucrase activity was 50% higher than, and the maltase activity double that, in animals fed a smaller amount or no creep diet (Kelly *et al.*, 1990). In neonatal guinea pigs, artificial milk formula significantly increased maltase and sucrase (Weaver *et al.*, 1991). In contrast to the effects on maltase and sucrase, artificial feeding usually did not significantly affect the development of lactase activity in piglets (Manners and Stevens, 1972; Kelly *et al.*, 1990). However, newborn guinea pigs fed artificial milk formula had significantly lower lactase activity between days 5 and 7 (Weaver *et al.*, 1991). In miniature piglets, early weaning by 1 week followed by artificial feeding induced a more rapid decline in lactase activity with age but there was no indication of any difference in lactase levels between artificially-reared and sow-reared animals by 4 weeks of age (Shulman *et al.*, 1988).

The neonatal decline in lactase activity can be delayed by the feeding regime e.g. prolonged nursing in rats (Lebenthal *et al.*, 1973), restricted feeding in neonatal piglets (2-week-old) (Tivey *et al.*, 1991) or malnutrition produced by expanding the litter size in neonatal rabbits (Butzner and Gall, 1988a). Starvation in adult rats caused a significant increase in lactase activity (Nsi-Emvo and Raul, 1984) and decreased the rate of enterocyte migration (Rudo *et al.*, 1976). This suggests that the rate of enterocyte migration might also change in malnourished neonatal animals.

1.2.2 Na^+-K^+-ATPase

Intestinal Na^+-K^+-ATPase is an integral membrane protein (Madsen *et al.*, 1992), located in the basolateral membrane of the enterocyte (Hamilton and Gall, 1982). The Na^+-K^+-ATPase ion pump plays a critical role in the fluid and electrolyte physiology of the small intestine. The Na^+ pump at the basolateral border of the epithelial cell works against a steep electrochemical gradient, the process needing ATP hydrolysis by Na^+-K^+-ATPase in the basolateral membrane. Under physiological conditions, the sodium gradient facilitates the absorption of nutrients (e.g. glucose and amino acids), fluid and ions from the lumen across the epithelial cell into the circulation.

1.2.2.1 Development of Na^+-K^+-ATPase

The developmental pattern of Na^+-K^+-ATPase in the intestine is not clearly established. Na^+-K^+-ATPase is present in the small intestinal tissue of foetal rats at 17 days with the activity gradually increasing up to one day after birth; then it declines at least until day 14 (Horvàth *et al.*, 1993). This enzyme is present in human foetuses between 7 and 22 weeks of age (Lev *et al.*, 1972) and in pig foetuses at 110 days and in 14-day-old piglets (Pauer *et al.*, 1991). Scattered data from control piglets in studies of experimental enteritis suggest that mucosal Na^+-K^+-ATPase activity may decline with age: Na^+-K^+-ATPase activity was 1.8-3.0 µmol per mg protein in 11- to 13-day-old York piglets (Davidson *et al.*, 1977) but 0.5-1.4 µmol per mg protein in 25- to 28-day-old York pigs (Kelly *et al.*, 1972). In contrast, the Na^+-K^+-ATPase activity of villus enterocytes gradually increased from 6.2 µmol/min/g protein in neonatal rabbits (3-5 days of age) to 8.6 and 13.9 µmol/min/g protein respectively at 10-14 and 24-28 days of age, and in adult rabbits it was 17.1 µmol/min/g protein (Shepherd *et al.*, 1980). Zemelman *et al.* (1992) also demonstrated an increase with age from week 1 to week 8 in rats, but no difference between proximal and distal portions of the small intestine. Cortisone acetate

injection enhanced the small intestinal Na^+-K^+-ATPase activity in neonatal rats (Zemelman *et al.*, 1992).

1.2.2.2 Distribution along the intestine

Na^+-K^+-ATPase is present in the whole intestine, but the location of the greatest activity varies with the species and even between studies in the one species. The highest activity of Na^+-K^+-ATPase is in the colon, followed by the jejunum and the ileum in rats (Charney *et al.*, 1975) and in calves (Landsverk, 1986). In the small intestine, the activity of this enzyme was greater in the proximal jejunum than in posterior regions of the intestine in 4-week-old pigs (Kelly *et al.*, 1972), whereas in rats it was highest in the proximal ileum, less in the jejunum and least in the distal ileum (Gnanaprakasam and Srivastava, 1973).

As the enterocyte migrates up the crypt-villus axis, it matures from a predominately secretory crypt cell to an absorptive villus tip cell (Madsen *et al.*, 1992), hence the activity of Na^+-K^+-ATPase is greater in the villus tip than in the crypt in the small intestine of rats (Gall *et al.*, 1977) and rabbits (Rowling and Sepulveda, 1984; Madsen *et al.*, 1992). The activity of this enzyme in the villi (22.8 ± 3.8 nmol/min/mg protein) is almost three times that in the crypts (7.7 ± 1.3 nmol/min/mg protein) in the rat jejunum (Gall *et al.*, 1977). Na^+-K^+-ATPase activity is located mainly in the basolateral membranes of enterocytes. Isolated basolateral membranes have a very high activity (618 ± 53 in the villus and 433 ± 26 nmol/min/mg protein in the crypt) compared with the activity in whole enterocyte homogenates (24.3 ± 4.5 in the villus and 17.6 ± 3.6 nmol/min/mg protein in the crypt) in New Zealand white rabbits (Madsen *et al.*, 1992).

1.3 ANTIMICROBIAL AGENTS IN COLOSTRUM AND MILK

Neonates are more resistant to enteric infections when suckled than when fed formula feeds, irrespective of whether they are born with immunoglobulins in their blood (human and rabbit) or whether they depend on the transfer of immunoglobulins into the blood from ingested colostrum through the temporarily permeable gut (piglets and calves) (Reiter, 1978). This indicates that colostrum and mature milk have an important role in intestinal protection of the neonate. Both colostrum and milk, particularly the former, contain immunoglobulins, leucocytes, lysozyme and lactoferrin. Milk from healthy mammary glands contains about 1-3 x 10^5 leucocytes/ml and 13-39 mg/100 ml of

lysozyme (Reiter, 1978). Milk lysozyme has been shown to lyse and kill several Gram-positive and Gram-negative microorganisms (Reiter, 1978; Ellison and Giehl, 1991).

1.3.1 Antibodies

Different species of mammals acquire antibodies in the perinatal period at different times, and in the case of pigs, they are usually obtained after birth. Mammalian foetuses normally do not synthesize their own antibodies. In some species, including guinea pigs, rabbits and humans, passive immunity is acquired before birth from the placental transfer of maternal antibodies. Rodents and dogs derive passive immunity in part from the intrauterine transfer of maternal antibodies and in part from the postpartum intestinal uptake of immunoglobulin. In contrast, pigs (Sibalin and Björkman, 1966), horses and ruminants (Davenport, 1977) are devoid of antibodies at birth. Since no immunologically significant amounts of maternal antibodies are transferred through the placenta to the pig foetus, all immunoglobulins are received in the neonatal period.

Colostrum contains large amounts of gamma-globulins (γ-globulins). In some species, e.g. humans, immunoglobulin A (IgA) predominates, whereas in others, e.g ruminants and pigs, immunoglobulin G (IgG) forms the largest part. Human colostrum primarily contains secretory IgA antibodies which are not readily transported across intestinal barriers because of the presence of a secretory component on the γ-globulin molecule (Johnson, 1981). IgA is generally regarded as being the most resistant to proteolysis (Reiter, 1978) and at least three-quarters of the IgA in the colostrum is excreted as protein in the faeces (Widdowson, 1984). The secretory IgA has been shown by gel filtration to be intact (Davidson and Lönnerdal, 1987). The function of IgA in humans is to act within the intestine to limit the multiplication of bacteria and viruses within the digestive tract. These specific antibodies in the intestine of the newborn can agglutinate bacteria, neutralize toxins and viruses, fix complement and interfere with bacterial adhesion to the intestinal epithelium (Reiter, 1978).

Guinea pigs, rabbits and humans obtain maternal antibodies before birth. Although γ-globulins are taken up into epithelial cells, transport from the cells into the circulation is considerably less than the γ-globulin transport in species depending on the gut for passive immunity e.g. pigs (Walker, 1981). Absorption of γ-globulin has been demonstrated in rabbits in the morphological study of Kraehenbuhl and Campiche (1969). Lysosomal breakdown of γ-globulins in epithelial cells may be greater in rabbits

and humans than in pigs (Walker, 1981) and would reduce the absorption of γ-globulins into the circulation.

Rodents and dogs obtain maternal antibodies in part while *in utero* and in part during the neonatal period. There is a prolonged period of selective transport of γ-globulins. The concept of selectivity of transport in the rat and the mouse is supported by the observation that uptake and transport of γ-globulin proteins is much greater than the absorption of other proteins, such as albumin. Among the γ-globulins absorbed, homologous immunoglobulins are taken up more readily by the neonatal rat intestine than are heterologous antibodies from other species. Absorption of antibody ceases at 20 days in rats, at 16 days in mice and at 10 days in dogs (Brambell, 1958).

Pigs, horses and ruminants obtain all maternal antibodies during early suckling. Porcine colostrum contains approximately 80% IgG, 15% IgA and 5% IgM (Hunter, 1986). In newborn piglets, both IgG and γ-globulin levels increase immediately after colostrum is ingested and reach the maternal antibody level during the first 1-2 days (Sibalin and Björkman, 1966; Johnson, 1981). Normally, newborn piglets and lambs lose the ability to absorb antibody at approximately 36 hours of age (Brambell, 1958; Lecce, 1966), but, when nursing is prevented for up to 4 or 5 days after birth, antibody absorption still occurs during this time in piglets which were maintained on parenteral nutrients (Lecce and Morgan, 1962; Payne and Marsh, 1962). Since the hydrolysis of protein to its constituent amino acids is inhibited in piglets during the first 36-48 hours by the presence of a trypsin inhibitor, the protein in colostrum is absorbed intact. After this time, gut closure occurs and proteins must be hydrolysed before absorption (Pond and Houpt, 1978). Absorption of macromolecules by newborn piglets is qualitatively non-selective, a large number of macromolecules e.g. egg white protein; bovine, equine, and human serum protein; enzymes and polyvinylpyrrolidone are absorbable (Lecce, 1966).

1.3.2 Lactoferrin

Lactoferrin, an iron-binding glycoprotein, is present in the colostrum and milk of mammals including humans, the guinea-pig, cow, goat, pig, horse and mouse (Masson *et al.*, 1966; Underwood, 1977). The concentration of lactoferrin is very high in human colostrum (5-7 mg/ml) and in mature milk (2-3 mg/ml) (Masson and Heremans, 1971) and in cow, goat and sow milk (0.02-0.2 mg/ml) (Underwood, 1977). Lactoferrin has a well known action against bacteria (Brock *et al.*, 1983; Bortner *et al.*, 1986; Ellison

and Giehl, 1991) and fungi (Soukka *et al.*, 1992). *In vitro*, lactoferrin also increases the greater uptake and faster killing of the intracellular parasite *Trypanosoma cruzi* by mouse peritoneal macrophages and human blood monocytes (Lima and Kierszenbaum, 1985).

1.3.2.1 Inhibition of bacteria

Both *in vitro* and *in vivo* experiments suggest that lactoferrin plays a role in the defence of the neonate against gastrointestinal infections. An enteropathogenic strain of *E. coli* inoculated into human milk, which contains lactoferrin, did not start to grow for about 10 hours, but this bacteriostatic effect had disappeared by 24 hours (Brock *et al.*, 1983). Similarly, sow milk and sow colostrum were bactericidal for a pig enteropathogenic strain of *E. coli in vitro* (Nagy, 1975). The antibacterial action of lactoferrin against strains of *E. coli, Vibrio cholera, Salmonella typhimurium* and *Legionella pneumophila* has also been demonstrated in media to which has been added purified lactoferrin from human or bovine milk (Brock *et al.*, 1983; Bortner *et al.*, 1986; Ellison and Giehl, 1991). Lactoferrin also inhibited the growth of the fungus *Candida albicans* at pH 7.0, the degree of inhibition increasing as the incubation temperature rose (Soukka *et al.*, 1992).

In vivo, the bacteriostatic effect of lactoferrin is not as consistent as might be expected from the *in vitro* experiments. Zagulski *et al.* (1986, 1989) demonstrated the protective effect of lactoferrin against bacteria in rabbits and mice. Lactoferrin-injected rabbits, which had been experimentally infected with *E. coli*, survived longer (9 days, range 0.5-30 days) than control animals (19 hours, range 6-20 hours) (Zagulski *et al.*, 1986). A single intravenous dose of lactoferrin in mice 24 hours before a challenge with *E. coli* improved the survival rate at 24 hours postinfection (92%) in comparison with controls receiving *E. coli* alone (11%). After two weeks, 71% of animals survived in the lactoferrin group but only 5% of controls (Zagulski *et al.*, 1989).

Recently, Teraguchi *et al.* (1993) demonstrated that 0.5% bovine lactoferrin in commercial milk (pasteurized at 130 ^{0}C for 2 seconds) administered orally to specific pathogen free mice reduced their faecal *Enterobacteriaceae*. Interestingly, the bacteriostatic effect did not occur, even at the high concentration of 5%, when the bovine lactoferrin was supplied in water for drinking and the animals were fed with commercial pellets (Teraguchi *et al.*, 1993). Newborn infants fed milk formula with

added bovine lactoferrin did not have the same faecal microflora as breast-fed babies (Balmer *et al.*, 1989). Hall *et al.* (1988) failed to show any bactericidal effects of bovine lactoferrin in neonatal piglets infected with a pathogenic strain of *E. coli.* No inhibitory action of lactoferrin on *Y. enterocolitica* has been reported either *in vitro* or *in vivo.*

1.3.2.2 Mechanism of inhibition of bacteria by lactoferrin

Iron is an essential growth factor for nearly all bacteria (Finkelstein at el., 1983). Administration of iron to animals can increase their susceptibility to experimental infection (Miles *et al.*, 1979; Robins-Browne *et al.*, 1985; Kramer *et al.*, 1986). Each molecule of lactoferrin chelates two ferric ions with high affinity (Groves, 1960), thus making iron unavailable to microorganisms in the intestine when sufficient lactoferrin is present in the intestinal lumen. *In vitro*, the bacteriostatic effect of human milk could be abolished by adding sufficient iron to saturate the lactoferrin (Brock *et al.*, 1983).

Other studies have indicated that the mechanism of lactoferrin-mediated, antimicrobial action is more complex than simple iron deprivation and is the result of a direct bactericidal effect on some bacteria. Arnold *et al.* (1977) demonstrated that *Streptococcus mutans* and *V. cholera*, but not an enteropathogenic strain of *E. coli*, were killed by incubation with purified human lactoferrin. At sublethal levels of lactoferrin, dose-dependent delays in growth initiation were observed, however, lactoferrin is inhibitory at levels significantly lower than those needed to bind all the available iron in the assay medium. The direct bactericidal effect of lactoferrin is by damage to the outer membrane of enteric Gram-negative bacteria by releasing lipopolysaccharides (Ellison *et al.*, 1988, 1990), which are complex outer membrane constituents of Gram-negative bacteria (Al-Hendy *et al.*, 1992). Observation by transmission electron microscopy showed that *E. coli* cells exposed to lactoferrin at 40 milliosmoles/l (mOsm) for 24 hours become quite enlarged, hypodense and rounded whereas the majority of bacterial cells were cylindrical when grown in media alone (Ellison and Giehl, 1991).

1.3.2.3 Factors influencing the bacteriostatic effect of lactoferrin

In vitro, the bacteriostatic effect of lactoferrin is reduced by calcium, magnesium, bicarbonate and citrate. High concentrations of calcium and magnesium in the medium inhibit the bacteriostatic effect of lactoferrin even though lactoferrin does not chelate

calcium and magnesium (Ellison and Giehl, 1991). Citrate can interfere with the inhibitory effect on bacterial growth of the lactoferrin in bovine colostrum (Reiter *et al.*, 1975), possibly because citrate competes with the lactoferrin for iron. However, it is known that the iron-citrate complex itself can be utilized by *E. coli* and promotes their growth in low-iron media (Rosenberg and Young, 1974). Bicarbonate is believed to be important for the bacteriostatic effect of lactoferrin on *E. coli* (Griffiths and Humphreys, 1977) because it is required for the binding of iron by transferrin and lactoferrin (Masson and Heremans, 1968). The degree of bacterial inhibition correlates with the medium osmolarity. *E. coli* were killed by lactoferrin at 40 mOsm but bacterial killing progressively decreased as the osmolarity was increased, and was completely absent in 100 mOsm media (Ellison and Giehl, 1991).

1.4 PATHOPHYSIOLOGY OF INFECTIOUS DIARRHOEA

The gastrointestinal tract is directly exposed to natural milk, artificial milk or other food, and is unavoidably exposed to the normal commensal flora and their products. At birth, the gastrointestinal tract of the newborn is sterile, but it is rapidly colonized by large numbers of bacteria, e.g. within 24 hours in piglets. Lactobacilli are present in large numbers throughout the tract, including the stomach, during the entire suckling period (Pond and Houpt, 1978). The newborn may be exposed to potentially or obligatorily pathogenic microorganisms, parasites, and toxins. If the gut is exposed to large numbers of enteropathogens, specific antibodies or non-specific substances, e.g. lactoferrin and lysozyme, may be insufficient to protect against those organism or toxins, and infectious diarrhoea may result.

Infectious diarrhoea is a major health problem throughout the world, particularly in developing countries. A review by the World Health Organization of community-based surveillance studies in various third world countries, but excluding China, estimated that in 1982 approximately 1 billion episodes of diarrhoea occurred in children under 5 years of age (Snyder and Maerson, 1982). Enteropathogens may be viral, bacterial or parasitic. The most common viral enteropathogens are rotavirus (Cohen, 1991) and transmissible gastroenteritis (TGE) virus (Hamilton, 1990). According to a review by Cohen (1991), *Salmonella* is the most common cause of bacterial diarrhoea in children, and *Shigella* is the second most commonly identified bacterial enteropathogen. *Yersinia enterocolitica* is a common cause of gastroenteritis in children in Europe, Canada and Japan.

Diarrhoea can be defined as increased frequency and/or fluidity of bowel movements, e.g. passage of stools containing excessive water. A number of factors are known to contribute to excessive fluid and electrolyte losses, including (i) abnormal transit, (ii) active secretion of fluid and electrolytes, and (iii) decreased digestion and absorption of nutrients (O'Loughlin *et al.*, 1991). Enteric organisms cause diarrhoea in different ways: some are noninvasive but secrete toxins that interact with intestinal epithelial cells to stimulate fluid secretion (e.g., *V. cholera*); others invade and destroy intestinal epithelial cells, thereby altering nutrient absorption and fluid transport.

1.4.1 Disordered Transit

Normal digestion and absorption are partly dependent on intestinal motor function. If transit through the small intestine were so rapid that mucosal contact time for digestion and absorption were inadequate, diarrhoea would occur (Moon, 1978). Therefore, diarrhoea is a very common sign of disturbed gastrointestinal function. Increased secretion or decreased absorption may stimulate motor activity. On the other hand, some enterotoxins, e.g cholera enterotoxin and *E. coli* enterotoxin, stimulate propulsive muscular contractions in rabbit small intestine (Moon, 1978).

1.4.2 Increased Secretion of Fluid and Electrolytes

A wide variety of pathogenic bacteria elaborate enterotoxins and cytotoxins capable of stimulating intestinal secretion. The activity of toxigenic bacteria depends on the adherence of the bacteria and the interaction of enterotoxins with receptors. More receptors are present on the immature intestinal surface than on the mature surface, which may account for an increased incidence of toxigenic diarrhoea in young infants (Walker, 1981). Cholera is a classical example of enterotoxigenic diarrhoea. *Vibrio cholera* colonizes the upper small intestine without inducing morphological alterations in the mucosa (lliott *et al.*, 1970). Following adherence to the mucosa, the organism elaborates a heat-labile toxin that, after binding to the apical membrane of the mucosal cell, induces secretion of fluid and electrolytes, whereas active transport processes for nonelectrolytes, such as glucose, are unimpaired. Studies of isolated ileal mucosa from rabbits and humans performed under short-circuited conditions in Ussing chambers revealed that luminal administration of cholera toxin induced electrogenic chloride secretion by a cyclic AMP-dependent process (O'Loughlin *et al.*, 1991). A number of other bacterial pathogens are known to produce enterotoxins. *Salmonella* produces a

cholera-like enterotoxin which is capable of stimulating fluid secretion in ligated rabbit ileal loops. This effect is inhibited by monospecific cholera antitoxin (O'Loughlin *et al.*, 1991). *Campylobacter jejuni* and *Aeromonas hydrophila* also produce cholera-like toxins, but their role in diarrhoeal production is less clear (O'Loughlin *et al.*, 1991).

Enterotoxigenic *E. coli* elaborates a cholera-like, heat labile toxin but also produces a heat stable toxin. After binding to the receptor on the apical membrane of the enterocytes on the mucosal surface of both the small and large intestine, the heat-stable toxin inhibits Na^+ and Cl^- absorption and induces Cl^- secretion.

Most pathogenic strains of *Y. enterocolitica*, including serotype 0:3 biotype 4, produce an enterotoxin which resembles *E. coli* heat-stable enterotoxin (Pai and Mors, 1978; Boyce *et al.*, 1979). It is unclear whether *Y. enterocolitica* enterotoxin is associated with diarrhoea *in vivo* since the enterotoxin is detected in broth supernatant fluid after 12 or 24 hours of incubation at 25 ^{0}C but not at 37 ^{0}C (Pai and Mors, 1978; Boyce *et al.*, 1979) and may not be elaborated by the bacterium *in vivo* at body temperature. Intestinal contents of the colon, ileum and jejunum from rabbits with diarrhoea after challenge with *Y. enterocolitica* were negative for enterotoxin (Pai *et al.*, 1980). Secretion of Cl^- in the small intestine was unimpaired in *Y. enterocolitica*-infected rabbits (O'Loughlin *et al.*, 1988). This suggests that *Y. enterocolitica* enterotoxin may not be important in causing diarrhoea *in vivo*.

1.4.3 Decreased Digestion and Absorption

Maldigestion and malabsorption occur in enteric infectious diseases once the epithelial surface is damaged by enteropathogens. Abnormal digestion and absorption of nutrients, particularly of dietary carbohydrates, can produce diarrhoea. Low disaccharidase activity, coupled with a normal carbohydrate intake, may result in unabsorbed sugar in the lumen of the gut which may cause an osmotic diarrhoea (Bailey *et al.*, 1956; Graham *et al.*, 1984).

1.4.3.1 Damage to the mucosa

Many enteropathogenic organisms - viruses and bacteria - damage the mucosa of the small intestine, resulting in a reduction of the mucosal surface area. Different types of damage are produced in the intestinal mucosa by different pathogens. Generally,

enteroviruses invade epithelial cells, whereas enterobacteria cause both mucosal invasion and an inflammatory response in the lamina propria.

Invasion by most enteroviruses, e.g rotavirus and TGE virus, is associated with loss of the mature absorptive cells, producing a proliferative response in the crypts that results in repopulation of the intestinal epithelial lining with virus-free, poorly differentiated cells on the villi (reviewed by Moon, 1978; Argenzio and Whipp, 1980; Hamilton, 1990; Cohen, 1991). Some viruses, e.g. parvovirus causing infectious feline panleukopenia, destroy the epithelium of the crypt (Kent and Moon, 1973).

There are two main histopathological characteristics of rotaviral and TGE viral enteritis: villus atrophy and crypt hyperplasia. Experimental infection of piglets with rotaviruses or transmissible gastroenteritis virus (TGE) causes profuse diarrhoea within 16-24 hours of infection and morphological changes within 24-48 hours (Pearson and McNulty, 1977). Virus replication results in desquamation of villus absorptive cells of the mucosa of the small intestine and progressively shortened villi (Davidson *et al.*, 1977; Snodgrass *et al.*, 1979; McAdaragh *et al.*, 1980). Epithelial cells divide in the crypts and migrate up the villi to be shed. During viral infections, the villi are shed more quickly and crypt cells divide faster than normally. Crypt hyperplasia commonly occurs after infections resulting in rapid replacement of villus absorptive cells, so that the number of cuboidal villus epithelial cells increases (Snodgrass *et al.*, 1979; McAdaragh *et al.*, 1980). In rotavirus-infected pigs, the ratio of the villus height to the crypt depth in the small intestine is decreased in the proximal and middle parts to 1.1, and in the distal part to 1.4, compared with control values of 3.9, 5.4 and 3.8 respectively (Woode *et al.*, 1978).

Bacterial invasion destroys the villus, but not as quickly as does viral invasion. Bacterial enteropathogens do not multiply in epithelial cells, although after ingestion they proliferate within the intestinal lumen and then adhere to the epithelial cells. After adherence, the bacteria invade and multiply in the lamina propria of the intestinal mucosa and elicit an acute mucosal inflammatory reaction that may result in ulceration (Cohen, 1991). In *Salmonella* enterocolitis, the typical enteric lesion is necrosis of crypt and surface enterocytes that varies from focal to diffuse. The lamina propria and submucosa contain numerous macrophages and moderate numbers of lymphocytes, but conspicuously few neutrophils. The necrosis frequently extends to involve the muscularis mucosa, submucosa, and lymphoid follicles. In the ileum, the necrosis is usually quite superficial and is often seen as villus atrophy (Wilcock, 1986).

Yersinia enterocolitica infection causes both epithelial destruction and brush border damage (O'Loughlin *et al.*, 1991). The pathological injury is characterized by patchy microabscess formation in experimentally-infected mice (Carter, 1975), piglets (Robins-Browne *et al.*, 1985) and rabbits (O'Loughlin *et al.*, 1986). Villus atrophy and crypt hyperplasia have been observed at 6 days postinfection in rabbits (O'Loughlin *et al.*, 1986).

1.4.3.2 Abnormalities of mucosal enzyme activity

Small intestinal maldigestion and malabsorption, particularly of carbohydrate, would be an important factor contributing to the production of diarrhoea (O'Loughlin *et al.*, 1991). Damage to the villus cells may result in decreased activity of both digestive enzymes and enzymes associated with absorption. Disaccharidase, which is usually unique to the small intestine, has a higher activity in the villi than in the crypt. Loss of mature absorptive cells and replacement with less differentiated cells decreases disaccharidase activity during viral infections. Maltase, sucrase and lactase activities decrease in children with rotavirus enteritis (Davidson and Barnes 1979) and in lambs (Ferguson *et al.*, 1981), piglets (Davidson *et al.*, 1977; Graham *et al.*, 1984) and mice (Collins *et al.*, 1988) experimentally infected with rotaviruses. Piglets infected with TGE viruses (Kelly *et al.*, 1972; Gall *et al.*, 1977; Kerzner *et al.*, 1977) also show lower lactase, maltase and sucrase activities. In human diarrhoea, the reduction in sucrase and lactase activities is greater than the loss of maltase activity (Lebenthal and Lee, 1980). Artificial *Y. enterocolitica* infection causes lower maltase and sucrase activities in both the jejunum and ileum of weanling rabbits (O'Loughlin *et al.*, 1986), and lower sucrase in both the jejunum and ileum of nursed rabbits (Butzner and Gall, 1988b). Surprisingly, lactase activity decreased in the jejunum only in undernourished rabbits infected with *Y. enterocolitica* at 17 days of age, but not in nursed rabbits (Butzner and Gall, 1988b).

The relationship between mucosal injury and the reduction in disaccharidase activity in the small intestine has not been well defined. Berg *et al.* (1973) demonstrated that disaccharidase (lactase, trehalase, maltase, isomaltase and sucrase) activity was correlated with the logarithm of the ratio of villus plus crypt to crypt in the mucosa of the small intestine in 178 adult human patients with various gastrointestinal complaints. In infants with protracted diarrhoea, Shulman *et al.* (1991), however, failed to show any correlation between the disaccharidase (lactase, maltase and sucrase) activity and the ratio of the villus to the crypt regardless of whether or not the data were logarithmically

transformed.

The effect of viral and bacterial infection on Na^+-K^+-ATPase, an enzyme associated with absorption, has not been consistent in various studies. In piglets infected with enteroviruses, Na^+-K^+-ATPase activity has been reported to decrease in different parts of the intestine. In 4-week-old pigs infected with TGE viruses or 11- to 13-day-old pigs infected with rotaviruses, the enzyme decreased in the proximal jejunum, but not in posterior regions of the intestine (Kelly *et al.*, 1972; Davidson *et al.*, 1977). In 7- to 9-day-old miniature piglets, reduced activity was found only in the ileum during rotavirus infection (Graham *et al.*, 1984). In neonatal rats infected with rotaviruses on day 7, small intestinal Na^+-K^+-ATPase did not change significantly during the 7 day infection period (Collins *et al.*, 1988). Furthermore, this enzyme increased in all regions of the small intestine, but was unchanged in the colon, in weanling rabbits infected with *Y. enterocolitica* (O'Loughlin *et al.*, 1986). These animals had a lower absorption of electrolyte in the jejunum and ileum (O'Loughlin *et al.*, 1988). Diminished activity of Na^+-K^+-ATPase would reduce active extrusion of sodium at the lateral membrane of the enterocyte, causing defective sodium transport (Davidson *et al.*, 1977).

1.5 ANIMAL MODELS OF *YERSINIA ENTEROCOLITICA* ENTERITIS

Among numerous enteropathogens, *Y. enterocolitica* has become an important cause of bacterial enteritis in infants and children and appears to be increasing in frequency in many temperate areas of the world (Delorme *et al.*, 1974; Black *et al.*, 1978; Maki *et al.*, 1980; Marks *et al.*, 1980; Lee *et al.*, 1991). Infection with *Y. enterocolitica* in humans has been reported in over 30 countries since 1970 (Marriott, 1987). Children under 5 years of age seem more sensitive to this organism, 80% of patients being in this age group, however, enteritis due to *Y. enterocolitica* is rarely fatal (Mair and Fox, 1986). Much of the information on the pathology of the disease has been obtained from experimental infection of animal models in which diarrhoea is produced after orogastric inoculation with *Y. enterocolitica*. A number of species, including monkeys (Maruyama, 1973), mice (Carter and Collins, 1974; Carter, 1975; Hanski *et al.*, 1989; Grützkau *et al.*, 1990), rabbits (Une, 1977; Pai *et al.*, 1980; O'Loughlin *et al.*, 1986; O'Loughlin *et al.*, 1988) and pigs (Robins-Browne *et al.*, 1985; Schiemann, 1988) have been infected experimentally with *Y. enterocolitica* resulting in enteritis. The investigation of experimental *Yersinia* enterocolitis in these animal models has produced much useful information which has increased the understanding of the human disease.

1.5.1 Mice

Carter and Collins (1974) found CD-1 mice to be more susceptible to *Y. enterocolitica* infection than either of two other strains of mice, leading to the development of a model in mice using intragastric infection of specific pathogen-free CD-1 mice (Carter, 1975). Histological evidence of infection could be observed as early as 24 hours postinfection, with the Peyer's patches in the ileum showing aggregations of neutrophils between the mucosa and the lymphoid nodule. There was also a marked influx of neutrophils into the lamina propria of the mucosa surrounding the Peyer's patches. The lamina propria in the intestinal mucosa opposite the side of the intestine containing the Peyer's patches remained free from neutrophil infiltration, and sections of the small intestinal wall not containing Peyer's patches also showed no signs of infection. The upper parts of small intestine were also uninfected. Lesions were also observed in the medullary portion of the mesenteric lymph nodes, spleen, liver and lungs. In several mice, the caecal Peyer's patches were heavily infected in the same manner as the Peyer's patches in the distal ileum. The colon and kidneys remained uninvolved throughout the 12 days postinfection (Carter, 1975).

The affinity of *Y. enterocolitica* for Peyer's patches arises from the attachment of *Yersinia* to M cells, which are special cells present in the follicle-associated epithelium covering the lymphoid follicles of Peyer's patches of the small intestine, followed by phagocytosis and transport from the lumen into the lamina propria (Hanski *et al.*, 1989; Grützkau *et al.*, 1990). The bacteria proliferate within the Peyer's patches, from which they spread into the lamina propria. The bacteria do not invade columnar absorptive cells in mice.

1.5.2 Rabbits

Rabbit models of yersiniosis have provided more information about the disease than have the mouse models. In the first rabbit model (Une, 1977), diarrhoea and the histological changes of enterocolitis were produced by direct inoculation of the bacteria into the duodenal lumen through the serosa under laparotomy, a method which could introduce the undesirable complications of peritonitis and systemic infections. Later, Pai *et al.* (1980) and O'Loughlin *et al.* (1986) improved the technique of challenge by inoculating orogastrically with *Y. enterocolitica* suspended in 10% sodium bicarbonate solution. Histopathological changes were present in the whole intestine of infected

rabbits, the most pronounced change generally being noted in the ileum, especially in sites rich in Peyer's patches. A spherical nidus of Gram-negative coccobacilli admixed with, or surrounded by, inflammatory cells was present in the lamina propria of the crypt region (Pai *et al.*, 1980; O'Loughlin *et al.*, 1986) and occasionally seen higher in the lamina propria of the villus, where distortion of the villus architecture was produced (Buret *et al.*, 1990). Villus height was significantly decreased and crypt depth increased in the ileum, but not the jejunum, at 6 days postinfection and both villus height and crypt depth, but not the microvillus height in the ileum, returned to control values by 14 days postinfection (Buret *et al.*, 1990). In the caecum and colon, microabscesses tended to be patchy in distribution, and to result more frequently in ulcer formation (O'Loughlin *et al.*, 1986).

During *Y. enterocolitica* infection, goblet cells significantly increased throughout the whole intestine (Mantle *et al.*, 1991). This increase developed more rapidly in the distal small intestine (day 1) than that in the mid- and the upper parts (days 3 and 6). The degree of goblet cell hyperplasia was also greater in the distal small intestine than in the upper and mid-regions. Goblet cell hyperplasia in the proximal colon of infected animals was similar to that in the distal small intestine, hence goblet cell hyperplasia developed more rapidly, and to a greater extent, in the ileocecal region where mucosal injury was most severe (Mantle *et al.*, 1991).

In preweanling rabbits, *Y. enterocolitica* infection at 17 days of age caused lactase activity to decline at 6 days postinfection in the jejunum of undernourished rabbits, produced by expanding litter size, but not in normally suckled rabbits of the same age (Butzner and Gall, 1988b). In weanling rabbits infected with *Y. enterocolitica*, sucrase and maltase activities significantly decreased in the entire small intestine at 3 days postinfection, remained depressed until day 10, and at 14 days after inoculation these enzymes returned to normal only in the jejunum, but not in the ileum (O'Loughlin *et al.*, 1986). Sucrase activity was also reduced in preweanling rabbits in both the jejunum and ileum at 6 days postinfection and remained lower in the ileum at 10 days postinfection (Butzner and Gall, 1988b). A significant increase in Na^+-K^+-ATPase activity has been found in the entire small intestine in infected animals (O'Loughlin *et al.*, 1986). Absorption of glucose and glucose-coupled sodium was decreased in the jejunum and ileum in both *in vivo* and *in vitro* studies, whereas there was no evidence of active ion secretion (O'Loughlin *et al.*, 1988).

1.5.3 Piglets

Colostrum-suckled piglets have not been useful as an animal model of *Y. enterocolitica* infection. Schiemann (1988) failed to produce the disease in full-term piglets which had sucked antibody-positive colostrum for 36 hours. Experimental infection with *Y. enterocolitica* also failed in fattening pigs (Fukushima *et al.*, 1984). Robins-Browne *et al.* (1985), and later Schiemann (1988), were successful in developing an animal model for human *Y. enterocolitica* enteritis using piglets delivered by caesarian section and raised artificially. Lesions comparable with those seen in rabbits were observed in the small intestine of infected piglets: lesions increased progressively from the proximal jejunum, where only one or two were found, to the mid- and terminal ileum where almost every villus was affected. The large intestine was less uniformly affected than the ileum, although clusters of discrete lesions similar to those in the small intestine were seen in several areas. A difference from both the mouse and rabbit model was the absence of any evidence in infected piglets of bacterial invasion of visceral organs (Robins-Browne *et al.*, 1985).

In both the successful piglet models, the experimental design involved examination of a number of serotypes and doses of *Y. enterocolitica* and few animals per group. In the study of Robins-Browne *et al.* (1985), 8 piglets, within 2 days of delivery by caesarian section, were divided into 4 groups and challenged with 2 pathogenic strains of *Y. enterocolitica* in 3 doses (2×10^9, 10^{10} and 4×10^{10}). Two animals infected with the high dose died on day 1 without showing clinical signs. Of the other animals, 3 developed diarrhoea, one showed anorexia and two showed no clinical signs. Schiemann (1988) inoculated 8 caesarean-delivered piglets with 4 serotypes (0:8, 0:2, 0:3, 0:13) of *Y. enterocolitica.* During the 8 day experimental period, 2 of the 8 infected pigs died on days 2 or 6 and 2 had to be killed on day 6 because of severe illness.

Animal models of *Y. enterocolitica* enteritis have made important contributions to the understanding of the pathology of the disease, particularly histopathological findings, however, there are some deficiencies in the models. In neither the mouse nor the piglet have morphometric or biochemical measurements been made on the intestine. The number of piglets used for bacterial infection was small, and some infected animals died during the periods of experiment in both pig models.

1.6 CONCLUSIONS

Most structures in the gastrointestinal tract develop during foetal life and continue to mature after birth. In long gestation (precocious) species of mammals, the gastrointestinal tract is relatively mature at birth, while in the short gestation species the gut is much less well developed. The gastrointestinal tract is sterile and safe while the foetus is in the uterus, and nutrients are supplied directly by the mother. After birth, the newborn mammal must adapt quickly from parenteral to enteral nutrition. Therefore, during neonatal life, the gastrointestinal tract grows markedly, particularly the small intestine, which rapidly increases in both weight and length in the first day after birth in naturally-suckled neonates.

The disaccharidases are important during the neonatal period for digestion of sugars, particularly lactose, which is present in high concentrations in milk. At birth, lactase activity is high and sucrase and maltase activities are low and maturation of digestion includes the well described decline in lactase and increase in maltase and sucrase activities. Only in humans are all disaccharidases well developed at birth.

With enteral nutrition, the gastrointestinal tract is unavoidably exposed to pathogenic microorganisms or their toxins. In colostrum and mature milk, mothers supply antibodies and non-specific substances to the newborn to protect the young against pathogenic microorganisms during the neonatal period when the immune system is not well developed. Very often pathogens are so virulent that the young develops diarrhoea. In humans, it is well documented that diarrhoea is the leading cause of childhood growth retardation and death.

Neonatal life is a time for rapid development of the gastrointestinal tract and one of vulnerability to infectious diseases. The impact of enteritis in the newborn on gut development and maturation, however, is poorly documented, nor are the long term effects after clinical recovery well known. The present studies were carried out in order to evaluate the effects of gut infection on the growth of internal organs, particularly the small intestine, and on the development of the small intestinal enzymes during neonatal life and to determine whether growth and development were unimpaired after recovery from infection.

Neonatal pigs, because of their anatomical and physiological similarities to humans

(Book and Bustad, 1974), have been used to study the effects on the gut structure and function of enteropathogens e.g rotavirus (Pearson and McNulty, 1977; Newport *et al.*, 1982), TGE virus (Shepherd *et al.*, 1979), *E. coli* (Moon *et al.*, 1970) and *Y. enterocolitica* (Robins-Browne *et al.*, 1985; Schiemann, 1988). Pigs have not been used so far as a model for studying the effects of gastroenteritis on the development of the gastrointestinal tract in the neonate. Compared with rotavirus or *E. coli*, *Y. enterocolitica* is less virulent and does not cause death in children (Mair and Fox, 1986), therefore, it would appear to be a suitable pathogen for use in an animal model of gastroenteritis. In the present studies, newborn, colostrum-deprived piglets were used for the model of *Y. enterocolitica* enteritis because of their susceptibility to diarrhoea after orogastric infection (Robins-Browne *et al.*, 1985; Schiemann, 1988). From 5 days after inoculation, some infected animals were treated with antibiotic to study recovery from the enteritis. The study has focused on the growth of the body and internal organs and development of the morphology and biochemistry of the small intestine. The final aspect of the study was examination of whether oral supplementation with two naturally occurring antimicrobial agents from bovine milk are capable of influencing the susceptibility of the piglets to *Y. enterocolitica* enteritis or of affecting the severity of damage to the intestinal structure and function.

Chapter 2

EXPERIMENTAL INFECTION OF NEWBORN PIGLETS WITH *YERSINIA ENTEROCOLITICA*

2.1 INTRODUCTION

Yersinia enterocolitica, a Gram-negative coccobacillus, was first described as a cause of enterocolitis in 1939 by Schleifstein and Coleman. There were few further reports for 30 years until 1970, when Sonnenwirth described a patient with *Y. enterocolitica* bacteraemia and meningitis and subsequently Sonnenwirth and Weaver (1970) re-emphasized the importance of this microorganism as a human pathogen. In the past three decades, publications from over 30 countries have now documented the importance of *Y. enterocolitica* as a human enteric pathogen (Marriott, 1987). The highest incidences of *Y. enterocolitica* infection in humans have been observed in Scandinavia (Ahvonen, 1972; Christensen, 1987), Belgium (Vandepitte and Wauters, 1979; De Groote *et al.*, 1982), Japan (Asakawa *et al.*, 1973; Fukushima *et al.*, 1983), Canada (Toma and Lafleur, 1974; Marks *et al.*, 1980) and the United States (Black *et al.*, 1978; Shayegani *et al.*, 1983; Lee *et al.*, 1991). Infections have also been reported in the United Kingdom, France (De Groote *et al.*, 1982), Australia (Marriott, 1987) and New Zealand (McCarthy and Fenwick, 1991). Nearly 80% of all patients with *Y. enterocolitica* enteritis are under 5 years of age (Mair and Fox, 1986).

The clinical picture of abdominal pain, fever, diarrhoea, nausea and vomiting cannot be distinguished from infection by organisms of the *Salmonella* or *Shigella* groups or by pathogenic *Escherichia coli*. Only by stool culture or serological tests can the causative agent be identified (Mair and Fox, 1986). *Yersinia enterocolitica* has been isolated from the stools of 0.5-1.1% of children with diarrhoea (Marriott, 1987; Lee *et al.*, 1991; Jertborn *et al.*, 1991; McCarthy and Fenwick, 1991; Metchock *et al.*, 1991; Morris *et al.*, 1991). In clinical cases of *Y. enterocolitica* gastroenteritis, biotype 4 serotype 0:3 is most commonly found (Toma and Lafleur, 1974; Marks *et al.*, 1980; Thompson and Gravel, 1986; McCarthy and Fenwick, 1991; Lee *et al.*, 1991).

The source of infection of *Y. enterocolitica* for human patients is generally considered

to be food or water which has been contaminated by a human or animal carrier. For example, in the USA, an outbreak of food poisoning due to *Y. enterocolitica*-contaminated chocolate milk was reported by Black *et al.* (1978), in which infected children in New York State suffered abdominal pain simulating appendicitis, diarrhoea and fever. Strains of the same serovar of *Y. enterocolitica* were isolated from the chocolate milk and rectal swabs of the patients. Another outbreak in the same State affected more than 200 campers and staff at a summer camp and was due to powdered milk and turkey chow mein contaminated with *Y. enterocolitica* (Shayegani *et al.*, 1983). A family outbreak of gastroenteritis due to *Y. enterocolitica* from well water has been reported in Canada (Thompson and Gravel, 1986).

In New Zealand, human infection with *Y. enterocolitica* was sporadic before 1979. Watson *et al.* (1979), in a survey of *Campylobacter* and *Y. enterocolitica* in Palmerston North, failed to isolate any *Y. enterocolitica*, leading them to conclude that *Y. enterocolitica* was an infrequent cause of enteritis. Recently, McCarthy and Fenwick (1991) reported a rise in the number of isolations of *Y. enterocolitica* in the Auckland area from 0.2% in 1987 to 0.7% in 1988 and 0.5% in 1989. Hepatic abscesses and arthritis due to *Y. enterocolitica* have also been reported in New Zealand (Beeching *et al.*, 1985; Ameratunga *et al.*, 1987; Lello *et al.*, 1992).

Yersinia enterocolitica has been isolated from the faeces of domestic livestock (pigs, sheep, deer) and pets (dogs and cats), in addition to well water and stream water (Thompson and Gravel, 1986; Christensen, 1987; Terzieva and McFeters, 1991). Human strains of *Y. enterocolitica* are not only isolated from pig faeces but also from a high proportion of pig tonsils (Christensen, 1987) and from retail pork (Shiozawa *et al.*, 1987). Pigs have been implicated as natural reservoirs of *Y. enterocolitica* and are thought to play an important role in the transmission of human yersiniosis (Tsubokura *et al.*, 1973; Toma and Deidrick, 1975; Chiesa *et al.*, 1987; Maruyama, 1987; Mazzotti and Mingrone, 1987). Most human pathogenic *Y. enterocolitica* are able to colonise the lymphoid tissue and gastrointestinal tract of pigs without causing any clinical signs (Fukushima *et al.*, 1984; Schiemann, 1988), however, a report from China (Zheng, 1987) recording the isolation of *Y. enterocolitica* from pigs with diarrhoea suggests that the bacteria may also be capable of producing naturally-acquired illness in pigs. In New Zealand, *Y. enterocolitica* has been isolated from a flock of Romney sheep in which there was an outbreak of diarrhoea characterised by a morbidity rate of 30% and a mortality rate of 0.88% (McSporran *et al.*, 1984). At necropsy, the most significant

feature of the infection was suppurative enteritis, which is recognised as the most characteristic lesion of *Y. enterocolitica* enteritis in cattle and sheep (Belton and McSporran, 1988). An outbreak of yersiniosis involving *Y. enterocolitica* in goat weanlings was reported by Orr *et al.* (1987) on an Otago farm. Yersiniosis is also the most common cause of enteritis in farmed deer in New Zealand, *Y. enterocolitica* being carried by up to 36% of normal deer on some farms (Hendersen, 1984). *Yersinia enterocolitica* was also isolated from 24.7% of young goats on 18 farms in the Manawatu (Lañada, 1990).

Experimental investigation of *Y. enterocolitica* infection in animal models has produced much useful information which has improved our understanding of the human disease (Chapter 1, 1.6). To parallel the events in infant and childhood infections, animal models have been developed in which diarrhoea has been produced and the architecture or enzyme activity of the intestine has been changed after orogastric inoculation with *Y. enterocolitica.* Enterocolitis has resulted from experimental infection with *Y. enterocolitica* in monkeys (Maruyama, 1973), mice (Carter and Collins, 1974; Carter, 1975; Hanski *et al.*, 1989; Grützkau *et al.*, 1990), rabbits (Une, 1977; Pai *et al.*, 1980; O'Loughlin *et al.*, 1986; O'Loughlin *et al.*, 1988) and pigs (Robins-Browne *et al.*, 1985; Schiemann, 1988). The pathogenesis of *Y. enterocolitica* has been studied in all these models, but only in rabbits have biochemical alterations to the mucosa of the small intestine been demonstrated (O'Loughlin *et al.*, 1986; Butzner and Gall, 1988b).

The aim of the present study was to develop an animal model for *Y. enterocolitica* enteritis in which oral challenge with a pathogenic strain would consistently produce diarrhoea but would not cause the death of the animals. These infected animals could then be used to study the extent of damage to gut structure and intestinal enzyme activity as well as to investigate the subsequent recovery from the disease after antibiotic therapy. Newborn pigs were chosen as the experimental species because of their anatomical and physiological similarities to humans (Book and Bustad, 1974) and their known susceptibility to diarrhoea after orogastric infection (Robins-Browne *et al.*, 1985; Schiemann, 1988).

2.2 MATERIALS AND METHODS

2.2.1. Experimental Design

Nineteen piglets were infected with *Y. enterocolitica* within 3 hours of birth and a further 19 litter mates acted as controls. From each group, 6 were euthanased at 3 days of age, 8 at 5 days and 5 were treated with antibiotics from day 5 to either day 10 or 14, and killed on day 14.

2.2.2. Animals

Thirty-eight Large White piglets from 11 litters were used, with equal numbers from each litter being assigned randomly to the control and infected groups. The piglets chosen from each litter were visually appraised and considered to be of average size and neither very large nor very small for that particular litter. The mean body weight at birth of the 19 infected piglets was 1.455 ± 0.030 kg (mean ± SE) and that of the 19 controls was 1.477 ± 0.040 kg. Piglets were sex matched in the first litter used, but not in later experiments as there was no apparent effect of sex on body growth or on the infection, and matching animals for both size and sex became difficult. All sows had full term pregnancies (114-115 days of gestation).

Just before farrowing, the vaginal orifice of the sow was cleaned with warm water and washed thoroughly with Biocil Surgical Scrub (BSS) (Formula Products Ltd, Northcote Auckland, NZ). At birth, the piglets were supported to prevent contact with the sow's skin or the floor and to prevent them sucking colostrum. They were immediately washed with BSS, then placed in separate sterilized plastic tubs containing a layer of sterilized newspaper. The paper on the bottom of the tubs was changed at every feeding. Those animals treated with antibiotics were washed daily in separate containers with 1% BSS in tap water from the start of antibiotic treatment on day 5 to the termination of the experiment. During this period, the pig tubs were also washed daily with the same solution. The animal room was prepared by fumigation with formalin and its temperature was maintained at 34-35 ^{0}C.

In a preliminary study, 2 newborn piglets were taken from their dam immediately after birth, kept in a tub and bottle-fed with 14% commercial human infant milk formula (SMA, John Wyeth and Brother Ltd, Auckland, NZ), prepared with freshly boiled water,

at 2 hourly intervals from 6 a.m. until midnight for 3 days. These piglets were allowed to drink until satiated and the milk intake of each piglet was recorded at each feeding. The feeding regime was selected on the basis of these milk intakes.

The piglets were bottle-fed following the above feeding method. The animals were first bottle-fed 30 minutes after oral administration of either the bacterial culture or the control solutions. The volume of the milk formula given to each piglet at each feeding was about 40 ml for the first 7 days. From day 8 to the termination of the experiments, piglets were fed each day at 3 hourly intervals from 6 a.m. to midnight and the volume of milk given at each feeding was increased to 60-80 ml. The volume of milk consumed by the piglets at each feeding was recorded. Consumption of milk varied between piglets and between feedings. Daily milk intakes are shown in Appendix 1. All bottles and teats were disinfected with 1% Virkon solution (International Antec), then boiled for at least 15 minutes before being used. Sterile gloves and gowns were always worn by the experimenter, and these were changed between feeding the control and infected animals.

2.2.3 Inoculum

2.2.3.1 Bacterial strain

Yersinia enterocolitica, serotype 0:3, biotype 4, used in the present study was originally isolated from the faeces of a one-year-old boy with moderate diarrhoea. This strain was kindly provided by Mr S. Fenwick of the Department of Veterinary Pathology and Public Health at Massey University.

2.2.3.2 Bacterial multiplication and storage

In a preliminary study, the bacteria were grown on 5% sheep blood agar plates at 29 ^{0}C for 48 hours, harvested in physiological saline and stored at -70 ^{0}C (Fukushima *et al.*, 1984). The number of viable bacteria decreased from the original count of 3 x 10^{10} viable cells/ml to 2 x 10^{9}/ml after 10 days storage, making this method of preparing bacterial suspensions from sheep blood agar plates unsuitable for challenging animals and requiring the development of a different procedure so that viable bacteria would be available whenever a sow farrowed.

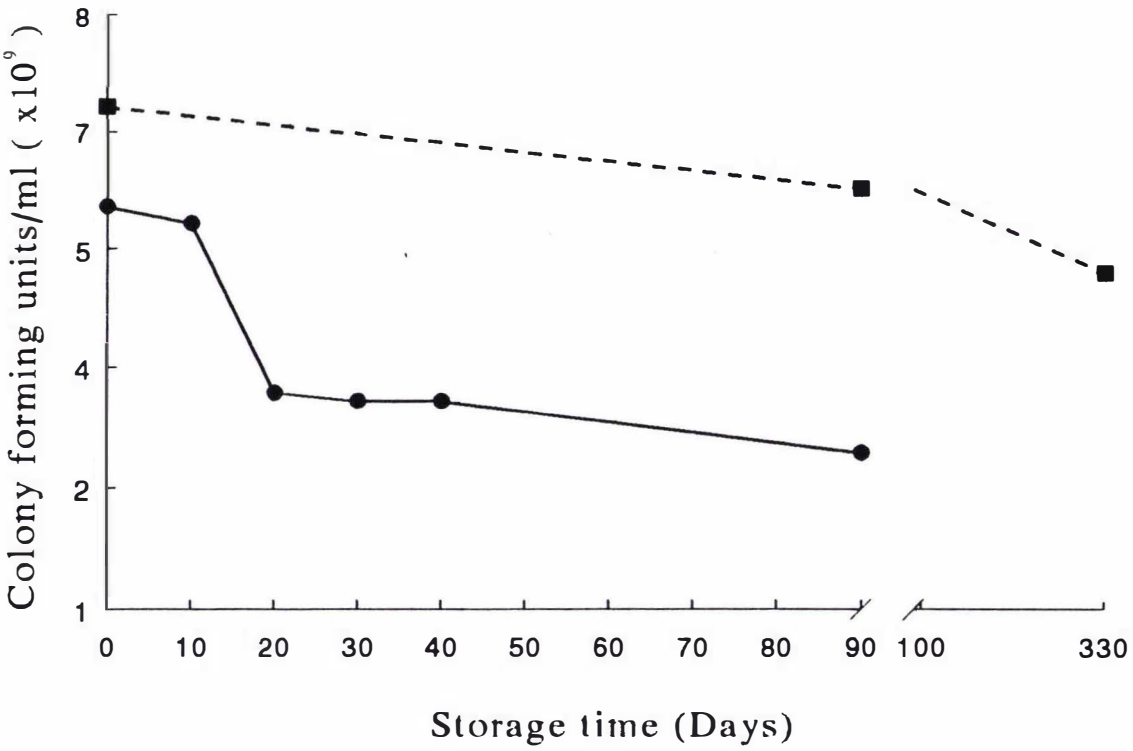

Figure 2.1 Survival curve of *Y. enterocolitica* in TSB stored at -70 ^{0}C for 330 days. The two batches of *Y. enterocolitica* suspensions are shown: batch 1 ——●——, batch 2 ---■---.

Trypticase soy broth (TSB) proved to be a suitable culture medium for long-term storage. A single colony of bacteria on a sheep blood agar plate was transferred into 10 ml of TSB and incubated at 29 ^{0}C for 24 hours. The bacteria were further multiplied in 200 ml of TSB shaken at 150 rpm on a shaker (Junior Orbit Shaker, Lab-line Instruments Inc. Melrose Park, ILL) at 29 ^{0}C for 24 hours. The bacterial suspension was divided into 10 ml aliquots and stored at -70 ^{0}C until used for challenging the animals. An aliquot was randomly taken for colony forming units (CFU) counting at 10, 20, 30 and 40 days and 3 and 11 months during storage.

2.2.3.3 Counting colony forming units

Tenfold dilutions of the bacterial suspension were made, and 0.1 ml of each dilution from 10^{-6} to 10^{-8} was placed on each of three sheep blood agar plates. The plates were inverted at 29 ^{0}C for 48 hours. The number of colonies on the plates (in the 30-300 range) was counted and averaged for the three plates for each dilution. The number of CFU per ml was calculated from the formula:

CFU/ml = CFU per plate x 10 x dilution factor

The survival curve of *Y. enterocolitica* in TSB stored at -70 ^{0}C for 330 days is shown in Figure 2.1. The CFU/ml did not drop below 3 x 10^9 throughout 330 days of storage. The bacterial suspensions grown in TSB were therefore used as the inoculum. The CFU in the stored bacterial suspension making up the inoculum was calculated from the bacterial survival curve before challenging the piglets. No bacterial suspensions stored over three months were used to inoculate piglets.

2.2.4 Experimental Infection of Piglets

Within 3 hours of birth, and before being fed, piglets assigned to the infected group were inoculated orogastrically with 10 ml of bacterial suspension containing about 3 x 10^{10} CFU of *Y. enterocolitica,* followed by 10 ml of sterile 10% $NaHCO_3$ solution (w/v), through a medical-grade tube with 1.57 mm (0.062 inch) interior diameter and 3.18 mm (0.125 inch) outer diameter (Dow Corning Corporation, Midland, Michigan, USA). Piglets in the control group were treated similarly, but with TSB replacing the bacterial suspension. The animals were first bottle-fed 30 minutes after challenge.

2.2.5. Clinical Observations

The pigs were observed at each feed for clinical signs of anorexia, vomiting or diarrhoea. Body weight was measured daily with electronic scales (Mettler PM, Switzerland). Faeces from all piglets were collected daily from the paper layer in the tub on to a sterilized swab and examined for *Y. enterocolitica.*

2.2.6 Antibiotic Treatment

Five piglets from each of the infected and control groups were given antibiotics from day 5 to either day 10 or day 13. The antibiotics used were Amphoprim, containing 40 mg trimethoprim and 200 mg of sulphadimethyl-pyrimidine per ml (Virbac Laboratories, France), and Terramycin Q-100, containing 100 mg oxytetracycline per ml (Pfizer Laboratories Ltd, New Zealand).

The 10 animals were from 2 litters. The first litter of 4 pigs (2 infected and 2 controls) was injected intramuscularly with Amphoprim (0.3 ml/kg body weight) daily from day 5 to day 9. Amphoprim was then replaced by oral Terramycin Q-100 (15 mg/kg body weight, 3 times daily in the milk formula) for another 4 days. Another 6 pigs, from the second litter, were treated with oral Terramycin Q-100 alone at the same dose rate for 5 days from day 5. All pigs treated with antibiotic were euthanased on day 14.

2.2.7 Necropsy and Sample Collection

The piglets were anaesthetized by intraperitoneal injection with 1-2 ml (depending on body weight) of sodium pentobarbitone (South Island Chemical Ltd., Christchurch, NZ), 250 mg/ml diluted in physiological saline. After collection of a blood sample, the animals were then killed by intracardiac administration of an overdose (2-3 ml) of sodium pentobarbitone. The abdominal cavity of the animal was opened immediately after death using sterile instruments, taking care to avoid bacterial contamination. Some mesenteric lymph nodes were removed and placed in a sterile vial and a sample of the contents of the small intestine were collected on a swab.

The gastrointestinal tract was clamped at the abdominal end of the oesophagus and at the pelvic inlet, excised and immediately placed in chilled physiological saline until dissection. The intestine was then carefully dissected from its mesentery on a cold tray

on ice. Tissues for histological examination were taken from the mid-portion of seven segments of the small intestine: the duodenum from the pylorus to the ligament of Treitz and six equal segments designated proximal, mid-and distal jejunum or ileum. The large intestine was divided into the caecum, and the colon which was in turn divided into two equal lengths designated the proximal and distal colon. Samples for histology were taken from the mid-point of these three segments and also from the pancreas, the liver and three regions of the stomach: cardiac, fundic and pyloric regions. All tissues, except the small intestine, were immediately placed in Bouin's solution. A 3 cm length of tissue was removed from the mid-portion of each of the small intestinal segments, ligated at both ends, filled with Bouin's solution using a syringe and fine hypodermic needle and then submerged in this solution.

2.2.8 Histopathology

2.2.8.1. Histological section preparation

Tissues were fixed in Bouin's solution overnight, changed into 70% alcohol, then dehydrated routinely through graded alcohols in an Automatic Tissue Processer (SE400, Shandon Scientific Co. Ltd., London) and embedded in paraffin wax. From all tissues, 5 µm thick sections were cut, mounted on slides and stained with haematoxylin and eosin. Gram-Twort (Drury and Wallinton, 1967) or Giemsa (Luna, 1968) stains were used only for some sections (e.g. the stomach sections which showed lesions) to confirm the presence of bacterial clumps.

2.2.8.2. Enumeration of the small intestinal lesions

The number of microabscesses in 8 non-serial sections was counted from each segment of the small intestine of infected piglets on days 3 and 5. In some sections, the lesions were not discrete, but there was a continuous area of damaged mucosa for part or all of the circumference of the section (Figure 2.7). These sections were assigned a score, which was considered to be similar to the lesion number which was counted in sections with discrete lesions, e.g. if 100% of the mucosa were involved, the maximal number was assigned, this being the highest number of discrete lesions recorded from any section from segments of the small intestine; if only part of the mucosa in the section was damaged (e.g. 50%), the corresponding proportion of the maximal lesion number was assigned.

2.2.9 Bacteriology

2.2.9.1 Isolation of *Y. enterocolitica*

To detect the presence of *Y. enterocolitica*, mesenteric lymph nodes were directly cultured on both Cefsulodin-irgasan-novobiocin (CIN) agar (Appendix 2.1) and sheep blood agar plates, and the swabs of small intestinal contents on CIN agar. All faecal samples were placed into 2.0 ml of 0.067 M phosphate buffered saline (PBS, pH 7.6) and enriched at 4 ^{0}C for 2-3 weeks before being plated on CIN agar plates and incubated at 29 ^{0}C for 48 hours (Fukushima *et al.*, 1984). *Yersinia*-like colonies on the CIN agar were subcultured on blood agar plates for identification of the biotype and serotype.

Heart blood and liver samples from one control pig, which died on day 2, were cultured aerobically on 5% sheep blood agar plates at 37 ^{0}C for 48 hours.

2.2.9.2 Identification of *Y. enterocolitica*

Serotyping was carried out by slide agglutination with *Y. enterocolitica* typing serum 0:3 (Eco-Bio N.V./S.A. Woudstraat, Belgium) according to the manufacturer's instructions (Appendix 2.2). Briefly, one drop of antiserum 0:3 was placed on a clean slide and a few colonies on a blood agar plate (which had been incubated at 29 ^{0}C for 44 hours) were picked off with a loop, added to the antiserum and mixed thoroughly with the loop until the mixture was cloudy. As a control, bacterial colonies were suspended in a drop of distilled water on the slide. After one minute, the mixture was examined for agglutination, which constituted a positive reaction, while a cloudy drop was negative.

The biotype of isolates was determined according to the method of Mair and Fox (1986). Isolates were tested on the following media: triple sugar iron (TSI) slope, lysine iron agar (LIA) slope, urea, ornithine decarboxylase, sucrose, trehalose, rhamnose, indole, salicin, esculin hydrolysis and D-xylose and Voges-Proskauer (VP) (Appendix 2.2).

2.2.10 Statistical Analysis

The body weights of control and infected piglets aged from birth to 14 days were analyzed by a two-way Analysis of Variance (ANOVA). The lesion numbers in seven

segments of the small intestine from infected piglets on days 3 and 5 were analyzed by a one-way ANOVA. Both ANOVA used the general linear model of the SAS computer programme (SAS Institute Inc., Cary, NC, USA, 1987). The data are presented as mean and standard error of the mean (SE) and the results of ANOVA presented as probability (p). A p value of < 0.05 was considered significant.

2.3 RESULTS

2.3.1 Clinical Signs

Most infected animals (16/19) developed transient anorexia lasting about 24 hours on days 1 or 2, after which the appetite of some pigs became variable. Vomiting occurred in 6 infected pigs, in 3 of these it was frequent and lasted for 3 days. Eighteen infected animals developed diarrhoea, in 16 it began on day 2 or day 3, and in 2 on day 4. Once diarrhoea had started, defaecation occurred often, with faeces being present in the tubs at almost every feed. The faeces were loose or watery and contained white-grey fibrin, and had an acid smell, but contained no visible blood. One of the infected pigs showed no signs of illness during the 3 day experimental period and only once produced faeces (meconium on day 2). Some infected animals were depressed and hunched up. No infected piglets died during the experimental period, however, one control piglet died suddenly on day 2. Bacterial cultures of samples of the heart blood and the liver inoculated on to sheep blood agar plates were negative. No *Y. enterocolitica* were isolated from the small intestinal contents.

After 4 days of injection with Amphoprim, one of the two infected animals had a good appetite but one remained depressed, hunched up, had severe diarrhoea and was hard to feed. By the second day of Terramycin Q-100 therapy, the piglet which refused to feed during Amphoprim treatment regained its appetite. After oral administration of Terramycin Q-100, all infected pigs became active and looked for food. They had good appetites and produced a smaller amount of faeces. All infected and control animals produced loose, brown coloured faeces during oral administration of Terramycin Q-100.

There was no anorexia among control piglets and vomiting occurred only once in three of them. Ten control animals had loose or occasionally watery faeces on days 3 or 4. The frequency of defaecation was less than that in the infected animals and the faeces were free of the acid smell and white-grey fibrin noted in the infected animals.

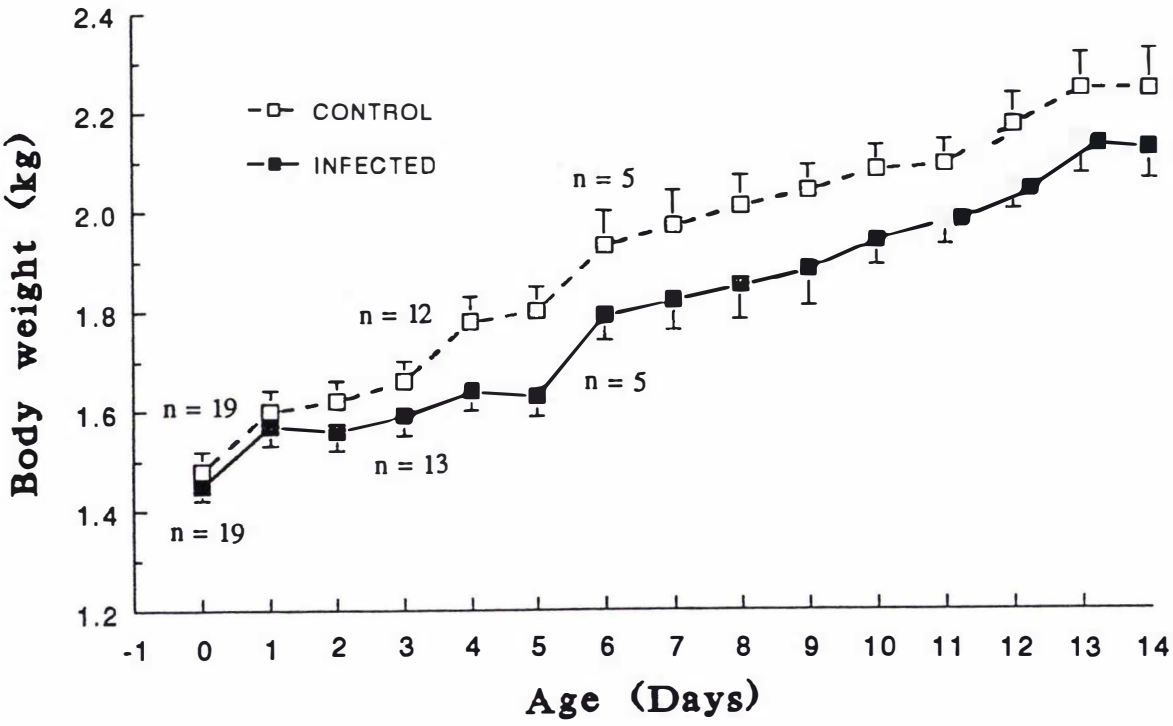

Figure 2.2 Daily body weight in *Y. enterocolitica*-infected and control piglets from 0 (birth, inoculation) to 14 days. Data are presented as Mean ± SE for individual groups. The number of animals day 0 was 19 in each group and the numbers in parenthesis represent the numbers of animals followed to that day. ANOVA used the pooled error mean squares with the greater degrees of freedom. The body weight was lower in the infected group ($p < 0.001$).

Table 2.1. Proportion of positive isolations of *Y. enterocolitica* from faeces, mesenteric lymph nodes and small intestinal contents (positive animal number/examined animal number).

Group	Age (days)	Faeces	Mesenteric lymph nodes	Intestinal contents
Infection	3	18/19	1/4	2/2
	5	13/13	2/8	8/8
	14	2/5	0/5	2/5
Control	3	0/18	0/4	0/2
	5	2/12	0/7	2/7
	14	0/5	0/5	0/5

The mean body weights of control and infected piglets throughout the 14 days are shown in Figure 2.2. Body weights in control and infected piglets diverged during the period of infection from birth to day 5, but after antibiotic therapy from day 5 the body weights of the infected piglets became parallel to those of the controls from day 6 to the termination of the experiment. The mean body weight of infected piglets was significantly lower than that of controls ($p < 0.001$).

2.3.2 Bacterial Isolation

The proportion of samples of faeces, small intestinal contents and mesenteric lymph nodes from which *Y. enterocolitica* were isolated is shown in Table 2.1. Eighteen of 19 infected piglets showed positive cultures for *Y. enterocolitica* from faecal samples by day 3. *Yersinia* was isolated from the faeces of all infected animals (13/13) by day 5. No *Y. enterocolitica* was isolated from the faeces (only meconium on day 2, no faeces on other days) of one infected animal which showed no clinical signs of diarrhoea during the first 3 days, however, its mesenteric lymph nodes were positive for *Y. enterocolitica.* In 2 of the 5-day-old infected animals, mesenteric lymph nodes were positive for *Y. enterocolitica.* The same strain of *Y. enterocolitica* was also isolated from the faeces of 2 control animals on day 5 (but not on previous days) and from small intestinal contents of these animals obtained at necropsy (Table 2.1).

During the period of oral Terramycin Q-100 administration, no *Y. enterocolitica* was isolated. Three days after Terramycin Q-100 therapy was terminated, *Y. enterocolitica* was recovered again from the faeces of infected animals (2/3), and was present in the small intestinal contents (2/3) postmortem. *Yersinia enterocolitica* was continually present in the faeces of infected animals injected with Amphoprim until administration of Terramycin Q-100 commenced.

All isolates had a positive serum 0:3 agglutination reaction. All isolates were identified as biotype 4, indicated by the following: positive for TSI agar, A/AG (acid/acid with) gas, LIA, K/A (alkali/acid), urea, ornithine decarboxylase, sucrose, and trehalose, and negative for rhamnose, indole, salicin, esculin hydrolysis and D-xylose; VP positive at 29 ^{0}C but not at 37 ^{0}C.

Table 2.2. Number of piglets showing histopathological changes in the gastrointestinal tract and the liver in piglets infected with *Y. enterocolitica* for 3 or 5 days

Tissue	Postinfection period (days)	
	3 (*n*=6)	5 (*n*=8)
Stomach	1	2
Duodenum	2	5
Proximal jejunum	5	6
Mid-jejunum	5	7
Distal jejunum	5	8
Proximal ileum	4	6
Mid-ileum	4	7
Distal ileum	6	8
Caecum	4	6
Proximal colon	0	2
Distal colon	1	1
Liver	0	7

Table 2.3. Number of lesions in the small intestine in *Y. enterocolitica*-infected piglets at 3 or 5 days postinfection and ANOVA of lesion number between 3 and 5 days postinfection

Tissue	Postinfection (days)				p
	3 (*n*=6)		5 (*n*=8)		
	Mean ± SE	Range	Mean ± SE	Range	
Duodenum	2.81 ± 2.71	0 - 16	8.52 ± 6.55	0 - 54	NS
Proximal jejunum	2.67 ± 1.91	0 - 12	8.34 ± 4.05	0 - 29	NS
Mid-jejunum	1.48 ± 0.48	0 - 3	6.13 ± 1.64	0 - 14	*
Distal jejunum	3.81 ± 1.94	0 - 13	6.98 ± 4.72	0 - 39	NS
Proximal ileum	0.90 ± 0.35	0 - 2	13.59 ± 6.54	0 - 52	NS
Mid-ileum	1.42 ± 0.61	0 - 4	10.92 ± 5.44	0 - 36	NS
Distal ileum	11.63 ± 2.87	4 - 21	12.63 ± 6.96	1 - 60	NS

NS $p > 0.05$; * $p < 0.05$

2.3.3 Histopathology

No histopathological changes were noted in the gastrointestinal tract of any control piglets, but lesions were present in all infected animals. The frequency of lesions in the gastrointestinal tract and the liver of infected piglets at 3 and 5 days postinfection is shown in Table 2.2. No lesions were seen in any segments of the gut or other organs from 14-day-old infected animals which had been treated with antibiotics.

Table 2.3 shows the number of lesions counted in 8 sections of each of the seven segments of the small intestine from infected animals at 3 and 5 days postinfection and the results of ANOVA of the lesion numbers between the two infection periods. A significant increase in lesion number from day 3 to day 5 after infection was present only in the mid-jejunum ($p < 0.05$). In other portions of the small intestine, there was no significant increase in lesion numbers although the mean values were greatly increased with the longer period of infection. The greater number of lesions in every segment of the small intestine on day 5 indicated that *Y. enterocolitica* continued to invade the tissue up to day 5 after inoculation.

Day 3. Bacterial clumps surrounded by a few neutrophils were present in the mucosa of the cardiac region of the stomach in one infected pig on day 3. Most sections of the small intestine from infected animals contained microabscesses which consisted of neutrophils surrounding bacterial colonies. The colonies of Gram-negative coccobacilli were most numerous in the distal ileum, which was involved in all infected piglets (6/6). The villi over Peyer's patches were most severely damaged in the majority of piglets (Figure 2.3). Most of the microabscesses in the small intestine were located in the bases of villi or in the crypt region (Figure 2.4). Occasionally, a microabscess extended through the muscularis mucosa and involved the submucosa. The microabscesses contained large colonies of bacteria surrounded by numerous neutrophils (Figure 2.5). Although bacterial clumps could be seen near the middle, or at the tip, of some villi, most villi adjacent to microabscesses were intact and extensive damage was noted only in a few villi lying between the microabscesses. Microabscesses were found in the lamina propria of the caecum, and occasionally in the lamina propria or submucosa of the colon. The visceral organs showed no lesions at this stage of infection.

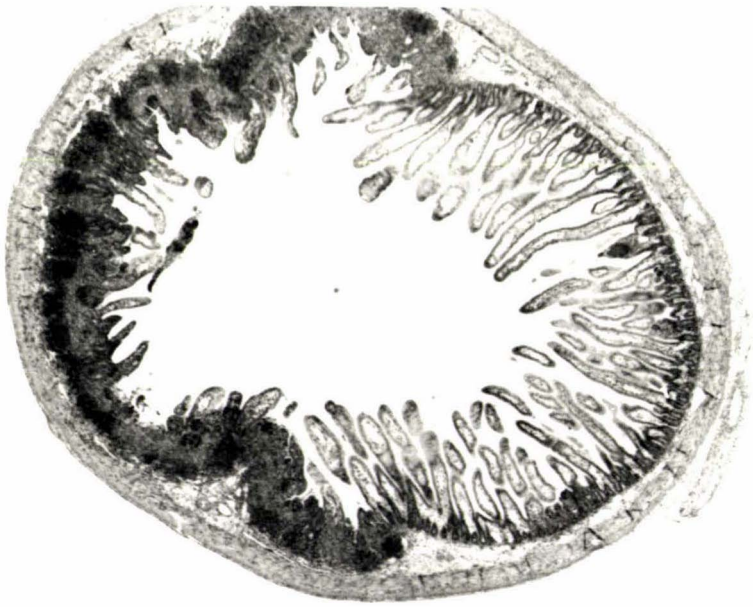

Figure 2.3 Photomicrograph of a section of the distal ileum of a *Y. enterocolitica*-infected piglet killed at 3 days postinfection. The villi over Peyer's patches were severely damaged by bacteria invasion. (HE, x26)

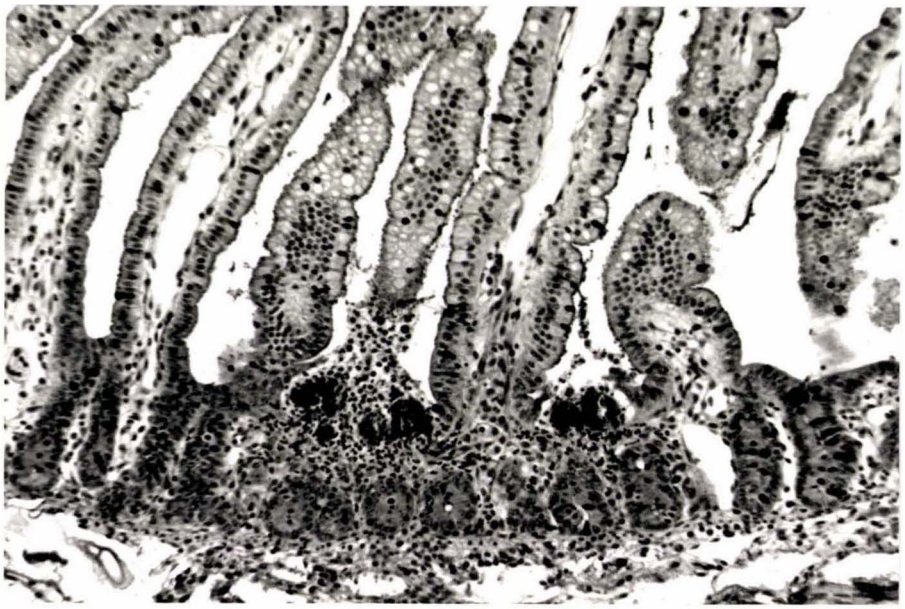

Figure 2.4 Photomicrograph of a section of the distal jejunum of a *Y. enterocolitica*-infected piglet killed at 3 days postinfection. Microabscesses are present in the base of villi and in the crypt region (AB/PAS, x136).

Day 5. Invasion of the gastrointestinal tract and liver by *Y. enterocolitica* was increased at 5 days postinfection (Table 2.2). Two piglets showed more extensive involvement of the stomach mucosa (Figure 2.6) than was seen in the 3-day-old pig. More microabscesses were also found in the duodenum and the jejunum (Table 2.3). These lesions were located in the lamina propria of both the crypts and villi. These microabscesses were extensive, and destroyed adjacent villi. In the jejunum, numerous microabscesses or bacterial clumps surrounded by a few neutrophils were also present in the middle or upper villi. In the ileum, most villi, particularly those over Peyer's patches, were completely destroyed, leaving only a few short villi intact. In the distal ileum of some piglets, the entire villus architecture was lost and only bacterial clumps, interspersed with many inflammatory cells, were observed in association with intact crypts (Figure 2.7). Lesions in the large intestine were similar to those seen on day 3. Seven of 8 infected piglets had liver microabscesses with neutrophils surrounding bacterial colonies (Figure 2.8). No abnormalities were found in mesenteric lymph nodes and other visceral organs including the pancreas, spleen and kidneys.

Day 14. No evidence of bacterial invasion in the intestine was observed in all infected piglets which were treated with antibiotics and killed on day 14, although two of them re-excreted *Y. enterocolitica* from day 13 (3 days after the termination of Terramycin Q-100 therapy).

Controls. No *Y. enterocolitica* invasion of the gastrointestinal tract was observed in any control piglets at 3, 5 and 14 days of age although two piglets showed positive faecal cultures on day 5.

2.4 DISCUSSION

The present study demonstrated that newborn, colostrum-deprived piglets can be used successfully as an experimental model of *Y. enterocolitica* enteritis. Infected piglets developed clinical manifestations and gastroenteritis which were comparable to those in humans. Children infected with *Y. enterocolitica* commonly show diarrhoea, fever, abdominal pain, nausea, vomiting and anorexia (Vantrappen *et al.*, 1977; Marks *et al.*, 1980; Lee *et al.*, 1991). Other manifestations of *Y. enterocolitica* infection occur less frequently. Marks *et al.* (1980) summarized the clinical signs in 57 children (median age 22 months, with the range of 2 months to 14 years) infected with *Y. enterocolitica* biotype 4, serotype 0:3, which was the same strain of organism used to inoculate piglets

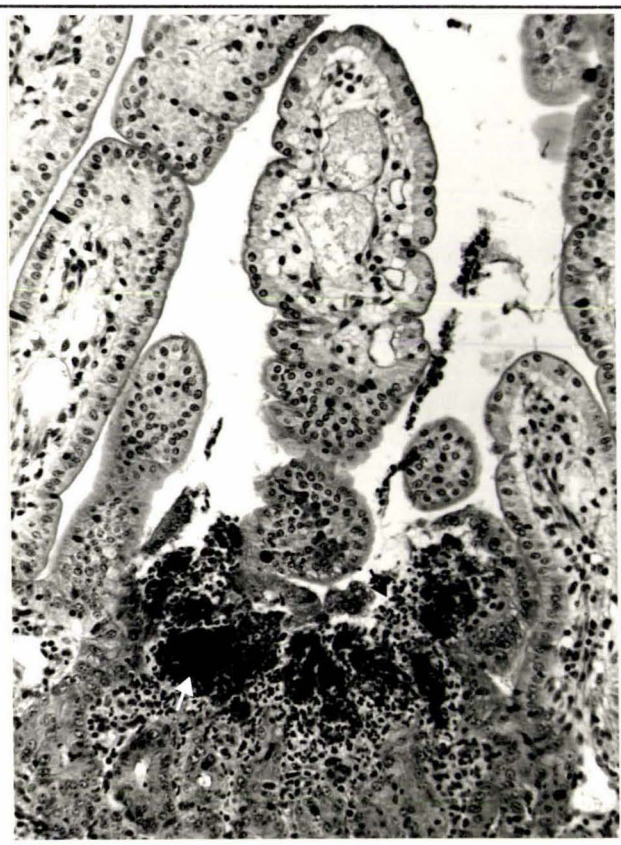

Figure 2.5 Photomicrograph of a section of the distal jejunum of a *Y. enterocolitica*-infected piglet killed at 3 days postinfection. The microabscess contains large colonies of bacteria (white arrow) surrounded by neutrophils (black arrow) (AB/PAS, x220).

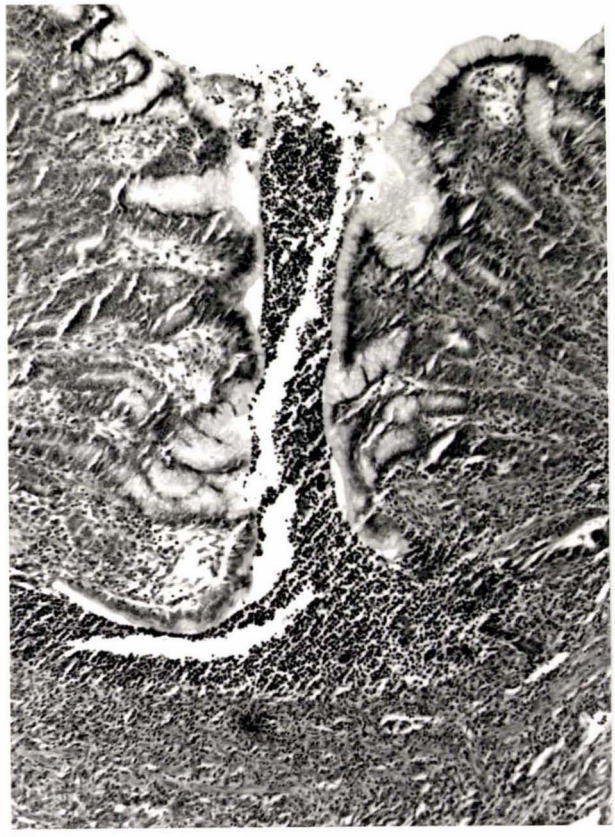

Figure 2.6 Photomicrograph of a section of the stomach of a *Y. enterocolitica*-infected piglet killed at 5 days postinfection demonstrating infiltration of the mucosa by neutrophils. (HE, x110)

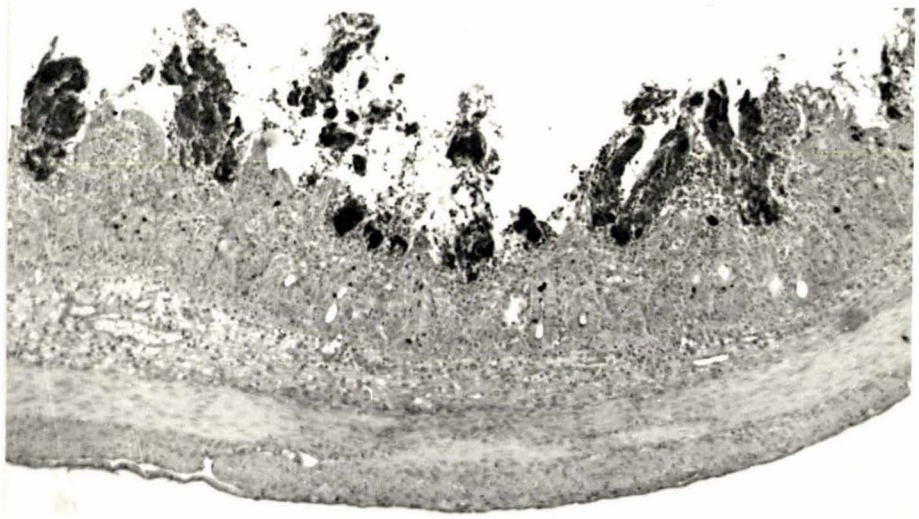

Figure 2.7 Photomicrograph of a section of the distal ileum of a *Y. enterocolitica*-infected piglet killed at 5 days postinfection showing complete destruction of the villi by *Y. enterocolitica* invasion (AB/PAS, x110).

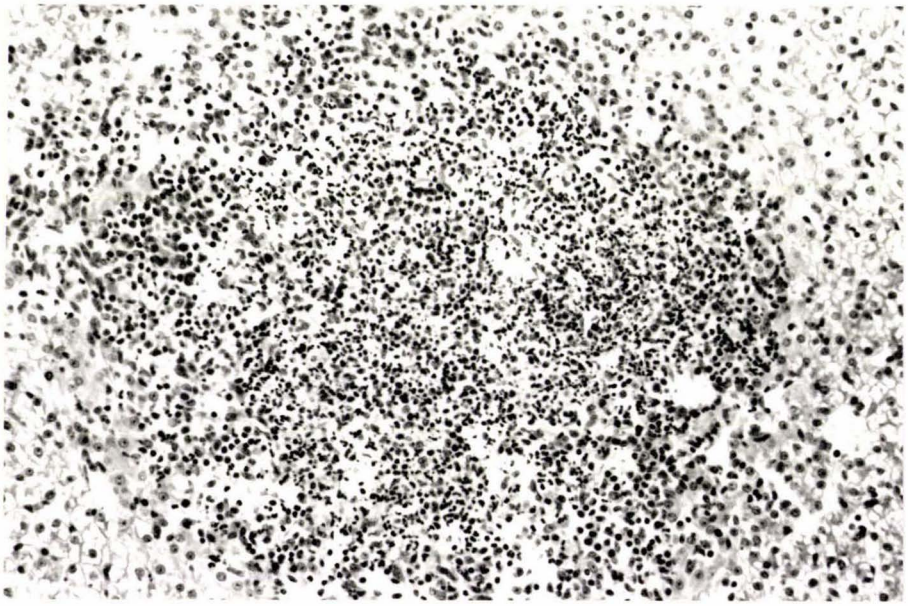

Figure 2.8 Photomicrograph of the liver of a *Y. enterocolitica*-infected piglet killed at 5 days postinfection displaying a microabscess (HE, x220).

in the present study. Diarrhoea, of an average duration of 14 days, was present in 56 children (98%), fever in 39 (68%), vomiting in 22 (38%) and abdominal pain in 31 of the 48 children who were old enough to be evaluated (64%). In older patients aged from 15 to 57 years, Vantrappen *et al.* (1977) reported diarrhoea in 78%, vomiting in 8%, anorexia in 22% and nausea in 13%. The frequencies of diarrhoea (94%, 18/19) and vomiting (31%, 6/19) in the *Y. enterocolitica*-infected piglets in the present study were similar to those in children, but higher than those in human adults. The occurrence of anorexia in 84% of piglets was higher than was reported in humans (Vantrappen *et al.*, 1977). In animal studies, there was also a high frequency of diarrhoea, 87-88%, (20/23 and 21/24) following infection of rabbits with two strains of *Y. enterocolitica* (Pai *et al.*, 1980). In piglets, diarrhoea and anorexia were reported by Robins-Browne *et al.* (1985). Clinical signs have not been reported in any of the mouse models of infection with *Y. enterocolitica.*

Three common clinical signs observed in acute *Y. enterocolitica* diarrhoea in children - fever, abdominal pain and nausea - were not evaluated in the present study. Nausea cannot be determined in animals and abdominal pain can only be assessed subjectively. Many of the infected piglets assumed a hunched-up posture suggesting that they had abdominal discomfort. Measurement of rectal temperature appeared to cause distress to the piglets. Since the major purpose of these experiments was to assess gut development, the benefits of measuring body temperature were considered to be less than the possible confounding effects of the stress the procedure might cause.

Morphological changes in the intestine, in particular diffuse enteritis, were accompanied by mucosal ulceration, which is similar to the findings described in humans (Vantrappen *et al.*, 1977), pigs (Robins-Browne *et al.*, 1985) and rabbits (O'Loughlin *et al.*, 1986) with *Y. enterocolitica* infection. *Yersinia enterocolitica* appeared to have a particular affinity for the distal ileum, which was involved in all infected piglets (6/6) by 3 days postinfection. Invasion by the bacteria became more extensive from 3 days to 5 days after inoculation. Not only did the number of pigs with enteritis increase during this short period but the lesion number also dramatically increased in all segments of the small intestine, particularly in the proximal and mid-ileum. The mean (± SE) number of lesions increased from 0.90 ± 0.35 and 1.42 ± 0.61 per section respectively in the proximal and mid-ileum on day 3 to 13.59 ± 6.54 and 10.92 ± 5.44 per section on day 5, although the results of the ANOVA did not show a significant difference between the two age groups. The reason why the ANOVA failed to show a significant difference

in lesion development from day 3 to day 5 was probably because there was very large variation within groups. The range of lesion numbers was 0 to 52 and 0 to 36 per section in the proximal and mid-ileum respectively. It was difficult in the present study to quantify the progression of the disease because only seven sites were sampled from the whole length of the small intestine and the sections examined histologically may not have been representative of the segment as a whole. Bacterial colonies mainly occurred at the base of villi and in crypts and occasionally also at the tip or the middle of the villi in the small intestine. This distribution of lesions is seen in all species except the mouse, in which bacterial invasion occurred only in Peyer's patches in the ileum and caecum but not in the duodenum and upper small intestine (Carter, 1975). The reason for this difference between mice and pigs or rabbits remains to be clarified.

The severity of damage to the ileum was a result of the well established affinity of *Y. enterocolitica* for the lymphoid tissue of Peyer's patches (Grützkau *et al.*, 1990). The sequence of *Y. enterocolitica* invasion has been demonstrated in rabbits and mice. Initially, *Y. enterocolitica* adhered to mucus and multiplied there at a high rate (Pærregaard *et al.*, 1991). The ability to adhere to tissue did not differ between the proximal and distal portions of the rabbit small intestine (Pærregaard *et al.*, 1990). Adhesion to mucus might facilitate bacterial colonization of the mucosal surface. The bacteria are generally capable of adhering to the absorptive epithelial cells but not of invading them. In contrast, *Y. enterocolitica* binds to M cells, which are specialised cells present in the follicle-associated epithelium covering the lymphoid follicles of Peyer's patches of the small intestine, and this results in uptake of the bacteria followed by transport into the Peyer's patch dome (Grützkau *et al.*, 1990), where the organisms multiply and spread into the lamina propria of the villi (Hanski *et al.*, 1989). Involvement of M cells explains the early presence of bacterial clumps in the Peyer's patches. The bacteria, which have multiplied in Peyer's patches, are then drained through lymphatics to mesenteric lymph nodes or to the systemic circulation resulting in systemic infection (Bottone, 1984; Beeching *et al.*, 1985).

The liver seemed to be an important target for *Y. enterocolitica* invasion in these newborn, colostrum-deprived pigs. Liver involvement at 5 days postinfection in the present study (87.5%) was much higher in frequency than that in humans (12%) (Sæbø and Lassen, 1991), in whom the antemortem diagnosis of liver abscesses remains difficult, despite the use of modern imaging techniques such as ultrasonography (Beeching *et al.*, 1985). Liver involvement in humans is thus difficult to diagnose

during acute *Y. enterocolitica* infection, particularly when there is no enlargement of the liver. In most cases, diarrhoea is the main symptom and once yersiniosis is diagnosed antibiotics are administered and microabcesses in the liver would quickly disappear. Microabscesses in the liver were no longer present after antibiotic therapy in the piglets in the present study, along with the absence of bacterial clumps in the mucosa of the gut. In humans, hepatic abscesses due to *Y. enterocolitica* have been diagnosed in the absence of gastroenteritis, e.g. a 3-month-old male infant with an enlarged liver was diagnosed by laparotomy as having hepatic abscesses. Culture of these abscesses showed a pure growth of *Y. enterocolitica*, however, the blood and faecal samples were negative (Ryan *et al.*, 1979). In other cases, multiple liver abscesses due to *Y. enterocolitica* were found at postmortem examination (Beeching *et al.*, 1985).

The absence of microabscesses in the liver on day 3 indicated that involvement of the liver by the bacteria was later than in the gut. This was also the case in other animal studies. Hepatic microabscesses were present in rabbits by days 6 and 14 and in mice by day 12 after oral infection with *Y. enterocolitica* (Carter, 1975; O'Loughlin *et al.*, 1986). Robins-Browne *et al.* (1985) did not report microabscesses in the liver of the one piglet that was examined 6 days after infection, nor were liver lesions observed by Schiemann (1988), although *Y. enterocolitica* were isolated from the liver on days 6 and 8.

The present study appears to be the first report of microabscesses, consisting of bacterial clumps surrounded by inflammatory cells, in the stomach of animals infected with *Y. enterocolitica*. Bacterial colonization of the mucosa of the stomach would be favoured by the less acid pH in the stomach in the newborn where there would likely be suitable conditions for bacterial growth. Brocklehurst and Lund (1990) demonstrated that *Y. enterocolitica* multiplied at pH 4.18 in culture medium. The gastric pH is higher in newborns than in older animals: in newborn dogs it was stable at a mean value of 5.85 until the 7th postnatal day, after which pH decreased to 3.45, then increased to 4.95 by the 18th day (Malloy *et al.*, 1979); in newborn piglets the gastric acid output was significantly lower than in older sucking pigs (Cranwell, 1985). In the present study, $NaHCO_3$ administered immediately following the inoculation, could have neutralized some or all of the acid produced by the stomach, making the conditions in the stomach more favourable for colonization of the mucosa by *Y. enterocolitica*.

Colostrum deprivation would increase the animal's susceptibility to *Y. enterocolitica*

infection. Schiemann (1988) compared full-term piglets which sucked antibody-positive colostrum for 36 hours with caesarian-delivered piglets deprived of colostrum. The former did not develop any signs of illness until 15 days. In the present study, all the animals challenged showed illness by 5 days postinfection. Although one piglet in the infected group did not develop diarrhoea by day 3 when it was killed, the positive *Y. enterocolitica* isolation and the typical histopathological lesions in the gut indicated that this animal was infected. This suggests that diarrhoea is not always a clinical sign in the early stage of *Yersinia* enteritis. The great susceptibility of newborn, colostrum-deprived piglets to yersiniosis may not be due solely to the colostrum deprivation, but may relate in part to their age and virtual absence of a normal intestinal flora. Robins-Browne *et al.* (1985) failed to show illness in one piglet delivered by caesarian section and maintained free of specific pathogens for 13 days before infection with 4 x 10^9 CFU of *Y. enterocolitica* (a dose which produced enteritis in 1- or 2-day-old piglets in the same study).

Full-term, naturally-born piglets, from sows which farrowed without induction, as used here, have some advantages for experimental infection when compared with piglets delivered by caesarean section (Robins-Browne *et al.*, 1985; Schiemann, 1988). First, naturally-born piglets are less expensive than caesarean-delivered piglets and the availability of animals for the model is greater. In the present study 38 piglets were taken from 11 litters. Using these naturally-born piglets, it was easier to match control and infected animals on the basis of body size. Secondly, in the present study all piglets infected with 3 x 10^{10} CFU of bacteria developed moderately severe gastroenteritis as judged by clinical signs and histopathological examination, but none died during the 5 day experimental period. In contrast, in one study of caesarian-delivered piglets, two of eight animals died after challenge with 10^{10} CFU of *Y. enterocolitica* (Schiemann, 1988), and in another study, two caesarian-delivered piglets inoculated with a high dose (4 x 10^{10}) died on day 1 without showing clinical signs (Robins-Browne *et al.*, 1985). Thus, the premature status of caesarian-delivered piglets may make them more susceptible to death from yersiniosis. Although naturally-born piglets were good to use as a model of *Y. enterocolitica* infection, there were two disadvantages: it was inconvenient when piglets were born during the night, and the bacterial inoculum had to be available for challenge whenever the sows farrowed. The latter problem was solved by the storage of the bacteria at -70 ^{0}C.

All but one control piglet remained healthy during the present study and there were no

lesions in any tissues examined. No diagnosis could be made after a *postmortem* examination of the control piglet that died suddenly on day 2 after exhibiting an 8 hour period of fever. No bacteria were cultured from heart blood or liver samples on blood agar plates nor were *Y. enterocolitica* cultured from the small intestinal contents of this animal. On day 5, two control animals showed positive cultures for *Y. enterocolitica* strain biotype 4 serotype 0:3 from faeces and small intestinal contents, but cultures from faeces were negative by day 4. There was no *Y. enterocolitica* invasion of the gut in these two animals. It was unclear from these results whether the level of bacterial contamination was low, whether the time was insufficient for development of lesions or whether the animals were old enough (5-day-old) to be resistant to *Y. enterocolitica* invasion and were only carriers of the bacteria.

The ideal experimental design would incorporate matching animals for both body weight and sex and also pair feeding. It was difficult to match both body size and sex in control and infected pairs from a particular litter, so the pairs were matched for body size only, except in the first two litters where they were also sex matched. No difference in clinical signs or pathological changes was observed between males and females in these litters. Mair and Fox (1986) reported both sexes of humans to be equally affected by enteritis due to *Y. enterocolitica*. Although matching the piglets on sex was discontinued after the second litter, the total numbers of males and females in control and infected groups were equal.

Strict pair feeding, which would allow the evaluation of reduced milk intake *per se*, poses important welfare issues. Pair feeding was not possible both on welfare grounds and because of the confounding effect of hungry control piglets expending energy when left unfed. When infected piglets became sick, they often completely rejected the milk offered. Healthy control piglets would have been left very hungry if they had been pair-fed. Sick infected piglets were depressed and were quiet or slept and expended little energy, whereas unfed control piglets were restless, moved around the tub looking for more food and consumed more energy than infected animals did. Although pair feeding was not carried out, the milk intake of some control piglets was restricted during the first 5 days (Appendix 1).

The purpose of the antibiotic therapy in the present study was to promote clinical recovery from the infection, so that subsequent effects on gut development could be investigated. Oral Terramycin Q-100 was more effective than intramuscular Amphoprim

injection in treating *Y. enterocolitica* infection in newborn piglets. Amphoprim contains trimethoprim 40 mg/ml, to which this strain of *Y. enterocolitica* has been shown to be sensitive (McCarthy and Fenwick, 1991). However, 1 of 2 infected piglets failed to respond to Amphoprim injection. Once Terramycin Q-100 was administered orally to an infected piglet, it began to recover on the next day. In contrast to human patients, piglets recovered quickly from the bacterial infection after antibiotic therapy. In 3 infected piglets from the second litter, Terramycin Q-100 therapy for 5 days resulted in the absence of bacterial clumps in the gut and liver by the time the animals were killed at 14 days of age. In human patients, treatment with antibiotics resulted in the disappearance of most symptoms and signs after 4 to 6 weeks and multiple focal defects in the liver were completely resolved on liver scan after 42 days of intravenous ampicillin sodium (Ryan *et al.*, 1979), although the radiographic pictures of "follicular ileitis" persisted for several months (Vantrappen *et al.*, 1977).

Although only 5 days of Terramycin Q-100 therapy caused no morphological lesions to be present and no *Y. enterocolitica* were isolated from the faeces of any infected piglets during the therapy, the faeces of 2 of the 3 piglets were positive for *Y. enterocolitica* again 3 days after termination of administration of the antibiotic. *Yersinia enterocolitica* was also cultured from the small intestinal contents collected at necropsy from the same animals. It is unclear whether the *Y. enterocolitica* isolated from these 2 piglets persisted in the gut in spite of antibiotic treatment, whether the negative culture results during the previous days were false because of the presence of Terramycin Q-100 in the samples cultured or whether the piglets were re-contaminated from the environment. The re-excretion of *Y. enterocolitica* from these two piglets which showed no illness or histopathological changes in the gut supports the belief that pigs can be natural reservoirs for human yersiniosis, carrying the bacteria without clinical signs.

A piglet model of *Y. enterocolitica* infection has been established. All control animals survived well except one, which died on day 2 but did not show any lesions in the gut and liver and was excluded from the following study. Using this model, it was possible to study in detail some pathophysiological effects of *Y. enterocolitica* infection on gut development in newborn piglets and subsequent recovery following antibiotic therapy. These studies are presented in the next two Chapters.

2.5 SUMMARY

Yersinia enterocolitica infection was studied in newborn, colostrum-deprived piglets. Nineteen were challenged with *Y. enterocolitica* biotype 4 serotype 0:3 within 1-3 hours of birth, and an equal number of litter mates served as controls. Six from each group were killed on day 3, and 8 infected and 7 controls (one died on day 2) were killed on day 5. Five of each group were administered antibiotics from day 5 to days 10 or 13 and were killed on day 14. Eighteen of 19 piglets (94%) in the infected group developed diarrhoea at 2 or 3 days postinfection, and the diarrhoea lasted until at least day 5. Six of 19 animals vomited (31%). *Yersinia enterocolitica* were isolated from the faeces of the 18 infected animals with diarrhoea and from the small intestinal contents of all infected animals at death.

Microabscesses surrounding bacteria were present in the mucosa of the stomach in 1 of 6 piglets at 3 days and in 2 of 8 piglets at 5 days postinfection. The liver was not involved by day 3, but 7 of 8 infected piglets had microabscesses by day 5. Similar lesions were present at the base of the villi in all parts of the small intestine, but not in every segment of every piglet, except the distal ileum, in which all animals developed lesions by 3 days postinfection. By day 5, microabscesses were more extensive and villi were involved, particularly in the ileum, where many villi were destroyed by large microabscesses. After antibiotic treatment, all infected animals recovered clinically from the infection by day 14 and lesions in the gut and liver were no longer present.

Chapter 3

MORPHOLOGICAL DEVELOPMENT OF NEWBORN PIGLETS INFECTED WITH *YERSINIA ENTEROCOLITICA*

3.1 INTRODUCTION

The growth of underprivileged children in developing countries usually falls substantially below that of children in more developed countries (Black, 1991). The inadequate dietary intake and frequent occurrence of infectious diseases are often implicated as the factors adversely affecting growth in infancy and early childhood. Gastroenteritis is one of the most frequent infectious diseases in children in developing countries (Guerrant and McAuliffe, 1986), not only causing childhood death but also growth retardation. Martorell *et al.* (1975) studied 716 Guatemalan children under 7 years of age for 23 months. Illness histories were collected every 2 weeks and related to 6 monthly and yearly increments in weight and height. A higher percentage of days ill with diarrhoea was found to be significantly associated with reduced weight and height gains. Investigation of children 6-36 months of age in The Gambia by Rowland *et al.* (1977) showed similar results. Reduction in weight gain in children under 3 years of age was significantly associated with diarrhoeal disease also in Uganda and Mexico (Cole and Parkin, 1977; Condon-Paoloni *et al.*, 1977).

The effects of gastroenteritis on the growth of internal organs have not been documented. The only available data from infants and children with gastroenteritis are morphological measurements of villus height and crypt depth from biopsy samples of the mucosa of the small intestine (Davidson and Barnes, 1979). It is impossible to carry out other investigations, such as measurement of intestinal length and weight and organ weights in humans, so studies must be made in animal models. The weight of the small intestine and its mucosa decreased in undernourished, neonatal rabbits, but not in normal, suckled rabbits, when both were infected with *Y. enterocolitica* (Butzner and Gall, 1988b). There are few reports on the effects of gastroenteritis on the growth of internal organs including the gastrointestinal tract. Most investigators focused only on changes in villus length and crypt depth rather than effects on the intestinal length or weight. The changes in structure of the small intestinal mucosa have been described in

many infectious diseases, the most obvious of which was a decrease in villus height and an increase in the crypt depth in most enteroviral infections in pigs (Kelly *et al.*, 1972; Davidson *et al.*, 1977; Pearson and McNulty, 1977) and calves (Mebus *et al.*, 1971), and in enterobacterial infection such as *Y. enterocolitica* infection in rabbits (O'Loughlin *et al.*, 1986) and *Salmonella* enterocolitis in many species (Wilcock, 1986).

Bacterial gastroenteritis has been repeatably produced in newborn, colostrum-deprived piglets infected with *Y. enterocolitica* (Chapter 2, 2.3.3). These animals have been used here as a model to investigate the effects of gastroenteritis on growth of the body, gastrointestinal tract and non-intestinal organs during the period of severe diarrhoea and after recovery following antibiotic therapy. In addition, the small intestinal villus height, crypt depth, crypt cell proliferation, number of goblet cells and structure of the enterocytes of the villi have been examined. The results are reported here.

3.2 MATERIALS AND METHODS

3.2.1 Experimental Design

The 37 piglets used to develop the animal model of bacterial gastroenteritis described in Chapter 2 (2.2.2) were the experimental animals for this study. Nineteen were infected with *Y. enterocolitica* and 18 served as controls. Animal housing, feeding, and inoculation with *Y. enterocolitica* have been described (Chapter 2, 2.2.2). Six infected and 6 control piglets were killed at 3 days, and 8 infected and 7 controls were killed on day 5. The remaining 10 animals, 5 from each group, were given antibiotic therapy from day 5 to either day 9 or 13, and killed at 14 days.

To study the effect of *Y. enterocolitica* on crypt cell proliferation, 13 of the 37 piglets, 3 infected and 4 controls on day 5, and 3 infected and 3 controls on day 14, were injected intraperitoneally twice with 5-Bromo-2'-deoxy-uridine (BrDU) (Sigma Chemical Co. USA) dissolved in sterile physiological saline to give a solution of 5 mg/ml. Ten mg of BrDU per kilogram body weight were injected on both occasions, at 1 and 2 hours before these animals were killed.

3.2.2 Measurement of Body Weight and Length

All animals were weighed daily after being fed during the period of the experiment.

The birth weight was recorded just before the animals were inoculated and the last weight immediately after euthanasia. After death, two measurements of body length were made. First, crown rump length was measured from a point between the ears to the root of the tail and secondly, nose rump length from a point on the top of the nose to the root of the tail, both following the contours of the skin along the dorsal mid-line.

3.2.3 Dissection and Internal Organ Measurement

The piglets were killed by an intracardiac injection of sodium pentobarbitone (Chapter 2, 2.2.7). The whole gastrointestinal tract, with the spleen, liver and pancreas attached, was carefully removed from the abdominal cavity and submerged immediately in chilled 0.85% NaCl. The stomach was emptied, washed in saline, blotted dry and weighed on an electric balance (Mettler AT 200, Switzerland). The liver, pancreas, spleen and kidneys were removed and weighed.

Dissection of the small intestine was performed on a tray on ice. The length of the duodenum, from the pylorus to the duodena-jejunal flexure where the mesentery starts to lengthen to support the jejunum (Williams *et al.*, 1989), was measured. The remainder of the small intestine, from the duodena-jejunal flexure to the ileocaecal fold, was dissected from its mesentery, and divided equally into two parts: the upper was designated jejunum and the lower ileum. Each part was then cut into three equal segments, so that the six segments were designated proximal, mid- and distal jejunum or ileum. The length of one of the six segments was measured by holding one cut end of the segment and allowing it to hang vertically next to a long ruler.

A 3 cm length of tissue was taken from the middle region of each segment of the small intestine for histological study. The remainder of the duodenum and a 10 cm portion of each of the other six segments, immediately adjacent to the area taken for histology, was removed, opened longitudinally and the intestinal contents gently *wiped* off with a glass slide. The mucosa was *scraped* off the underlying muscle with a glass slide always by the same person. The mucosa and muscle were placed in separate tared containers and weighed. The total weights of mucosa and muscle in each segment were calculated from the weight of mucosa and muscle from the 10 cm length. The weight of the duodenal mucosa and muscle included that taken for histological study. The weights of the mucosa of all seven segments were added together to give the total weight of mucosa. Total muscle weight of the small intestine was determined in the

same way. Total small intestinal weight was the sum of the weights of total mucosa and total muscle.

The lengths of the caecum and colon were measured. The colon was divided into two portions of equal length, designated proximal and distal colon. The caecum and the proximal and distal colon were weighed after their contents had been gently wiped off. The mucosa was scraped from the caecum and from 5 cm lengths of the proximal and distal colon and stored at -20 ^{0}C for later measurements of Na^+-K^+-ATPase and protein concentration (Chapter 4, 4.2).

3.2.4 Histological Section Preparation

The 3 cm long tissue sample taken from the middle region of each segment of the small intestine for histological study was ligated at each end, injected with Bouin's solution via a fine needle to form a partially inflated tube, and then submerged into Bouin's solution overnight. The tissue was then transferred to 70% alcohol. Each segment was cut into a 0.5 cm length for transverse sectioning and dehydrated routinely through graded alcohol in an Automatic Tissue Processor (SE400, Shandon Scientific Co. Ltd., London). Tissues were embedded in paraffin wax. Sections 5 µm thick were cut and mounted on slides. From each block, 20 sections were cut. In general, serial sections were used unless some sections were damaged during processing, each 4 adjacent sections being mounted on single slides to give a total of 5 slides per block. Three slides, not serial, were stained with haematoxylin and eosin (HE), 1 slide was stained with both Alcian blue and periodic acid Schiff (AB/PAS, Appendix 3.1) and 1 processed for immunostaining (Appendix 3.2).

In samples from piglets injected with BrDU, immunostaining to visualize BrDU was performed according to the technique of Holle and Birtles (1990) (Appendix 3.2). This method used monoclonal antibodies to detect the incorporation of the thymidine analogue 5-bromo-2'-deoxyuridine into DNA during the S-phase of the cell cycle.

3.2.5 Morphometric Measurements

Morphological measurements were made using a digitising tablet (Sketchmaster, GTCO Corporation, Columbia, MD, USA) and an associated computer programme (SigmaScan: Jandel Scientific, California, USA). The image of the histological slides was projected

on to the digitising tablet using a microprojector (Leitz, Wetslar). Using a stage micrometer, the final magnifications for measuring the length of villi and crypts were 180 x, but 360 x for cell number counting and measurement of the area of the crypts. Slides were coded instead of being labelled, so that measurements were made while the source of the tissue was unknown.

3.2.5.1 Measurements of villus height and crypt depth

The villus height, from the tip to the crypt mouth (the crypt-villus junction), and crypt depth, from the crypt mouth to the base, were determined in 30-50 well-orientated villi in HE stained slides. The datum for each sample was the mean of all measurements from the sample. The villus height/crypt depth ratio was calculated.

3.2.5.2 Counting goblet cells

Goblet cells were stained either red (PAS positive-neutral mucin), blue (Alcian blue positive-acidic mucin) or shades of purple (a mixture of neutral and acid glycoproteins) (Figure 3.1). All positive-staining goblet cells were counted together in 15 well orientated crypt-villi in each segment of the small intestine (Brown *et al.*, 1988). The mean population of positive-staining goblet cells on the villus was adjusted to 1 mm of villus length by using the ratio of goblet cell numbers to the villus height. Goblet cell counts in the intestinal crypt area were expressed as the number of positive-staining cells per 0.01 mm^2 of intestinal crypt area (Dunsford *et al.*, 1990, 1991).

3.2.5.3 Counting BrDU-labelled cells

Crypt nuclei which reacted with the monoclonal antibody against BrDU were clearly stained dark brown due to the incorporation of BrDU into cellular DNA during the S-phase of the cell cycle, followed by binding of the monoclonal antibody. Crypt cells with dark brown nuclei were counted as the labelled cells and the area of the crypt in which the cells were counted was determined. Counting and measurement were performed on 40 crypts which were well orientated with the plane of the section passing vertically down the length of the crypt from the mouth right to the base (Sawada *et al.*, 1991). Cell proliferation was expressed as the number of BrDU-labelled cells per crypt and as number per 0.01 mm^2 crypt area.

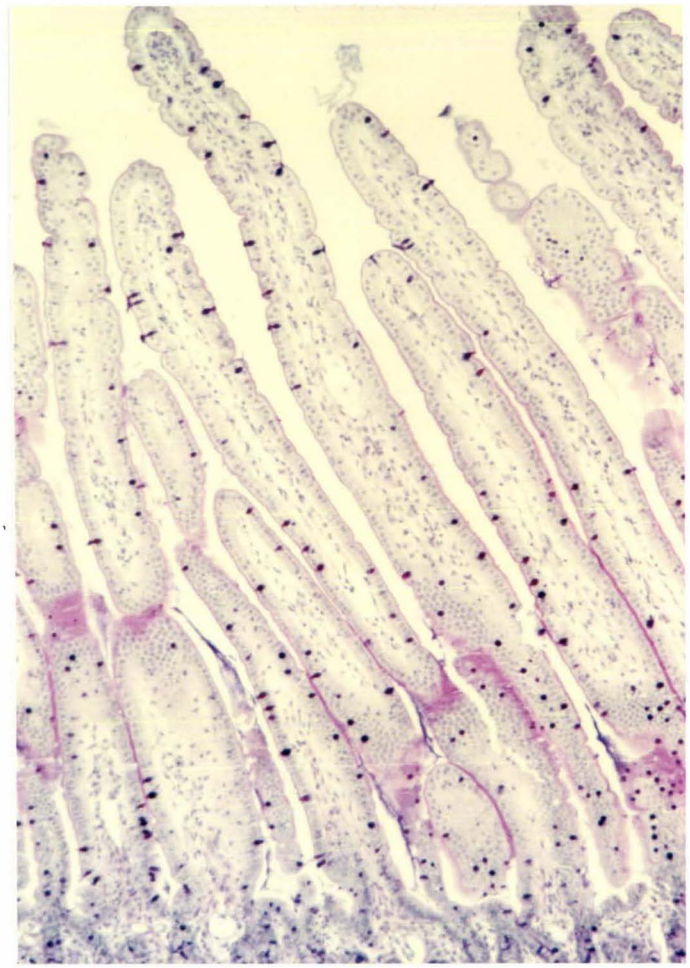

Figure 3.1 Photomicrograph of a section of the mid-jejunum of a control piglet killed at 3 days of age showing goblet cells staining positive for Alcian blue and periodic acid-Schiff in the villi and crypts (AB/PAS, x90)

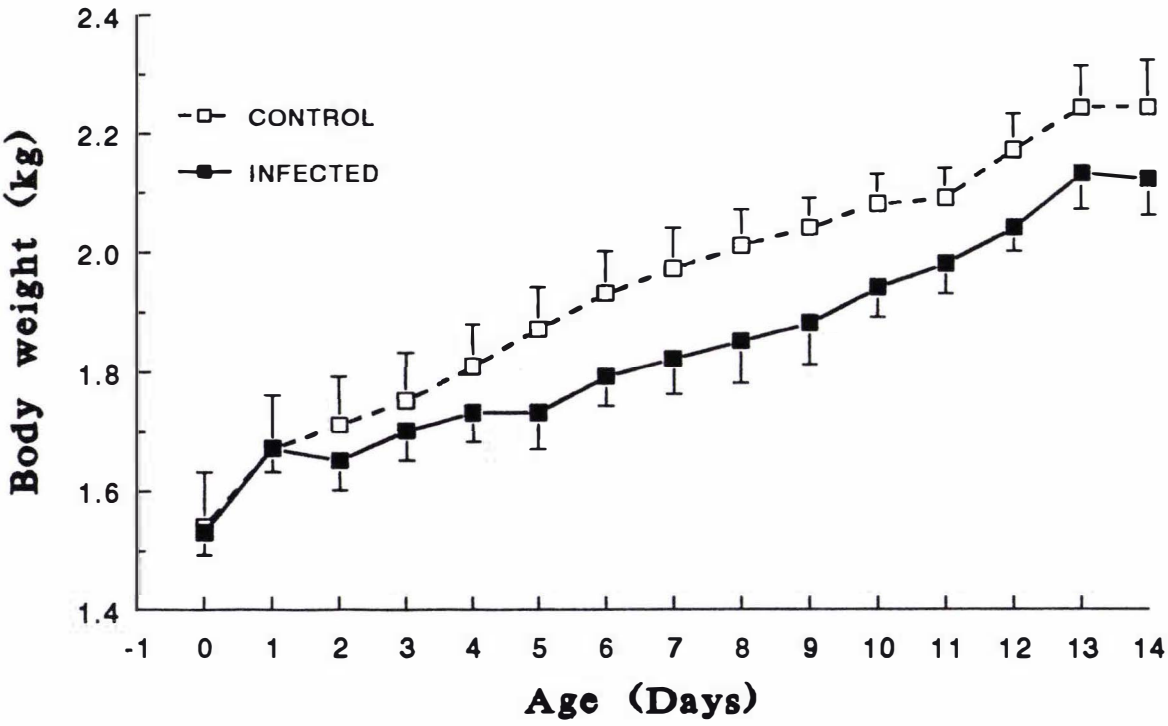

Figure 3.2 Body weight in the control and *Y. enterocolitica*-infected piglets from day 0 to day 14. Data were from 5 animals in each group and are presented as Mean ± SE. ANOVA used the pooled error mean square with the greater degrees of freedom. Body weight was lower in infected piglets than in controls ($p < 0.001$).

Table 3.1. Body weight gain in control and *Y. enterocolitica*-infected piglets between days 0-3, 0-5, 0-14 and 5-14 and ANOVA

	Days			
	0-3	0-5	0-14	5-14
Treatment	Body weight gain, g (Mean ± SE)			
Control	174 ± 17 *n=18*	269 ± 21 *n=12*	702 ± 49 *n=5*	377 ± 44 *n=5*
Infected	133 ± 18 *n=19*	163 ± 23 *n=13*	589 ± 61 *n=5*	390 ± 65 *n=5*
	Analyses of Variance			
Source	MS p	MS p	MS p	MS p
Treatment	15 NS	71 **	32 NS	0.4 NS
Error	6	6	15	16

NS $p > 0.05$; ** $p < 0.01$ MS = Mean Square

Table 3.2. Body length, crown rump length (CRL) and nose rump length (NRL) in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			CRL	NRL
Treatment	Age (Days)	n	Length, cm (Mean ± SE)	
Control	3	2	36.5 ± 1.5	43.0 ± 1.5
	5	7	36.1 ± 0.6	43.8 ± 0.6
	14	5	39.1 ± 1.2	45.9 ± 1.4
Infected	3	2	37.0 ± 2.0	43.7 ± 2.2
	5	8	35.7 ± 0.4	42.7 ± 0.4
	14	5	39.2 ± 0.6	46.2 ± 0.7
			Analyses of Variance	
Source		DF	MS p	MS p
Age		2	31.78 **	24.86 **
Linear		1	22.79 *	25.80 *
Quadratic		1	16.93 *	6.84 NS
Treatment		1	0.05 NS	0.01 NS
Interaction		2	0.31 NS	2.38 NS
Error		23	3.40	3.97

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$ MS = Mean Square

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Table 3.2-A. Daily milk intake on days 1 to 14. Data are presented as Mean ± SE for each feed. One way ANOVA compared control and *Y. enterocolitica*-infected piglets on each day.

Age (day)	n	Control	n	Infected	p
1	18	39 ± 1	19	37 ± 2	NS
2	18	36 ± 1	19	28 ± 2	***
3	18	37 ± 1	19	33 ± 1	**
4	12	39 ± 2	13	32 ± 1	**
5	12	39 ± 3	13	29 ± 2	**
6	5	47 ± 1	5	45 ± 1	NS
7	5	46 ± 2	5	48 ± 3	NS
8[a]	5	59 ± 3	5	52 ± 9	NS
9	5	69 ± 2	5	58 ± 7	NS
10	5	75 ± 1	5	60 ± 10	NS
11	5	76 ± 2	5	64 ± 10	NS
12	5	83 ± 3	5	73 ± 9	NS
13	5	85 ± 5	5	79 ± 8	NS
14	5	81 ± 3	5	75 ± 8	NS

*** $p < 0.001$; ** $p < 0.01$; NS $p > 0.1$

[a]: Number of feed was reduced from original 10 to 7 from day 8.

3.2.6 Statistics

For comparison between control and infected animals, or between ages, two-way analysis of variance (ANOVA) was performed using the general linear model of the SAS programme (SAS Institute Inc., Cary, NC, USA, 1987). The data are presented as mean and standard error of the mean (SE) and results of the ANOVA are presented as probability (p). Both mean square (MS) and p are tabled. A p value of < 0.05 was considered significant. To test whether the body weight growth curves of the control and infected piglets were divergent, two linear regressions were calculated for each group using different assumed points of inflection (days 4, 5, and 6). The slopes of the regression lines were tested using *t*-tests (Appendix 4).

3.3 RESULTS

3.3.1 Body Weight and Length

Body weight in the 5 control and 5 *Y. enterocolitica*-infected piglets which were studied for the whole 14 day period is shown in Figure 3.2. The mean gain in body weight, from all 18 control and 19 infected piglets over the periods 0-3, 0-5, 0-14 and 5-14 days is shown in Table 3.1. There was a rapid increase in body weight in both the control and infected groups on the first day, and body weight increased with advancing age ($p < 0.001$) but the body weight increased more slowly in infected piglets ($p < 0.01$). The body weight gain of infected piglets was lower than that of the controls during the first 5 days ($p < 0.01$), but not in other periods (Table 3.1). The total weight gain from 0-14 days in the infected group (589 ± 61 g) was 19% lower than in the control group (702 ± 49 g) (Table 3.1). Over the period of infection of 0-5 days, the daily gain in body weight of infected piglets was less than in the controls, which can also be seen in Figure 3.2 where the 2 curves were divergent by days 5 and 6 ($p < 0.05$). Once antibiotic therapy had been instituted, the gain in body weight was similar in the two groups from 5-14 days (Table 3.1) and the two growth lines became parallel (Figure 3.2, Appendix 4). Infected piglets took significantly less milk than controls from days 2 to 5 ($p < 0.001$ or < 0.01) but not on other days (Table 3.2-A).

Body length, both crown rump length and nose rump length, increased with age ($p < 0.01$) but was not affected by *Y. enterocolitica* infection (Table 3.2).

Table 3.3. Non-intestinal organ weight in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Stomach	Liver	Pancreas	Spleen	Kidneys
Treatment	Age (days)	n	Weight, g (Mean ± SE)				
Control	3	6	7.0 ± 0.4	65.8 ± 4.6	2.2 ± 0.1	1.5 ± 0.1	10.1 ± 0.6
	5	7	8.8 ± 0.5	83.7 ± 3.7	3.1 ± 0.2	2.4 ± 0.2	11.1 ± 0.5
	14	5	11.2 ± 0.5	72.0 ± 3.1	4.8 ± 0.4	3.5 ± 0.3	14.6 ± 0.6
Infected	3	6	7.1 ± 0.4	64.3 ± 5.9	2.5 ± 0.2	1.8 ± 0.1	11.1 ± 0.7
	5	8	8.4 ± 0.4	61.6 ± 3.1	2.5 ± 0.2	2.3 ± 0.2	9.7 ± 0.4
	14	5	11.2 ± 0.4	84.3 ± 3.8	4.5 ± 0.3	3.5 ± 0.4	15.8 ± 1.2

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	46.35	***	478.22	*	16.14	***	9.66	***	80.63	***
Linear	1	92.65	***	879.52	**	30.95	***	19.28	***	133.02	***
Quadratic	1	0.00	NS	63.20	NS	1.70	*	0.01	NS	31.70	**
Treatment	1	0.16	NS	126.81	NS	0.37	NS	0.00	NS	0.61	NS
Age*treatment	2	0.19	NS	938.63	***	0.81	NS	0.15	NS	7.11	NS
Error	31	1.15		106.35		0.35		0.36		2.62	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 3.4. Non-intestinal organ weights relative to body weight in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA. Data are expressed as gram organ weight per kilogram body weight.

			Stomach	Liver	Pancreas	Spleen	Kidneys
Treatment	Age (days)	n	Weight relative to body weight, g/kg (Mean ± SE)				
Control	3	6	4.7 ± 0.1	44.3 ± 3.8	1.5 ± 0.03	1.0 ± 0.04	6.7 ± 0.3
	5	7	5.0 ± 0.1	47.7 ± 1.7	1.8 ± 0.10	1.4 ± 0.10	6.3 ± 0.3
	14	5	5.0 ± 0.1	32.3 ± 2.1	2.1 ± 0.10	1.6 ± 0.10	6.5 ± 0.1
Infected	3	6	4.7 ± 0.2	42.3 ± 2.3	1.7 ± 0.2	1.2 ± 0.1	7.3 ± 0.1
	5	8	5.4 ± 0.2	39.5 ± 2.0	1.6 ± 0.1	1.5 ± 0.1	6.2 ± 0.3
	14	5	5.3 ± 0.2	39.9 ± 2.0	2.1 ± 0.1	1.6 ± 0.2	7.5 ± 0.6

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	0.86	*	196.44	**	0.97	***	0.81	**	2.35	*
Linear	1	0.86	NS	324.50	**	1.84	***	1.41	***	0.05	NS
Quadratic	1	0.82	NS	76.78	NS	0.12	NS	0.17	NS	4.67	*
Treatment	1	0.46	NS	7.16	NS	0.00	NS	0.08	NS	1.98	NS
Age*treatment	2	0.12	NS	187.89	*	0.15	NS	0.01	NS	0.98	NS
Error	31	0.23		36.14		0.08		0.10		0.66	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 3.5. Intestinal length in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Small intestine	Caecum	Colon
Treatment	Age (days)	n	Length, cm (Mean ± SE)		
Control	3	6	396 ± 17	2.9 ± 0.1	74.8 ± 2.8
	5	7	427 ± 20	3.7 ± 0.3	80.4 ± 3.9
	14	5	600 ± 27	6.9 ± 0.2	111.8 ± 1.3
Infected	3	6	407 ± 8	2.9 ± 0.1	77.3 ± 0.9
	5	8	423 ± 18	3.9 ± 0.3	81.3 ± 2.8
	14	5	572 ± 33	6.7 ± 0.2	106.0 ± 3.5

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p
Age	2	108902	***	44.39	***	3404.30	***
Linear	1	202633	***	86.91	***	6410.35	***
Quadratic	1	18371	*	2.63	**	490.90	**
Treatment	1	382	NS	0.00	NS	5.83	NS
Age*treatment	2	1030	NS	0.14	NS	52.14	NS
Error	31	2609		0.34		54.18	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 3.6. Intestinal length relative to body weight in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA. Data are expressed as centimetre length per kilogram body weight.

			Small intestine	Caecum	Colon
Treatment	Age (days)	n	Length relative to body weight, cm/kg (Mean ± SE)		
Control	3	6	272 ± 6	2.0 ± 0.1	50.0 ± 1.7
	5	7	244 ± 10	2.1 ± 0.2	45.7 ± 1.7
	14	5	267 ± 7	3.1 ± 0.2	50.0 ± 1.3
Infected	3	6	272 ± 14	1.9 ± 0.1	51.7 ± 3.0
	5	8	271 ± 12	2.5 ± 0.2	52.2 ± 2.2
	14	5	271 ± 18	3.2 ± 0.1	50.3 ± 2.7

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p
Age	2	600	NS	3.96	***	12.90	NS
Linear	1	44	NS	7.09	***	1.02	NS
Quadratic	1	1168	NS	0.04	NS	24.48	NS
Treatment	1	1581	NS	0.22	NS	72.09	NS
Age*treatment	2	536	NS	0.16	NS	33.54	NS
Error	31	867		0.17		30.50	

NS $p > 0.05$; *** $p < 0.001$ MS = Mean Square

3.3.2 Non-Intestinal Organ Weights

Non-intestinal organ weights and the organ weights relative to body weight in control and *Y. enterocolitica*-infected pigs on days 3, 5 and 14 are shown in Tables 3.3 and 3.4. There was linear growth with age in the absolute weight of the stomach, pancreas, spleen and kidneys ($p < 0.001$), however, there was also a quadratic component to the growth of the pancreas and the kidney which appeared to be faster in the later period of the experiment ($p < 0.05$ for pancreas and $p < 0.01$ for kidneys). The pancreas ($p < 0.001$), spleen ($p < 0.01$) and stomach ($p < 0.05$) increased in weight faster than the body as a whole, whereas growth of the liver was less than that of the body ($p < 0.01$). The minimum weight relative to body weight of the kidneys occurred on day 5 ($p < 0.05$). *Yersinia enterocolitica* infection did not significantly affect the growth of non-intestinal organs other than the liver during the period of the experiment.

In contrast to the changes in the other organs, in the liver the effects of age and *Y. enterocolitica* infection were complex. The ANOVA (Table 3.3) showed a linear increase in absolute weight of the liver with age from days 3 to 14 ($p < 0.05$) but growth of the liver was less than that of the body ($p < 0.01$) (Table 3.4). The significant interaction between age and treatment showed that the liver weight, both absolute ($p < 0.001$) and relative to body weight ($p < 0.05$), during the 14 day neonatal period was affected differently by the *Y. enterocolitica* infection at different ages. The liver weight (61.6 ± 3.1 g) of infected piglets was much less than that of the controls (83.7 ± 3.7 g) on day 5, but by day 14, after antibiotic therapy, the infected animals had a greater liver weight than litter mate controls. The reduction in liver weight in the control groups between days 5 and 14 (means 83.7 g and 72.0 g respectively) was unexpected, since over the same time period the corresponding liver weights in the *Y. enterocolitica*-infected groups were 61.6 g and 84.3 g respectively (Table 3.3).

3.3.3 Length and Weight of the Intestine

3.3.3.1 Length of the intestine

Intestinal length, and its length relative to body weight, in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 are shown in Tables 3.5 and 3.6. Both the small and large intestinal lengths increased highly significantly with age ($p < 0.001$). The age effect on the length of the intestine was more linear than curvilinear because the age

Table 3.7. Intestinal weight in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Small intestine			Caecum	Colon	
			Mucosa	Muscle	Total		Proximal	Distal
Treatment	Age (days)	n	Weight, g (Mean ± SE)					
Control	3	6	39.5 ± 3.5	14.3 ± 1.1	53.8 ± 4.4	0.7 ± 0.04	3.7 ± 0.3	3.0 ± 0.3
	5	7	40.1 ± 3.5	16.9 ± 0.9	56.9 ± 4.0	0.9 ± 0.07	4.8 ± 0.3	3.9 ± 0.3
	14	5	54.0 ± 2.8	25.6 ± 1.9	79.6 ± 4.6	1.7 ± 0.10	6.5 ± 0.6	5.9 ± 0.8
Infected	3	6	41.4 ± 3.2	15.5 ± 1.6	56.9 ± 4.7	0.7 ± 0.10	4.0 ± 0.3	3.7± 0.7
	5	8	39.3 ± 2.7	18.2 ± 1.0	57.5 ± 3.6	1.0 ± 0.06	5.1 ± 0.5	4.2 ± 0.4
	14	5	49.5 ± 1.8	28.5 ± 0.8	78.0 ± 2.1	2.0 ± 0.10	7.5 ± 0.9	6.7 ± 0.8

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	505.36	**	435.71	***	1839.21	***	3.97	***	26.94	***	26.13	***
Linear	1	810.58	***	847.38	***	3315.60	***	7.79	***	53.85	***	51.59	***
Quadratic	1	222.80	NS	32.52	NS	425.55	*	0.21	*	0.01	NS	1.13	NS
Treatment	1	11.24	NS	29.00	NS	4.13	NS	0.24	*	2.58	NS	2.54	NS
Age*treatment	2	28.32	NS	2.52	NS	15.37	NS	0.09	NS	0.44	NS	0.19	NS
Error	31	60.48		9.24		101.99		0.04		1.46		1.96	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 3.8. Intestinal weights relative to body weight in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA. Data are expressed as gram tissue weight per kilogram body weight.

			Small intestine			Caecum	Colon	
			Mucosa	Muscle	Total		Proximal	Distal
Treatment	Age (days)	n	Weight relative to body weight, g/kg (Mean ± SE)					
Control	3	6	25.0 ± 1.5	9.8 ± 0.4	34.8 ± 1.7	0.46 ± 0.01	2.5 ± 0.2	2.0 ± 0.1
	5	7	23.9 ± 1.2	9.4 ± 0.4	33.3 ± 1.2	0.55 ± 0.03	2.8 ± 0.2	2.3 ± 0.2
	14	5	24.0 ± 0.5	11.3 ± 0.5	35.4 ± 1.0	0.76 ± 0.06	2.9 ± 0.2	2.6 ± 0.3
Infected	3	6	27.1 ± 1.2	10.0 ± 0.5	37.0 ± 1.4	0.49 ± 0.02	2.7 ± 0.3	2.5 ± 0.5
	5	8	25.5 ± 1.4	11.3 ± 0.4	36.9 ± 1.7	0.66 ± 0.03	3.3 ± 0.2	2.7 ± 0.2
	14	5	23.4 ± 0.7	13.5 ± 0.6	36.9 ± 1.1	0.98 ± 0.06	3.6 ± 0.5	3.2 ± 0.4

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	35.33	*	19.30	***	17.86	NS	0.4375	***	1.24	NS	1.27	NS
Linear	1	39.88	NS	38.42	***	0.01	NS	0.8737	***	2.01	*	2.54	NS
Quadratic	1	28.87	NS	0.36	NS	35.46	NS	0.0037	NS	0.39	NS	0.00	NS
Treatment	1	8.28	NS	22.40	***	57.92	NS	0.1233	***	1.81	NS	0.82	NS
Age*treatment	2	6.99	NS	2.21	NS	8.79	NS	0.0245	NS	0.15	NS	0.02	NS
Error	31	10.19		1.39		13.96		0.0078		0.45		0.64	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 3.9. Weight of the segments of the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Segment weight, g (Mean ± SE)						
Control	3	6	1.7 ± 0.1	9.6 ± 0.9	8.9 ± 0.7	8.5 ± 0.8	8.6 ± 0.5	8.7 ± 0.5	8.5 ± 0.8
	5	7	2.1 ± 0.2	10.4 ± 0.6	9.7 ± 0.7	9.1 ± 0.8	9.2 ± 0.8	8.7 ± 0.8	8.8 ± 0.6
	14	5	2.8 ± 0.2	12.0 ± 0.7	12.4 ± 0.9	11.6 ± 0.5	13.2 ± 0.9	13.6 ± 0.7	13.9 ± 1.2
Infected	3	6	1.6 ± 0.1	10.9 ± 0.9	9.4 ± 0.6	8.8 ± 0.7	8.7 ± 0.7	9.2 ± 1.1	9.2 ± 0.9
	5	8	1.8 ± 0.1	9.3 ± 0.7	9.2 ± 0.5	9.5 ± 0.7	9.5 ± 0.8	9.1 ± 0.7	9.3 ± 0.6
	14	5	3.0 ± 0.1	12.6 ± 1.0	12.0 ± 0.3	12.2 ± 0.7	11.6 ± 0.7	13.3 ± 1.0	13.3 ± 0.9
			Analyses of Variance						
Source	DF		MS p	MS p	MS p	MS p	MS p	MS p	MS p
Age	2		4.55 ***	18.86 *	30.11 ***	31.96 ***	43.02 ***	75.70 ***	79.12 ***
Linear	1		8.95 ***	26.70 *	55.52 ***	62.05 ***	82.55 ***	128.32 ***	140.32 ***
Quadratic	1		0.22 NS	11.97 NS	5.62 NS	2.50 NS	4.48 NS	26.16 *	20.78 *
Treatment	1		0.03 NS	0.67 NS	0.12 NS	1.69 NS	1.35 NS	0.30 NS	0.26 NS
Age*treatment	2		0.24 NS	4.60 NS	0.91 NS	0.04 NS	3.31 NS	0.47 NS	1.37 NS
Error	31		0.12	4.02	2.55	3.62	3.77	4.40	4.17

NS $p > 0.05$; * $p < 0.05$; *** $p < 0.001$ MS = Mean Square

Table 3.10. Weight of the mucosa of the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Mucosal weight, g (Mean ± SE)						
Control	3	6	1.04 ± 0.08	7.2 ± 0.7	6.5 ± 0.5	6.3 ± 0.7	6.3 ± 0.5	6.3 ± 0.4	5.7 ± 0.6
	5	7	1.17 ± 0.10	7.8 ± 0.6	7.3 ± 0.6	6.6 ± 0.7	6.5 ± 0.7	6.0 ± 0.7	5.3 ± 0.5
	14	5	1.52 ± 0.13	8.6 ± 0.6	9.0 ± 0.6	8.3 ± 0.4	9.5 ± 0.8	9.5 ± 0.4	7.6 ± 0.6
Infected	3	6	0.87 ± 0.05	8.0 ± 0.6	7.1 ± 0.4	6.5 ± 0.5	6.4 ± 0.6	6.5 ± 0.9	6.1 ± 0.5
	5	8	0.97 ± 0.07	6.5 ± 0.5	6.5 ± 0.3	7.0 ± 0.6	6.7 ± 0.7	6.3 ± 0.7	5.3 ± 0.4
	14	5	1.71 ± 0.10	8.9 ± 0.8	8.5 ± 0.3	8.2 ± 0.4	7.6 ± 0.3	8.4 ± 0.8	6.3 ± 0.4

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	1.3324	***	7.94	NS	12.95	***	10.22	*	15.32	*	25.96	**	8.17	*
Linear	1	2.5315	***	8.98	NS	22.68	***	19.82	**	28.29	**	40.81	**	8.05	*
Quadratic	1	0.1671	NS	7.33	NS	3.70	NS	0.84	NS	2.83	NS	12.31	NS	8.74	*
Treatment	1	0.0296	NS	0.05	NS	0.52	NS	0.28	NS	2.52	NS	0.51	NS	1.02	NS
Age*treatment	2	0.1299	NS	3.75	NS	1.50	NS	0.17	NS	3.93	NS	1.62	NS	2.25	NS
Error	31	0.0512		2.56		1.44		2.60		2.90		3.25		1.60	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 3.11. Weight of the muscle of the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Muscle weight, g (Mean ± SE)						
Control	3	6	0.68 ± 0.04	2.4 ± 0.2	2.4 ± 0.2	2.1 ± 0.2	2.3 ± 0.1	2.4 ± 0.1	2.7 ± 0.2
	5	7	0.94 ± 0.09	2.6 ± 0.2	2.5 ± 0.1	2.5 ± 0.1	2.7 ± 0.2	2.6 ± 0.2	3.6 ± 0.3
	14	5	1.25 ± 0.10	3.4 ± 0.2	3.4 ± 0.3	3.3 ± 0.2	3.8 ± 0.2	4.1 ± 0.3	6.3 ± 0.8
Infected	3	6	0.70 ± 0.06	2.9 ± 0.4	2.3 ± 0.2	2.3 ± 0.2	2.3 ± 0.2	2.6 ± 0.3	3.1 ± 0.5
	5	8	0.84 ± 0.04	2.8 ± 0.2	2.8 ± 0.2	2.5 ± 0.1	2.8 ± 0.1	2.8 ± 0.2	4.0 ± 0.4
	14	5	1.32 ± 0.06	3.7 ± 0.2	3.5 ± 0.1	3.9 ± 0.4	4.0 ± 0.4	4.9 ± 0.4	7.0 ± 0.5
			Analyses of Variance						
Source	DF		MS p	MS p	MS p	MS p	MS p	MS p	MS p
Age	2		0.9809 ***	2.62 **	3.68 ***	6.03 ***	7.15 ***	13.27 ***	41.49 ***
Linear	1		1.9611 ***	4.75 **	7.23 ***	11.73 ***	14.19 ***	24.39 ***	81.15 ***
Quadratic	1		0.0042 NS	0.57 NS	0.20 NS	0.44 NS	0.19 NS	2.58 *	2.57 NS
Treatment	1		0.0000 NS	1.08 NS	0.13 NS	0.60 NS	0.18 NS	1.57 NS	2.30 NS
Age*treatment	2		0.0236 NS	0.05 NS	0.10 NS	0.31 NS	0.05 NS	0.34 NS	0.11 NS
Error	31		0.0269	0.42	0.33	0.28	0.26	0.39	1.37

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 3.12. Mucosal weight as a percentage of the segment weight in the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA.

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	% mucosa (Mean ± SE)						
Control	3	6	60.0 ± 1.2	74.8 ± 1.4	73.5 ± 1.4	74.6 ± 1.5	72.7 ± 1.3	72.4 ± 0.9	67.1 ± 1.3
	5	7	55.3 ± 2.1	74.6 ± 2.5	74.4 ± 1.2	71.6 ± 2.2	69.8 ± 2.2	68.9 ± 2.1	59.1 ± 3.4
	14	5	54.7 ± 0.3	61.5 ± 1.0	72.5 ± 1.0	71.6 ± 0.7	71.2 ± 1.9	70.0 ± 1.0	55.2 ± 2.5
Infected	3	6	55.6 ± 1.7	73.9 ± 1.3	75.5 ± 1.4	74.0 ± 1.2	73.1 ± 1.0	70.7 ± 2.2	67.1 ± 1.8
	5	8	53.0 ± 2.4	69.4 ± 0.8	70.2 ± 1.5	73.2 ± 1.5	68.8 ± 2.9	67.6 ± 3.6	57.4 ± 2.9
	14	6	56.2 ± 1.8	70.3 ± 1.1	70.7 ± 1.3	68.0 ± 2.0	65.5 ± 1.8	62.6 ± 2.3	39.8 ± 7.9
			Analyses of Variance						
Source	DF		MS p	MS p	MS p	MS p	MS p	MS p	MS p
Age	2		45.23 NS	207.45 NS	26.04 NS	55.25 NS	68.72 NS	78.05 NS	1045.86 ***
Linear	1		19.90 NS	410.04 *	40.94 NS	109.68 *	101.22 NS	138.12 NS	2059.23 ***
Quadratic	1		68.45 NS	7.62 NS	9.98 NS	0.38 NS	32.93 NS	15.32 NS	19.77 NS
Treatment	1		27.87 NS	8.37 NS	16.09 NS	6.72 NS	39.20 NS	107.75 NS	292.48 NS
Age*treatment	2		24.58 NS	143.77 NS	31.56 NS	20.36 NS	27.42 NS	31.10 NS	193.98 NS
Error	31		23.35	86.64	11.45	17.74	29.02	40.64	80.95

NS $p > 0.05$; * $p < 0.05$; *** $p < 0.001$ MS = Mean Square

mean square for the linear relationship was much greater than that for the quadratic relationship (Table 3.5). The length of the caecum by day 14 was more than double its length on day 3 (Table 3.5). The caecal length, relative to body weight, also increased with age ($p < 0.001$), whereas the length of the small intestine and colon in relation to body weight remained unchanged during the 14 day neonatal period (Table 3.6). The infected piglets had a similar intestinal length and intestinal length relative to body weight to controls.

3.3.3.2 Weight of the intestine

The weight of the entire small intestine and of the separated mucosa and muscle linearly increased with age ($p < 0.01$) (Table 3.7), as did the weights of each of the seven segments ($p < 0.05$ for the proximal jejunum and $p < 0.01$ for the others) (Table 3.9). Both the mucosa and muscle became heavier with age in all segments ($p < 0.001$ to < 0.05), the exception being the mucosa of the proximal jejunum ($p > 0.05$) (Tables 3.10 and 3.11). Over the whole length of the small intestine, the mucosal weight decreased ($p < 0.05$) but the muscle weight increased ($p < 0.001$) more than the body as a whole, so that the total weight was not disproportionate to body weight from days 3 to 14 (Table 3.8). A marked decrease in mucosal weight as a percentage of segment weight was seen in the distal ileum ($p < 0.001$) where the mean percentages at 14 days were 40% and 55% (infected and control) compared with about 70% in the younger piglets (Table 3.12). In the proximal and distal jejunum, the percentage of mucosa to the segment weight also decreased linearly ($p < 0.05$) (Table 3.12).

The weights of all parts of the large intestine (the caecum and the proximal and distal colon) linearly increased with age ($p < 0.001$) (Table 3.7) and, relative to body growth, positive linear effects were found in the caecum ($p < 0.001$) and the proximal colon ($p < 0.05$) (Table 3.8).

Yersinia enterocolitica infection did not affect the weight of the whole small intestine, mucosa or muscle (Table 3.7), nor the weight of any of the seven segments (Table 3.9) or their mucosa (Table 3.10) or muscle (Table 3.11). The muscle weight relative to body weight in the small intestine, however, was greater in the infected groups than in controls ($p < 0.001$) whereas the relative weight of mucosa was comparable between the two groups (Table 3.8). *Yersinia enterocolitica* infection increased the weight of the caecum ($p < 0.05$) (Table 3.7) and also its weight relative to body weight ($p < 0.001$)

(Table 3.8). In the colon, the absolute weight and its weight relative to body weight were comparable in the infected group and the controls (Tables 3.7 and 3.8).

3.3.4 Histological Observations

Histological observations were made on HE stained slides. Histopathological changes in the small intestine in infected piglets have been described previously (Chapter 2, 2.3.3). All observations in infected small intestine presented here were made on the intact villi where no lesions were observed. The seven segments of the small intestine could be divided into four regions on the basis of two major morphological features of the villus epithelial cells: (i) the position of the nucleus and (ii) the presence in the cytoplasm of vacuoles.

Region 1: Duodenum

The nuclei were located in the basal position and no vacuoles were present in any control or infected piglets at 3, 5 and 14 days of age.

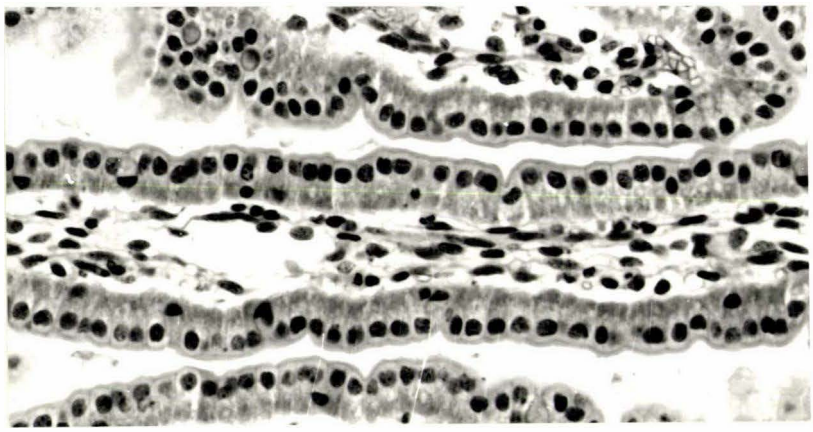
Region 2: Proximal jejunum to the proximal ileum

There were very occasional vacuoles in the cytoplasm in all piglets at 3, 5 and 14 days. The position of the nucleus changed with advancing age. On day 3, the nuclei of most epithelial cells were apical (Figure 3.3 A) but occasionally in the middle of the cell. By day 5, the nuclei were variable in location, being present in the apical, middle or basal positions in the cell (Figure 3.3 B). By day 14, no nuclei were apical, most were basal and a very few remained in the middle (Figure 3.3 C). The structure of the epithelium in infected piglets did not differ from that in controls.

Region 3: Mid-ileum.

In control piglets, vacuolation was present in the upper half of the villi at all three ages. The position of the nucleus differed between ages. Most nuclei were located in the middle or the base of the epithelial cells in 3 day-old-piglets. On days 5 and 14, nuclei were located variably: in the apex, the middle or the base.

A



B

C

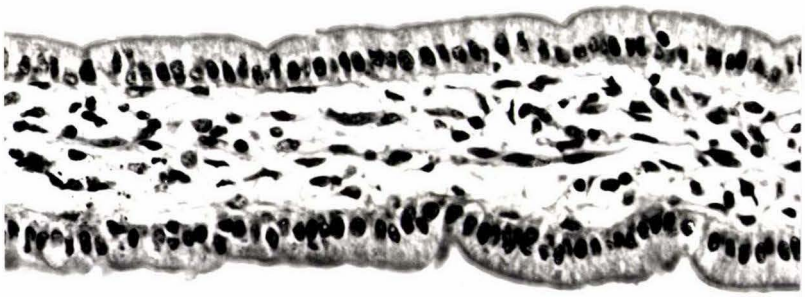


Figure 3.3 Photomicrographs of sections of the mid-jejunum in control piglets killed at 3, 5 and 14 days displaying epithelial nuclei in different positions. A: 3 days. Nuclei apical in epithelial cells. B: 5 days. Nuclei in variable positions either the middle or base. C: 14 days. Nuclei basal in epithelial cells. (HE, x440)

Table 3.13. Villus height in the jejunum and ileum in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Villus height, µm (Mean ± SE)					
Control	3	6	893 ± 75	801 ± 57	826 ± 99	782 ± 64	740 ± 45	651 ± 47
	5	7	982 ± 67	867 ± 101	863 ± 119	736 ± 84	678 ± 67	627 ± 38
	14	5	679 ± 15	709 ± 28	709 ± 54	859 ± 82	913 ± 52	667 ± 80
Infected	3	6	835 ± 83	775 ± 55	699 ± 52	713 ± 53	703 ± 57	625 ± 50
	5	8	708 ± 76	639 ± 54	732 ± 94	684 ± 108	695 ± 62	579 ± 75
	14	5	637 ± 39	653 ± 47	700 ± 68	688 ± 63	746 ± 102	515 ± 75

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	123	*	32	NS	25	NS	12.8	NS	63.6	NS	6.9	NS
Linear	1	202	*	64	NS	24	NS	0.2	NS	80.4	NS	10.7	NS
Quadratic	1	48	NS	0	NS	27	NS	19.9	NS	50.2	NS	2.7	NS
Treatment	1	183	*	96	NS	68	NS	84.8	NS	35.3	NS	50.7	NS
Age*treatment	2	45	NS	40	NS	13	NS	11.5	NS	25.8	NS	12.1	NS
Error	31	30		27		47		44.6		26.6		24.5	

NS $p > 0.05$; * $p < 0.05$ MS = Mean Square

Table 3.14. Crypt depth in the jejunum and ileum in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Crypt depth, µm (Mean ± SE)					
Control	3	6	93 ± 8	92 ± 5	88 ± 9	84 ± 6	85 ± 7	85 ± 7
	5	7	86 ± 5	98 ± 7	91 ± 7	93 ± 7	81 ± 6	84 ± 6
	14	5	91 ± 3	101 ± 5	92 ± 3	78 ± 4	76 ± 2	86 ± 5
Infected	3	6	126 ± 11	117 ± 5	120 ± 7	107 ± 5	109 ± 4	123 ± 6
	5	8	165 ± 18	182 ± 12	161 ± 14	147 ± 14	138 ± 12	138 ± 7
	14	5	120 ± 10	122 ± 7	127 ± 6	109 ± 6	93 ± 6	103 ± 7
			Analyses of Variance					
Source		DF	MS p	MS p	MS p	MS p	MS p	MS p
Age		2	1.0 NS	4.7 ***	1.7 NS	2.9 **	2.0 *	0.8 NS
Linear		1	0.2 NS	0.0 NS	0.0 NS	0.2 NS	1.4 NS	0.7 NS
Quadratic		1	3.0 NS	9.3 *	3.4 *	5.7 **	2.6 *	1.0 NS
Treatment		1	20.0 ***	17.2 ***	17.6 ***	11.5 ***	9.5 ***	11.7 ***
Age*treatment		2	2.0 NS	4.1 ***	1.4 NS	0.9 NS	1.5 *	1.1 *
Error		31	1.0	0.4	0.6	0.5	0.4	0.3

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

In infected piglets, the epithelium was vacuolated and the nuclei varied on day 3. Vacuoles were fewer or absent and nuclei were basal by 5 days postinfection. By day 14, when the animals had recovered clinically, there were few vacuolated cells.

Region 4: Distal ileum

Nuclei were basal in all piglets, both control and infected, at all three ages. Vacuolation was present in almost the whole villus in 3- and 5-day-old control piglets, in which the vacuoles were large, occupied almost the whole cytoplasm and the nuclei were basal (Figure 3.4 A). In 14-day-old piglets (both control and infected), vacuolated cells were limited to the upper two-thirds of the villi. Vacuoles were apical and nuclei uniformally basal (Figure 3.5 A). Vacuolation was absent on villi overlying Peyer's patches in all piglets (Figure 3.6).

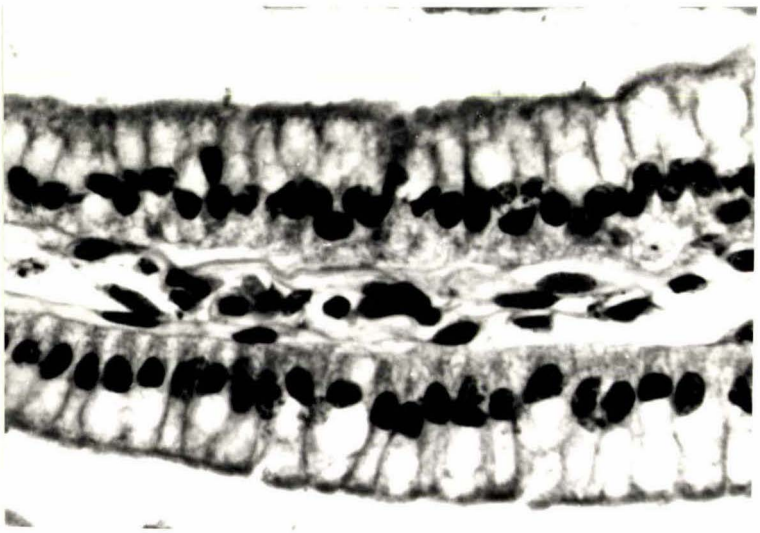
In infected piglets, small vacuoles were present in the whole villus at 3 days. There were almost no vacuolated cells at 5 days postinfection (Figure 3.4 B). By day 14, vacuoles were present again but much fewer in number and also smaller in the infected piglets compared with those in non-infected piglets of the same age (Figure 3.5 B).

3.3.5 Villus Height and Crypt Depth

Villus height and crypt depth in the jejunum and ileum of control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14, and results of ANOVA of villus height and crypt depth between ages and treatments are shown in Tables 3.13 and 3.14. There was no difference in the pooled means of villus height between the infected group and the controls and between ages in all segments of the small intestine except the proximal jejunum in which villus height showed a significant decrease in infected animals ($p < 0.05$) and in which the pooled mean of villus height decreased during the period between day 3 and day 14 ($p < 0.05$) (Table 3.13).

Crypt depth in the whole jejunum and ileum was markedly greater in *Y. enterocolitica*-infected piglets than in the controls over the period of the experiment ($p < 0.001$) (Table 3.14). The increase in crypt depth in infected pigs was greater in the distal ileum (45%) compared with other segments of the small intestine (about 30%) at 3 days postinfection. The age effect has to be interpreted in the light of the interaction between age and treatment. The age effect was generated by the greater mean crypt depth in infected

A



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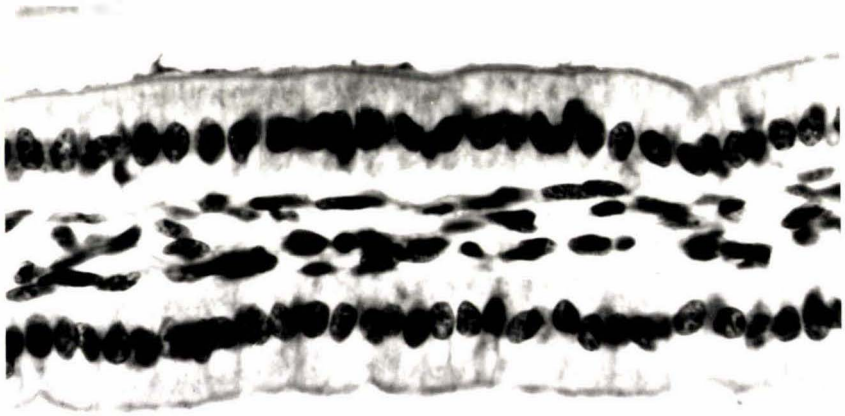
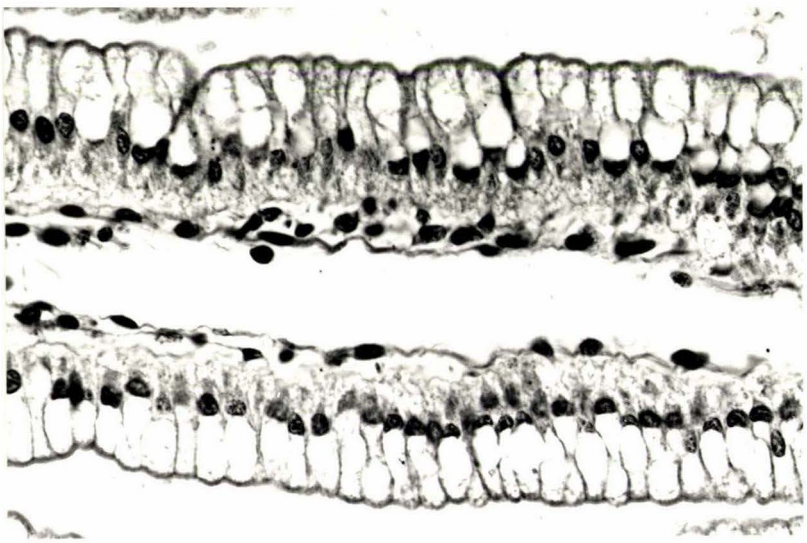


Figure 3.4 Photomicrographs of sections of the distal ileum of a control and a *Y. enterocolitica*-infected piglet killed on day 5, displaying a lower density of vacuoles in the infected piglet. A: Control. Vacuoles of varying size in the epithelium with a basal nuclei. B: Infected. No vacuoles in the epithelium (HE, x700)

A



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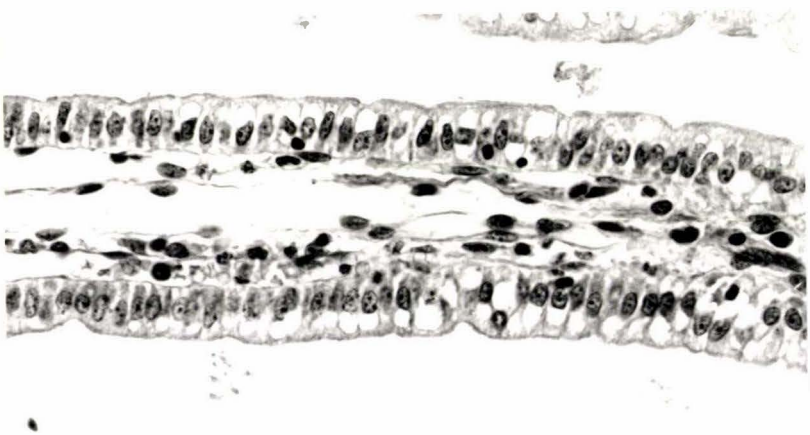


Figure 3.5 Photomicrographs of sections of the distal ileum of a control and a *Y. enterocolitica*-infected piglet killed on day 14. A: Control. Uniform vacuoles in the epithelial cells with basal nuclei. B: Infected. Many fewer vacuoles. (HE, x440)

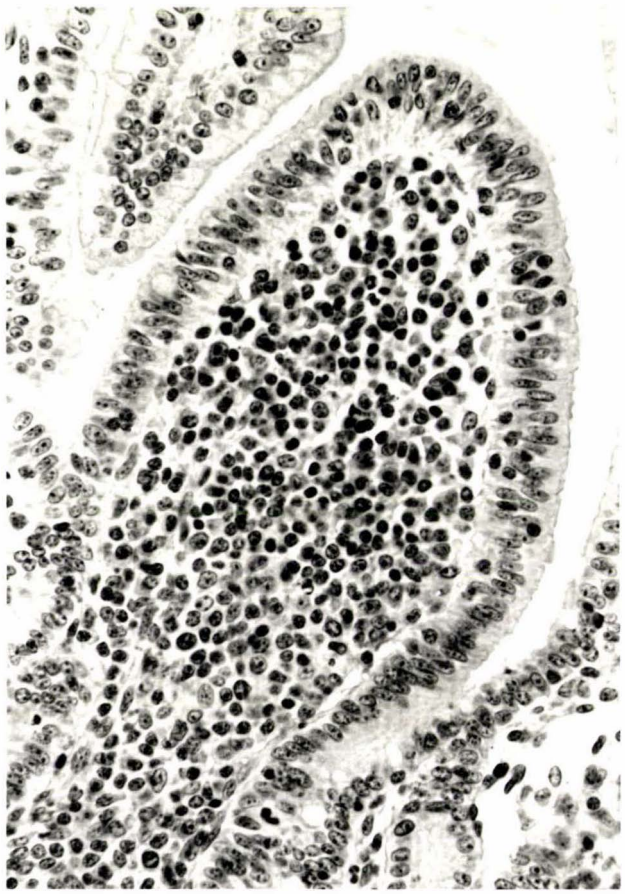


Figure 3.6 Photomicrograph of the distal ileum of a 14-day-old control piglet displaying the absence of vacuoles on the villi over the area of Peyer's patches. The short blunt villus with abundant lymphatic tissue in the lamina propria does not contain vacuolated cells (HE, x440)

piglets on day 5. A difference in crypt depth between ages was present in infected piglets, in which the deepest crypts were present on day 5 ($p < 0.001$ for the mid-jejunum, < 0.05 for the mid- and distal ileum). A significant interaction between age and infection was present in 3 of the 6 segments, suggesting a maximal difference in crypt depth between control and infected piglets at 5 days and also suggesting a difference in crypt depth between ages in infected animals (Figure 3.7). In the other 3 segments, the interaction mean square was greater than the error mean square although these did not reach significance (Table 3.14). The crypt depth in infected pigs did not return to the level of that in the control animals of the same age although by day 14 after antibiotic therapy the animals had clinically recovered from infection with the bacteria. In control piglets, crypt depth remained constant in all parts of the small intestine over the period of the experiment (Table 3.14).

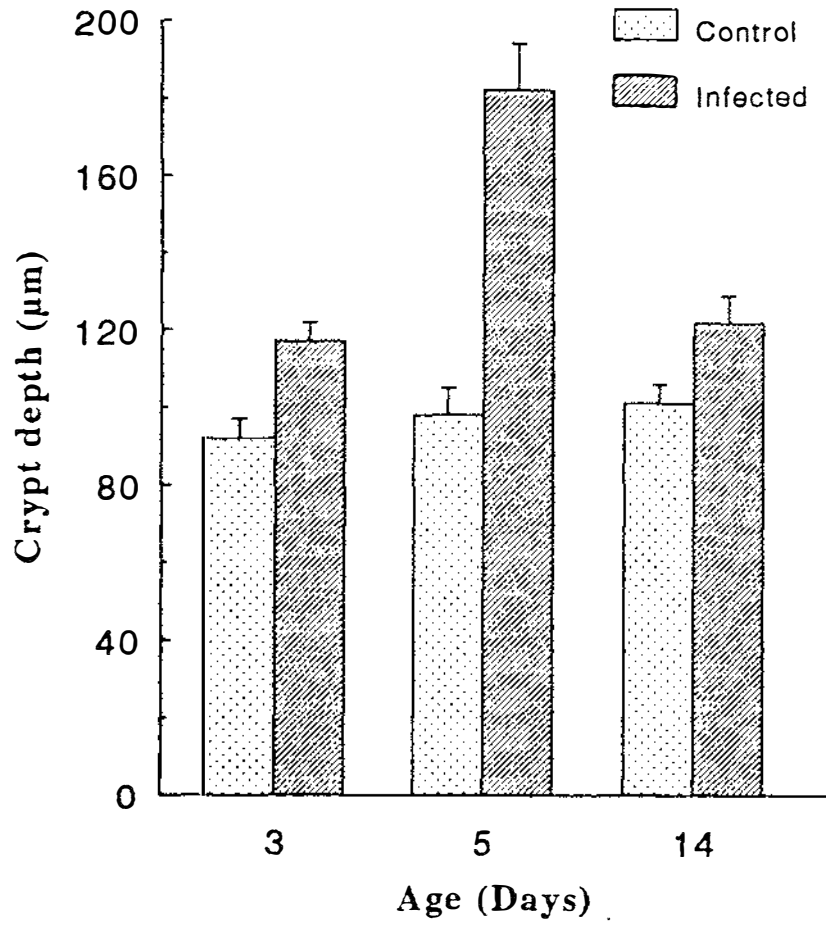


Figure 3.7 Crypt depth (Mean ± SE) of the mid-jejunum in *Y. enterocolitica*-infected and control piglets on days 3, 5 and 14. The crypt in infected piglets was deeper than that in controls at all ages. The age difference in crypt depth was in the infected group in which the deepest crypts were at 5 days postinfection.

Table 3.15. Ratio of villus height to crypt depth in the jejunum and ileum in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	VH/CD ratio (Mean ± SE)					
Control	3	6	10.1 ± 1.4	9.0 ± 1.0	10.2 ± 2.1	9.8 ± 1.5	9.0 ± 1.1	8.1 ± 1.1
	5	7	11.4 ± 1.1	9.2 ± 1.7	10.0 ± 1.4	8.6 ± 1.2	8.9 ± 0.8	7.8 ± 0.3
	14	5	7.5 ± 0.3	7.1 ± 0.5	7.8 ± 0.7	10.9 ± 0.9	12.1 ± 0.7	7.7 ± 0.6
Infected	3	6	6.4 ± 0.9	6.6 ± 0.4	6.1 ± 0.7	6.7 ± 0.6	6.5 ± 0.7	5.1 ± 0.4
	5	8	4.5 ± 0.5	3.6 ± 0.3	4.8 ± 0.7	5.2 ± 1.0	5.5 ± 0.8	4.3 ± 0.6
	14	5	5.4 ± 0.4	5.4 ± 0.4	5.6 ± 0.6	6.3 ± 0.5	7.9 ± 0.6	4.9 ± 0.5

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	10.05	NS	8.88	NS	5.52	NS	11.22	NS	24.32	*	1.06	NS
Linear	1	19.37	*	11.22	NS	10.79	NS	2.14	NS	32.35	*	0.23	NS
Quadratic	1	0.95	NS	6.07	NS	0.25	NS	20.67	NS	17.56	NS	1.86	NS
Treatment	1	159.64	***	94.56	***	126.05	***	121.59	***	100.49	***	84.48	***
Age*treatment	2	18.60	*	14.05	*	6.15	NS	1.69	NS	1.83	NS	0.50	NS
Error	31	3.95		3.17		8.43		8.06		5.67		3.33	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 3.16. Number of proliferating cells in the small intestine in control and *Y. enterocolitica*-infected piglets on days 5 and 14 and ANOVA. Data are expressed as cells per crypt and as cells per crypt area.

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Number of cells/crypt									
Treatment	Age (days)	n			Cells/crypt (Mean ± SE)				
Control	5	3	7.2 ± 0.9	7.7 ± 0.2	7.9 ± 0.4	7.5 ± 0.2	6.4 ± 0.3	7.9 ± 1.0	8.3 ± 0.7
	14	3	6.3 ± 0.9	6.3 ± 0.6	6.4 ± 0.5	7.4 ± 0.7	6.1 ± 0.2	6.8 ± 0.8	10.3 ± 1.6
Infected	5	4	11.9 ± 1.8	15.3 ± 2.1	19.5 ± 1.5	16.3 ± 2.3	13.0 ± 1.2	12.6 ± 1.0	15.9 ± 0.9
	14	3	9.0 ± 0.9	7.9 ± 0.6	8.2 ± 1.6	9.6 ± 1.2	9.2 ± 1.5	7.7 ± 0.9	6.6 ± 0.7
					Analyses of Variance				
Source		DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Age		1	11.63 NS	63.26 *	130.97 ***	36.63 NS	12.81 NS	29.42 *	42.65 **
Treatment		1	44.99 *	67.21 *	143.26 ***	95.05 **	74.58 **	24.06 *	12.85 NS
Age*treatment		1	3.15 NS	28.92 NS	77.28 **	35.63 NS	10.42 NS	11.54 NS	102.91 ***
Error		9	6.35	6.67	5.11	8.80	3.44	3.08	3.50
Number of cells/crypt area									
Treatment	Age (days)	n			Cells/0.01 mm² area, (Mean ± SE)				
Control	5	3	24.1 ± 0.6	26.6 ± 2.0	29.1 ± 3.5	30.7 ± 3.9	30.1 ± 1.0	37.0 ± 3.9	32.3 ± 3.7
	14	3	23.5 ± 2.8	27.9 ± 2.3	25.1 ± 0.3	33.9 ± 3.4	27.6 ± 1.3	31.9 ± 2.8	30.6 ± 3.2
Infected	5	4	32.2 ± 4.1	39.0 ± 4.2	35.9 ± 2.9	40.1 ± 1.3	42.6 ± 1.9	42.7 ± 1.9	45.9 ± 4.2
	14	3	30.8 ± 3.3	31.5 ± 2.5	33.5 ± 7.1	37.0 ± 4.8	37.5 ± 3.8	37.0 ± 3.5	29.5 ± 1.9
					Analyses of Variance				
Source		DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Age		1	3.01 NS	30.33 NS	33.30 NS	0.00 NS	46.10 NS	94.67 NS	264.71 *
Treatment		1	188.32 *	202.01 *	187.16 NS	125.55 NS	401.59 ***	93.31 NS	127.95 NS
Age*treatment		1	0.49 NS	61.96 NS	2.25 NS	30.97 NS	5.30 NS	0.25 NS	174.16 NS
Error		9	34.76	33.89	53.79	35.73	16.30	28.68	41.37

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

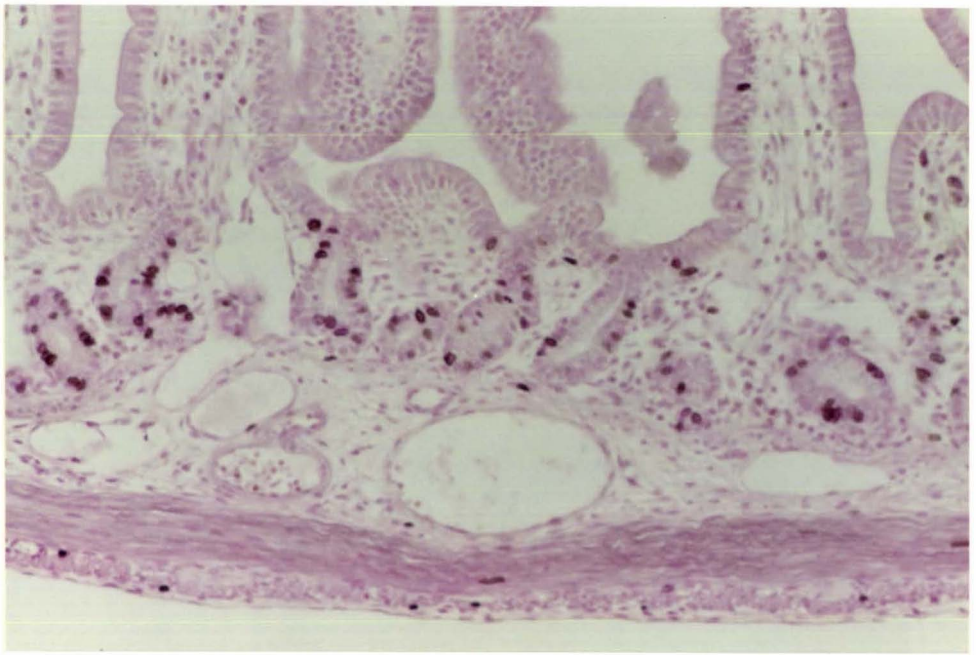
The ratio of villus height to crypt depth in the jejunum and the ileum in control and infected animals is shown in Table 3.15. An age effect occurred only in 2 segments: a decrease in the proximal jejunum ($p < 0.05$) but an increase in the mid-ileum ($p < 0.05$). Overall, the infected piglets had a lower ratio of villus height to crypt depth than controls in all segments ($p < 0.001$). A significant interaction between age and infection ($p < 0.05$) in the proximal and mid-jejunum resulted from the lowest ratio being present on day 5 in the infected animals. Although not reaching a level of statistical significance, a similar pattern in the mean values also occurred in the other five segments of the small intestine.

3.3.6 Crypt Cell Proliferation

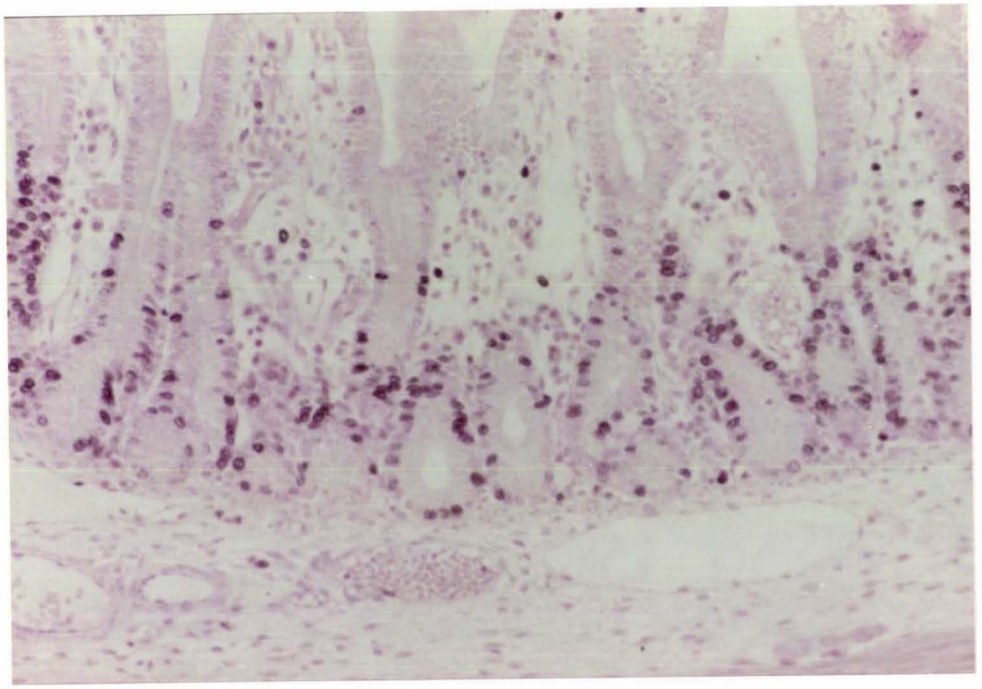
Histological sections from the jejunum of control and *Y. enterocolitica*-infected piglets at 5 days postinfection (Figure 3.8) show the different density of BrDU-labelled cells in the crypts. Infected piglets had a greater density of labelled cells than controls. The number of BrDU-labelled cells per intestinal crypt and the cells per 0.01 mm^2 of intestinal crypt area in control and *Y. enterocolitica*-infected piglets on days 5 and 14 are shown in Table 3.16. In infected piglets, a conspicuous increase in proliferating cells per crypt occurred in most segments of the small intestine, particularly at 5 days postinfection (p value from < 0.05 to < 0.001) except in the distal ileum ($p > 0.05$) (Table 3.16). When proliferating crypt cell number was expressed per 0.01 mm^2 of crypt area, a considerable increase in the numbers of these cells in infected piglets was demonstrated in the proximal ileum ($p < 0.001$), the duodenum and proximal jejunum ($p < 0.05$) (Table 3.16), and the treatment mean square was three times the error mean square in the other segments ($p < 0.1$).

A significant interaction between age and infection in the mid-jejunum ($p < 0.01$) and the distal ileum ($p < 0.001$) indicated that the increase in density of proliferating cells in infected animals was most marked on day 5. The significant age effect in the proximal and mid-jejunum and the mid- and distal ileum has to be interpreted in the light of the effect of the treatment and the age-treatment interaction (Table 3.16). In other segments of the jejunum and ileum, the interaction mean square also was larger than the error mean square (Table 3.16). In the control animals, age had no consistent effect, but this was not the case in the infected group where the mean values at 5 days were greatly elevated. The age effect is likely to result from this, and not to be due to age itself when the two groups are taken together.

A



B



Figue 3.8 Photomicrographs of sections of the mid-ileum of a control (A) and a *Y. enterocolitica*-infected piglet (B) killed at 5 days showing a higher density of BrDU-labelled cells in the crypts of the infected piglet (B) than the control (A) (ICC, x180)

Table 3.17. Goblet cell populations on the villi staining positive for Alcian blue and periodic acid-Schiff in the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA. Data are expressed as number of cells per millimetre of villus length.

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Cells/mm villus length, (Mean ± SE)						
Control	3	4	28.2 ± 6.1	19.4 ± 2.0	18.3 ± 0.7	16.7 ± 3.9	20.3 ± 1.5	26.2 ± 2.8	33.8 ± 2.5
	5	7	41.6 ± 1.5	24.0 ± 1.8	22.6 ± 1.8	20.0 ± 1.8	16.8 ± 3.4	13.3 ± 4.3	14.9 ± 4.4
	14	5	41.7 ± 5.1	21.6 ± 1.3	18.1 ± 2.4	19.7 ± 1.5	14.4 ± 0.8	9.0 ± 0.4	13.0 ± 1.8
Infected	3	4	30.5 ± 4.3	23.0 ± 3.6	19.5 ± 0.5	24.8 ± 3.2	18.0 ± 3.4	20.8 ± 4.7	25.1 ± 5.6
	5	8	37.0 ± 3.6	21.9 ± 2.3	21.2 ± 0.9	21.0 ± 1.7	21.8 ± 2.8	28.1 ± 2.9	31.0 ± 5.0
	14	5	43.6 ± 1.7	23.9 ± 3.2	17.8 ± 4.4	17.6 ± 4.6	13.7 ± 3.8	8.8 ± 2.3	19.1 ± 5.6

Analyses of Variance

Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Age	2	422.22 **	8.42 NS	52.33 NS	13.23 NS	93.33 NS	586.08 ***	402.61 NS
Linear	1	731.65 **	8.46 NS	9.98 NS	22.46 NS	128.16 NS	1046.48 ***	804.60 *
Quadratic	1	187.46 NS	10.33 NS	86.45 NS	2.07 NS	30.95 NS	54.41 NS	16.67 NS
Treatment	1	0.10 NS	12.32 NS	0.15 NS	41.44 NS	3.07 NS	72.16 NS	154.52 NS
Age*treatment	2	46.36 NS	25.69 NS	4.50 NS	59.58 NS	41.49 NS	320.05 *	401.57 NS
Error	27	76.22	32.31	26.01	40.39	52.88	63.93	124.29

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 3.18. Goblet cell populations in the intestinal crypts staining positive for Alcian blue and periodic acid-Schiff in the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA. Data are expressed as number of cells per 0.01 mm² of intestinal crypt area.

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Cells/0.01 mm² crypt area, (Mean ± SE)						
Control	3	4	24.9 ± 1.8	20.9 ± 1.2	20.1 ± 1.9	18.8 ± 5.3	23.9 ± 3.2	24.9 ± 3.1	31.2 ± 2.0
	5	7	25.4 ± 2.7	24.7 ± 2.4	21.6 ± 2.8	21.8 ± 1.7	22.8 ± 2.7	25.0 ± 1.3	25.8 ± 2.8
	14	5	26.2 ± 1.7	24.6 ± 2.1	23.8 ± 2.3	24.0 ± 1.0	24.3 ± 2.2	23.9 ± 1.0	28.5 ± 2.1
Infected	3	4	19.6 ± 1.3	19.0 ± 2.7	13.8 ± 1.5	17.6 ± 1.3	18.3 ± 1.2	19.9 ± 1.5	24.1 ± 1.9
	5	8	22.0 ± 1.0	18.6 ± 1.8	17.9 ± 1.0	20.3 ± 1.6	19.8 ± 1.7	21.8 ± 1.9	23.8 ± 1.5
	14	5	24.0 ± 1.9	26.6 ± 2.9	23.3 ± 1.4	24.3 ± 1.7	24.2 ± 0.7	27.9 ± 2.2	32.0 ± 2.6
			Analyses of Variance						
Source	DF		MS p	MS p	MS p	MS p	MS p	MS p	MS p
Age	2		18.45 NS	80.17 NS	98.77 *	78.25 NS	30.95 NS	31.27 NS	88.77 NS
Linear	1		36.78 NS	153.61 *	196.95 **	156.40 *	47.04 NS	59.09 NS	48.37 NS
Quadratic	1		1.08 NS	1.29 NS	0.76 NS	3.06 NS	6.78 NS	0.90 NS	109.83 NS
Treatment	1		99.85 *	30.72 NS	93.86 NS	5.20 NS	61.09 NS	15.09 NS	26.29 NS
Age*treatment	2		5.02 NS	49.72 NS	18.94 NS	2.82 NS	15.52 NS	54.18 NS	63.55 NS
Error	27		19.90	29.51	22.41	26.27	24.65	19.60	28.37

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$ MS = Mean Square

3.3.7 Goblet Cells

Populations of goblet cells staining positive for AB/PAS on the intestinal villi and in intestinal crypts in the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 are shown in Tables 3.17 and 3.18. Significant differences with age in the number of goblet cells per mm intestinal villus were found in the duodenum ($p < 0.01$) and the mid-ileum ($p < 0.001$) (increased in the duodenum but decreased in the mid-ileum) but not in the jejunum and the proximal ileum. Unlike the number of goblet cells on the villi, those in the crypts linearly increased with age in all segments of the jejunum ($p < 0.05$ or < 0.01), but there were no significant effects of age in the duodenum and the ileum.

ANOVA did not show any difference between control and infected piglets in the number of goblet cells on the villi in all segments of the small intestine (Table 3.17). A significant interaction between age and treatment ($p < 0.05$) suggested that *Y. enterocolitica*-infected piglets had a larger number of goblet cells on the villi in the mid-ileum (mean 28.1 ± 2.9, compared with the control mean 13.3 ± 4.3) on day 5 (Table 3.17). In the distal ileum, the mean number of goblet cells (31.0 ± 5.0) in infected piglets on day 5 was also greater than in controls (14.9 ± 4.4), and the interaction mean square was greater than the error mean square although not significantly different. In contrast, there was a lower density of goblet cells in the intestinal crypts in the duodenum in infected piglets than in controls at all ages ($p < 0.05$) (Table 3.18).

3.4 DISCUSSION

Neonatal life is a period of rapid growth of the body and of maturation of many organs. Gastroenteritis during this vulnerable period might be expected to have a serious impact on the growth of the young animal. In the present study, *Y. enterocolitica* infection in the newborn piglet impaired body growth, reduced the size of the liver and altered the structure of the intestine.

3.4.1 Gastroenteritis Affects Body Growth

The rate of gain of body weight, but not of body length, was less in the infected pigs than in the control group. A similar pattern was seen in childhood diarrhoea in which

body weight gain was significantly affected but not increments in height (Black, 1991). A smaller weight gain in infected animals is likely to be caused by a combination of fluid loss through diarrhoea and reduced intake and availability of nutrients. Loss of body fluid in infected animals may be a contributing factor, although there were no overt signs of dehydration (Chapter 2, 2.3.1). Growth impairment might result from reduced availability of nutrients because of the decreased milk intake (Appendix 1) during the infection, as well as from impaired function of the gastrointestinal tract. After antibiotic therapy, the milk intake of infected piglets was equal to that of controls and defaecation became less frequent in infected animals. The rate of weight gain was similar in the two groups during the 9 days following the start of antibiotic therapy. The gap in body weight which occurred during the 5 day period of illness was not overcome by the end of the experiment.

The mean body weight on each day of control and infected piglets shows a more rapid increase from birth to day 1 than on other days (Figures 2.3 and 3.2). This was probably the result of the gut having little contents at birth, but on subsequent days the animals were either euthanased an hour after feeding or the others which were being studied for a longer period were weighed immediately after feeding. The body weight of the 10 piglets which were studied for the full 14 days was not affected in this way and their mean values produced a more consistent growth curve (Figure 3.2) than did the means for all pigs which included values from those killed at 3 and 5 days (Figure 2.3).

3.4.2 Organ Growth during Gastroenteritis

Impaired body growth, which accompanied the diarrhoea caused by *Y. enterocolitica*, was likely to be due partly to reduced nutrient availability for growth resulting from several sources: reduced intake, impaired digestibility, reduced absorption or altered carbohydrate metabolism. There is likely to be reduced glucose delivery to the liver because of lower food intake and increased transit time through the intestine associated with diarrhoea. The lower liver weight at 5 days in the infected piglets suggests that carbohydrate availability was impaired and that the liver carbohydrate content may be decreased. The lower liver weight in infected piglets (about 20 g less than in controls on day 5) may have a similar origin to that in starved or malnourished animals. Newborn piglets, starved for 24 hours, showed a reduction in liver weight and in carbohydrate content (Widdowson and Crabb, 1976). The liver weight fell from 37.7

g at birth to 23.2 g after 24 hours starvation in newborn piglets compared with an increase to 44.5 g in colostrum-suckled piglets of the same age (Widdowson and Crabb, 1976). Analyses of the liver composition showed that the main change during this time without food was a fall in the amount of carbohydrate from 3.8 g at birth to 0.5 g at 24 hours in unsuckled piglets, whereas protein, fat, DNA and the ratio of protein to DNA did not change significantly (Widdowson and Crabb, 1976). During the period of severe diarrhoea, piglets in the present study had less appetite and the effect on the liver may be similar to that produced by starvation.

The lower liver weight in the *Y. enterocolitica*-infected piglets, on the other hand, may be different from that in undernourished experimental animals. In the present study, of all the abdominal organs examined, only the liver was reduced in weight by the *Y. enterocolitica* infection, whereas in studies of malnutrition, the liver was not the only organ affected. Malnutrition, produced in neonatal rabbits by combining two litters at 7 days after birth to increase the litter size to 13-16 pups, significantly impaired growth of the body and the liver, as well as the pancreas, small intestine and the small intestinal mucosa (Butzner and Gall, 1988a). Malnutrition decreased cell number and cell size in the liver, as well as in other organs, in newborn rats subjected to restricted feeding for 21 days from birth, and after the animals were refed these reductions were not overcome by adulthood (Winick and Noble, 1966). DNA content, as an indicator of the cell number, and the ratio of protein to DNA, as an indicator of cell size, were 12% and 43% higher respectively in the liver in suckled piglets than in unfed pigs during the first day (Widdowson and Crabb, 1976). Whether the lower liver weight in infected piglets was associated with either reduced cell division or cell size was not examined in the present study. Since the liver was the only organ whose weight decreased in the *Y. enterocolitica*-infected piglets, malnutrition during the period of diarrhoea may not be the main reason for the lower liver weight. As well as possible depletion of glycogen stores and reduction in cell number and size, other factors associated with the infection may be involved in reduction of the liver weight. Microabscesses were present in the liver in 7 of 8 infected piglets by day 5 so that the possibility that bacterial invasion of the liver reduced liver growth cannot be excluded.

The decreased liver weight in control piglets from 5 to 14 days was unexpected and the reason is not apparent. It may be associated with the administration of oxytetracycline. A diet containing 0.5 g oxytetracycline induced a significant reduction in the weight of the liver in New Hampshire cockerels (Franti *et al.*, 1972). In contrast to the control

group, in previously-infected animals given the same antibiotic therapy to produce clinical recovery from the *Y. enterocolitica* gastroenteritis, the liver weight increased over the same period to be higher than in the controls at 14 days. It is possible that the effect of this antibiotic on the liver may be different in animals with and without concurrent infection. Certainly, the infected animals displayed greater appetite than the controls, and although the milk intake was similar in the two groups, the previously-infected piglets fed more rapidly and appeared to be hungrier. Antibiotic therapy in healthy control animals may have altered the normal gut flora to be greater extent, and possibly affected digestion, and have resulted in usage of liver glycogen.

3.4.3 Gastroenteritis Affects Growth of the Intestine

Milk intake was lower in the infected piglets, but the amount offered to the control group was also reduced, although not to the same extent, for the reasons discussed in the previous Chapter. It is likely that reduced food intake is not the only cause of the inadequate nutrient supply, but that altered gastrointestinal function is also involved. In the present study, there is evidence of altered gastrointestinal structure. Although microscopic changes in the gut were apparent (Chapter 2, 2.3.3), this was not reflected in gross measurements of length or weight of whole tissue from any segment of the small intestine or colon. The caecum was greater in weight in the infected piglets than in the control group. It could be due to severe *Y. enterocolitica* invasion of the caecum (Chapter 2, 2.3.3) stimulating proliferation of scattered reticuloendothelial cells, Peyer's patches and connective tissue.

The total weight of muscle in the small intestine was close to being significantly higher in infected piglets than in controls (Table 3.7), and when the weight was expressed as weight relative to body weight the difference became highly significant ($p < 0.001$), probably related to the increased mechanical load (gut motility) associated with diarrhoea as well as to the lower body weight in the infected group. Very distinct hyperplasia and hypertrophy in smooth muscle have been demonstrated in the small intestine of rats infected with the nematode parasite *Trichinella spiralis* (Blennerhassett *et al.*, 1992).

The weight of the mucosa of the small intestine was unaffected by the bacterial infection. Mucosal damage was evident by days 3 and 5 in the infected pigs (Chapter 2, 2.3.3) but this could not be detected from whole tissue weights. The mean weights

were lower, but this did not reach statistical significance. This may be because the difference was too small to be detected by gross measurement, the animal variation was large, or that the area of damage was only a small part of the total mucosa. In addition, crypt hyperplasia, bacterial clumps and inflammatory cells in the mucosa may balance cellular losses elsewhere in the mucosa.

3.4.4 Effect of *Y. enterocolitica* on the Structure of the Small Intestine

In spite of the histological damage evident to the microstructure of the villi of infected piglets (Chapter 2, 2.3.3), morphometric measurements of the villus height were lower only in the proximal jejunum. In the small intestinal mucosa of infected pigs when the animals had severe diarrhoea, numerous villi had been destroyed by large microabscesses and had been shed into the lumen, leaving only a fewer intact villi in some areas of the small intestine (Chapter 2, 2.3.3). It is difficult to find the most appropriate way to quantify villus damage. The present method of quantitating villus height, by measuring the long villi, has been used in studies of experimental viral infectious diseases in pigs (Kelly *et al.*, 1972; Davidson *et al.*, 1977; Pearson and McNulty, 1977), *Y. enterocolitica* infection in rabbits (O'Loughlin *et al.*, 1986) and also used in the assessment of gut function in human gastroenteritis (Berg *et al.*, 1973). Where a transverse section of the intestine contains no intact villi, no measurement of villus length can be made. When only the longest villi are measured, the effect on the remaining villi, often the worst affected, is ignored and the damage is underestimated. Any method attempting to measure surface area is fraught with difficulty, particularly as many villi are sectioned at an angle so that they appear shorter than they actually are. Visual examination of sections from all parts of the small intestine in *Y. enterocolitica*-infected pigs showed that villus damage was extensive, but this was not reflected in the measurement of the height of the longest villi. A significant difference in the intact long villus length between control and infected animals was demonstrated, however, only in the proximal jejunum and not in other segments of the small intestine. In rabbits experimentally infected with *Y. enterocolitica*, shorter villi were present only in the ileum (O'Loughlin *et al.*, 1986). Measurement of the length of the longest villi was not a good indicator of the extent of injury to the small intestinal mucosa in *Y. enterocolitica* enteritis in the present study. Other morphometric measurements, e.g. surface area of the epithelium or numbers of villi, are also not appropriate to determine the mucosal injury, in acute *Y. enterocolitica* enteritis. The surface line was not continuous due to the presence of large numbers of lesions and it was impossible to cut

sections of the small intestine in which all villi were aligned at right angles.

Longer crypts, accompanied by a larger number of proliferating crypt cells, indicated that crypt hyperplasia occurred in *Y. enterocolitica*-infected piglets. Marked crypt hyperplasia is likely to reflect the damage to villus cells. The crypt depth was dramatically increased in the entire small intestine of all infected piglets (Table 3.14). Significant acceleration of cell proliferation in the crypt further confirmed crypt hyperplasia in those animals (Table 3.16). Crypt elongation was more clearly seen in neonatal piglets infected with *Y. enterocolitica* than in infected rabbits, in which the increase in the crypt length was significant only in the distal small intestine (O'Loughlin *et al.*, 1986). Crypt hyperplasia in the small intestine is common in infectious diarrhoea (Pearson and McNulty, 1977; Shepherd *et al.*, 1979) but the mechanism responsible has not been established. One hypothesis is that a "feedback control" increases crypt cell production in response to the loss of villi. Feedback control has been demonstrated in mice following transient ischaemia (Rijke *et al.*, 1976), where more cells in the crypt undergo cell division to replace cells lost from the villus until the number of cells on the villus returns to normal. Another hypothesis is "adaptive control". Adaptation of crypt cell production has been found after resection of the small intestine, resulting in an increase in DNA synthesis, number of cells per crypt and cells per villus column (Hanson and Osborne, 1971). Recent studies suggest that an increase in crypt cell proliferation is a result of T-cell activation. Activation of T cells has been shown to induce crypt epithelial proliferation in foetal human small intestine and colon in organ culture and the effect is inhibited by the immunosuppressive drug cyclosporin A (Ferreira *et al.*, 1990; Evans *et al.*, 1992). These findings suggest that T-cell factors may account for the early response to bacterial infection. Acute inflammation would be the first factor to cause crypt proliferation in the lamina propria of the small intestine in *Y. enterocolitica*-infected piglets. Crypt hyperplasia had occurred by 3 days postinfection when the villi had not been badly damaged, although there were many inflammatory cells in the lamina propria of the crypts.

Vacuolation of villus epithelial cells is a morphological feature occurring during neonatal development in piglets, as in other mammals. At birth, the epithelium of the duodenum and jejunum contains no vacuoles and the nuclei are in an apical position (Staley *et al.*, 1969; Clarke and Hardy, 1971; Moon, 1972) but in the terminal ileum, the epithelial cells usually contain one large vacuole and the nucleus is at the base of the cells (Hardy *et al.*, 1971; Moon, 1972). Once the newborn has sucked colostrum, the epithelium of

the duodenum and jejunum becomes vacuolated and nuclei occupy different positions in the cell. By the time the vacuoles disappear and nuclei are located in the base of the cells, the epithelium is mature. Maturation of the epithelium occurs in the duodenum at 36-48 hours (Clarke and Hardy, 1971), in the jejunum at 10-11 days (Clarke and Hardy, 1971; Moon, 1972) and in the ileum vacuolated cells persist for 3 weeks and progressively disappear during the fourth week (Moon, 1972).

The vacuolation of the epithelium of the small intestine of neonatal mammals is associated with absorption of macromolecules which are transferred intact from colostrum in the intestinal lumen to the blood. Cessation of antibody transfer (closure) during the second day after birth in piglets coincides with the loss of vacuoles from the duodenum and jejunum (Staley *et al.*, 1968, 1969). Closure time has been demonstrated to be related to the total colostrum intake. Supplying more colostrum to newborn piglets causes early closure (Lecce and Morgan, 1962). The present control piglets at 3 days of age still possessed the immature feature of apical nuclei in the epithelial cells of the jejunum (Staley *et al.*, 1968). The position of the nucleus also was suggestive of relative immaturity since in naturally-suckled, 3-day-old piglets, the nuclei were either basal (Xu *et al.*, 1992a) or in varying positions (Clarke and Hardy, 1971). It is not known whether the jejunal epithelium had been vacuolated during the preceding 3 day period in these colostrum-deprived piglets. Since the piglets were colostrum-deprived and fed milk formula containing 1.68% protein instead of sow colostrum (17.8% protein) or mature milk (5.8% protein) (Pond and Houpt, 1978) the lower protein concentration in the milk formula may have delayed maturation of the epithelium by decreasing the rate of enterocyte migration (Rudo *et al.*, 1976). It is unclear whether the jejunal epithelium, which has cells with no vacuoles but apical nuclei, retains its ability to transfer macromolecules.

Epithelial cells on intact villi from the duodenum to the proximal ileum in *Y. enterocolitica*-infected piglets did not differ from those in controls. The bacterial infection, however, accelerated the appearance of more mature features in the epithelial cells in the mid- and distal ileum, probably due to greater proliferation of crypt cells. Absence of vacuolation at 5 days postinfection in the distal ileum was coincident with the presence of the deepest crypts and greatest crypt cell proliferation. Rapid proliferation during the infection period may make the life span of the new epithelial cells too short for vacuoles to develop. Moon (1972) has related the presence of vacuoles in the distal ileum to the age of the cells, since most vacuolated cells examined

after exposure to tritiated thymidine had synthesized their DNA more than 72 hours previously. By day 14, when the mucosa of the small intestine was no longer invaded by the bacteria, vacuoles were again present, although much fewer in number and smaller in size than in the enterocytes in controls. The presence of vacuoles again coincided with a lower crypt size than during the infection period, and once again a similar rate of BrDU incorporation to that in the controls. The presence of vacuoles on day 14 suggests that the life span of the epithelial cells at this stage might have lengthened enough for vacuoles to develop. These cells with fewer vacuoles than controls are likely to be more physiologically mature. Normally, vacuoles in the ileum decreased from the second to third weeks after birth in piglets (Moon, 1972).

The rate of cell migration up the villi increases with age, taking 7-10 days in 1-day-old piglets but only 2-4 days in 21-day-old pigs (Moon, 1971). The disappearance of ileal vacuoles, when pigs are about 3-week-old, coincides with the disappearance of cells that survive more than 4 days after synthesis of DNA (Moon, 1972). Ileal vacuoles also disappeared in rats at about 3 weeks of age (Clarke and Hardy, 1969), at which age the long-lived epithelial cells disappeared in this species (Koldovský *et al.*, 1966b). Faster proliferation of crypt cells in infected piglets probably makes the survival time shorter than in controls.

Goblet cells secrete mucin, which protects the mucosa from bacterial overgrowth and penetration (Specian and Oliver, 1991). Hyperplasia of goblet cells has been reported in bacterial infection in rabbits (Mantle *et al.*, 1991) and in puppies heavily infected with the parasite *Toxocara canis* (Lloyd *et al.*, 1991). Whereas in weanling rabbits the increase in goblet cell number occurred at a very early stage of the infection (1 day postinfection), in the distal ileum in the *Y. enterocolitica*-infected piglets, the hyperplasia of the goblet cells was apparent by day 5, but not by day 3, and statistically significant only in the mid-ileum, although the mean values were also greater in the distal ileum. Since *Y. enterocolitica* infection caused more damage in the ileum than in the duodenum and jejunum (Chapter 2, 2.3.3), it is not surprising that goblet cell hyperplasia developed in the ileum where mucosal injury was most severe. There may be several reasons for the less marked hyperplasia in the piglets compared with that in the infected weanling rabbits: (i) species difference, (ii) age difference or (iii) difference of histological methods of fixation and staining. The difference in methods is probably the most important.

First, Carnoy's solution was used as the fixative in the rabbit study, whereas Bouin's solution was used in the present study. However, there have been no direct comparisons made of the number of the goblet cells in tissues treated with different fixative solutions.

Secondly, Mantle *et al.* (1991) used only PAS, not both AB and PAS, to stain the cells in the rabbit study. In general, immature goblet cells in the crypts of the small intestine produce neutral mucin containing little sialic acid, so that more cells are stained by PAS. As the cells mature and migrate to the villus tip, the mucin becomes increasingly sialated, so that more cells are stained by AB (Specian and Oliver, 1991). In a study of normal pigs using both AB and PAS staining, more goblet cells stained with AB than PAS on the villi whereas there were more goblet cells stained with PAS than AB in the crypts (Dunford *et al.*, 1990). During the period of *Y. enterocolitica* infection, crypt hyperplasia occurred in both the rabbit and piglet studies. Longer crypts would produce more goblet cells staining with PAS (containing neutral mucin), so that counting only PAS stained goblet cells along the crypt-villus axis may suggest there was a bigger increase of goblet cell number in infected rabbits than in infected piglets.

The *Y. enterocolitica*-infected piglets clearly have impaired soft tissue growth compared with the controls. Over the 5 days of infection, body weight increased slowly, although the growth of organs was not compromised more than that of the body as a whole, except the liver which appeared to be particularly vulnerable. Gross measurement of intestinal tissue did not reflect the histological effects of the infection. Following institution of effective antibiotic therapy, the piglets showed a remarkable recovery in body weight gain and organ growth and the disappearance of gastrointestinal lesions. It is of interest to note that skeletal growth, indicated by the crown-rump length and nose-rump length, was apparently unaffected by the *Y. enterocolitica* infection in the piglets, which confirmed reports in children with diarrhoea (Black, 1991).

3.4.5 Evaluation of Development of the Control Piglets

A question to be addressed is whether the piglets in the present study were growing and maturing over the two week experimental period in a manner comparable to piglets raised naturally by their dam or under other artificial rearing conditions. A study of the impact of gastroenteritis on development requires that the severity of the disease be as uniform as possible, which dictated that both the infected and control animals were reared artificially, and that the piglets had to be deprived of colostrum. They also may

not have acquired the normal gut flora by not contacting their dam. It is therefore necessary to compare the growth and development of the control piglets with others naturally-reared before it can be considered valid to extrapolate from the present model to animals with a naturally-acquired infection.

For evaluation of the effect of the infection on body and gut development, control piglets must be prevented from contamination with *Y. enterocolitica* and all piglets should ideally be kept free of other pathogens. This necessitated prevention of contact of the piglet with the skin of its dam after birth, and probably resulted in the piglets failing to acquire some of the normal gut flora. Colostrum deprivation would appear to be an important condition in setting up a repeatable model of *Y. enterocolitica* enteritis in the newborn piglet, since earlier studies (Schiemann, 1988) were less successful when maternal antibodies were available to the piglets. Because of their lack of protective maternal antibodies, strict hygiene was required, making artificial feeding necessary, and a commercial milk formula the practical solution. Because many infected piglets developed anorexia for a short period, some control animals were subjected to a short period of restricted feed intake (Appendix 1). It is, therefore, likely that the artificial rearing conditions would make growth different from that in suckled piglets.

The body weight gain during the first 24 hours in the present study (8%) was the same as that in piglets nursed by sows (Xu *et al.*, 1992a) but lower than the 12.6% reported by Widdowson and Crabb (1976). By days 3 and 14, there were only 12.2% and 51% increases over birth weight in the present piglets, whereas increases of 23% by day 3 (Xu *et al.*, 1992a) and 100% by day 10 (Widdowson and Crabb, 1976) have been reported in suckled piglets.

Along with the slower body growth, organ and gut growth in the present piglets was also less than in suckled piglets over the first three days after birth in the study by Xu *et al.* (1992a,b): the stomach weight was 85% less, the small and the large intestinal weights 14% and 89% less, the small intestinal length 12% less, and the weight of mucosa and muscle of the small intestine 17% and 6% less. The effect would appear to be less in the small intestine than in the stomach and large intestine. Similar comparisons in rodents have produced conflicting results. Newborn rats, fed for 40 hours on an artificial formula isocaloric with expressed rat milk, had significantly lighter stomachs than those fed natural milk (Berseth, 1987b), whereas isocaloric milk formula fed rats and guinea pigs had greater stomach and caecal weight (Tonkiss *et al.*, 1985;

Weaver *et al.*, 1991).

The weight of most organs examined was much lower than that in naturally-suckled piglets except for the pancreas, which had almost the same weight. The liver, spleen and kidneys were 72 g, 3.5 g, and 14.6 g respectively in the present control group at 14 days compared with 129 g, 7 g and 23.7 g in suckled piglets (Widdowson and Crabb, 1976).

Whilst recognising that artificial feeding could impact on growth and development of both the control and infected piglets, it is not possible to quantify the relative importance of the different factors. Nutrient value of the diet, quantity of milk consumed and its digestibility should all be considered. Colostrum, in addition to its nutrient value, is an important source of growth factors. Beagle puppies fed artificial bitch milk gained as much body weight as those that were suckled, but there was no appreciable growth of the intestinal mucosa (Heird *et al.*, 1984). Epidermal growth factor (EGF) is a major growth-promoting agent in human milk (Carpenter, 1980), stimulating cellular proliferation in intestinal mucosa (Klein and McKenzie, 1980; Al-Nafussi and Wright, 1982). EGF-fed animals have heavier and longer intestines and their intestines contain more DNA and RNA than animals not fed EGF (Berseth, 1987a). EGF also influences the maturation and the proliferation of enterocytes in the suckled mouse (Malo and Ménard, 1982). Insulin is also believed to influence small intestinal growth and development. The concentration of insulin in human and pig colostrum is 3- to 30-fold that in serum (Kulski and Hartmann, 1983; Slebodzinski *et al.*, 1986; Weström *et al.*, 1987). Oral insulin increases the weight of the mucosa and the protein and RNA content in the mucosa of the small intestine in newborn miniature pigs (Shulman, 1990). These valuable growth factors were absent from the milk formula used in the present study.

The slower growth in body and in internal organs in the present piglets than in the suckled piglets is likely to be due in large part to the lower nutrient value in milk formula than in sow milk, particularly in protein concentration. Sow colostrum contains about 17.8% protein and mature milk 5.8%. The piglets were deprived of colostrum, and fed milk formula (containing only 1.68% protein) during the period of the experiment. A low protein concentration in the milk reduced the growth rate of the gastrointestinal tract in piglets (Simmen *et al.*, 1990) and rabbits (Butzner and Gall, 1988a). It was decided not to add extra protein to raise it to the level in porcine milk,

because a very high protein concentration in the milk formula may have exacerbated the diarrhoea in infected piglets, since dipeptidase activities in the small intestinal mucosa may have decreased, as occurs in human patients with diarrhoea (Berg *et al.*, 1973).

The presence of the bacterial flora in the intestine is believed to influence gut morphology, since both germ-free rearing and the administration of antibiotics alter structural features of the gut. Germ-free rats and pigs have a significantly lower weight of the wall of the intestinal canal (Gordon *et al.*, 1966; Miniats *et al.*, 1973), mainly due to a reduction in scattered reticuloendothelial cells, Peyer's patches and connective tissue in the mucosa and submucosa (Gordon and Bruckner-Kardoss, 1961). Renewal rates of the intestinal epithelium were lower in germ-free mice (Abrams *et al.*, 1963). It has been reported that antibiotic treatment reduces the thickness of the intestinal wall (Pond and Houpt, 1978). Antibiotics were administered to a group of infected piglets in the present study to examine the extent of recovery of the gastrointestinal tract from bacterial gastroenteritis, however, when the infected piglets were administrated antibiotic from day 5 to days 9 or 13, all the control piglets were treated in the same way.

It is clear that piglets reared colostrum-deprived and fed human milk formula grew more slowly than piglets nursed by sows, the greatest difference being in the weight of the body, the stomach and large intestine. However, as a model for biomedical research, overall growth rate is not important as long as the animals are healthy and their internal organs grow in proportion to the body. In the present study, all control piglets were healthy and growth of almost all internal organs was proportional to the body as a whole during the period of the experiment. The growth ratio was comparable with, or slightly higher than, that in piglets suckled by sows. The weight of the small intestine relative to body weight was similar to that in piglets nursed by sows (Cranwell and Moughan, 1989). The lengths of the small and large intestines in the 14-day-old piglets (respectively 268 and 53 cm/kg) were much greater than in suckled piglets (186 and 36 cm/kg) (Widdowson and Crabb, 1976; Widdowson *et al.*, 1976). Stomach, liver and kidney weights relative to body weight (5.0, 32.1 and 6.52 g/kg respectively) were almost the same as those in sow-nursed piglets (4.8, 35.5 and 6.5 g/kg respectively) (Widdowson and Crabb, 1976; Widdowson *et al.*, 1976; Braude *et al.* 1981). The relative pancreatic weight was greater than in suckled animals (Cranwell and Moughan, 1989).

The villus epithelium of the small intestine in the control piglets appeared less mature than in piglets nursed by sows. The apical position of the nuclei in the jejunum in 3-

day-old piglets was different from that in suckled piglets at the same age, as discussed earlier. The villus length and goblet cell number were comparable with those reported in suckled, neonatal piglets. Artificial feeding did not affect the height of the longest villi of the small intestine. The average height of the longest villi (782 μm) from six segments of the jejunum and ileum on day 3 in the present piglets was no shorter than that (718 μm) in suckled piglets aged 2 days studied by Cera *et al.* (1988), using similar methods of measurement. The crypt depth in control animals was similar at all ages. Apart from the differences in some morphological characteristics of the enterocytes, the microstructure of the intestine was comparable in control piglets and naturally-suckled animals.

3.4.6 Conclusions

Diarrhoea caused by *Y. enterocolitica* infection in neonatal piglets resulted in slower growth of the body and some organs and changes to the structure of the small intestinal mucosa, villus damage and crypt hyperplasia and early maturation of epithelial cells in neonatal piglets. Infectious diarrhoea may change not only the morphology but also the biochemistry of the small intestine, e.g. mucosal enzyme activity, cell size and cell protein synthetic capacity. Alterations to the biochemistry of the mucosa of the small intestine in piglets with diarrhoea are described in the next Chapter.

3.5 SUMMARY

The newborn, colostrum-deprived piglets fed with milk formula were healthy and grew linearly in both weight and length of body during the first 14 days after birth. Organ and intestinal growth was proportional to body growth as a whole except the liver whose relative growth rate was slower from days 5 to 14.

Diarrhoea, caused by *Y. enterocolitica*, significantly reduced the gain in the body weight and liver weight, compared with the controls, during the acute infection period, and increased caecal weight through the period of the experiment, but did not affect the length of the body or intestine or the weight of other parts of the intestine or non-intestinal organs. The *Y. enterocolitica* infection stimulated crypt growth through the entire small intestine. Crypt hyperplasia had occurred by 3 days, was greatest at 5 days postinfection and did not return to the value in the controls by day 14 when the animals had clinically recovered after antibiotic therapy. Acceleration of crypt cell proliferation confirmed crypt hyperplasia. In contrast, villus atrophy in infected piglets occurred only in the proximal jejunum. *Yersinia enterocolitica* infection caused an early decrease in the number of vacuolated epithelial cells in the distal part of the small intestine. A larger number of goblet cells appeared on the villi only in the mid- and distal ileum on day 5, whereas a lesser number was seen in the intestinal crypts of the duodenum. A more mature epithelium was evident in the distal ileum: early disappearance of vacuolated cells during acute infection and many fewer vacuoles were present by day 14 after clinical recovery.

Chapter 4

BIOCHEMICAL CHANGES IN THE INTESTINE OF NEWBORN PIGLETS INFECTED WITH *YERSINIA ENTEROCOLITICA*

4.1 INTRODUCTION

Enteric infections are very common in infants and young animals (Marks *et al.*, 1980; Cohen, 1991; Lee *et al.*, 1991). Epithelial cells in the small intestine divide in the crypts and migrate up the villi to be shed. During viral infections, cells are lost from the villi more quickly than normal and crypt cells divide faster (Kelly *et al.*, 1972; McAdaragh *et al.*, 1980). The desquamation of epithelial cells causes a reduction in, or even loss of, the activities of membrane-bound enzymes, particularly disaccharidases, resulting in malabsorption (McClung *et al.*, 1976; Davidson *et al.*, 1977; Kerzner *et al.*, 1977; O'Loughlin *et al.*, 1988).

Disaccharidases comprise one of the major groups of digestive enzymes. In the jejunal mucosa, the activities of lactase, maltase and sucrase in the villus are normally 4 to 10 times that in the crypt (Fortin-Magana *et al.*, 1970). Because disaccharidase activity is unique to the mucosa of the small intestine, reduction in these enzymes would result in less complete digestion of sugar. Normal sugar intake, coupled with low disaccharidase activity, might be expected to cause diarrhoea because of the increased osmotic concentration due to unabsorbed sugars in the lumen of the gut (Bailey *et al.*, 1956; Graham *et al.*, 1984). Disaccharidase activity has, therefore, been used as an indicator of digestive and absorptive function in the small intestine in human clinical cases (Lebenthal and Lee, 1980) as well as in experimental enteritis after infection with enteroviruses in pigs (Davidson *et al.*, 1977; Davidson and Barnes, 1979; Graham *et al.*, 1984) and in mice (Collins *et al.*, 1988) or with enterobacteria, e.g. *Y. enterocolitica*, in nursed (Butzner and Gall, 1988b) and weanling (O'Loughlin *et al.*, 1986) rabbits.

Another intestinal enzyme, sodium-potassium dependent adenosine triphosphatase (Na^+-K^+-ATPase), which is associated with the absorption of sodium and glucose in the gut, decreases when there is a loss of villus enterocytes in acutely enterovirus-infected pigs (Kelly *et al.*, 1972; Davidson *et al.*, 1977). The activity of this enzyme is also lower

in biopsies from the proximal jejunum in humans with acute cholera (Hirschhorn and Rosenberg, 1968). Depression of Na^+-K^+-ATPase activity would cause defective sodium absorption by active transport processes.

During early postnatal development in the pig there are marked changes in lactase, maltase and sucrase activities in the small intestine. Lactase activity is higher in newborn pigs than in adults, whereas maltase activity is present at very low levels and sucrase is almost undetectable in the small intestine of newborn pigs (Bailey *et al.*, 1956; Walker, 1959a; Hartman *et al.*, 1961; Manners and Stevens, 1972; Widdowson *et al.*, 1976; James *et al.*, 1987). The high lactase activity is maintained during the first two weeks after birth (Manners and Stevens, 1972; Widdowson *et al.*, 1976; James *et al.*, 1987), then declines with age, but, in contrast, maltase and sucrase activities remain low during the first week after birth, then increase (Bailey *et al.*, 1956; Manners and Stevens, 1972). Almost all information about the development of disaccharidases in pigs has been obtained from piglets nursed by sows or weaned early then artificially fed.

In biomedical research in which piglets are used, colostrum deprivation is sometimes necessary to obtain a successful animal model (Schiemann, 1988). Any effect of colostrum deprivation on enzyme development, however, does not appear to be known.

Previous studies (Chapter 2 and 3) have demonstrated a reduced body weight gain and lower liver weight in *Y. enterocolitica*-infected piglets during acute diarrhoea and that the small intestine is the main target for the bacterial invasion during *Y. enterocolitica* infection in newborn piglets. In all infected piglets, some villi were partly or completely destroyed, accompanied by crypt hyperplasia, while in other areas the villi were intact and long. These alterations to the structure of the mucosa of the small intestine may change its protein, DNA and RNA contents, as well as the small intestinal enzyme activity. The ratios of tissue weight to DNA and protein to DNA have both been used as indicators of cell size and the RNA/DNA ratio has been considered to reflect the capacity to synthesize protein (Winick and Nobles, 1966, 1967; Majumdar, 1984). In the experiment described in this Chapter, to assess the impact of *Y. enterocolitica* infection on the intestinal function of the piglets, the activities of disaccharidases and Na^+-K^+-ATPase, and the concentration of protein, DNA and RNA in the mucosa of the small intestine have been determined during the infection period and subsequent recovery following antibiotic therapy.

4.2 MATERIALS AND METHODS

4.2.1 Experimental Design

The 37 piglets used to develop the animal model of bacterial gastroenteritis described in Chapters 2 and 3 were the experimental animals for the present investigation. Nineteen piglets were inoculated with *Y. enterocolitica* and 18 litter mates served as controls. Piglets were killed at three different times: 6 from each group at 3 days postinfection, 8 infected piglets and 7 controls after 5 days, and the remainder, 5 from each group, were treated with antibiotic from day 5 to day 10 or 14 (Chapter 2, 2.2.6) then killed on day 14.

4.2.2 Dissection and Samples

4.2.2.1 Blood sampling

The piglets were anaesthetized by intraperitoneal injection of 1-2 ml (according to body weight) of sodium pentobarbitone (South Island Chemical Ltd., Christchurch, NZ) (250 mg/ml, diluted in physiological saline). Five ml of blood were then collected by intracardiac puncture using a needle and syringe and placed in a Neo-tube (Nipro Medical Industries Ltd., Tokyo, Japan) containing sodium heparin. The blood samples were centrifuged at 500 g for 15 min, and the plasma aspirated and stored at -20 ^{0}C for later measurement of concentrations of Na^{+}, K^{+}, Cl^{-} and glucose.

4.2.2.2 Dissection

The animals were euthanased by intracardiac administration of an overdose (2-3 ml) of sodium pentobarbitone (250 mg/ml). Immediately after death, the animals were dissected (Chapter 3, 3.2.3).

4.2.2.3 Tissue for parallel cryostat sections

A length of 1.5 cm of the mid-jejunum and mid-ileum from both control and infected animals, killed on either day 5 or day 14 (n = 3 and n = 5 respectively), was opened through the conjunction with the mesentery, rinsed with cold physiological saline and laid over the surface of a 0.1% agar block with the villi pointing upwards from the

block. The tissue block was frozen and stored at -20 ^{0}C. Lactase activity was estimated on all tissue, but maltase and sucrase activities only on those from animals killed on day 14.

4.2.2.4 Mucosa

Mucosa was scraped from the whole duodenum and from a 10 cm length of each of the other six segments of the small intestine (Chapter 3, 3.2.3), from the remainder of the caecum after sampling for histology and from 4 cm lengths of the proximal and distal colon. The mucosae were stored at -20 ^{0}C until homogenization immediately before assay for protein, DNA, RNA, disaccharidases and Na^+-K^+-ATPase.

4.2.3 Electrolyte and Glucose Measurements

Plasma sodium and potassium were measured by indirect potentiometry using a Synchron EL-1SE electrolyte system (Beckman Instructions Inc., Fullerton, California, USA). An ion selective electrode (a lithium aluminum silicate glass electrode for sodium and an electrode containing a valinomycin membrane for potassium) was used in conjunction with a reference electrode to measure the changes in ion activity in diluted samples. The ions fill the surface of the electrode and the change in potential (voltage) is converted to concentration units in the sample.

Plasma chloride and glucose were determined using an automated system (Cobas Mira, Roche, Nutley, New Jersey, USA). Measurement of chloride is based upon a modification of the procedure of West and Coll (1956) in which chloride and ferric ions form a complex which absorbs strongly near 340 nm in an acid environment. The method for determination of glucose is based on the hexokinase glucose-6-phosphate dehydrogenase reaction.

4.2.4 Mucosal Homogenates

4.2.4.1 Homogenates for disaccharidases, protein, DNA and RNA

One hundred mg wet weight of the frozen mucosa from seven segments of the small intestine were weighed, suspended in 1.0 ml of chilled physiological saline and homogenised for about 90 seconds with an ultrasonic disintegrator (MSE Soniprep 150,

MSE Scientific Instruments, Manor Royal, England). Four ml of chilled physiological saline were added to the homogenate to give a 1:50 (w:v) mucosal homogenate.

4.2.4.2 Homogenates for Na^+-K^+-ATPase

Fifty mg of wet mucosal tissue from each of ten segments of the intestine (seven small intestine and three large intestine) were homogenized in 0.5 ml of 2.5 mM di-sodium ethylenediaminetetra-acetate (EDTA, May & Baker Ltd., Gagenham, England) to prepare a 1:10 homogenate. 0.1 ml of 0.1% sodium deoxycholate in 2.5 mM EDTA was added to 0.1 ml of homogenate to give a final concentration of 1:20.

4.2.5 Cryostat Section Homogenates

To determine the disaccharidase activity along the crypt-villus axis, homogenates were made of parallel cryostat sections cut at right angles to the crypt-villus axis from the top of the villus to the base of the crypt in the mid-jejunum and ileum, according to the method of Dahlqvist and Nordström (1966). The edges of the frozen tissue from the mid-jejunum and mid-ileum were trimmed to leave a tissue block with a surface of about 8 mm x 10 mm. The tissue block was then attached to a chuck in an electric microtome cryostat (Lipshaw Manufacturing Company, Detroit, Michigan, USA). The tissue was cut at right angles to the crypt-villus axis into parallel 14 µm thick sections from the top of the villi to the base of the crypts. Every 5 sections were combined in a vial with 1.0 ml of chilled physiological saline. The tissue suspension were homogenized in the same way as was the mucosal tissue.

4.2.6 Biochemical Measurements

4.2.6.1 Protein, DNA and RNA

The protein concentration in homogenates of the small intestinal mucosa was determined by the method of Lowry *et al.* (1951) using bovine serum albumen (BSA, Fraction V, Boehringer Manheim, Germany) as the standard (Appendix 5.1).

DNA and RNA were extracted from the tissue homogenate using different concentrations of perchloric acid (0.2N, 0.6N, 1.0N and 1.2N) following the procedures of Johnson and Chandler (1973) (Appendix 5.2). RNA concentration was estimated

from the absorbances of the extract of the tissue homogenate, read at 232 nm and 260 nm in a spectrophotometer (SP 6-550 UV/Vis, PYE Unicam Ltd, Cambridge, England), using the following formula (Fleck and Begg, 1965):

$$C_{RNA}\ (\mu g/ml) = (3.4\ OD_{260} - 1.44\ OD_{232}) / 0.068$$

Measurement of DNA content was performed using the diphenylamine method of Burton (1956) with the modification of Giles and Myers (1965) (Appendix 5.2).

4.2.6.2 Disaccharidases

The activities of three disaccharidase: lactase, maltase and sucrase, were measured by incubation with the appropriate disaccharide followed by the assay of liberated glucose with a tris-glucose oxidase reagent (Dahlqvist, 1964, 1984) (Appendix 5.3). Tris serves the double function of interrupting the activity of the intestinal disaccharidase at the end of the incubation period and inhibiting contaminant disaccharidases in the glucose oxidase (Dahlqvist, 1984). In any disaccharidase assay, if the amount of glucose liberated was greater than 1000 µmol, the sample was diluted and re-assayed.

To assess the repeatability of the lactase and maltase assays, replicate analyses were carried out in duplicate on a control sample of the mid-jejunal mucosa in 10 lactase assays and 5 maltase assays. For lactase, the inter-assay coefficient of variation was 16.1% and the intra-assay variation was 2.6%. For maltase, the inter-assay coefficient of variation was 18.5% and the intra-assay variation was 2.9%.

4.2.6.3 Na^+-K^+-ATPase

Na^+-K^+-ATPase was measured according to procedures described by Kelly *et al.* (1972) and Shepherd *et al.* (1979), in which samples were incubated with adenosine 5'-triphosphate (ATP) followed by the assay of inorganic phosphate (P_i) produced using a molybdate-sulphuric reagent and metol solution (Appendix 5.4). Since ouabain selectively inhibits the Na^+-K^+-ATPase activity stimulated by sodium and potassium, the difference in P_i produced with and without ouabain represents the Na^+-K^+-ATPase activity, and was expressed as micromoles of P_i produced per hour per g of wet tissue or per mg protein.

All biochemical assays were performed in duplicate. The concentration of protein was expressed as mg per g wet mucosa tissue or as total protein content (mg). Data for

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Table 4.1. Electrolyte and glucose concentrations in plasma of control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Na^+	K^+	Cl^-	Glucose
Treatment	Age (days)	n	Concentration, mmol/l (Mean ± SE)			
Control	3	2	140 ± 1	5.1 ± 0.3	97 ± 1	11.5 ± 0.1
	5	7	141 ± 2	4.3 ± 0.3	103 ± 1	6.3 ± 0.5
	14	5	138 ± 1	3.3 ± 0.1	101 ± 1	6.2 ± 0.4
Infected	3	2	140 ± 4	4.8 ± 0.3	99 ± 3	8.0 ± 0.2
	5	8	134 ± 2	4.4 ± 0.3	98 ± 1	3.6 ± 0.5
	14	5	135 ± 2	3.7 ± 0.1	102 ± 1	5.6 ± 0.6

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p
Age	2	18	NS	3.55	**	18	NS	36.27	***
Linear	1	34	NS	6.37	**	34	NS	34.64	***
Quadratic	1	12	NS	0.03	NS	13	NS	62.74	***
Treatment	1	55	NS	0.06	NS	1	NS	28.50	***
Age*treatment	2	24	NS	0.23	NS	41	*	4.34	NS
Error	23	21		0.57		9		1.73	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

DNA and RNA were expressed as ratios of wet mucosal weight to DNA, protein to DNA and total DNA content of each segment and the ratio of RNA to DNA. There were three expressions of enzyme activity in the mucosa of the intestine: µmol per g wet mucosa, µmol per g protein (for both disaccharidases and Na^+-K^+-ATPase), and total activity (µmol/min) of each segment (only for disaccharidases). Disaccharidase activities in cryostat section homogenates were expressed as µmol per g protein per min.

4.2.7 Statistical Analysis

Three analyses were used in the present study: correlation, one-way Analysis of Variance (ANOVA) and a two-way ANOVA. All analyses were carried out using the computer statistical package SAS (SAS Institute Inc., Cary, NC, USA, 1987). The data are presented as mean and standard error of the mean (SE) and results of the ANOVA are presented as probability (p). Both mean square (MS) and p are tabled. A p value of < 0.05 was considered significant. Correlations between lactase and villus height/crypt depth ratio (Chapter 3, 3.3.5) or lactase and lesion numbers (Chapter 2, 2.3.3) were analyzed by SAS correlation analysis. Comparison of disaccharidase activity in parallel sections along the crypt-villus axis in the jejunum and ileum between control and infected piglets was made using one-way ANOVA. Other data were analyzed by a two-way ANOVA for effects between the control and infected groups and between ages using the general linear model of SAS. Since some of the enzyme data showed heterogeneous variance between ages or between the control and infected groups, all data for lactase and maltase activities per gram protein were logarithmically transformed and retested. No consistent changes were found in the results of ANOVA of these logarithmically transformed data, therefore, ANOVA for all data presented in this chapter used non-logarithmically transformed data.

4.3 RESULTS

4.3.1 Plasma Electrolyte and Glucose Concentration

Plasma electrolyte and glucose concentrations in the control and infected piglets on days 3, 5 and 14 are shown in Table 4.1. Plasma glucose concentration in control piglets decreased with age, both the linear and quadratic components being highly significant ($p < 0.001$). The marked decrease in glucose concentration occurred between days 3 (11.5 ± 0.1 mmol/l) and 5 (6.3 ± 0.5 mmol/l) whereas it did not change from days 5 to

Table 4.2. Protein concentration in wet mucosal tissue of the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Protein concentration, mg/g (Mean ± SE)						
Control	3	6	121 ± 6	110 ± 7	125 ± 5	127 ± 5	108 ± 6	114 ± 6	102 ± 5
	5	7	119 ± 5	126 ± 4	122 ± 5	111 ± 4	100 ± 5	109 ± 3	108 ± 6
	14	5	105 ± 6	110 ± 7	101 ± 3	100 ± 6	90 ± 3	97 ± 3	89 ± 4
Infected	3	6	120 ± 7	108 ± 4	116 ± 5	121 ± 5	114 ± 6	106 ± 5	101 ± 5
	5	8	118 ± 4	116 ± 3	113 ± 6	110 ± 4	100 ± 5	101 ± 4	103 ± 2
	14	5	97 ± 9	113 ± 6	116 ± 6	110 ± 4	106 ± 6	92 ± 3	93 ± 4

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	1290	**	566	*	404	NS	1107	**	548	NS	679	**	631	*
Linear	1	2394	**	1	NS	796	NS	1802	***	743	*	1351	**	773	*
Quadratic	1	225	NS	1128	*	17	NS	365	NS	325	NS	12	NS	523	*
Treatment	1	122	NS	57	NS	7	NS	4	NS	494	NS	412	NS	6	NS
Age*treatment	2	42	NS	135	NS	545	NS	173	NS	173	NS	11	NS	56	NS
Error	31	240		156		193		138		173		105		123	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 4.3. Total protein content in the mucosa of the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Protein content, mg (Mean ± SE)						
Control	3	6	127 ± 11	800 ± 104	806 ± 52	799 ± 86	669 ± 43	710 ± 33	575 ± 53
	5	7	140 ± 18	983 ± 96	878 ± 69	721 ± 65	648 ± 84	663 ± 80	584 ± 74
	14	5	158 ± 13	935 ± 70	900 ± 52	831 ± 65	855 ± 89	923 ± 61	673 ± 42
Infected	3	6	105 ± 9	865 ± 71	826 ± 76	785 ± 5	726 ± 76	707 ± 125	613 ± 54
	5	8	113 ± 8	753 ± 50	738 ± 73	769 ± 75	666 ± 76	628 ± 64	546 ± 40
	14	5	167 ± 23	988 ± 69	991 ± 83	905 ± 65	794 ± 26	773 ± 87	582 ± 51

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	6416	**	47823	NS	65903	NS	45206	NS	86853	NS	124125	NS	11727	NS
Linear	1	12548	**	94735	NS	106397	NS	42936	NS	110305	NS	138681	NS	8688	NS
Quadratic	1	398	NS	1494	NS	28338	NS	49962	NS	68047	NS	116387	NS	15387	NS
Treatment	1	1614	NS	12257	NS	796	NS	11310	NS	198	NS	35040	NS	8363	NS
Age*treatment	2	1098	NS	93159	NS	44603	NS	5824	NS	9734	NS	16062	NS	11769	NS
Error	31	1150		38470		30679		32519		33136		39420		188707	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 4.4. Ratio of mucosal tissue to DNA in the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (Days)	n	Tissue/DNA ratio (Mean ± SE)						
Control	3	6	196 ± 19	182 ± 12	175 ± 14	163 ± 17	192 ± 18	161 ± 16	163 ± 20
	5	7	242 ± 33	164 ± 15	176 ± 17	180 ± 14	182 ± 12	190 ± 8	158 ± 16
	14	5	184 ± 13	186 ± 10	194 ± 9	203 ± 6	198 ± 12	218 ± 11	168 ± 18
Infected	3	6	175 ± 19	202 ± 18	192 ± 20	160 ± 10	175 ± 14	172 ± 18	148 ± 13
	5	8	161 ± 11	158 ± 8	176 ± 7	168 ± 11	179 ± 12	183 ± 10	159 ± 10
	14	5	199 ± 12	168 ± 9	179 ± 8	182 ± 9	190 ± 18	184 ± 16	147 ± 7

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	940.6	NS	3229.6	*	395.3	NS	2677.4	NS	560.8	NS	3346.4	NS	29.1	NS
Linear	1	67.3	NS	629.3	NS	126.3	NS	5343.6	*	742.2	NS	6142.8	*	10.5	NS
Quadratic	1	1793.3	NS	5720.5	*	679.9	NS	1.8	NS	409.0	NS	452.8	NS	46.5	NS
Treatment	1	7456.9	NS	17.6	NS	5.0	NS	1277.4	NS	819.2	NS	996.9	NS	1263.6	NS
Age*treatment	2	7224.2	NS	1098.2	NS	678.2	NS	210.3	NS	186.3	NS	1395.1	NS	376.7	NS
Error	31	2626.2		956.7		1139.5		958.1		1286.8		1073.6		135.7	

NS $p > 0.05$ and * $p < 0.05$

Table 4.5. Ratio of mucosal protein to DNA in the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (Days)	n	Protein/DNA ratio (Mean ± SE)						
Control	3	6	23.8 ± 2.6	19.8 ± 1.1	21.6 ± 1.5	20.6 ± 2.0	20.4 ± 1.5	18.2 ± 1.7	16.5 ± 2.0
	5	7	29.1 ± 4.8	20.5 ± 1.6	21.2 ± 1.7	19.9 ± 1.6	18.1 ± 1.3	20.9 ± 1.1	17.1 ± 1.9
	14	5	19.2 ± 1.6	20.2 ± 0.8	19.6 ± 1.1	20.2 ± 1.1	17.9 ± 1.3	21.0 ± 0.7	14.7 ± 0.9
Infected	3	6	20.6 ± 2.0	21.6 ± 1.6	21.9 ± 2.1	19.5 ± 1.9	19.4 ± 0.9	18.3 ± 2.2	14.8 ± 1.4
	5	8	18.9 ± 1.2	18.5 ± 1.3	19.7 ± 1.0	18.3 ± 1.1	17.8 ± 1.0	18.5 ± 1.3	16.4 ± 1.3
	14	5	18.9 ± 1.4	18.9 ± 1.1	20.8 ± 1.0	20.0 ± 1.2	20.1 ± 2.3	16.8 ± 1.1	13.6 ± 0.8

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	74.07	NS	5.88	NS	8.65	NS	4.44	NS	13.16	NS	7.02	NS	20.70	NS
Linear	1	74.21	NS	5.94	NS	12.18	NS	0.27	NS	2.34	NS	1.15	NS	17.40	NS
Quadratic	1	78.01	NS	5.50	NS	4.69	NS	8.70	NS	23.55	NS	12.67	NS	25.11	NS
Treatment	1	187.22	NS	2.00	NS	0.06	NS	8.47	NS	0.88	NS	41.71	NS	11.00	NS
Age*treatment	2	81.70	NS	13.68	NS	5.96	NS	1.30	NS	7.13	NS	12.70	NS	0.75	NS
Error	31	47.34		11.36		13.20		15.17		11.78		13.63		14.60	

NS $p > 0.05$ MS = Mean Square

Table 4.6. Total DNA content of the mucosa of the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	DNA content, mg (Mean ± SE)						
Control	3	6	5.4 ± 0.3	40.0 ± 4.0	38.0 ± 4.2	39.0 ± 2.4	33.7 ± 3.1	40.5 ± 3.8	36.5 ± 3.5
	5	7	5.2 ± 0.5	48.3 ± 3.4	42.8 ± 4.0	37.0 ± 3.7	36.1 ± 4.3	32.2 ± 4.1	34.4 ± 3.8
	14	5	8.3 ± 0.3	46.4 ± 2.9	46.7 ± 4.4	41.2 ± 2.6	48.2 ± 4.4	44.0 ± 3.1	46.6 ± 4.2
Infected	3	6	5.2 ± 0.4	41.8 ± 5.6	40.1 ± 6.5	42.0 ± 5.1	38.0 ± 4.9	39.9 ± 6.5	42.5 ± 4.4
	5	8	6.2 ± 0.6	42.6 ± 5.0	37.5 ± 3.1	41.9 ± 2.9	37.0 ± 3.9	35.5 ± 4.6	34.5 ± 3.0
	14	5	8.8 ± 0.8	52.8 ± 3.9	47.6 ± 0.7	45.5 ± 2.6	40.8 ± 2.7	46.2 ± 4.7	42.6 ± 2.4

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	33.39	***	207.55	NS	207.26	NS	46.02	NS	241.60	NS	392.64	NS	311.56	*
Linear	1	61.37	***	395.89	NS	388.17	NS	54.63	NS	445.60	*	211.60	NS	214.40	NS
Quadratic	1	6.45	NS	14.68	NS	32.20	NS	39.92	NS	45.03	NS	592.63	*	424.88	*
Treatment	1	1.64	NS	5.76	NS	5.52	NS	147.10	NS	2.65	NS	24.01	NS	4.75	NS
Age*treatment	2	1.19	NS	117.32	NS	52.20	NS	2.95	NS	100.30	NS	13.21	NS	69.72	NS
Error	31	1.86		122.22		112.49		73.97		104.03		136.90		83.16	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 4.7. Ratio of RNA/DNA in the mucosa of the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (Days)	n	RNA/DNA ratio (Mean ± SE)						
Control	3	6	1.28 ± 0.12	1.04 ± 0.04	1.12 ± 0.08	1.11 ± 0.11	1.12 ± 0.08	0.96 ± 0.07	0.90 ± 0.07
	5	7	1.01 ± 0.21	0.85 ± 0.09	0.92 ± 0.09	0.91 ± 0.10	0.91 ± 0.10	1.03 ± 0.12	0.77 ± 0.09
	14	5	0.86 ± 0.05	0.90 ± 0.06	0.87 ± 0.05	0.93 ± 0.06	0.86 ± 0.05	1.00 ± 0.03	0.73 ± 0.04
Infected	3	6	1.30 ± 0.16	1.14 ± 0.11	1.23 ± 0.08	1.15 ± 0.07	1.15 ± 0.07	1.08 ± 0.12	0.93 ± 0.10
	5	8	1.06 ± 0.09	1.12 ± 0.08	1.17 ± 0.12	1.05 ± 0.08	1.10 ± 0.05	1.09 ± 0.09	0.91 ± 0.07
	14	5	0.95 ± 0.11	0.87 ± 0.06	0.94 ± 0.06	0.93 ± 0.05	0.89 ± 0.16	0.81 ± 0.06	0.70 ± 0.04
			Analyses of Variance						
Source	DF		MS p	MS p	MS p	MS p	MS p	MS p	MS p
Age	2		0.4373 *	0.1151 NS	0.1904 *	0.1257 NS	0.1811 *	0.0761 NS	0.1096 NS
Linear	1		0.7427 *	0.2200 *	0.3697 *	0.2014 *	0.3510 **	0.0953 NS	0.2192 *
Quadratic	1		0.1151 NS	0.0078 NS	0.0078 NS	0.0447 NS	0.0080 NS	0.0609 NS	0.0002 NS
Treatment	1		0.0264 NS	0.1142 NS	0.1826 NS	0.0299 NS	0.0653 NS	0.0001 NS	0.0200 NS
Age*treatment	2		0.0036 NS	0.0730 NS	0.0296 NS	0.0180 NS	0.0307 NS	0.0733 NS	0.0218 NS
Error	31		0.1209	0.0393	0.0526	0.0449	0.0461	0.0550	0.0384

NS $p > 0.05$, * $p < 0.05$ and $p < 0.01$ MS = Mean Square

14. K^+ was the only plasma electrolyte which decreased with age from days 3 to 14 ($p < 0.01$). Infection with *Y. enterocolitica* significantly reduced the glucose concentration in neonatal piglets ($p < 0.001$). For Cl^-, the significant interaction between age and infection was interpreted as a reduced concentration in infected piglets at 5 days postinfection. Plasma Na^+ and K^+ concentrations were not affected during the period of the experiment.

4.3.2 Mucosal Protein

Protein concentration (per g wet mucosal tissue) and total mucosal protein in each segment of the small intestine of infected and control piglets are shown in Tables 4.2 and 4.3. Tissue protein concentration decreased with age (p value from < 0.05 to < 0.01) except in the mid-jejunum (Table 4.2). Total mucosal protein content significantly increased only in the duodenum ($p < 0.01$) (Table 4.3). *Yersinia enterocolitica* infection did not significantly change either the protein concentration or total protein content in the small intestine (Tables 4.2 and 4.3).

4.3.3 Mucosal DNA and RNA

The ratios of mucosal tissue weight to DNA and of protein to DNA in the small intestine are shown in Tables 4.4 and 4.5, the total DNA content per segment in Table 4.6 and the RNA/DNA ratio in Table 4.7.

Yersinia. enterocolitica infection did not alter the mucosal tissue weight to DNA ratio, the protein to DNA ratio, the total DNA content or the ratio of RNA to DNA (Tables 4.4 - 4.7), but a significant effect of age was apparent in some segments. The ratio of protein to DNA did not change over the first two weeks after birth (Table 4.5). The ratio of mucosal tissue weight to DNA increased linearly with age in the distal jejunum and mid-ileum ($p < 0.05$) but the lowest value in the proximal jejunum was on day 5 ($p < 0.05$) (Table 4.4). Total mucosal DNA content linearly increased with age in the duodenum ($p < 0.001$) and the proximal ileum ($p < 0.05$) but showed a quadratic change, with the lowest value on day 5, in the mid- and distal ileum ($p < 0.05$) (Table 4.6). The ratio of RNA/DNA also decreased linearly with age in most segments of the small intestine ($p < 0.05$), except the mid-ileum (Table 4.7).

Table 4.8. Lactase activity per gram wet mucosal tissue of the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Lactase activity, µmol/g wet tissue/min (Mean ± SE)						
Control	3	6	12.7 ± 2.8	20.1 ± 3.3	25.1 ± 0.9	22.7 ± 2.3	14.0 ± 2.2	12.3 ± 2.1	4.9 ± 1.1
	5	7	11.6 ± 1.8	23.2 ± 1.1	25.3 ± 1.4	19.9 ± 2.9	13.9 ± 3.5	11.8 ± 3.2	3.0 ± 0.9
	14	5	4.6 ± 1.0	12.1 ± 1.2	10.5 ± 1.0	8.8 ± 1.5	8.6 ± 0.9	7.2 ± 0.5	1.5 ± 0.9
Infected	3	6	7.2 ± 1.8	17.2 ± 1.8	21.1 ± 3.1	17.0 ± 2.7	13.7 ± 3.1	11.5 ± 1.8	3.9 ± 0.8
	5	8	5.6 ± 0.9	9.8 ± 1.4	11.5 ± 1.6	11.6 ± 3.0	10.2 ± 2.3	8.8 ± 2.3	1.7 ± 0.6
	14	5	3.0 ± 1.0	10.1 ± 1.0	10.0 ± 0.8	7.9 ± 0.7	6.1 ± 0.3	4.8 ± 1.4	0.3 ± 0.3

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	113.05	**	162.83	**	456.40	***	368.85	**	119.31	NS	100.45	NS	35.31	**
Linear	1	220.81	**	323.11	***	912.50	***	737.68	***	236.45	*	199.19	*	64.45	***
Quadratic	1	7.34	NS	4.38	NS	0.08	NS	0.40	NS	3.59	NS	2.90	NS	5.11	NS
Treatment	1	168.89	**	334.25	***	337.65	***	218.50	*	41.68	NS	40.08	NS	13.20	NS
Age*treatment	2	16.60	NS	134.44	**	152.59	**	40.46	NS	9.56	NS	3.85	NS	0.13	NS
Error	31	18.66		21.05		19.61		42.68		41.60		31.91		4.34	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 4.9. Lactase activity per gram mucosal protein in the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Lactase activity, µmol/g protein/min (Mean ± SE)						
Control	3	6	102 ± 19	181 ± 27	203 ± 11	179 ± 19	132 ± 22	106 ± 14	50 ± 12
	5	7	95 ± 13	186 ± 13	209 ± 13	181 ± 28	141 ± 36	108 ± 30	28 ± 8
	14	5	45 ± 11	112 ± 13	106 ± 12	89 ± 14	97 ± 11	75 ± 5	19 ± 13
Infected	3	6	61 ± 15	158 ± 14	179 ± 21	140 ± 21	119 ± 23	107 ± 15	40 ± 9
	5	8	47 ± 8	85 ± 13	100 ± 9	108 ± 27	107 ± 27	88 ± 23	17 ± 6
	14	5	30 ± 9	89 ± 8	86 ± 5	71 ± 5	58 ± 4	52 ± 15	3 ± 3
			Analyses of Variance						
Source		DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Age		2	5583 *	13033 **	24860 ***	19288 **	8196 NS	5724 NS	3360 **
Linear		1	10899 **	25281 ***	49638 ***	37140 **	14344 NS	11068 NS	5738 **
Quadratic		1	367 NS	558 NS	9 NS	1866 NS	2356 NS	500 NS	854 NS
Treatment		1	10855 **	21371 ***	23169 ***	16803 *	7130 NS	1846 NS	1463 NS
Age*treatment		2	805 NS	6735 *	8492 **	2534 NS	543 NS	497 NS	30 NS
Error		31	1084	1557	1022	333	4146	2725	490

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 4.10. Total lactase activity of the mucosa of the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Total lactase activity, µmol/min (Mean ± SE)						
Control	3	6	13.8 ± 3.2	151.9 ± 29.7	164.0 ± 15.1	147.1 ± 23.6	91.4 ± 19.2	76.5 ± 11.1	31.2 ± 9.7
	5	7	13.6 ± 2.7	182.0 ± 19.2	185.8 ± 25.1	139.0 ± 28.0	104.5 ± 34.1	85.0 ± 30.1	17.5 ± 6.3
	14	5	7.3 ± 1.9	105.6 ± 15.0	94.4 ± 11.1	74.1 ± 14.1	84.8 ± 15.8	69.3 ± 7.1	13.6 ± 9.6
Infected	3	6	6.2 ± 1.4	135.8 ± 16.0	152.4 ± 28.4	116.2 ± 23.4	91.0 ± 23.9	77.4 ± 17.1	22.3 ± 3.5
	5	8	5.4 ± 1.0	63.7 ± 10.5	77.1 ± 15.7	93.3 ± 27.1	75.4 ± 19.7	61.6 ± 18.4	9.2 ± 3.3
	14	5	5.6 ± 2.1	90.3 ± 13.0	85.3 ± 9.6	64.6 ± 6.2	46.1 ± 3.2	44.0 ± 15.6	2.0 ± 2.0
				Analyses of Variance					
Source	DF	MS p		MS p	MS p	MS p	MS p	MS p	MS p
Age	2	40.92 NS		5741.64 NS	12817.59 *	11321.01 NS	2298.08 NS	1255.59 NS	1080.80 *
Linear	1	76.29 NS		11280.32 *	25573.72 **	22267.52 *	4061.33 NS	2405.40 NS	1764.36 *
Quadratic	1	6.74 NS		127.97 NS	11.74 NS	550.34 NS	618.77 NS	135.34 NS	352.12 NS
Treatment	1	305.20 **		22371.75 **	16747.41 *	7417.00 NS	4628.39 NS	2290.52 NS	829.14 NS
Age*treatment	2	35.36 NS		11714.48 **	10718.42 *	981.93 NS	1142.54 NS	635.23 NS	8.33 NS
Error	31	29.16		2062.88	2442.08	3616.11	3362.04	2429.35	239.46

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 4.11. Maltase activity per gram wet mucosal tissue of the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Maltase activity, µmol/g wet tissue/min, (Mean ± SE)						
Control	3	6	0.2 ± 0.1	2.6 ± 0.6	4.6 ± 0.8	3.5 ± 0.9	1.1 ± 0.4	0.8 ± 0.2	0.9 ± 0.1
	5	7	2.1 ± 1.4	5.6 ± 1.6	6.4 ± 1.8	4.4 ± 1.6	2.8 ± 1.0	1.7 ± 0.6	1.6 ± 0.8
	14	5	0.7 ± 0.2	8.1 ± 1.5	7.8 ± 0.6	6.4 ± 0.2	3.3 ± 1.1	1.0 ± 0.1	1.5 ± 0.2
Infected	3	6	1.0 ± 0.3	5.1 ± 0.9	5.6 ± 0.9	3.5 ± 0.5	1.5 ± 0.3	1.0 ± 0.2	0.8 ± 0.1
	5	8	3.7 ± 1.3	5.5 ± 0.7	8.3 ± 1.7	6.1 ± 1.2	4.2 ± 1.4	2.1 ± 0.8	1.6 ± 0.6
	14	5	0.8 ± 0.2	7.8 ± 0.8	12.2 ± 2.1	11.8 ± 1.7	8.5 ± 1.3	3.9 ± 0.9	3.9 ± 0.8

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	21.83	*	47.00	**	66.95	*	88.14	***	56.16	**	7.07	NS	9.34	*
Linear	1	0.28	NS	93.31	**	131.82	**	175.97	***	109.50	***	12.04	*	18.48	**
Quadratic	1	43.54	*	0.31	NS	1.26	NS	0.83	NS	1.93	NS	1.84	NS	0.10	NS
Treatment	1	7.02	NS	4.18	NS	52.60	NS	49.79	*	49.36	*	11.76	*	5.53	NS
Age*treatment	2	1.52	NS	7.27	NS	8.37	NS	20.87	NS	17.03	NS	6.33	NS	5.51	NS
Error	31	6.13		7.40		14.93		9.30		7.40		2.52		2.22	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 4.12. Maltase activity per gram mucosal protein in the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Maltase activity, µmol/g protein/min (Mean ± SE)						
Control	3	6	1.3 ± 0.5	22.8 ± 4.7	38.3 ± 8.6	27.8 ± 7.7	10.6 ± 3.8	6.4 ± 1.7	8.5 ± 0.9
	5	7	16.6 ± 11.1	43.5 ± 12.2	49.7 ± 13.0	40.9 ± 15.4	28.1 ± 10.6	16.2 ± 6.1	13.6 ± 6.9
	14	5	6.3 ± 1.8	74.6 ± 11.8	78.2 ± 7.8	64.7 ± 5.8	36.2 ± 12.4	9.9 ± 1.2	16.0 ± 2.5
Infected	3	6	8.5 ± 2.9	46.6 ± 8.9	47.5 ± 7.8	28.5 ± 4.2	13.1 ± 2.2	8.9 ± 2.3	7.6 ± 1.4
	5	8	30.2 ± 11.1	47.2 ± 5.6	71.9 ± 11.3	57.0 ± 11.8	43.8 ± 16.0	20.8 ± 8.7	16.0 ± 4.6
	14	5	8.6 ± 2.5	69.9 ± 8.9	106.4 ± 19.9	108.9 ± 17.1	78.9 ± 10.9	42.1 ± 10.4	43.0 ± 10.3
			Analyses of Variance						
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Age	2	1357.3 *	4031.1 **	6756.9 **	9542.9 ***	5743.4 **	950.8 *	1297.6 **	
Linear	1	0.6 NS	8002.8 ***	13511.9 ***	19085.8 ***	10932.8 ***	1736.4 *	2588.7 **	
Quadratic	1	2714.2 *	103.5 NS	3.4 NS	12.6 NS	426.2 NS	136.9 NS	15.5 NS	
Treatment	1	531.5 NS	521.2 NS	3542.4 NS	3716.9 *	3717.5 *	1540.9 *	813.8 NS	
Age*treatment	2	98.9 NS	609.9 NS	266.4 NS	1309.8 NS	1134.0 NS	746.8 NS	629.2 NS	
Error	31	402.3	497.4	884.5	857.9	811.6	266.4	215.8	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 4.13. Total maltase activity of the mucosa of the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Duodenum	Jejunum			Ileum		
				Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Total maltase activity, µmol/min (Mean ± SE)						
Control	3	6	0.2 ± 0.1	18.5 ± 4.1	31.9 ± 7.8	21.6 ± 5.9	6.5 ± 1.9	4.4 ± 1.1	5.1 ± 0.8
	5	7	2.5 ± 1.7	42.0 ± 11.2	45.8 ± 13.6	28.8 ± 12.3	18.3 ± 7.1	11.8 ± 5.2	9.8 ± 5.2
	14	5	1.1 ± 0.3	72.1 ± 15.7	69.9 ± 6.8	53.0 ± 3.6	28.6 ± 8.4	9.2 ± 1.4	10.5 ± 1.2
Infected	3	6	1.0 ± 0.3	43.0 ± 9.3	40.1 ± 8.1	21.7 ± 2.9	10.0 ± 2.3	6.4 ± 1.5	4.7 ± 0.8
	5	8	3.3 ± 1.2	35.1 ± 4.0	55.8 ± 15.6	46.4 ± 12.4	34.1 ± 14.2	16.1 ± 7.5	9.0 ± 3.5
	14	5	1.5 ± 0.5	68.4 ± 9.3	102.6 ± 16.5	97.4 ± 15.3	62.8 ± 8.8	30.4 ± 5.4	24.2 ± 5.1
			Analyses of Variance						
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Age	2	19.34 NS	4722.63 ***	7173.78 **	8124.62 ***	3825.87 **	584.56 *	428.40 **	
Linear	1	0.83 NS	9116.31 ***	14272.55 ***	16173.63 ***	7457.09 ***	1065.23 *	856.73 **	
Quadratic	1	37.53 *	431.09 NS	142.85 NS	148.83 NS	133.83 NS	86.30 NS	0.30 NS	
Treatment	1	3.86 NS	196.53 NS	2599.32 NS	3854.16 *	2869.02 *	757.71 *	155.73 NS	
Age*treatment	2	0.09 NS	924.17 NS	502.80 NS	1351.58 NS	646.48 NS	295.22 NS	187.34 NS	
Error	31	7.25	517.74	1024.26	688.81	537.96	165.00	79.40	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 4.14. Sucrase activity per gram wet mucosal tissue of the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Sucrase activity, µmol/g wet tissue/min (Mean ± SE)					
Control	3	6	0.1 ± 0.1	2.1 ± 0.1	0.7 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	5	7	2.6 ± 0.9	6.2 ± 1.8	1.3 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	14	5	5.0 ± 0.9	7.4 ± 1.2	5.0 ± 0.7	1.4 ± 0.6	0.0 ± 0.0	0.0 ± 0.0
Infected	3	6	1.1 ± 0.4	1.7 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	5	8	0.5 ± 0.3	2.4 ± 0.5	0.6 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	14	5	5.8 ± 0.7	10.3 ± 1.0	8.7 ± 0.9	4.4 ± 0.9	0.8 ± 0.5	1.3 ± 0.5

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	68.84	***	134.10	***	139.74	***	30.71	***	0.54	*	1.50	**
Linear	1	133.08	***	268.15	***	254.15	***	52.23	***	0.90	*	2.42	**
Quadratic	1	6.06	NS	0.31	NS	29.91	***	10.43	***	0.20	NS	0.63	NS
Treatment	1	0.13	NS	1.66	NS	5.36	NS	9.33	**	0.62	*	1.90	**
Age*treatment	2	9.77	*	34.00	*	18.01	***	8.52	***	0.54	*	1.50	**
Error	31	2.36		7.54		1.77		0.76		0.14		0.21	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 4.15. Sucrase activity per gram mucosal protein in the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Sucrase activity, µmol/g protein/min (Mean ± SE)					
Control	3	6	0.9 ± 0.6	17.8 ± 5.7	5.7 ± 3.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	5	7	20.4 ± 7.4	48.3 ± 13.9	11.7 ± 5.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	14	5	44.6 ± 5.5	72.5 ± 10.4	48.8 ± 4.9	14.9 ± 6.5	0.0 ± 0.0	0.0 ± 0.0
Infected	3	6	9.8 ± 3.5	13.9 ± 4.9	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.8 ± 0.8
	5	8	4.2 ± 2.7	20.6 ± 3.6	5.8 ± 1.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	14	5	50.3 ± 3.8	90.1 ± 11.1	79.7 ± 8.0	41.7 ± 7.3	8.6 ± 5.2	15.0 ± 7.2

Analyses of Variance

Source	DF	MS	p	MS	p	MS	p	MS	p	MS	p	MS	p
Age	2	5493.46	***	12245.35	***	12454.46	***	2922.46	***	66.70	*	197.20	**
Linear	1	10438.87	***	24310.42	***	22726.97	***	4970.60	***	112.08	*	320.88	**
Quadratic	1	687.45	*	314.03	NS	2585.78	***	992.36	***	24.05	NS	82.17	NS
Treatment	1	2.41	NS	196.60	NS	372.60	NS	718.66	**	76.16	*	248.94	*
Age*treatment	2	627.36	*	1571.80	NS	1224.16	***	655.87	***	66.69	*	197.20	**
Error	31	129.91		490.76		120.34		62.00		19.70		34.51	

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 4.16. Total sucrase activity of the mucosa of the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 and ANOVA

			Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	Age (days)	n	Total sucrase activity, µmol/min (Mean ± SE)					
Control	3	6	0.6 ± 0.4	14.6 ± 5.2	5.6 ± 3.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	5	7	18.3 ± 6.3	41.8 ± 11.1	7.4 ± 3.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	14	5	43.2 ± 9.1	64.1 ± 7.4	41.5 ± 6.8	11.5 ± 4.4	0.0 ± 0.0	0.0 ± 0.0
Infected	3	6	9.2 ± 3.3	12.8 ± 5.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.5
	5	8	3.3 ± 2.1	16.1 ± 3.9	4.7 ± 1.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	14	5	49.7 ± 5.2	87.5 ± 9.7	71.3 ± 6.5	33.8 ± 7.1	5.6 ± 3.2	8.2 ± 3.7
			Analyses of Variance					
Source		DF	MS p	MS p	MS p	MS p	MS p	MS p
Age		2	5466.28 ***	11259.50 ***	9803.18 ***	1875.34 ***	28.53 *	57.64 **
Linear		1	10255.40 ***	22133.36 ***	17470.80 ***	3189.62 ***	47.90 *	92.41 **
Quadratic		1	829.72 *	563.25 NS	2484.00 ***	636.80 ***	10.34 NS	25.45 NS
Treatment		1	0.01 NS	16.58 NS	461.91 *	495.54 **	32.70 *	75.77 **
Age*treatment		2	575.64 *	1820.56 *	1052.64 ***	452.25 ***	28.53 *	57.64 **
Error		31	143.24	344.71	92.11	45.17	6.99	9.07

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

4.3.4 Disaccharidase Activity

4.3.4.1 Mucosal disaccharidase activities

Over the period of 3 to 14 days after birth, there was, in general, a decline in lactase and an increase in maltase and sucrase activity. Disaccharidase activity was greatest in the mid-jejunum and declined both orally and aborally.

Lactase activity in all segments of the small intestine in control and *Y. enterocolitica*-infected piglets on days 3, 5 and 14 has been expressed in three ways: per gram mucosal tissue (Table 4.8), per gram protein (Table 4.9) and total lactase activity of the whole segment (Table 4.10). Lactase activity in control piglets remained at a high level for the first 5 days, then decreased to about half by 14 days in most regions of the small intestine except the distal ileum in which it was declining by day 5 (Tables 4.8 - 4.10). The linear reduction in lactase activity with age was significant in the entire small intestine when expressed per gram wet tissue (Table 4.8) but when expressed as activity per gram protein it did not show significance in the proximal and mid-ileum (Table 4.9). A significant linear decline with age in total lactase activity occurred in the entire jejunum ($p < 0.05$ or < 0.01) as well as in the distal ileum ($p < 0.05$) (Table 4.10).

Maltase and sucrase activities have been expressed in the same three ways as was lactase in Tables 4.11 to 4.16. Maltase activity was present throughout the entire small intestine by day 3 and continued to increase with age in the jejunum and ileum (p from < 0.05 to < 0.001), but in the duodenum it was greatest on day 5 ($p < 0.05$) (Tables 4.11 - 4.13). Sucrase activity remained undetectable in the duodenum of all pigs and also in the mid- and distal ileum of control animals up to day 14. This enzyme was present in the jejunum on day 3 and increased progressively during the following days. There were both significant linear and quadratic ($p < 0.001$) components to the increase with age in sucrase activity in the distal jejunum and the proximal ileum, regardless of the method of expression, but in the proximal jejunum the increase was only demonstrated in activity per g protein and total activity ($p < 0.05$). The mean values were similar at days 3 and 5 but had increased markedly by day 14.

Infection with *Y. enterocolitica* caused a significant depression of lactase activity in the duodenum and jejunum in neonatal piglets when expressed both as per gram wet tissue and per gram protein (p value from < 0.05 to < 0.001) (Tables 4.8 and 4.9). Total

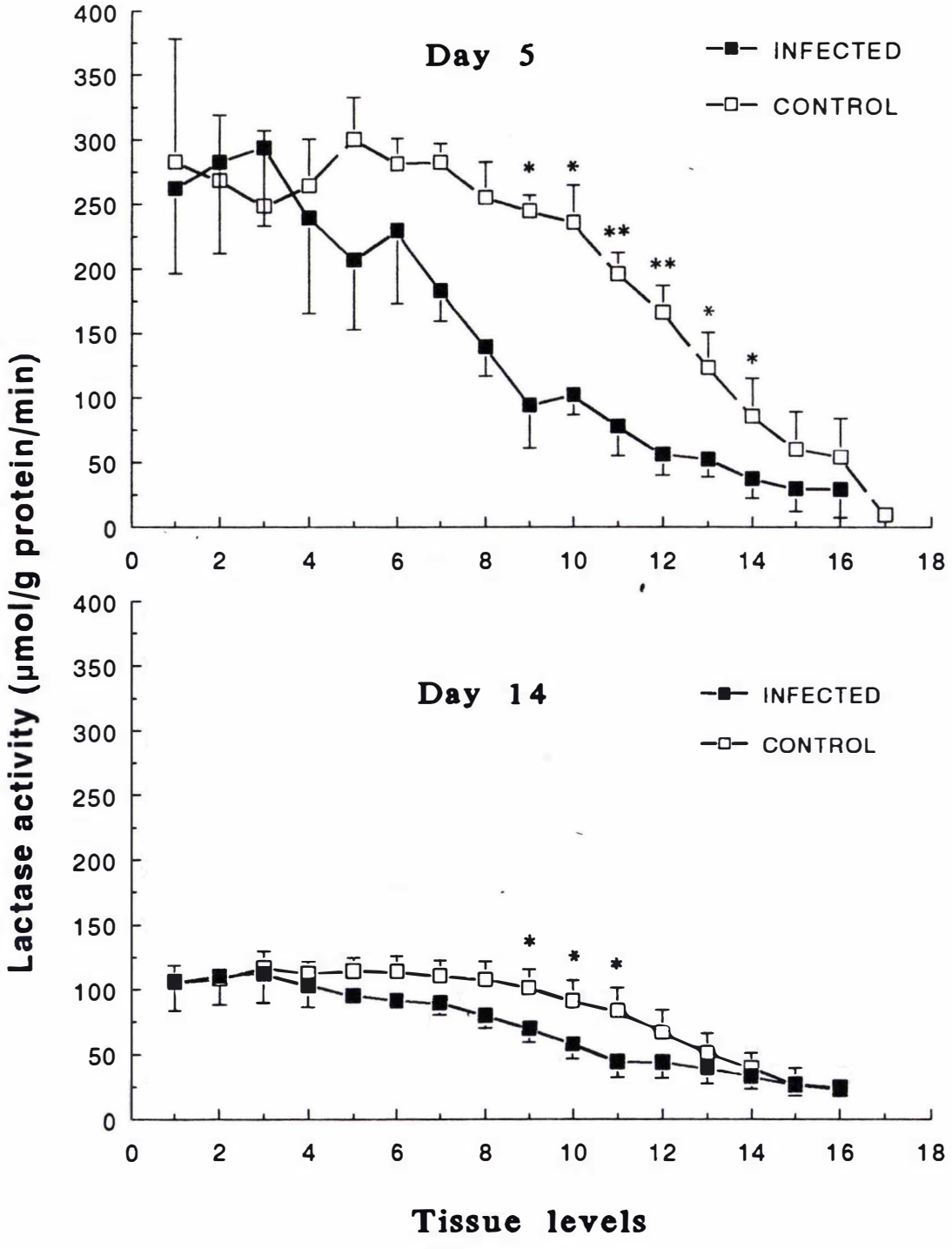

Figure 4.1 Distribution of lactase activity (Mean ± SE) along the crypt-villus axis in the mid-jejunum of control and *Y. enterocolitica*-infected piglets at 5 and 14 days of age. The tissue level was taken from the top of the villi (1) down to the base of the crypts (17). Significant differences between the two groups are indicated (*) $p < 0.05$; (**) $p < 0.01$.

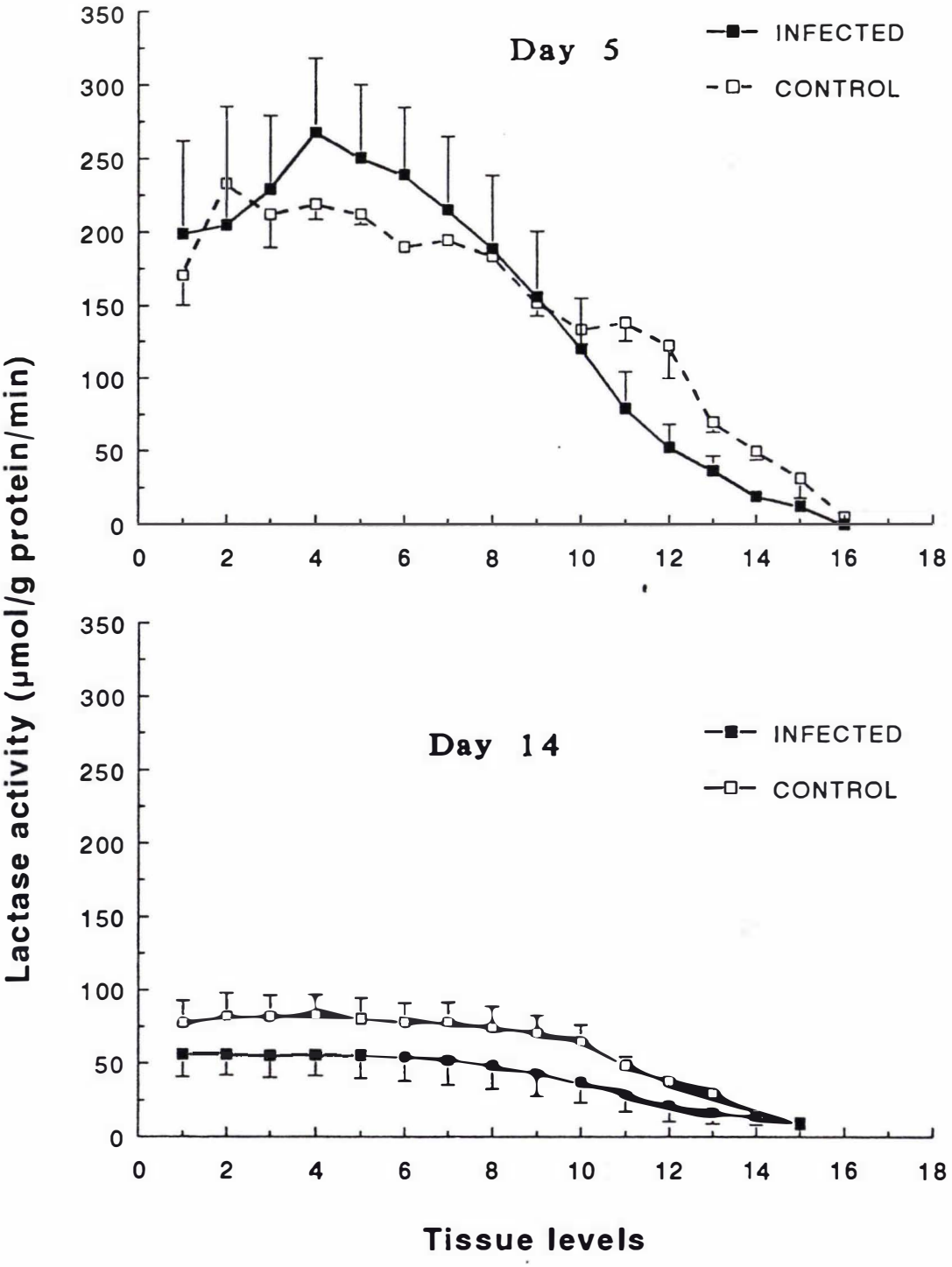

Figure 4.2 Distribution of lactase activity (Mean ± SE) along the crypt-villus axis in the mid-ileum of control and *Y. enterocolitica*-infected piglets at 5 and 14 days of age. The tissue level was taken from the top of the villi (1) to the base of the crypts (16).

lactase activity showed a significant decrease in the duodenum, proximal jejunum ($p < 0.01$) and the mid-jejunum ($p < 0.05$) (Table 4.10). The infection reduced lactase activity in the proximal and mid-jejunum more on day 5 than on other days ($p < 0.01$) regardless of the method of expression (Tables 4.8 - 4.10). After antibiotic therapy, although clinical signs of the infection had disappeared, lactase activity did not return to the level of controls. Maltase activity, usually low in the small intestine of the newborn pigs, was significantly increased overall from the distal jejunum to the mid-ileum in *Y. enterocolitica*-infected piglets ($p < 0.05$). Mean maltase activity was greater after clinical recovery following antibiotic therapy (Tables 4.11 - 4.13).

The development of sucrase activity in infected animals was different from that of both lactase and maltase activity. The infection reduced sucrase activity by day 5. After the piglets had recovered clinically following antibiotic therapy, sucrase activity dramatically increased in the jejunum and appeared early in the ileum (Tables 4.14 - 4.16).

4.3.4.2 Distribution of disaccharidase activity along the crypt-villus axis

To study the distribution of enzyme activity and to find where the loss of the enzyme activity occurred along the crypt-villus axis, disaccharidase activities were measured from homogenates of parallel cryostat sections from the top of the villus to the base of the crypt. According to the villus length (Chapter 3, 3.3.5), the base of the villus was defined as the 11th or 12th level (each level comprises 5 sections) from the top of the villus to the base of the crypt in the jejunum, and at level 10 in the ileum. Lactase activity in enterocytes located within different levels of the crypt-villus axis in the jejunum and ileum of control and *Y. enterocolitica*-infected piglets aged 5 and 14 days is shown in Figures 4.1 and 4.2. The highest lactase activity was located in the upper villus just below the tip, and the lowest activity in the crypt, in both the jejunum and ileum. The distribution of lactase activity along the crypt-villus on day 5 was similar to that on day 14. A significant reduction in lactase activity from the lower villus downward to the upper crypt (tissue level from 9 to 14) was found in the jejunum of infected piglets on day 5 ($p < 0.05$ or < 0.01) (Figure 4.1). The reduced activity of the enzyme persisted in the lower villus (tissue level from 9 to 11) of the jejunum in 14-day-old piglets which had clinically recovered from the infection as a result of antibiotic therapy (Figure 4.1). In the ileum, lactase activity at all tissue levels was not significantly different between control and infected piglets on both days 5 and 14 (Figure 4.2).

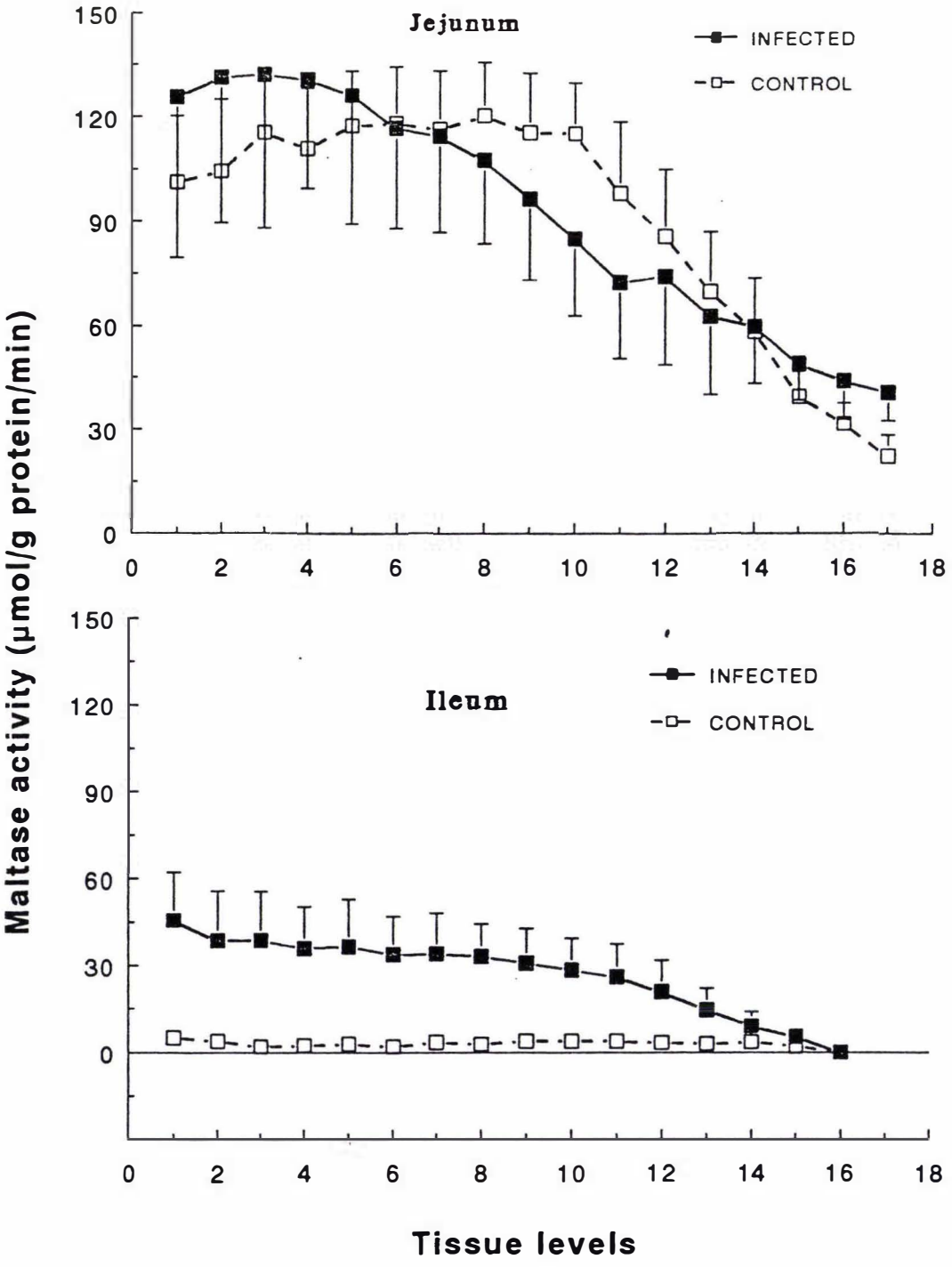

Figure 4.3 Distribution of maltase activity (Mean ± SE) along the crypt-villus axis in the mid-jejunum and mid-ileum of control and *Y. enterocolitica*-infected piglets at 14 days of age. The tissue level was taken from the top of the villi (1) to the base of the crypts (17).

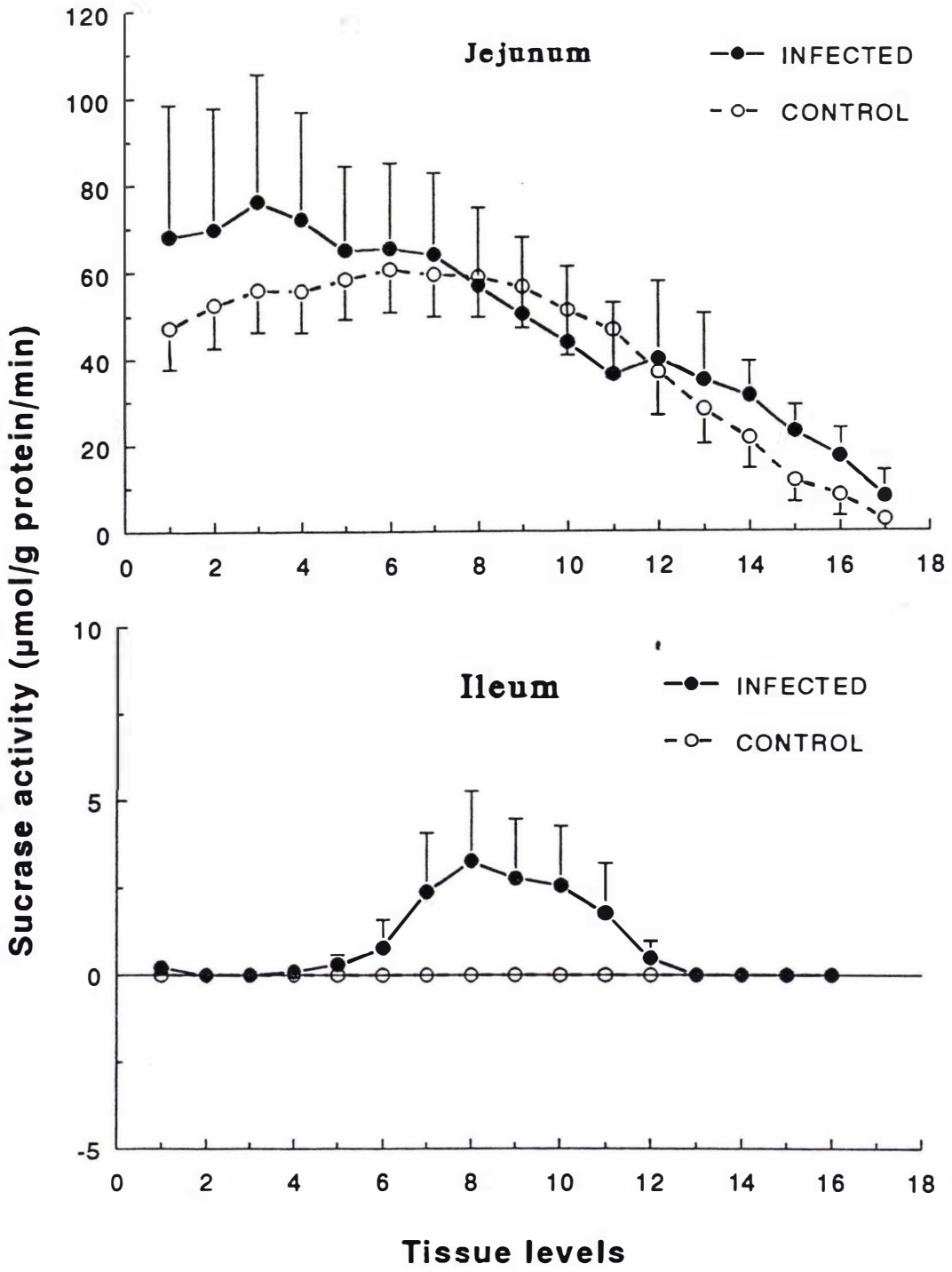

Figure 4.4 Distribution of sucrase activity (Mean ± SE) along the crypt-villus axis in the mid-jejunum and mid-ileum of control and *Y. enterocolitica*-infected piglets at 14 days of age. The tissue level was taken from the top of the villi (1) to the base of the crypts (17).

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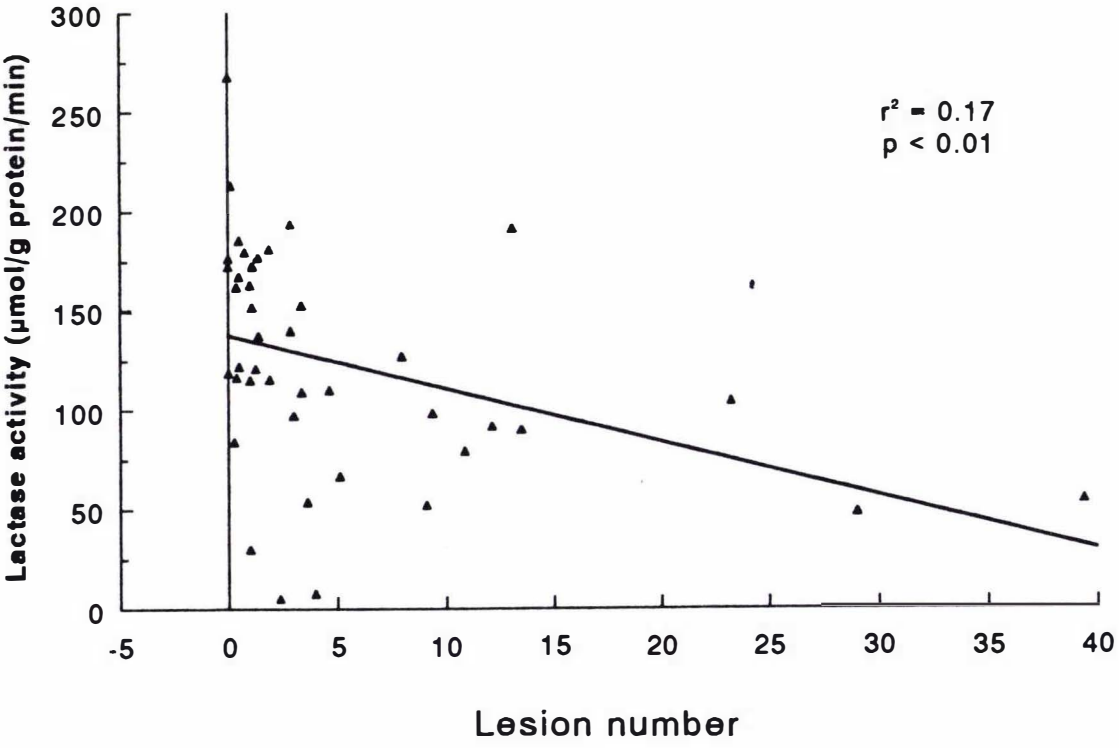


Figure 4.5 Graph of lactase activity against lesion number in three segments of the jejunum in *Y. enterocolitica*-infected piglets at 3 and 5 days postinfection. Lactase activity was negatively correlated with lesion number, r^2=0.17, $p < 0.01$.

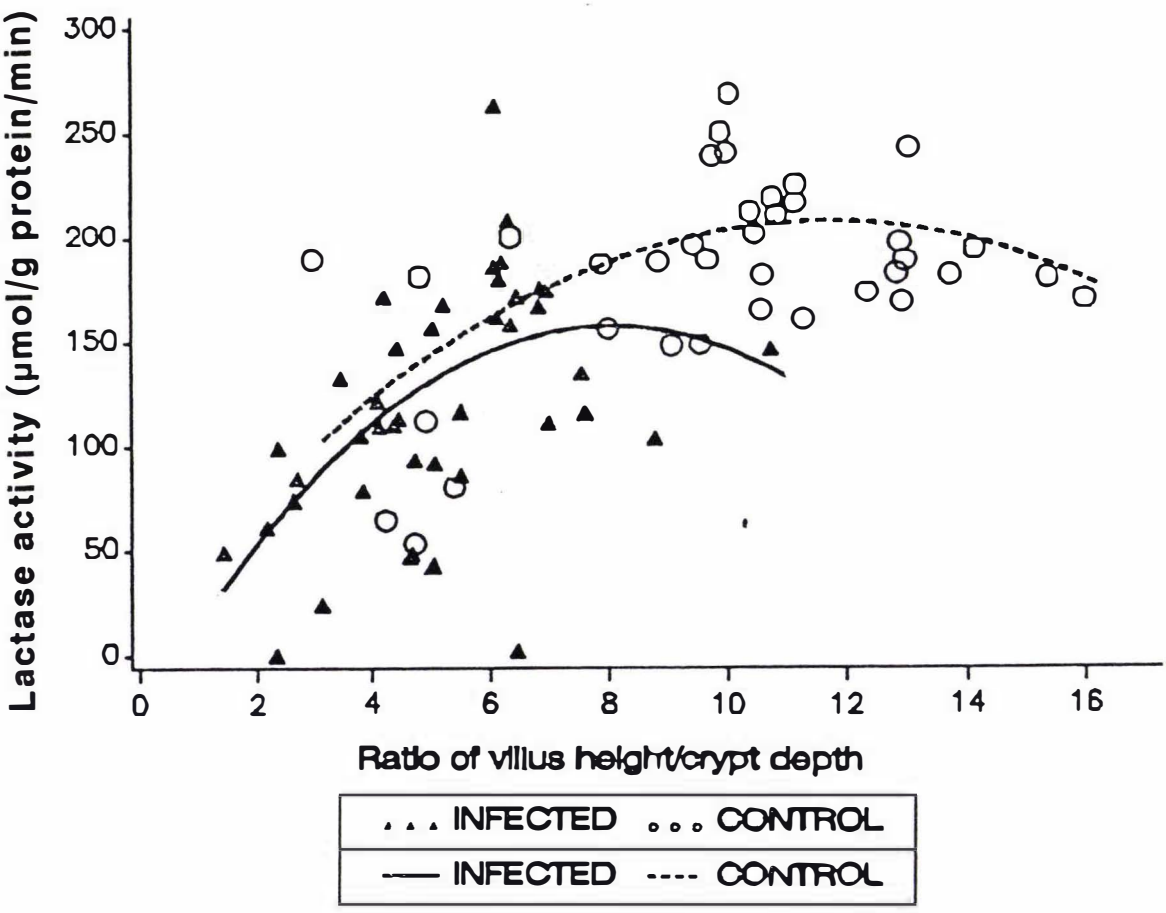


Figure 4.6 Graph of lactase activity against the ratio of villus height/crypt depth in three segments of the jejunum in control and *Y. enterocolitica*-infected piglets at 3 and 5 days postinfection. The correlation between lactase activity and the ratio of villus height to crypt depth was positive in both control and infected piglets, showing significant curvilinear relationships. The regression equation for control group was $Y = 10.5 + 33.8x - 1.5x^2$, $r^2 = 0.34$, $p < 0.001$ and for infected group $Y = -27.5 + 45.8x - 2.8x^2$, $r^2 = 0.30$, $p < 0.001$.

The maltase activity in enterocytes along the crypt-villus axis in the jejunum and ileum in 14-day-old piglets is shown in Figure 4.3 and sucrase activity in Figure 4.4. Like lactase activity, maltase and sucrase activities were high in the villus and low in the crypt in the jejunum in both control and infected pigs. No significant difference between the two groups was found in either maltase (Figure 4.3) or sucrase activities (Figure 4.4) in any regions of the crypt-villus in either the jejunum or ileum. The mean maltase activity in enterocytes in the ileum of previously-infected piglets was always greater than in controls. The enzyme activity was low, but evenly distributed, from the tip of the villus downward to the crypt in the previously-infected piglets (Figure 4.3). In contrast, the early appearance of sucrase activity in the ileum in those piglets occurred mainly in the lower villus enterocytes (tissue levels from 6 to 12) where no sucrase was detectable in control piglets.

4.3.4.3 Correlation between lactase activity and histopathological findings

Jejunal lactase activity has been plotted against number of lesions in infected piglets in Figure 4.5 and against the ratio of villus height/crypt depth in both control and infected piglets in Figure 4.6. Forty-two individual datum points from three regions of the jejunum of 14 piglets infected with *Y. enterocolitica* (6 at 3 days and 8 at 5 days postinfection) and 37 from 13 controls (6 at 3 days, 7 at 5 days) were used. Lactase activity in infected piglets was negatively correlated with lesion number ($r^2 = 0.17$, $p < 0.01$). The correlation between lactase activity and the ratio of villus height to crypt depth was positive in both control and infected piglets, calculated separately and also for the pooled data. The three sets of data, for control, infected and pooled values, presented in the table below, showed both significant linear and curvilinear relationships, but in all cases the curvilinear fit was better. Nonlinear regression equations revealed a significant quadratic relationship between lactase activity and the ratio of villus height to crypt depth in both control and infected piglets. This relationship suggests that lactase activity in the jejunum changed less when the ratio of villus height to crypt depth increased above 11 in control and above 8 in infected piglets.

	Curvilinear			Linear		
	Regression equation	r^2	p	Regression equation	r^2	p
Control	$y = 10.5 + 33.8x - 1.5x^2$	0.34	< 0.001	$y = 12.7 + 6.6x$	0.20	< 0.01
Infected	$y = -27.5 + 45.8x - 2.8x^2$	0.30	< 0.001	$y = 48.9 + 14.5x$	0.23	< 0.01
Pooled	$y = -4.0 + 33.7x - 1.4x^2$	0.48	< 0.0001	$y = 71.7 + 11.1x$	0.40	< 0.0001

Table 4.17. Na^+-K^+-ATPase activity in the intestine in control and *Y. enterocolitica*-infected piglets on days 3 and 5 and ANOVA

Treatments	Infected		Control		Two-way ANOVA		
Age (days)	3 n=6	5 n=7	3 n=6	5 n=6	Age	Treatment	Age*Treatment
	Mean ± SE						
Na^+-K^+-ATPase activity (µmol/mg protein/h)							
Duodenum	5.43 ± 0.53	5.84 ± 0.34	5.00 ± 0.33	4.27 ± 0.84	NS	NS	NS
Proximal jejunum	3.72 ± 0.39	4.31 ± 0.82	3.78 ± 1.01	3.66 ± 1.00	NS	NS	NS
Mid-jejunum	3.48 ± 0.57	4.21 ± 0.67	3.31 ± 0.17	3.46 ± 0.81	NS	NS	NS
Distal jejunum	3.44 ± 0.51	3.24 ± 0.71	2.61 ± 0.43	2.66 ± 0.69	NS	NS	NS
Proximal ileum	2.33 ± 0.29	3.93 ± 0.78	1.60 ± 0.24	2.77 ± 0.96	NS	NS	NS
Mid-ileum	2.70 ± 0.32	2.64 ± 0.57	2.15 ± 0.24	4.03 ± 1.09	NS	NS	NS
Distal ileum	2.83 ± 0.67	2.14 ± 0.43	1.81 ± 0.33	2.08 ± 0.57	NS	NS	NS
Ceacum	2.41 ± 0.72	2.54 ± 0.31	1.39 ± 0.02	2.39 ± 0.21	NS	NS	NS
Proximal colon	2.06 ± 0.44	2.88 ± 0.30	4.11 ± 0.48	2.55 ± 0.37	NS	NS	*
Distal colon	2.41 ± 0.88	2.85 ± 0.28	4.17 ± 0.29	2.63 ± 0.83	NS	NS	NS
Na^+-K^+-ATPase activity (µmol/g wet tissue/h)							
Duodenum	637 ± 44	700 ± 43	615 ± 68	496 ± 82	NS	NS	NS
Proximal jejunum	406 ± 48	492 ± 87	426 ± 120	448 ± 112	NS	NS	NS
Mid-jejunum	411 ± 82	499 ± 92	415 ± 31	411 ± 77	NS	NS	NS
Distal jejunum	410 ± 59	353 ± 83	339 ± 66	294 ± 80	NS	NS	NS
Proximal ileum	263 ± 36	414 ± 91	170 ± 25	282 ± 93	NS	NS	NS
Mid-ileum	285 ± 33	283 ± 68	243 ± 30	435 ± 114	NS	NS	NS
Distal ileum	275 ± 55	221 ± 44	184 ± 32	226 ± 55	NS	NS	NS
Ceacum	264 ± 67	281 ± 21	168 ± 2	275 ± 28	NS	NS	NS
Proximal colon	233 ± 71	311 ± 31	448 ± 49	286 ± 44	NS	NS	*
Distal colon	298 ± 105	304 ± 33	473 ± 4	290 ± 98	NS	NS	NS

NS $p > 0.05$; * $p < 0.05$

4.3.5 Na^+-K^+-ATPase Activity

The activity of Na^+-K^+-ATPase in the mucosa of ten segments of the intestine of control and infected piglets on days 3 and 5 is listed in Table 4.17. In neonatal piglets, the greatest activity of Na^+-K^+-ATPase was found in the duodenum, followed by the upper jejunum. There was no difference either between 3 and 5 days or between control and infected groups in all segments of the small intestine. In the proximal colon, however, there was a significant interaction ($p < 0.05$), since the enzyme activity was lower at 3 days in infected piglets (2.06 ± 0.44, compared with control 4.11 ± 0.48 μmol/mg protein/hour) but there was no difference between the two groups at 5 days.

4.4 DISCUSSION

4.4.1 Effects of *Y. enterocolitica* Infection

The reduced body growth, lower liver weight and morphological damage evident in the intestine of *Y. enterocolitica*-infected piglets suggests that intestinal function might be impaired by the infection. Three indices of gut function have been examined: digestive enzymes (disaccharidases), an absorptive enzyme (Na^+-K^+-ATPase) and biochemical indicators of cell size, number and protein synthetic capacity. The major impact of the *Y. enterocolitica* infection was on the digestive enzymes, whereas there was no demonstrable effect on either the cell characteristics or on an absorptive enzyme in the small intestine.

4.4.1.1 Tissue DNA, RNA and protein

The constancy of the tissue to DNA and protein to DNA ratios, the total DNA content and the ratio of RNA to DNA in infected piglets suggests that *Y. enterocolitica* infection did not change epithelial cell size, total cell number or protein synthetic ability in the mucosa of the small intestine. Neither protein concentration nor total protein content of the small intestine was altered by the infection. Protein synthesis in the digestive tract was less affected than in other body tissue during periods of nutrient insufficiency in young pigs (Sève *et al.*, 1986). It should be pointed out that the methods used in the present study for assaying protein, DNA and RNA were unable to distinguish the host protein, DNA and RNA from those of bacterial origin. If the protein and DNA from the bacteria can be ignored, the rapid epithelial cell division in the crypts and the

presence of a number of inflammatory cells in the mucosa in infected animals presumably just balanced the lost epithelial cells, resulting in no detectable change in protein and DNA contents between control and infected piglets.

4.4.1.2 Disaccharidases

During the first two weeks after birth, the activities of the three disaccharidases, lactase, maltase and sucrase, undergo marked changes. The control piglets exhibited the typical enzyme distribution and developmental changes seen in piglets nursed by sows (Manner and Stevens, 1972). The highest disaccharidase activity was in the jejunum and this declined both orally and aborally whether the specific activity of enzyme was high or low. Lactase activity remained high for at least 5 days and declined between days 5 and 14, whereas maltase activity was low on day 3 and significantly increased with age during the period of the experiment. Sucrase activity was low throughout the experimental period, being present only in the jejunum on day 3 but also in the proximal ileum by day 14. *Yersinia enterocolitica* infection affected the three disaccharidases in newborn piglets in different manners in the periods of infection and clinical recovery. Lactase was most sensitive to *Y. enterocolitica* infection being lower than in controls during the whole period of the experiment with the most marked difference being on day 5. Sucrase was the next most sensitive to the infection. The activity of this enzyme was reduced in the jejunum during the infection period, but markedly exceeded the control levels in the jejunum and appeared early in the ileum after recovery. Mean maltase activity was higher than in controls during the infection period, but after recovery there was a more dramatic increase.

A reduction in lactase activity is seen in many infectious diarrhoeal diseases regardless of the animal species and age: humans (Lebenthal and Lee, 1980), rabbits (O'Loughlin *et al.*, 1986), lambs (Ferguson *et al.*, 1981), mice (Hammond and Rosenberg, 1972; Collins *et al.*, 1988) and in piglets at 2 days of age (Newport *et al.*, 1982), at 8-10 days of age (Davidson *et al.*, 1977) and at 23-26 days of age (Kelly *et al.*, 1972). In contrast, the effect of infectious diarrhoea on maltase and sucrase seems to relate to the age of the animal and the level of the enzyme activity. In children (Lebenthal and Lee, 1980), weanling rabbits (O'Loughlin *et al.*, 1986), and piglets aged over 11 days (Kelly *et al.*, 1972; Davidson *et al.*, 1977), diarrhoea reduced both maltase and sucrase activities. In neonatal mice, which have low maltase and undetectable sucrase, Coxsackie virus (Hammond and Rosenberg, 1972) or rotavirus infection (Collins *et al.*, 1988) caused the

premature appearance of maltase and sucrase activities. In the present study, piglets with *Y. enterocolitica* diarrhoea had lower lactase and sucrase activities, but not maltase activity, during the infection. The reduction in lactase and sucrase activities is more marked than the loss of maltase activity in infants and children with diarrhoea (Lebenthal and Lee, 1980) and in rotavirus-infected piglets at 8-10 days of age (Davidson *et al.*, 1977). When the infected piglet in the present study had clinically recovered following antibiotic therapy, there was premature development of maltase and sucrase as in the neonatal mice with infectious diarrhoea.

The reduction in lactase activity in the *Y. enterocolitica*-infected piglets was significant in the proximal small intestine, where there is high enzyme activity, but not in the ileum, although the mean values there were lower. A similar effect was observed in 23-day-old suckled, undernourished rabbits which had been infected with *Y. enterocolitica* at 17 days of age (Butzner and Gall, 1988b). In contrast, the bacterial infection in the present study increased maltase activity in both the infection period and the recovery period in the mid-parts of the small intestine but not in the proximal small intestine and the distal ileum. Sucrase activity was modified by the infection in a different manner from either lactase or maltase. During the period of infection, sucrase activity decreased in the jejunum, but during the recovery period it markedly increased in the jejunum and appeared early in the ileum. Reduction in sucrase activity was also observed in both the jejunum and ileum in naturally-suckled 17-day-old rabbits (Butzner and Gall, 1988b) and weanling rabbits (O'Loughlin *et al.*, 1986) infected with the same organism, and also in 5- to 7-day-old miniature piglets infected with porcine rotavirus (Graham *et al.*, 1984). This effect on sucrase activity probably results from damage to the enterocytes.

Altered disaccharidase activity could be the result of (i) microscopically visible damage to the mucosa, (ii) ultrastructural damage to microvilli or (iii) a shortened life span of the villus epithelium because of faster crypt cell proliferation. It may be that the importance of the different factors varies along the length of the small intestine and could explain why the impact of the infection on each of the three enzymes is not the same.

During the period of infection, damage to the mucosa, evident from the large number of microabscesses (Chapter 2, 2.3.3), in general, appears to result in a decline in disaccharidase activity although this reduction in activity applies to lactase and sucrase but not to maltase. Lactase activity has received particular attention in the infected

piglets in the present study because the activity is high in neonatal piglets. Parallel sections of the jejunum showed that there was a loss of lactase activity in the lower villi and upper crypts where most lesions occurred (Chapter 2, 2.3.3). Damage to the mucosa and shedding of enterocytes could play significant roles in the loss in lactase activity. Calculation of the correlation coefficients for lactase activity with lesion number and with villus height:crypt depth in the jejunum showed that 17% and 30% respectively of the variance in lactase activity in the infected piglets was in common with the two histological findings (Figures 4.5 and 4.6). Morphological findings during the *Y. enterocolitica* infection could, therefore, be used, in part, as a predictor of altered digestive function of the small intestine. Of the two parameters, the ratio of villus height/crypt depth had a greater degree of correlation with lactase activity than lesion number and would appear to be the more useful as a predictor. The jejunum was selected for the examination of any correlation between lactase activity and histological findings because the lactase activity in the jejunum of control piglets remained high from day 3 to day 5 and a significant reduction in lactase activity in the jejunum was found in infected piglets in the present study.

The decrease in lactase and sucrase activities may be caused by mechanisms other than obvious damage to the mucosa of the small intestine. Not all damage may be observed under the light microscope, but may be confined to the microvilli where the enzymes are located. A significant reduction in mucosal lactase activity occurred in the proximal parts of the small intestine (duodenum and jejunum) although the injury to the small intestinal mucosa by *Y. enterocolitica* was in the entire small intestine at 5 days postinfection (Chapter 2, 2.3.3). Decreased lactase and sucrase activity may have also resulted from injury to the brush border by metabolic products of the bacteria, a mechanism that has been described in the bacterial overgrowth syndrome (Jonas *et al.*, 1977, 1978). Increased degradation of brush border glycoproteins, which include disaccharidases, was found to be due to the action of proteases released by the bacteria (Jonas *et al.*, 1978).

In addition to damage to both the mucosa and the enterocyte brush border, accelerated proliferation of crypt cells and migration of enterocytes up the villi may play a significant role in the modified development of all three disaccharidases in infected piglets. This would be particularly so for the lower lactase activity in 14-day-old piglets. Following antibiotic therapy, they had clinically recovered, were gaining weight at the same rate as controls during the last 9 days, and there was a normal villus height.

High lactase activity is associated with a long life span of the epithelium in newborns. Reduced lactase activity in previously-infected piglets probably was associated with a shortening of the time available for lactase expression in brush-border membranes because of faster proliferation of enterocytes in the crypts and cell migration up the villi during the period of infection (Chapter 3, 3.3.6). Lactase activity increases as enterocytes migrate over the basal region of the villus, remains constant in the mid-villus region and tends to fall as enterocytes approach the villus tip (Smith and James, 1987). In the present study, on day 14, the proliferation of crypt cells in previously-infected piglets had returned to the level seen in the controls but the epithelial cells may have been more physiologically mature and may have been replaced faster than those in controls. Those cells, therefore, would have had a shorter time to express lactase activity. A decline with age in lactase activity in mucosal homogenates of the jejunum of rats was accompanied by a corresponding increase in enterocyte migration rate (Smith and James, 1987), enterocytes migrating on to the base of villi in young rats (15-day-old) having twice the lactase activity of those in weaned rats (23-day-old).

The patterns of development of maltase and sucrase activities were similar in neonatal piglets, but the effects of the infection on these two enzymes were different. This may be because 20% of maltase activity is provided by a maltase-glucoamylase complex which may not have the same distribution in the crypt-villus axis as the sucrase-isomaltase complex. Maltase-glucoamylase does not display any marked qualitative or quantitative modifications either along the crypt-villus axis or during postnatal development (Simon *et al.*, 1979). In both these respects, this enzyme differs from lactase, and from sucrase-isomaltase which accounts for 80% of maltase activity (Galand, 1989). In the suckled, neonatal rat, high lactase activity was present mainly in the mid-villi. Low sucrase-isomaltase activity was present initially in the lower crypt, then appeared also in the upper crypt and the villi, continuing to be higher in the lower villi and upper crypts than in the lower crypts (Simon *et al.*, 1979).

The higher maltase activity in the infected piglets may have resulted from a greater number of crypt cells being produced during the *Y. enterocolitica* infection and those new cells containing more maltase activity when they migrated upward, so that maltase activity particularly in the mid-parts of the small intestine was higher at 14 days. When maltase activity was low, the difference in activity between the crypt and the villus (Figure 4.3 ileum) was not as greater as when it was high (Figure 4.3 jejunum). This was also observed in neonatal rats (Simon *et al.*, 1979). During the infection period, the

villus maltase activity would be reduced, but compensated for by crypt enlargement, so that the total activity was unchanged in the jejunum. In the ileum, the effects of *Y. enterocolitica* infection on maltase activity in the villus probably would be small because of very low activity, but the activity present in the longer crypts would be much greater than in normal crypts, resulting in higher total maltase activity in the infected piglets.

Increased maltase activity in the present study seems to be opposite to that found in rabbits infected with *Y. enterocolitica* (O'Loughlin *et al.*, 1986). Weanling rabbits, being older, had high maltase activity (250 μmol/gm protein/min in the proximal small intestine). If the distribution of maltase is the same in these rabbits as has been reported in adult rats (Simon *et al.*, 1979), then the maltase would be located mostly in the villi and less in the crypt. In the weanling rabbits, the primary effect of the bacterial infection would be to reduce maltase activity, which was high and located in the villi. In contrast, in the piglets, the primary effect is likely to be crypt enlargement. Cells, which had a low maltase activity, would be replaced by new crypt cells capable of developing a much higher maltase content as they migrated upward.

The early appearance of sucrase was confined to the ileum, where it still had not developed in 14-day-old control piglets, but was detected in previously-infected piglets. The dramatic increase in sucrase activity in the mid- and distal ileum after antibiotic treatment is consistent also with the early maturation of the epithelium because of increased cell proliferation. Premature development of maltase and sucrase has been reported in mucosal homogenates of rotavirus-infected neonatal mice at 4 days postinfection (11-day-old) (Collins *et al.*, 1988). The early appearance of sucrase activity in the lower villus and upper crypt enterocytes in the present study was similar to the findings of Collins *et al.* (1990). The initial appearance of sucrase and maltase activity is mainly in the immature cells in the lower villi and in the crypts both in the piglets (Figures 4.3 and 4.4) and in mice (Collins *et al.*, 1990). In their histochemical study, the activity of α-glucosidase (a combination of sucrase, isomaltase, maltase I, maltase III, and trehalase), when first detectable, was present in both villus and crypt cells, followed by increased enzyme activity in villus enterocytes, but less in crypt enterocytes (Collins *et al.*, 1990). Sucrase was also located in the cells in the crypt and lower villus compartments in sucrose-refed rats that had been fasted for 48 hours (Raul *et al.*, 1980) and in rats which were injected with cortisone (50 μg/g body weight) in which sucrase was induced precociously (Yeh *et al.*, 1991).

It has been suggested that increased adrenal corticoid secretion is involved in the precocious development of disaccharidase in enteritic infections. Injection of cortisone increased cellular migration, mitotic index, and depth of the crypt column (Herbst and Sunshine, 1969) coincident with the precocious development of intestinal sucrase, maltase, isomaltase (Doell and Kertchmer, 1964; Lebenthal *et al.*, 1972; Yeh *et al.*, 1991) and precocious decrease of lactase (Henning and Leeper, 1982) in suckled rats. Increased adrenal corticoids have been observed in neonatal calves with *E. coli* infection (Lopez *et al.*, 1975), in neonatal mice infected with Coxsackie virus (Hammond and Rosenberg, 1972) and in suckled rats with sugar-induced diarrhoea (Goda *et al.*, 1985). In the *Y. enterocolitica*-infected piglets, however, comparison of cortisol levels in plasma samples failed to show any difference in the hormone levels between control and infected groups either at 3 or 5 days postinfection (personal communication, N. Petrie).

4.4.1.3 Na^+-K^+-ATPase

The reported effects of enteritis on Na^+-K^+-ATPase activity in the intestine are very variable. No significant change in the Na^+-K^+-ATPase activity in the mucosa of the small intestine was observed in neonatal piglets infected with *Y. enterocolitica* (3 or 5 days of age) in the present study, whereas the enzyme activity significantly increased in rabbits infected with the same organism (O'Loughlin *et al.*, 1986). The present finding is similar to that in neonatal mice infected with rotaviruses (Collins *et al.*, 1988). Na^+-K^+-ATPase activity decreased in the small intestine in young pigs infected with TGE viruses (York breed pigs aged 25 to 28 days, Kelly *et al.*, 1972) or rotaviruses (York breed conventional piglets aged 11 to 13 days, Davidson *et al.*, 1977; miniature piglets aged 7 to 9 days, Graham *et al.*, 1984) and in rats infected with the nematode *Nippostrongylus brasiliensis* (Wild and Murray, 1992). Differences in experimental findings may be due to species variation or because different species react in different ways to the same organism or the same species to different organisms. *Yersinia enterocolitica* infection did not alter the small intestinal Na^+-K^+-ATPase activity, however, this enzyme was lower in the proximal colon of infected piglets only at 3 days. Overall, there was no significant change in the activity of this enzyme.

4.4.1.4 Glucose

Reduction in lactase activity coupled with an unchanged Na^+-K^+-ATPase activity in the small intestine of *Y. enterocolitica*-infected piglets may indicate that the lower plasma

glucose during severe diarrhoea was due mainly to maldigestion by disaccharides. The lower plasma chloride concentration at 5 days postinfection suggests that the absorptive function of the gut may have been affected during the acute phase of *Y. enterocolitica* infection although the plasma sodium and potassium concentrations were unchanged. Therefore, the lower concentration of plasma glucose in piglets during the infection period (days 3 and 5) may result from both maldigestion of lactose and malabsorption of glucose. This has been shown in pigs with diarrhoea caused by rotavirus infection, in which there was lower lactase activity in both the jejunum and ileum and a high lactose concentration in the faeces (Graham *et al.*, 1984).

By day 14, the plasma glucose concentration remained lower in previously-infected piglets although they no longer showed evidence clinically or morphologically of abnormality in the gut and had a similar body weight gain and total disaccharidase activity to uninfected piglets. The lower plasma glucose probably was due to decreased glucose absorption resulting from the earlier maturity of the small intestine because of faster proliferation of crypt cells. Reduced glucose absorption with increasing age has been observed in piglets (Puchal and Buddington, 1992) and rabbits (Gall and Perdue, 1980) during postnatal development and also lower in adult rats (Ferraris *et al.*, 1993). The maximal rates of transport of glucose by the proximal small intestine of 10-day-old piglets (2.6 ± 0.1 nmol/mg tissue/min) is only half that of suckled piglets at 6-8 hour old (5.2 ± 0.2 nmol/mg tissue/min) (Puchal and Buddington, 1992). A lower blood glucose concentration was also observed during accelerated growth of neonatal rabbits as a result of reducing litter size (Gall and Chung, 1982).

4.4.2 Development of Control Piglets

In the control group, the biochemical development of the piglets under the present experimental regime appeared to be no different from that in naturally suckled piglets with respect to both enzyme activity, cell size and capacity to synthesize protein.

4.4.2.1 Number and size of mucosal cells

Mucosal cell number, indicated by DNA content, in the present control piglets at 3 days was similar to that in piglets nursed by sows (Xu *et al.*, 1992a) and significantly increased with age during the first 2 weeks in some segments of the small intestine. It seems that feeding neonatal piglets for 3 days with an artificial milk formula rather than

either colostrum or mature milk does not affect the mucosal cell number in the small intestine. Newborn piglets fed 5% lactose for 24 hours had a similar DNA content in the small intestinal mucosa to that in piglets fed colostrum or mature milk (Simmen *et al.*, 1990).

Biochemical estimates of cell size gave equivocal results. Overall the cell size in the mucosa of the small intestine may increase with age during the first 2 weeks. Both the ratios of tissue weight to DNA and protein to DNA have been used as expressions of the cell size (Winick and Nobles, 1966 and 1967; Majumdar, 1984). The constancy of the ratio of protein to DNA suggested that cell size did not change from 3 to 14 days, however, the tissue weight to DNA ratio increased in some regions.

Compared with the ratio of protein to DNA (29-39) in 3-day-old suckled piglets (Xu *et al.*, 1992a), the ratio of protein to DNA in the mucosa of the small intestine in the present piglets (17-23) was much lower. This may be due partly to lower protein synthesis and partly to less protein absorption into epithelial cells. Piglets fed sow colostrum had a higher rate of intestinal protein synthesis than those fed mature milk (Burrin *et al.*, 1992). The artificial milk formula fed to the present piglets may have resulted in a lower protein synthesis in the natural-suckled piglets in the study of Xu *et al.*, 1992a). The intact protein absorption may also have been less in the present piglets than in those fed sow milk which contains 6.8% protein. It is not known how much protein is retained in the epithelial cells in 3-day-old piglets. Epithelial cells in the terminal small intestine of neonatal piglets can take up macromolecules for 12-14 days (Clarke and Hardy, 1971), so that the epithelial cells in 14-day-old piglets might still retain protein.

The capacity for protein synthesis appeared to decline with age over the period of 3-14 days. If the RNA:DNA ratio is used as an index of protein synthesizing capacity (Winick & Noble, 1965; Sarkar *et al.*, 1977), which in turn might be regarded as an index of growth potential, the intensity of protein synthesis in the mucosa of the small intestine was apparently reduced with advancing age in neonatal piglets in the present study. That decrease in capacity for protein synthesis in the mucosa, if it did occur, was coincident with a decreased tissue protein concentration, which has also been reported in young pigs (Kidder and Manners, 1980; Shulman *et al.*, 1988). This suggests that the concentration of non-protein components was increased with age.

4.4.2.2 Disaccharidases

The pattern of development of the disaccharidase activity in the control piglets, similar to that in piglets nursed by sows, was a declining lactase but increasing maltase and sucrase activity over the first two weeks after birth. A similar decrease in lactase activity has been reported between days 2 and 7 in piglets which were sow-reared or early weaned (at 2- or 7-day-old) and then artificially reared (Hartman *et al.*, 1961; Manners and Stevens, 1972).

Using the combined data from other authors and from the present study, it is suggested that lactase activity probably began to decline between days 5 and 7 after birth. High lactase activity is well-known in piglets at birth (Bailey *et al.*, 1956; Hartman *et al.*, 1961; Manners and Stevens, 1972; Ekstrom *et al.*, 1975) and at 24 hours (Widdowson *et al.*, 1976), but there does not appear to be any data reported for the activity of this enzyme in piglets between 24 hours and one week of age. Lactase activity in the proximal half of the small intestine is considerably higher in unsuckled newborns, than in 1-week-old piglets (Manners and Stevens, 1972). In the present study, lactase activities at 3 and 5 days were similar, but higher than those in the jejunum in 7-day-old Large white piglets (Manners and Stevens, 1972). Therefore, the decline in lactase activity is likely to be between 5 and 7 days in piglets.

The development of sucrase activity in the present study, in both distribution in the small intestine and increase with age, was comparable to that in piglets nursed by sows or early weaned (at 2 days of age) and then reared artificially (Manners and Stevens, 1972). Bailey *et al.* (1956) and Manners and Stevens (1972) have demonstrated that sucrase activity rose steadily from negligible levels in the jejunum at birth to significant levels at one week. In the second week, the enzyme continued to increase in the jejunum and extended into the proximal ileum. However, these authors did not examine when this enzyme rose during the first week after birth. The present study has further demonstrated that sucrase activity had appeared in the jejunum on day 3, and continued to increase by days 5 and 14. The lack of detectable sucrase in the duodenum and the mid- or distal ileum by day 14 was similar to findings in the same aged piglets nursed by sows or early weaned (at 2 days of age) and then reared artificially (Manners and Stevens, 1972).

Low maltase activity was present in the whole small intestine on day 3 and increased

with age in the present study, as in suckled piglets (Hartman *et al.*, 1961). The present findings confirm the observations of Shulman *et al.* (1988) that maltase activity consistently increases with age only in the jejunum and ileum but not in the duodenum, where maltase increases only during neonatal life.

4.4.2.3 Na^+-K^+-ATPase

In contrast to disaccharidases, Na^+-K^+-ATPase development in neonatal piglets is not well known. A histological study by Pauer *et al.* (1991) demonstrated that this enzyme is present in the small intestine both in 110-day-old fetuses and in 14-day-old piglets, but did not quantify the enzyme activity. Data from control piglets from studies of experimental enteritis suggest that Na^+-K^+-ATPase activity decreases with age. In the present study, Na^+-K^+-ATPase activity in the mucosa of the intestine ranged from 1.4 to 5.8 µmol per mg protein in 3- or 5-day-old piglets, which is comparable with the range of 1.8-3.0 µmol per mg protein in the small intestine of 11- to 13-day-old York piglets (Davidson *et al.*, 1977), but much higher than the range of 0.5-1.4 µmol per mg protein in the small intestinal mucosa in 25- to 28-day-old York pigs (Kelly *et al.*, 1972). Therefore Na^+-K^+-ATPase activity is likely to decrease with age. This hypothesis is supported by observations in Sprague-Dawley rats in which Na^+-K^+-ATPase activity sharply rises before birth to reach a peak on day 1 after birth, then declines with age at least until day 14 (Horváth *et al.*, 1993).

4.4.3 Conclusions

Yersinia enterocolitica infection in newborn piglets caused a lower body weight gain, slower growth of some organs and a marked reduction in plasma glucose concentration and lactase activity in the small intestine. After antibiotic therapy, piglets appeared to have recovered because their small intestinal morphology and body weight gain were similar to those in controls. The final body weight, however, was still below that of the controls and lactase activity and plasma glucose concentration also remained lower than in controls.

Although antibiotic therapy is an effective treatment for gastroenteritis, there were residual effects on body weight, and the disaccharidase profile in the small intestine was different from that in controls. It would be useful to develop an oral supplement of protein which are normal milk components, which might be effective in preventing

gastroenteritis. These proteins are unlikely to be present in commercial milk formulas and their concentration in milk may be not high enough to be effective. Lactoferrin and other bovine milk fractions have potential to protect the newborn against bacterial diarrhoea. The effects of lactoferrin and a bovine milk fraction, supplied by the New Zealand Dairy Research Institute, on *Y. enterocolitica* infection in newborn piglets are described in the next Chapter.

4.5 SUMMARY

In control piglets, lactase activity remained high for at least 5 days in almost all segments of the small intestine and then declined with age. Maltase activity was present in the entire small intestine at 3 days, but higher in the jejunum, increased with age in the jejunum and ileum but had a peak activity on day 5 in the duodenum. Sucrase activity was present only in the jejunum by day 3 and increased by day 14 but was undetectable in the duodenum and the mid- and distal ileum. Na^+-K^+-ATPase activity remained unchanged in the entire intestine from day 3 to day 5. Protein concentration and the ratio of RNA/DNA linearly decreased with age in most segments of the small intestine.

Yersinia enterocolitica infection reduced the normally high neonatal lactase activity in the duodenum and jejunum but not in the ileum. This was most apparent on day 5. After 5 days of antibiotic therapy, lactase activity did not recover to the level seen in control animals. Lactase activity in homogenates of parallel cryostat sections of the mid-jejunal mucosa indicated that there was a significant reduction in the activity of this enzyme at the base of the villi in both 5- and 14-day-old piglets. *Yersinia enterocolitica*-infected pigs had significantly higher maltase activity from the distal jejunum to mid-ileum throughout the duration of the experiment. Sucrase activity was reduced during the period of infection but increased to exceed that of the control pigs after antibiotic therapy. There was an early appearance of sucrase activity in the mid- and distal ileum of the infected piglets after antibiotic therapy. *Yersinia enterocolitica* infection did not impact upon Na^+-K^+-ATPase activity, protein and DNA contents, protein to DNA ratio or RNA to DNA ratio.

Chapter 5

EFFECTS OF ORAL LACTOFERRIN AND A BOVINE MILK FRACTION ON NEWBORN PIGLETS INFECTED WITH *YERSINIA ENTEROCOLITICA*

5.1 INTRODUCTION

Neonates are more resistant to enteric infections when suckled than when fed formula feeds, irrespective of whether they are born with immunoglobulins in their blood (humans and rabbits) or whether they depend on the transfer of immunoglobulins into the blood from the ingested colostrum through the temporarily permeable gut (piglets and calves) (Reiter, 1978). This indicates that immunoglobulins and other components of colostrum and mature milk have an important role in intestinal protection of the neonate. It is well known that lactoferrin and lysozyme act against enterobacteria *in vitro* (Brock *et al.*, 1983; Bortner *et al.*, 1986; Ellison and Giehl, 1991). Recently, a bovine milk fraction (BMF), a protein component purified from bovine milk by the New Zealand Dairy Research Institute, in an *in vitro* study inhibited *Escherichia coli* haemagglutination (personal communication Dr. L. Schollum), suggesting that the BMF might be effective *in vivo* as an oral antimicrobial agent.

Lactoferrin, an iron-binding glycoprotein, is present in milk (Masson *et al.*, 1966; Underwood, 1977) as well as in neutrophils (Baggiolini *et al.*, 1970) and exocrine secretions (Masson *et al.*, 1966; Masson and Heremans, 1968). The lactoferrin content of human milk is very high, with concentrations ranging from a high of 5-7 mg/ml in colostrum to a low of 2-3 mg/ml in mature milk (Masson and Heremans, 1971), and is present in concentrations of 0.02-0.2 mg/ml in cow, goat and sow milk (Underwood, 1977).

The bacteriostatic effects of lactoferrin, which have been well demonstrated *in vitro* against strains of *E. coli, Vibrio cholera, Salmonella typhimurium* and *Legionella pneumophila*, are mediated largely by its iron-binding capacity (Brock *et al.*, 1984; Bortner *et al.*, 1986; Ellison and Giehl, 1991), each molecule chelating two ferric ions with high affinity (Groves, 1960). Iron is an essential growth factor for nearly all bacteria (Finkelstein *at el.*, 1983). In the intestine, the host and microbes compete for

iron and, with its strong affinity for iron, lactoferrin binds iron and makes it unavailable to microorganisms. The opposite is also true, as administration of iron to animals increases their susceptibility to experimental infection (Miles *et al.*, 1979; Robins-Browne and Prpic, 1985; Kramer *et al.*, 1986). Lactoferrin has another antibacterial action of binding to bacteria (Arnold *et al.*, 1977) and directly damaging the outer membrane of enteric Gram-negative bacteria by releasing lipopolysaccharides (Ellison *et al.*, 1988, 1990).

In vivo protection against bacterial enteritis by lactoferrin has been demonstrated by Zagulski *et al.* (1986, 1989) in rabbits and mice. Lactoferrin prolonged the survival time of rabbits experimentally infected with *E. coli* to 9 days compared with 19 hours in controls (Zagulski *et al.*, 1986). In *E. coli*-infected mice, the survival rates in the lactoferrin group were 92% and 71% at 24 hours and 2 weeks postinfection respectively compared with 11% and 5% in controls (Zagulski *et al.*, 1989). In both these studies in which lactoferrin was effective against *E. coli*, the administration of lactoferrin was by intravenous injection. When lactoferrin was supplied orally, the bacteriostatic effect was not consistent. Teraguchi *et al.* (1993) demonstrated that 0.5% bovine lactoferrin in commercial milk, pasteurized at 130 ^{0}C for 2 seconds, when orally administered to specific pathogen-free mice reduced their faecal *Enterobacteriaceae* but lactoferrin, even at the high concentration of 5%, was ineffective when supplied in drinking water and the animals were fed with commercial pellets. Newborn infants, fed a milk formula to which bovine lactoferrin had been added, did not have the same faecal microflora as did breast-fed babies (Balmer *et al.*, 1989). Hall *et al.* (1988) failed to show any bactericidal effects of bovine lactoferrin in neonatal piglets infected with a pathogenic strain of *E. coli.* No inhibitory action of lactoferrin on *Y. enterocolitica* has been reported either *in vitro* or *in vivo.*

Oral supplementation is a natural and easy method of administration to human infants and other neonatal mammals, therefore, the oral route was chosen for lactoferrin and BMF supplementation in the present study. A piglet model has been developed in which *Y. enterocolitica* produced severe diarrhoea accompanied by slow growth, a badly damaged mucosa and alterations to the digestive enzymes in the small intestine (Chapters 2, 3, 4). In the experiment reported in this Chapter, newborn, colostrum-deprived piglets infected with *Y. enterocolitica* were given oral supplements of either lactoferrin or the BMF to examine whether these components of bovine milk have a protective effect against a bacterial enteritis.

5.2 MATERIALS AND METHODS

5.2.1 Lactoferrin and Bovine Milk Fraction

Lactoferrin and the BMF used in the present study were purified from bovine milk. The lactoferrin powder contained 74% apo-lactoferrin (iron free), 23% iron-saturated lactoferrin and 0.02 units of lactoperoxidase per mg. Both proteins were kindly provided by Dr. L. Schollum of the New Zealand Dairy Research Institute, Palmerston North, N.Z. The BMF is a protein component of bovine milk which is owned by the NZDRI and the method of preparation is commercially sensitive.

5.2.2 Bacteria

Yersinia enterocolitica, serotype 0:3 biotype 4 (Chapter 2, 2.2.3) was used. The preparation of the *Y. enterocolitica* suspension for animal challenge has been described in Chapter 2 (2.2.3).

5.2.3 Inhibition of *Y. enterocolitica* Growth *in vitro*

This was a preliminary study in order to find first, whether or not lactoferrin inhibited *Y. enterocolitica* growth *in vitro* and secondly, a concentration of lactoferrin which effectively inhibited *Y. enterocolitica in vitro* which would provide a basis for selection of the lactoferrin concentration for the *in vivo* study. The effect of the BMF on *Y. enterocolitica* growth *in vitro* was not carried out, since the BMF would not completely dissolve in either TSB or 1% peptone water and the BMF suspension could not be passed through a 0.45 μm filter.

Two media, trypticase soy broth (TSB) and 1% peptone water, were used to study the effects of lactoferrin on *Y. enterocolitica* growth *in vitro*. Lactoferrin powder was dissolved in TSB to give solutions of 0.3%, 0.6%, 1% and 5% lactoferrin, and in 1% peptone water to give solutions of 1% and 5% lactoferrin (w/v). All solutions were passed through a 0.45 μm filter (Micron Separations Inc., Westborough, Massachusetts, USA) before use.

Yersinia enterocolitica was grown on a sheep blood agar plate at 29 ^{0}C for 48 hours. One bacterial colony from the plate was placed in 10 ml of TSB and another colony in

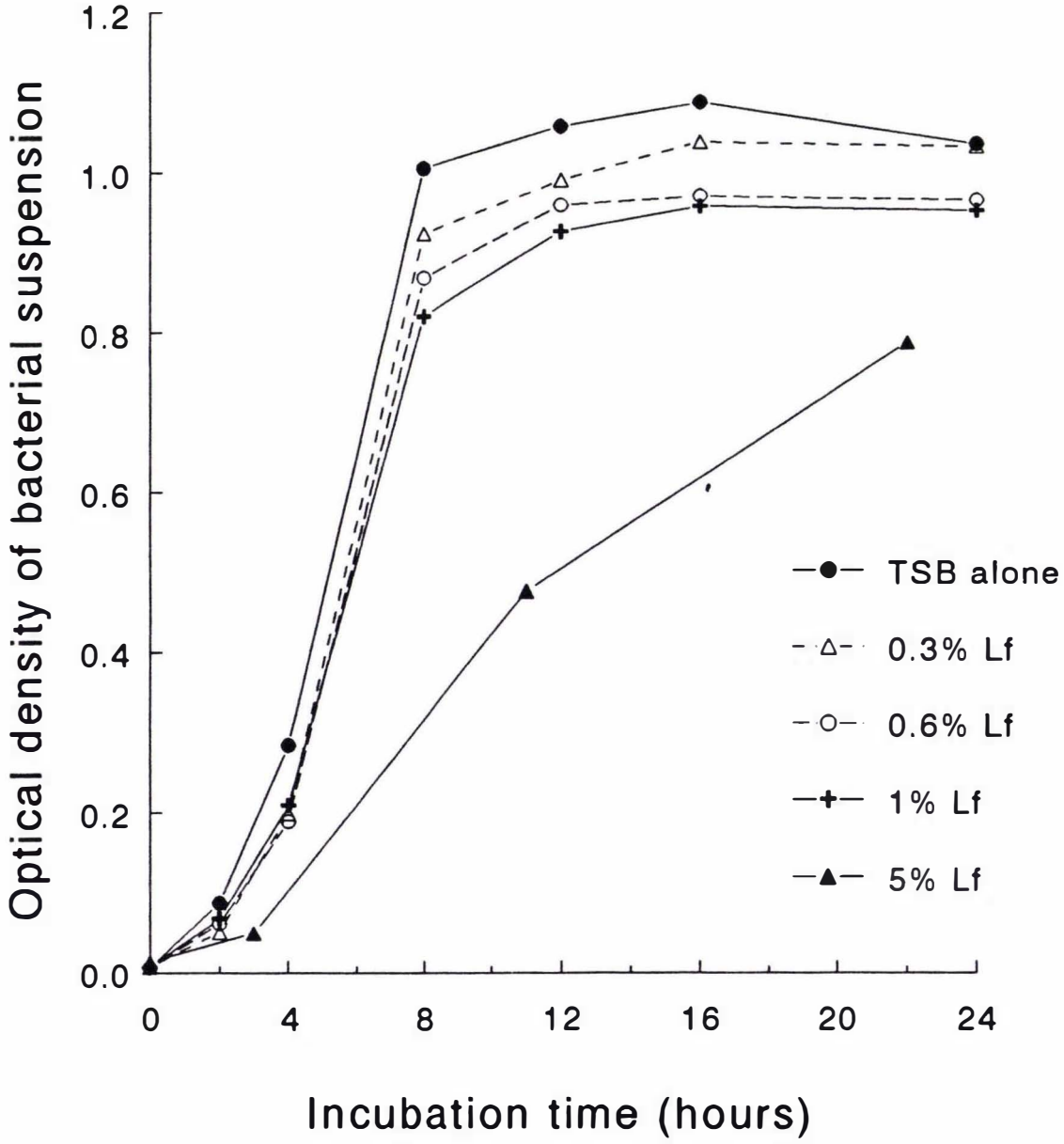


Figure 5.1 Optical density of *Y. enterocolitica* suspensions in Trypticase soy broth (TSB) containing 0, 0.3%, 0.6%, 1% and 5% lactoferrin (Lf) during incubation at 29 ^{0}C.

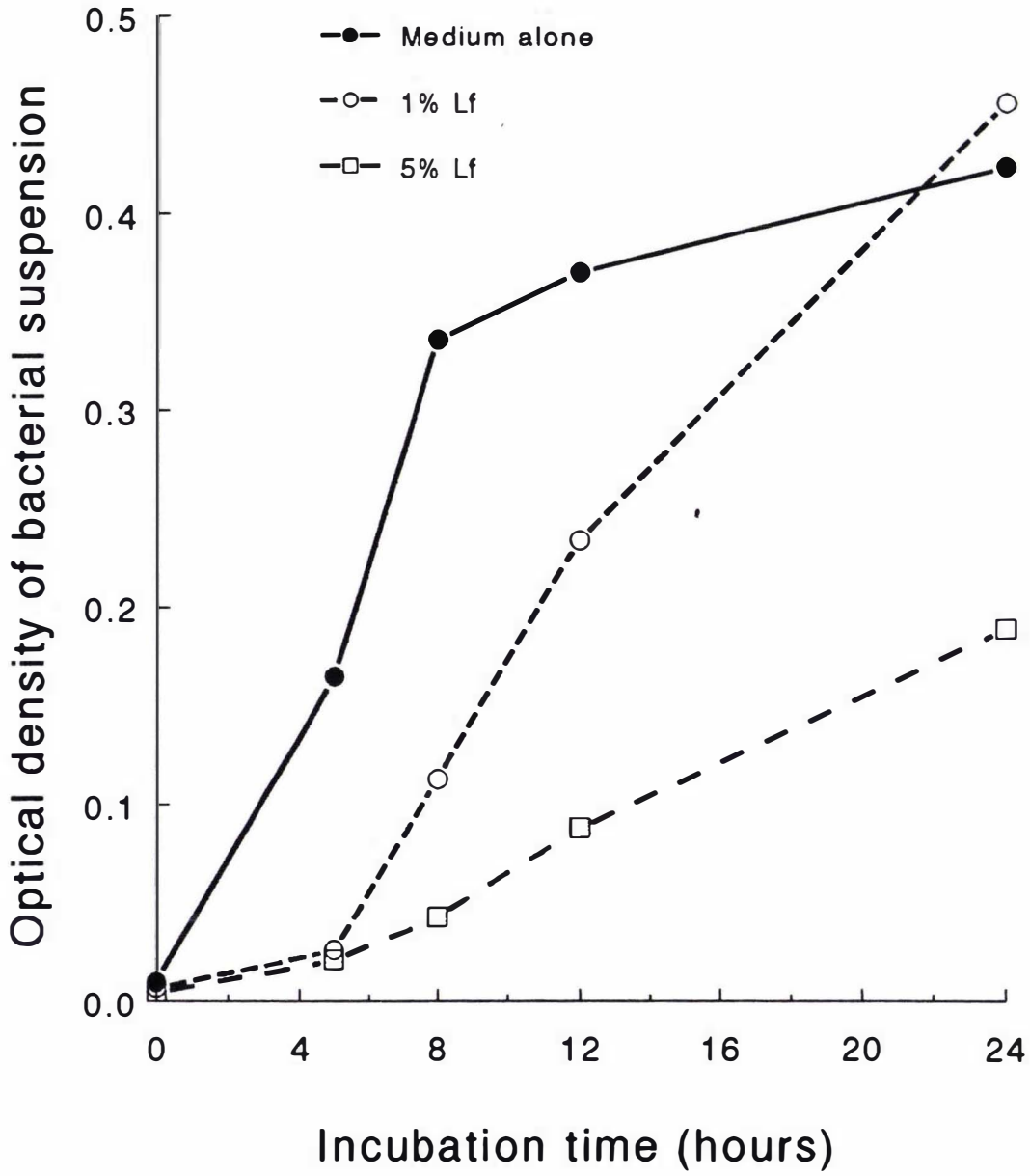


Figure 5.2 Optical density of *Y. enterocolitica* suspensions in 1% peptone water containing 0, 1% and 5% lactoferrin (Lf) during incubation at 29 ^{0}C.

3 ml of 1% peptone water; both cultures were then incubated at 29 ^{0}C for 24 hours. One ml of bacterial suspension in TSB was added to each of four aliquots of 80 ml of TSB containing 0, 0.3, 0.6 and 1% lactoferrin respectively. Similarly, 0.2 ml of bacterial suspension in 1% peptone water was added to 20 ml of each of three 1% peptone water media prepared to contain no lactoferrin, 1% and 5% lactoferrin powder. Each bottle of bacterial suspension was divided into 2 ml aliquots, followed by incubation at 29 ^{0}C. During incubation for 24 hours, bacterial growth was assessed in 2 aliquots per group at 2, 4, 8, 12, 16 and 24 hours by reading the optical density against a TSB blank at 540 nm using a spectrophotometer (SP8-400 UV/VIS, PYE Unicam Ltd., Cambridge, England). Bacterial growth in TSB containing 5% lactoferrin, and in 1% peptone water containing 1% and 5% lactoferrin, was assessed similarly. The optical density of the bacterial suspension was read after 3, 11 and 22 hours incubation for 5% lactoferrin in TSB and after 5, 8 ,12 and 24 hours for the peptone water.

The effects of lactoferrin on *Y. enterocolitica* growth in TSB and in 1% peptone water are shown in Figures 5.1 and 5.2. The optical density of the bacterial suspensions indicated that after 24 hours incubation there was a much higher bacterial number in TSB (maximal optical density 1.2) (Figure 5.1) than in 1% peptone water (maximal optical density 0.5) (Figure 5.2). Inhibition of *Y. enterocolitica* growth by lactoferrin in both TSB and peptone water was concentration dependent. In TSB, 5% lactoferrin had the greatest inhibitory effect, and lower concentrations inhibited bacterial growth very poorly (Figure 5.1). Both 1% and 5% lactoferrin in 1% peptone water inhibited bacterial growth for 5 hours postinoculation, after which the inhibition was maintained only in the 5% lactoferrin (Figure 5.2).

Since there was effective inhibition of *Y. enterocolitica* growth by 1% lactoferrin in peptone water and by 5% lactoferrin in both peptone water and TSB *in vitro*, 5% lactoferrin was chosen as the oral supplement for the *Y. enterocolitica*-infected piglets.

5.2.3 Experimental Design

Twenty newborn, colostrum-deprived piglets from 4 litters (5 animals per litter) were assigned to 5 groups (described in the table below), there being one piglet from each litter in each group. Piglets in groups 1, 4 and 5 were inoculated with 3 x 10^{10} *Y. enterocolitica* in TSB (Chapter 2, 2.2.4). For group 5, 0.2 ml of 4.5% BMF stock solution was added to the inoculum before challenge. Litter mate controls in groups 2

and 3 received TSB medium only. Groups 1 and 2 were fed milk formula, groups 3 and 4 milk formula containing 5% bovine lactoferrin powder (w/v) and group 5 milk formula containing 0.15% BMF (w/v).

Group	Treatment	n	Feeding
1	Infected	4	Milk formula alone
2	Control	4	Milk formula alone
3	Control + lactoferrin	4	Milk formula containing 5% lactoferrin powder
4	Infected + lactoferrin	4	Milk formula containing 5% lactoferrin powder
5	Infected + BMF	4	Milk formula containing 0.15% BMF

Milk formula containing 5% lactoferrin powder was made up in two steps. First, 20 g of lactoferrin powder was dissolved completely in 100 ml of cooled, boiled water to make a lactoferrin stock solution which was kept at room temperature for no longer than 6 hours. Next, the lactoferrin stock solution was added to milk formula to give a final concentration of 5% lactoferrin. The 14% milk formula contained 1.68% protein (60% lactalbumin and 40% casein) and 178 μM iron (1.0 mg per 100 ml). Milk formula with 5% lactoferrin contained 463 μM apo-lactoferrin. The BMF stock solution, made up by adding 4.5 g BMF powder to 100 ml of sterile water, was stored as 10 ml aliquots at --20 ^{0}C until used (processed by Dr. L. Schollum). The BMF stock solution was added to milk formula immediately before feeding in a ratio of 1:30 (BMF stock solution : milk formula, v:v) to give a final concentration of 0.15% BMF.

All the methods of animal inoculation and feeding, observation of clinical signs, *Y. enterocolitica* isolation from faeces, mesenteric lymph nodes and contents of the small intestine, animal dissection, sampling, histological processing and lesion number counting in sections of the small intestine from all infected piglets have been described in Chapter 2 (2.2). Measurement of body weight and length, organ weight, intestinal length and all morphological measurements (Chapter 3, 3.2) and all biochemical assays (Chapter 4, 4.2) of the small intestinal mucosa have been previously described.

5.2.6 Statistical Analysis

Data were analyzed by two-way Analysis of Variance (ANOVA) and by individual comparisons using a least significant difference form of the *t*-test by the General Linear

Table 5.1. Number of piglets showing clinical signs, positive *Y. enterocolitica* isolations and the presence of lesions in *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) or with added bovine milk fraction (BMF)

	Infected *n=4*	Infected + Lf *n=4*	Infected + BMF *n=4*
Clinical signs			
Anorexia	4	2	4
Vomiting	2	4	1
Diarrhoea	4	4	4
***Y. enterocolitica* isolation**			
Faeces	4	4	4
Mesenteric lymph nodes	2	3	4
Small intestinal contents	4	4	4
Occurence of lesions			
Stomach	1	0	1
Duodenum	2	4	3
Proximal jejunum	3	4	3
Mid-jejunum	3	3	1
Distal jejunum	4	3	2
Proximal ileum	2	4	4
Mid-ileum	3	4	3
Distal ileum	4	4	4
Caecum	3	2	3
Proximal colon	1	1	0
Distal colon	1	1	1
Liver	4	3	4

Table 5.2. Number of lesions in the small intestine of *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) or with added bovine milk fraction (BMF) and ANOVA with individual comparisons

		Duodenum	Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	n			Lesion number (Mean ± SE)				
Infected	4	1.94 ± 1.62	10.38 ± 6.47	7.34 ± 2.57	1.28 ± 0.91	2.94 ± 1.90	7.66 ± 7.45	8.06 ± 2.79
Infected + Lf	4	22.75 ± 14.23	20.53 ± 8.95	2.03 ± 1.03	0.75 ± 0.41	3.28 ± 1.39	7.69 ± 5.90	5.47 ± 1.15
Infected + BMF	4	3.97 ± 2.01	2.66 ± 1.07	1.25 ± 1.25	1.78 ± 1.07	1.09 ± 0.34	2.44 ± 1.37	11.50 ± 1.62
				Analyses of Variance				
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Treatment	2	526.6810 NS	321.4961 NS	43.9779 NS	1.0638 NS	5.5352 NS	36.5326 NS	36.6133 *
Litter	3	285.8589 NS	222.1962 NS	11.9688 NS	4.3247 NS	4.9670 NS	231.9596 NS	34.0777 *
Error	6	275.5595	135.1401	12.4987	2.0968	8.8615	68.2565	6.3824
				Comparison of individual treatments				
Infected + Lf vs Infected		NS	NS	NS	NS	NS	NS	NS
Infected + BMF vs Infected		NS	NS	NS	NS	NS	NS	NS

NS $p > 0.05$; * $p < 0.05$ MS = Mean Square

Table 5.3. Plasma electrolyte and glucose concentration in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA with individual comparisons

		Na^+	K^+	Cl^-	Glucose
Treatment	n	Concentration, mmol/l, (Mean ± SE)			
Infected	4	133.4 ± 3.9	3.7 ± 0.2	98.1 ± 2.3	3.8 ± 0.9
Control	4	141.4 ± 2.2	3.9 ± 0.4	104.4 ± 1.3	6.9 ± 0.7
Control + Lf	4	142.2 ± 1.1	3.3 ± 0.0	101.5 ± 1.1	5.1 ± 0.3
Infected + Lf	4	132.7 ± 0.3	3.1 ± 0.1	97.4 ± 1.4	2.7 ± 0.4
Infected + BMF	4	136.5 ± 0.7	3.6 ± 0.1	100.1 ± 0.4	4.2 ± 0.5
		Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p
Litter	3	8.00 NS	0.37 NS	12.10 NS	4.29 *
Treatment	4	77.30 *	0.42 NS	31.60 *	9.91 ***
Error	12	20.20	0.21	7.80	0.85
		Comparison of individual treatments			
Control vs Infected		*	NS	**	***
Control + Lf vs Control		NS	NS	NS	*
Infected + Lf vs Infected		NS	NS	NS	NS
Infected + BMF vs Infected		NS	NS	NS	NS

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Models Procedure of SAS (SAS Institute Inc., Cary, NC, USA, 1989). After ANOVA, 4 comparisons were made in each instance (1) control vs infected, (2) control + lactoferrin vs control, (3) infected + lactoferrin vs infected, (4) infected + BMF vs infected. Data for sucrase activity (μmol/g protein/min) were logarithmically transformed and retested because the data showed heterogeneous variance between groups. Data are presented as mean and standard error of the mean (SE), and results of the ANOVA are presented as probability (p). Both mean square (MS) and p are tabled. A p value of < 0.05 was considered significant. In individual comparisons, p values between 0.05 and 0.1 were designated as NS^+, and $p > 0.1$ as NS.

5.3 RESULTS

5.3.1 Clinical Signs and Histomorphology

The number of animals showing clinical signs, positive *Y. enterocolitica* isolations and histopathological lesions in the gastrointestinal tract and the liver are shown in Table 5.1, and the mean lesion number in the small intestine of *Y. enterocolitica*-infected piglets fed with or without lactoferrin or BMF in Table 5.2. Neither lactoferrin nor BMF reduced the occurrence of clinical signs in *Y. enterocolitica*-infected piglets. In both the lactoferrin- and BMF-fed groups, invasion by *Y. enterocolitica* involved almost all segments of the small intestine. There were no statistically significant effects of either lactoferrin or BMF on the number of lesions in the small intestine from the ANOVA, however, comparison of the group means showed that, in the BMF-fed piglets, there was a lower number of lesions in most segments except the distal jejunum and the distal ileum, whereas in lactoferrin-fed piglets the mean lesion number was larger in the duodenum and the proximal jejunum than in the milk formula-fed group of piglets.

5.3.2 Plasma Electrolyte and Glucose Concentration

Plasma Na^+ ($p < 0.05$), Cl^- ($p < 0.01$) and glucose ($p < 0.001$) were all lower in infected than control animals; lactoferrin and BMF administration did not reverse this effect (Table 5.3). Control piglets given lactoferrin had a lower plasma glucose concentration than piglets fed milk formula alone ($p < 0.05$). Potassium concentrations were similar in all groups.

Table 5.4. Body length (Crown rump length, CRL, and Nose rump length, NRL) on day 5 and body weight gain from birth to day 5 in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA

		CRL	NRL	Body weight gain
		Mean ± SE		
Treatment	n	Length, cm		weight, g
Infected	4	35.6 ± 0.4	42.2 ± 0.4	169 ± 65
Control	4	36.7 ± 1.0	43.8 ± 0.9	238 ± 29
Control + Lf	4	37.6 ± 1.4	44.5 ± 1.6	259 ± 13
Infected + Lf	4	37.8 ± 0.9	44.1 ± 1.2	170 ± 58
Infected + BMF	4	38.5 ± 1.6	44.7 ± 1.9	213 ± 23
		Analyses of Variance		
Source	DF	MS p	MS p	MS p
Litter	3	10.71 NS	13.90 NS	6.4 NS
Treatment	4	4.98 NS	3.86 NS	8.1 NS
Error	12	4.18	5.39	7.0

NS $p > 0.05$ MS = Mean Square

Table 5.5. Weight of non-intestinal organs in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA with individual comparisons

		Stomach	Liver	Pancreas	Spleen	Kidney
Treatment	n		Weight, g (Mean ± SE)			
Infected	4	8.30 ± 0.80	62.9 ± 5.4	2.37 ± 0.38	1.78 ± 0.19	9.52 ± 0.53
Control	4	8.69 ± 0.32	85.4 ± 6.4	3.18 ± 0.25	2.11 ± 0.21	11.13 ± 0.81
Control + Lf	4	8.85 ± 0.80	52.6 ± 6.7	2.80 ± 0.28	1.78 ± 0.17	13.27 ± 1.61
Infected + Lf	4	9.50 ± 1.11	55.4 ± 6.6	2.75 ± 0.25	2.16 ± 0.19	12.72 ± 0.98
Infected + BMF	4	8.37 ± 0.72	66.6 ± 7.4	2.61 ± 0.27	2.18 ± 0.46	9.52 ± 1.07
			Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p
Litter	3	6.1533 NS	394.88 NS	0.2380 NS	0.2366 NS	12.0620 *
Treatment	4	0.9244 NS	670.30 **	0.3508 NS	0.1676 NS	12.2485 *
Error	12	1.6111	113.58	0.3611	0.2888	2.6371
			Comparison of individual treatments			
Control vs Infected		NS	*	NS	NS	NS
Control + Lf vs Control		NS	***	NS	NS	NS^{+}
Infected + Lf vs Infected		NS	NS	NS	NS	*
Infected + BMF vs Infected		NS	NS	NS	NS	NS

NS $p > 0.05$; NS^{+} $0.1 > p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 5.6. Weight of non-intestinal organs relative to body weight in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA with individual comparisons

		Stomach	Liver	Pancreas	Spleen	Kidney
Treatment	n	Weight relative to body weight, g/kg (Mean ± SE)				
Infected	4	5.34 ± 0.32	40.7 ± 2.7	1.52 ± 0.19	1.15 ± 0.09	6.17 ± 0.32
Control	4	4.94 ± 0.09	48.3 ± 1.5	1.81 ± 0.16	1.19 ± 0.07	6.33 ± 0.50
Control + Lf	4	5.27 ± 0.25	30.9 ± 1.0	1.72 ± 0.06	1.26 ± 0.09	7.82 ± 0.41
Infected + Lf	4	5.78 ± 0.46	33.4 ± 1.2	1.67 ± 0.05	1.32 ± 0.07	7.86 ± 0.64
Infected + BMF	4	5.50 ± 0.49	43.1 ± 1.9	1.69 ± 0.02	1.38 ± 0.17	6.15 ± 0.25
		Analyses of Variance				
Source	DF	MS p	MS p	MS p	MS p	MS p
Litter	3	1.7100 **	12.25 NS	0.0477 NS	0.0290 NS	2.0923 *
Treatment	4	0.3874 NS	202.98 ***	0.0466 NS	0.0365 NS	3.1653 **
Error	12	0.2011	12.70	0.0584	0.0477	0.4623
		Comparison of individual treatments				
Control vs Infected		NS	**	NS	NS	NS
Control + Lf vs Control		NS	***	NS	NS	**
Infected + Lf vs Infected		NS	*	NS	NS	**
Infected + BMF vs Infected		NS	NS	NS	NS	NS

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 5.7. Length and length relative to body weight of the small intestine, caecum and colon in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA

		Small intestine	Caecum	Colon
Length (cm)				
Treatment	n		Mean ± SE	
Infected	4	461 ± 9	4.5 ± 0.3	84.0 ± 4.7
Control	4	443 ± 35	4.0 ± 0.4	83.1 ± 6.9
Control + Lf	4	474 ± 25	4.4 ± 0.4	87.3 ± 6.1
Infected + Lf	4	486 ± 23	4.9 ± 0.2	91.8 ± 4.6
Infected + BMF	4	445 ± 13	4.5 ± 0.2	81.8 ± 6.6
			Analyses of Variance	
Source	DF	MS p	MS p	MS p
Litter	3	6141 NS	0.28 NS	432.15 **
Treatment	4	1396 NS	0.39 NS	64.05 NS
Error	12	1062	0.38	62.66
Relative length to body weight (cm/kg)				
Treatment	n		Mean ± SE	
Infected	4	299 ± 10	2.9 ± 0.3	54.6 ± 3.7
Control	4	251 ± 14	2.3 ± 0.3	47.0 ± 2.6
Control + Lf	4	288 ± 28	2.7 ± 0.3	52.9 ± 5.2
Infected + Lf	4	300 ± 18	3.0 ± 0.2	57.1 ± 5.6
Infected + BMF	4	294 ± 20	3.0 ± 0.1	53.7 ± 4.1
			Analyses of Variance	
Source	DF	MS p	MS p	MS p
Litter	3	2749 NS	0.50 NS	243.41 NS
Treatment	4	1691 MS	0.36 NS	55.91 NS
Error	12	1106	0.20	33.94

NS $p > 0.05$; ** $p < 0.01$ MS = Mean Square

Table 5.8. Weight and weight relative to body weight of the small intestine and its mucosa and muscle, the caecum and colon in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA

		Small intestine			Caecum	Colon	
		Mucosa	Muscle	Total		Proximal	Distal
Weight (g)							
Treatment	n			Mean ± SE			
Infected	4	38.9 ± 2.3	18.2 ± 1.8	57.1 ± 4.1	1.00 ± 0.12	5.43 ± 0.98	4.74 ± 0.82
Control	4	39.6 ± 3.5	16.7 ± 1.7	56.3 ± 4.7	0.92 ± 0.08	6.15 ± 1.61	4.60 ± 1.18
Control + Lf	4	42.8 ± 5.6	18.3 ± 2.5	61.2 ± 8.1	0.93 ± 0.16	3.91 ± 0.55	3.62 ± 0.57
Infected + Lf	4	43.7 ± 4.7	20.3 ± 1.4	63.9 ± 5.6	1.31 ± 0.04	7.28 ± 0.58	5.27 ± 0.39
Infected + BMF	4	39.3 ± 4.7	16.3 ± 1.8	55.6 ± 6.5	1.03 ± 0.14	4.94 ± 1.04	4.08 ± 0.87
				Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	142.29 NS	40.67 *	331.52 NS	0.0360 NS	1.3052 NS	0.7682 NS
Treatment	4	19.88 NS	9.91 NS	51.78 NS	0.0702 NS	6.4376 NS	1.6108 NS
Error	12	57.16	7.48	93.49	0.0587	4.9602	3.1108
Weight relative to body weight (g/kg)							
Treatment	n			Mean ± SE			
Infected	4	25.1 ± 0.5	11.7 ± 0.8	36.8 ± 1.2	0.64 ± 0.05	3.5 ± 0.5	3.0 ± 0.4
Control	4	22.3 ± 1.0	9.4 ± 0.7	31.7 ± 1.2	0.54 ± 0.05	3.5 ± 1.0	2.6 ± 0.7
Control + Lf	4	25.5 ± 2.6	10.8 ± 0.9	36.4 ± 3.5	0.54 ± 0.05	2.3 ± 0.1	2.1 ± 0.1
Infected + Lf	4	26.8 ± 2.4	12.4 ± 0.5	39.3 ± 2.7	0.78 ± 0.07	4.5 ± 0.5	3.3 ± 0.1
Infected + BMF	4	25.3 ± 1.1	10.5 ± 0.3	35.8 ± 1.1	0.61 ± 0.05	3.1 ± 0.4	2.6 ± 0.3
				Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	12.18 NS	1.09 NS	19.22 NS	0.0044 NS	1.17 NS	0.38 NS
Treatment	4	10.82 NS	5.32 NS	29.49 NS	0.0399 NS	2.55 NS	0.81 NS
Error	12	12.04	1.88	18.68	0.0136	1.28	0.79

NS $p > 0.05$; * $p < 0.05$ MS = Mean Square

5.3.3 Growth of the Body and Non-intestinal Organs

Body length at 5 days was no different in the 5 groups (Table 5.4). The body weight gain from birth to day 5 also showed no significant difference in the ANOVA. The group mean in infected piglets (169 ± 65 g), however, was 29% lower than in the controls (238 ± 29 g); BMF-fed infected piglets had a 26% higher body weight gain (213 ± 23 g) than those fed milk formula alone. Lactoferrin feeding did not change the body weight gain in infected piglets (170 ± 58 g) but increased it by 9% in non-infected piglets (259 ± 13 g) (Table 5.4).

The absolute weights and weights relative to body weight of the non-intestinal organs are shown in Tables 5.5 and 5.6. The liver and kidney, but not the stomach, pancreas or spleen, showed significant differences between the 5 groups. *Yersinia enterocolitica* infection reduced the liver weight, but lactoferrin had an even greater effect. The lowest mean liver weight was in the control piglets receiving lactoferrin (52.6 ± 6.7 g), followed by that in infected animals given oral lactoferrin supplementation (55.4 ± 6.6 g), both weights being even lower than in infected piglets fed milk formula alone (62.9 ± 5.4 g). *Yersinia enterocolitica* infection reduced the liver weight, both absolute ($p < 0.05$) and relative to body weight ($p < 0.01$), while oral lactoferrin reduced the liver weight, both absolute and relative to body weight, in non-infected piglets ($p < 0.001$), but only the relative weight in infected piglets ($p < 0.05$).

In contrast, oral lactoferrin increased the kidney weight both absolute ($p < 0.05$) and relative to body weight ($p < 0.01$) in infected piglets, and the relative weight in non-infected piglets ($p < 0.01$) (Tables 5.5 and 5.6). Control animals given oral lactoferrin had a mean absolute weight of the kidneys 19% greater than that of animals fed milk formula alone although the increase was not significantly different ($0.05 < p < 0.1$). Neither lactoferrin nor BMF affected the stomach, pancreas and spleen weights, both absolute and relative to body weight.

5.3.4 Intestinal Length and Weight

The length and weight of the whole small intestine, caecum and colon were similar in all groups (Tables 5.7 and 5.8), nor were there any differences in total mucosal or muscle weight of the whole small intestine (Table 5.8). When the seven segments of the small intestine were considered individually (Tables 5.9 and 5.10), no differences

Table 5.9. Weight of the mucosa and muscle of seven segments of the small intestine in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA with individual comparisons

		Doudenum	Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Mucosa weight (g)								
Treatment	n				Mean ± SE			
Infected	4	1.1 ± 0.1	6.5 ± 0.6	6.5 ± 0.3	7.2 ± 0.7	6.5 ± 0.3	5.9 ± 0.7	5.2 ± 0.2
Control	4	1.1 ± 0.1	7.6 ± 0.6	7.1 ± 0.6	7.0 ± 0.8	6.5 ± 0.8	6.2 ± 0.9	5.0 ± 0.9
Control + Lf	4	1.3 ± 0.2	7.3 ± 1.2	6.9 ± 0.9	8.4 ± 1.4	7.5 ± 1.1	6.9 ± 0.6	4.5 ± 0.7
Infected + Lf	4	1.3 ± 0.1	7.7 ± 1.3	7.6 ± 1.2	8.3 ± 1.1	7.3 ± 0.9	6.6 ± 0.2	4.8 ± 0.7
Infected + BMF	4	1.2 ± 0.2	6.7 ± 0.8	6.5 ± 0.5	7.0 ± 1.1	6.6 ± 0.8	6.0 ± 1.0	5.3 ± 0.5
					Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	0.16 NS	8.31 NS	5.87 *	6.09 NS	4.93 NS	1.73 NS	1.50 NS
Treatment	4	0.05 NS	1.16 NS	0.84 NS	1.94 NS	0.88 NS	0.76 NS	0.43 NS
Error	12	0.06	2.49	1.56	3.92	2.18	2.33	1.66
Muscle weight (g)								
Treatment	n				Mean ± SE			
Infected	4	0.8 ± 0.1	2.9 ± 0.2	2.7 ± 0.4	2.4 ± 0.1	2.7 ± 0.2	2.6 ± 0.2	3.9 ± 0.8
Control	4	0.9 ± 0.1	2.4 ± 0.2	2.4 ± 0.2	2.5 ± 0.3	2.6 ± 0.3	2.5 ± 0.3	3.5 ± 0.4
Control + Lf	4	0.9 ± 0.2	2.7 ± 0.3	2.5 ± 0.3	3.0 ± 0.4	2.9 ± 0.4	2.9 ± 0.4	3.4 ± 0.6
Infected + Lf	4	1.1 ± 0.2	3.4 ± 0.2	2.7 ± 0.2	2.8 ± 0.2	2.8 ± 0.2	3.6 ± 0.1	3.9 ± 0.6
Infected + BMF	4	0.8 ± 0.2	2.6 ± 0.1	2.1 ± 0.2	2.4 ± 0.4	2.9 ± 0.5	2.3 ± 0.2	3.3 ± 0.4
					Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	0.23 *	0.39 *	0.70 NS	0.82 *	1.57 **	0.49 NS	2.69 NS
Treatment	4	0.04 NS	0.62 **	0.27 NS	0.27 NS	0.05 NS	1.01 *	0.37 NS
Error	12	0.05	0.09	0.24	0.21	0.20	0.25	1.07
					Comparison of individual treatments			
Control vs Infected		NS	*	NS	NS	NS	NS	NS
Control + Lf vs Control		NS	NS	NS	NS	NS	NS	NS
Infected + Lf vs Infected		NS	*	NS	NS	NS	*	NS
Infected + BMF vs Infected		NS	NS	NS	NS	NS	NS	NS

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$ MS = Mean Square

Table 5.10. Weight of segments and percentage of mucosa in the small intestine in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA

		Duodenum	Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Segment weight (g)								
Treatment	n				Mean ± SE			
Infected	4	1.9 ± 0.1	9.4 ± 0.8	9.3 ± 0.6	9.6 ± 0.7	9.3 ± 0.4	8.5 ± 0.9	9.1 ± 0.8
Control	4	2.1 ± 0.1	9.9 ± 0.8	9.5 ± 0.8	9.5 ± 1.0	9.2 ± 1.0	8.7 ± 1.2	8.5 ± 0.9
Control + Lf	4	2.3 ± 0.4	10.0 ± 1.4	9.5 ± 1.3	11.3 ± 1.8	10.4 ± 1.5	9.8 ± 1.0	7.8 ± 1.3
Infected + Lf	4	2.4 ± 0.2	11.1 ± 1.4	10.3 ± 1.3	11.1 ± 1.2	10.1 ± 1.0	10.2 ± 0.3	8.7 ± 0.9
Infected + BMF	4	2.0 ± 0.3	9.3 ± 0.8	8.6 ± 0.7	9.4 ± 1.4	9.5 ± 1.3	8.3 ± 1.2	8.6 ± 1.0
					Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	0.64 *	11.46 *	10.25 *	11.29 NS	11.77 *	4.05 NS	7.29 NS
Treatment	4	0.15 NS	2.12 NS	1.50 NS	3.58 NS	1.20 NS	2.94 NS	0.89 NS
Error	12	0.17	3.07	2.31	5.26	3.12	3.77	3.28
Mucosa percentage to segment weight (%)								
Treatment	n				Mean ± SE			
Infected	4	57.0 ± 2.0	68.9 ± 1.0	70.9 ± 2.1	74.3 ± 1.9	70.6 ± 1.8	68.6 ± 1.2	58.0 ± 4.2
Control	4	54.5 ± 3.4	76.2 ± 0.4	74.9 ± 1.4	73.3 ± 1.7	71.0 ± 2.1	70.7 ± 2.2	58.0 ± 5.7
Control + Lf	4	59.8 ± 1.1	72.3 ± 1.6	73.4 ± 0.9	73.4 ± 1.0	71.9 ± 1.7	70.6 ± 1.6	57.6 ± 1.3
Infected + Lf	4	55.6 ± 5.3	67.9 ± 4.1	73.1 ± 2.4	74.1 ± 2.3	71.8 ± 2.1	64.8 ± 0.8	55.4 ± 5.4
Infected + BMF	4	59.8 ± 2.3	71.6 ± 2.7	75.7 ± 0.5	74.7 ± 1.7	70.0 ± 1.7	71.8 ± 1.3	61.9 ± 0.9
					Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	113.16 *	45.38 NS	4.70 NS	1.82 NS	19.00 NS	2.04 NS	68.01 NS
Treatment	4	23.26 NS	42.65 NS	13.55 NS	1.47 NS	2.48 NS	30.40 NS	21.97 NS
Error	12	23.95	16.64	12.17	14.82	12.58	10.50	64.93

NS $p > 0.05$; * $p < 0.05$ MS = Mean Square

between the 5 groups were demonstrated in total segment weight, mucosal weight or percentage of mucosa to segment weight. The only significant difference between treatments was in muscle weight, and then only in the proximal jejunum and mid-ileum ($p < 0.05$ or < 0.01, Table 5.9). In these two segments, oral lactoferrin increased the muscle weight in infected piglets but not in uninfected animals. In infected animals given lactoferrin, in the other five segments of the small intestine, the group means were equal to, or higher than, those in the infected piglets fed milk formula alone. In the proximal jejunum, the muscle weight was also greater in the infected piglets when compared with the controls ($p < 0.05$).

5.3.5 Histological Observation of the Small Intestinal Epithelium

In both control and *Y. enterocolitica*-infected piglets, there were almost no vacuolated cells on the villi of the small intestine except in the distal ileum, where the epithelium contained many vacuoles in control piglets but very few vacuoles in infected animals. The morphology of the enterocytes in control and infected piglets was no different from that previously described (Chapter 3, 3.3.4). In infected piglets, neither oral lactoferrin nor BMF affected the vacuolation of the small intestinal epithelium, there being in all 3 groups almost no vacuolated cells from the distal jejunum to the mid-ileum, while there was a very small number of vacuolated cells in the distal ileum.

Control piglets fed lactoferrin had markedly increased vacuolation of the epithelium of the small intestine. These animals had numerous vacuolated cells from the mid-jejunum to the distal ileum, but not in the duodenum or the proximal jejunum. In the upper part of the villi, the enterocytes were always vacuolated and sometimes the vacuolated cells extended to the base of the villi but never into the crypts (Figure 5.3). In general, most vacuolated cells in the mid- and distal jejunum contained large vacuoles which distended the cytoplasm and the nuclei were located at the apex of the cells (Figure 5.4 A). In the proximal and mid-ileum, vacuolated cells were more abundant, the vacuoles were large and the location of the nuclei was variable: they were usually located at the apex of the cells, but occasionally in the middle or the base of the cells (Figure 5.5 A). In the distal ileum, most nuclei in vacuolated cells were at the base of the cells. The presence of vacuoles in non-infected piglets fed lactoferrin contrasts with the lack of vacuoles from the mid-jejunum to the mid-ileum in control piglets fed milk formula alone (Figures 5.4 B and 5.5 B).

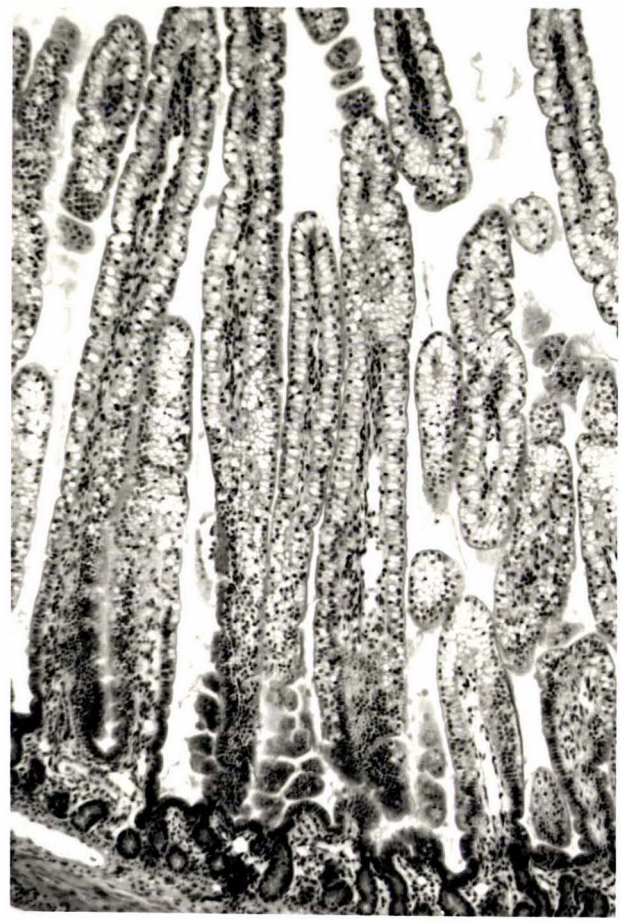
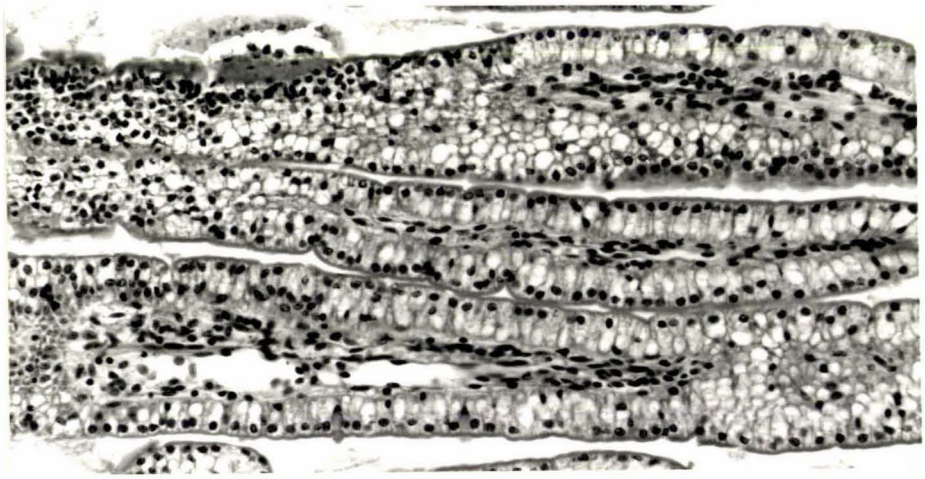


Figure 5.3 Photomicrograph of a section of the distal jejunum of a non-infected, 5-day-old piglet fed milk formula containing 5% lactoferrin showing the presence of vacuoles on the villi but not in the crypts. (HE, x110)

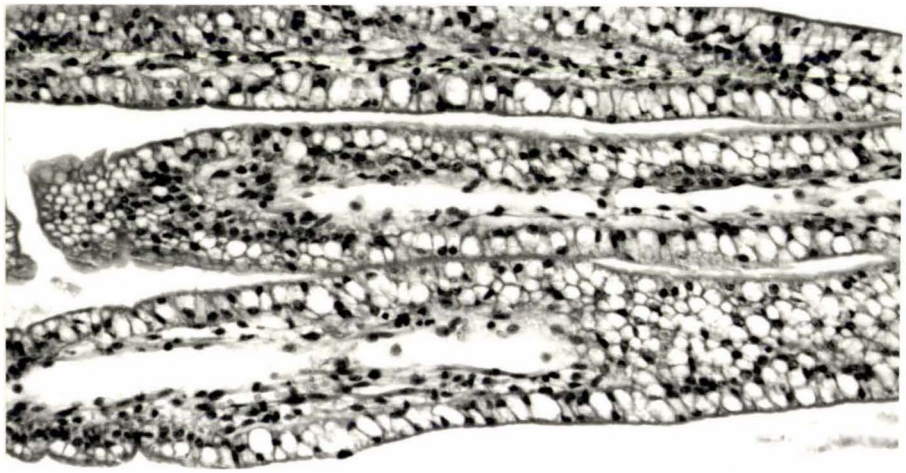
A



B

Figure 5.4 Photomicrographs of sections of the distal jejunum of non-infected, 5-day-old piglets fed milk formula containing 5% lactoferrin (A) and fed milk formula alone (B). A: Vacuolated cells were present on the whole villi and nuclei were in the apex of the cells B: No vacuoles on the villi. (HE, x220)

A



B

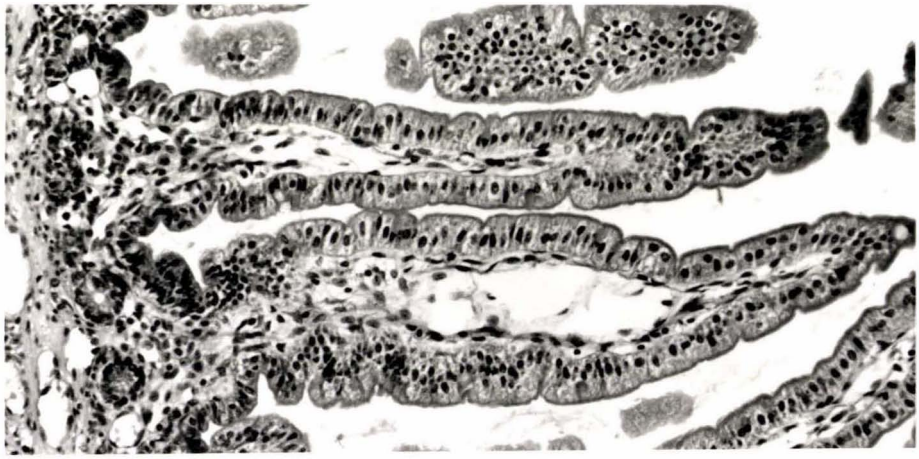


Figure 5.5 Photomicrographs of sections of the mid-ileum of non-infected, 5-day-old piglets fed milk formula containing 5% lactoferrin (A) and fed milk formula alone (B). A: Vacuoles were large and empty, the position of the nuclei was variable. B: No vacuoles on the villi and the nuclei were basal. (HE, x220)

Table 5.11. Villus height and crypt depth in the jejunum and ileum in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA with individual comparisons

		Jejunum			Ileum		
		Proximal	Mid-	Distal	Proximal	Mid-	Distal
Villus height (µm)							
Treatment	n			Mean ± SE			
Infected	4	862 ± 99	721 ± 61	836 ± 70	826 ± 97	728 ± 42	617 ± 61
Control	4	1063 ± 75	986 ± 100	1041 ± 62	849 ± 43	767 ± 47	678 ± 53
Control + Lf	4	907 ± 86	825 ± 105	1133 ± 18	1077 ± 121	973 ± 124	671 ± 71
Infected + Lf	4	953 ± 393	769 ± 80	914 ± 147	831 ± 65	879 ± 113	596 ± 54
Infected + BMF	4	814 ± 109	773 ± 111	917 ± 103	839 ± 56	759 ± 38	701 ± 47
				Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	20.9 NS	98.9 *	34.2 NS	3.8 NS	5.7 NS	3.6 NS
Treatment	4	40.7 NS	42.0 NS	55.4 NS	46.7 NS	41.9 NS	7.8 NS
Error	12	41.1	18.8	33.0	32.2	31.9	15.9
Crypt depth (µm)							
Treatment	n			Mean ± SE			
Infected	4	164 ± 27	189 ± 19	138 ± 15	130 ± 21	129 ± 16	138 ± 13
Control	4	94 ± 6	93 ± 9	83 ± 2	77 ± 2	71 ± 3	74 ± 1
Control + Lf	4	107 ± 5	100 ± 5	100 ± 6	82 ± 5	73 ± 4	83 ± 5
Infected + Lf	4	217 ± 33	172 ± 7	157 ± 11	143 ± 5	148 ± 8	145 ± 7
Infected + BMF	4	156 ± 9	143 ± 10	128 ± 11	110 ± 8	121 ± 17	141 ± 11
				Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	0.2 NS	1.4 *	0.3 NS	0.6 NS	0.5 NS	0.5 NS
Treatment	4	6.7 *	7.2 ***	3.5 **	3.3 **	4.8 **	4.8 ***
Error	12	1.4	0.2	0.4	0.4	0.5	0.2
				Comparison of individual treatments			
Infected vs Control		*	***	**	**	**	***
Control + Lf vs Control		NS	NS	NS	NS	NS	NS
Infected + Lf vs Infected		NS	NS	NS	NS	NS	NS
Infected + BMF vs Infected		NS	**	NS	NS	NS	NS

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 5.12. Ratio of villus height to crypt depth in the mucosa of the jejunum and ileum in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA with individual comparisons

		Jejunum			Ileum		
		Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	n	Ratio of villus height/crypt depth (Mean ± SE)					
Infected	4	5.4 ± 0.5	3.9 ± 0.4	6.1 ± 0.2	6.8 ± 1.2	6.0 ± 0.9	4.5 ± 0.3
Control	4	11.4 ± 1.0	10.6 ± 0.1	12.6 ± 1.0	11.1 ± 0.9	10.8 ± 0.7	9.1 ± 0.8
Control + Lf	4	8.5 ± 0.9	8.2 ± 0.8	11.5 ± 0.8	13.1 ± 1.3	13.4 ± 1.5	8.0 ± 0.5
Infected + Lf	4	4.0 ± 1.1	4.5 ± 0.4	6.0 ± 1.2	5.9 ± 0.7	5.9 ± 0.7	4.1 ± 0.2
Infected + BMF	4	5.2 ± 0.6	5.3 ± 0.6	7.2 ± 0.4	7.7 ± 0.6	6.6 ± 1.0	5.1 ± 0.7
		Analyses of Variance					
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	0.9 NS	2.8 *	1.5 NS	3.0 NS	0.9 NS	1.7 NS
Treatment	4	30.8 **	32.1 ***	39.1 ***	37.2 ***	45.7 ***	20.5 ***
Error	12	3.1	0.7	3.0	3.8	4.7	0.9
		Comparison of individual treatments					
Infected vs Control		***	***	***	**	**	***
Control + Lf vs Control		*	**	NS	NS	NS	NS
Infected + Lf vs Infected		NS	NS	NS	NS	NS	NS
Infected + BMF vs Infected		NS	*	NS	NS	NS	NS

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 5.13. Number of proliferating cells per crypt in the small intestine in control and *Y.enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA with individual comparisons

		Duodenum	Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	n	Cells/crypt (Mean ± SE)						
Infected	4	11.9 ± 1.8	15.3 ± 2.1	19.5 ± 1.5	16.3 ± 2.3	13.0 ± 1.2	12.6 ± 1.0	16.0 ± 0.9
Control	3	7.2 ± 0.9	7.8 ± 0.2	7.9 ± 0.4	7.5 ± 0.2	6.4 ± 0.3	8.0 ± 1.0	8.3 ± 0.7
Control + Lf	4	7.6 ± 0.4	9.6 ± 0.9	9.1 ± 1.0	9.1 ± 1.2	7.9 ± 0.5	8.4 ± 1.1	10.1 ± 0.9
Infected + Lf	4	16.7 ± 1.7	21.0 ± 2.4	19.4 ± 1.1	17.9 ± 3.5	20.0 ± 1.4	17.5 ± 1.8	13.5 ± 2.6
Infected + BMF	4	10.5 ± 1.6	13.5 ± 1.7	11.6 ± 0.7	13.8 ± 0.3	10.5 ± 1.7	10.7 ± 1.0	13.9 ± 3.2
		Analyses of Variance						
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	8.05 NS	12.43 NS	1.62 NS	12.76 NS	1.33 NS	10.00 NS	27.05 NS
Treatment	4	56.43 **	94.37 **	117.25 ***	69.58 *	107.30 ***	55.00 ***	40.23 NS^{+}
Error	11	8.07	11.98	5.05	17.88	6.52	4.85	12.95
		Comparison of individual treatment						
Control vs Infected		*	*	***	*	**	*	**
Control + Lf vs Control		NS	NS	NS	NS	NS	NS	NS
Infected + Lf vs Infected		*	*	NS	NS	**	**	NS
Infected + BMF vs Infected		NS	NS	NS	NS	NS	NS	NS

NS $p > 0.05$; NS^{+} $0.1 > p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 5.14. Number of proliferating cells per crypt area in the small intestine in control and *Y.enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA with individual comparisons

		Duodenum	Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	n	Cells/0.01 mm² crypt area (Mean ± SE)						
Infected	4	32.2 ± 4.1	39.0 ± 4.2	35.9 ± 3.0	40.1 ± 1.3	42.6 ± 1.9	42.7 ± 1.9	46.0 ± 4.2
Control	3	24.1 ± 0.6	26.7 ± 2.0	29.1 ± 3.5	30.8 ± 3.9	30.1 ± 1.0	37.0 ± 3.9	32.3 ± 3.7
Control + Lf	4	30.0 ± 1.4	31.4 ± 1.1	32.7 ± 0.7	31.8 ± 4.0	33.9 ± 2.6	35.0 ± 5.8	36.3 ± 4.2
Infected + Lf	4	29.1 ± 0.7	37.8 ± 1.7	40.8 ± 1.5	38.2 ± 2.1	41.2 ± 0.9	40.5 ± 3.3	34.7 ± 4.8
Infected + BMF	4	24.1 ± 0.5	28.6 ± 1.7	28.9 ± 3.0	37.6 ± 1.5	35.0 ± 3.9	33.4 ± 3.0	32.0 ± 4.6
		Analyses of Variance						
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	7.50 NS	35.55 NS	3.12 NS	30.23 NS	18.19 NS	105.70 NS	105.00 NS
Treatment	4	48.66 NS⁺	114.31 **	96.90 *	59.33 NS	99.57 *	60.12 NS	131.77 NS
Error	11	18.90	19.43	28.01	26.16	24.56	41.83	64.89
		Comparison of individual treatments						
Control vs Infected		*	**	NS	*	**	NS	*
Control + Lf vs Control		NS	NS	NS	NS	NS	NS	NS
Infected + Lf vs Infected		NS	NS	NS	NS	NS	NS	NS⁺
Infected + BMF vs Infected		*	**	NS⁺	NS	NS⁺	NS⁺	*

NS $p > 0.05$; NS⁺ $0.1 > p > 0.05$; * $p < 0.05$; ** $p < 0.01$ MS = Mean Square

5.3.6 Morphometric Measurement of the Small Intestine

5.3.6.1 Villus height and crypt depth

Villus height, crypt depth and the ratio of villus height to crypt depth are shown in Tables 5.11 and 5.12. Villus height did not significantly differ between the 5 treatments, whereas differences in crypt depth between the 5 groups were present in all segments of the jejunum and ileum (p values from < 0.05 to < 0.001). Significantly deeper crypts resulted from the *Y. enterocolitica* infection. Lactoferrin did not significantly change the crypt depth in either the control or infected animals, whereas infected piglets fed oral BMF had shallower crypts in the mid-jejunum compared with those in infected pigs fed milk formula alone ($p < 0.01$). In other segments, except the distal ileum, the group mean for the crypt depth was also lower in the BMF-fed group, although not reaching statistical significance.

There was a significant difference in the ratio of villus height to crypt depth in all segments of the jejunum and ileum ($p < 0.01$ or < 0.001, Table 5.12). Infected piglets had a lower ratio of villus height to crypt depth than controls in all segments ($p < 0.01$ or < 0.001). Comparison of the means suggested there may be a trend of increasing crypt depth with lactoferrin feeding both in control and infected piglets but this was not significant. The significance of the villus height/ crypt depth ratio probably results from the greater mean crypt depth and the lower mean villus height. Lactoferrin reduced the ratio of villus height to crypt depth in control piglets in the duodenum ($p < 0.05$) and the proximal jejunum ($p < 0.01$) compared with non-lactoferrin-fed animals, again probably because of the greater mean crypt depth. BMF feeding significantly increased the ratio of villus height to crypt depth in the mid-jejunum in infected piglets compared with infected animals fed milk formula alone ($p < 0.05$) and a similar trend occurred in the rest of the small intestine but was not significant.

5.3.6.2 Crypt cell proliferation

In infected piglets, the number of proliferating cells was significantly greater using two methods of expressing the data: per crypt, in all segments of the small intestine ($p < 0.05$ to < 0.001, Table 5.13) and per crypt area, in the duodenum, the proximal and distal jejunum and the proximal and distal ileum ($p < 0.01$, Table 5.14). Oral lactoferrin caused infected piglets to have more proliferating crypt cells in the duodenum, proximal

Table 5.15. Goblet cell populations in the villi staining positive for Alcian blue and periodic acid-Schiff in the small intestine in *Y. enterocolitica*-infected and control piglets fed milk formula with or without lactoferrin (Lf) and infected piglets with added bovine milk fraction (BMF) and ANOVA with individual comparisons. Data are expressed as number of cells per millimeter of villus length.

		Duodenum	Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	n	Cells/mm villus length (Mean ± SE)						
Infected	4	35.3 ± 7.1	24.2 ± 4.1	22.2 ± 1.2	23.6 ± 2.7	24.0 ± 5.2	30.9 ± 3.8	35.3 ± 7.7
Control	4	40.5 ± 1.1	25.3 ± 3.0	23.8 ± 2.5	19.6 ± 2.3	18.3 ± 5.1	16.1 ± 7.2	13.3 ± 5.6
Control + Lf	4	41.6 ± 4.3	25.0 ± 2.3	26.4 ± 4.2	19.4 ± 3.7	17.5 ± 7.2	15.6 ± 7.3	27.4 ± 10.9
Infected + Lf	4	29.1 ± 3.8	21.7 ± 0.5	23.9 ± 1.0	25.8 ± 3.3	23.0 ± 1.6	24.7 ± 6.1	27.0 ± 9.7
Infected + BMF	4	31.0 ± 1.9	24.1 ± 2.6	26.1 ± 2.7	24.2 ± 1.3	20.1 ± 6.2	21.1 ± 7.5	30.3 ± 4.4
		Analyses of Variance						
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	184.60 *	70.71 NS	38.28 NS	36.61 NS	215.54 NS	230.03 NS	526.36 NS
Treatment	4	123.81 NS	7.96 NS	12.35 NS	33.29 NS	32.62 NS	163.04 NS	266.45 NS
Error	12	42.27	20.47	23.84	29.42	90.51	155.41	189.54
		Comparison of individual treatments						
Infected vs Control		NS	NS	NS	NS	NS	NS	*
Control + Lf vs Control		NS	NS	NS	NS	NS	NS	NS
Infected + Lf vs Infected		NS	NS	NS	NS	NS	NS	NS
Infected + BMF vs Infected		NS	NS	NS	NS	NS	NS	NS

NS $p > 0.05$; * $p < 0.05$ MS = Mean Square

Table 5.16. Goblet cell populations in the intestinal crypts staining positive for Alcian blue and periodic acid-Schiff in the small intestine in *Y. enterocolitica*-infected and control piglets fed milk formula with or without lactoferrin (Lf) and infected piglets with added bovine milk fraction (BMF) and ANOVA with individual comparisons. Data are expressed as number of cells per 0.01 mm² of intestinal crypt area.

		Duodenum	Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	n	Cells/0.01 mm² crypt area (Mean ± SE)						
Infected	4	23.7 ± 1.0	21.7 ± 2.0	18.6 ± 1.6	22.5 ± 2.7	21.1 ± 2.4	23.2 ± 2.8	26.0 ± 2.5
Control	4	28.3 ± 3.4	28.1 ± 2.9	25.5 ± 3.9	24.7 ± 1.9	26.6 ± 3.7	26.9 ± 1.4	28.9 ± 4.3
Control + Lf	4	22.5 ± 1.0	21.7 ± 3.2	24.0 ± 1.4	23.0 ± 3.4	27.2 ± 1.2	24.6 ± 1.3	31.7 ± 2.6
Infected + Lf	4	20.6 ± 2.0	19.6 ± 1.4	19.4 ± 2.3	18.9 ± 2.4	22.3 ± 3.3	21.3 ± 1.5	30.2 ± 1.8
Infected + BMF	4	17.9 ± 1.8	17.0 ± 1.7	18.5 ± 1.2	19.1 ± 0.7	18.4 ± 2.2	21.1 ± 0.9	22.8 ± 1.7
		Analyses of Varianc						
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	21.17 NS	17.87 NS	52.13 *	44.10 NS	57.99 NS	19.06 NS	79.87 *
Treatment	4	59.37 *	67.43 NS	43.81 *	26.61 NS	55.16 NS	23.17 NS	51.02 NS
Error	12	15.63	23.01	13.31	17.99	21.81	9.65	17.35
		Comparison of individual treatments						
Infected vs Control		NS	NS	*	NS	NS	NS	NS
Control + Lf vs Control		NS	NS	NS	NS	NS	NS	NS
Infected + Lf vs Infected		*	NS	NS	NS	NS	NS	NS
Infected + BMF vs Infected		*	NS	NS	NS	NS	NS	NS

NS $p > 0.05$; * $p < 0.05$ MS = Mean Square

Table 5.17. Protein concentration and total protein content of the mucosa of seven segments of the small intestine in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA

		Duodenum	Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Protein concentration (mg/g tissue)								
Treatment	n				Mean ± SE			
Infected	4	113 ± 5	114 ± 3	108 ± 9	111 ± 6	101 ± 8	106 ± 7	101 ± 2
Control	4	115 ± 5	121 ± 5	118 ± 8	113 ± 4	96 ± 7	110 ± 1	104 ± 11
Control + Lf	4	116 ± 4	119 ± 5	118 ± 6	112 ± 2	106 ± 6	99 ± 9	102 ± 4
Infected + Lf	4	102 ± 5	108 ± 6	115 ± 5	102 ± 3	102 ± 5	99 ± 6	106 ± 5
Infected + BMF	4	106 ± 8	108 ± 2	118 ± 4	107 ± 9	98 ± 7	101 ± 4	99 ± 6
					Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	10.1 NS	86.8 NS	63.1 NS	112.1 NS	372.1 NS	394.0 *	206.7 NS
Treatment	4	155.4 NS	158.0 NS	73.6 NS	77.2 NS	56.2 NS	99.3 NS	28.4 NS
Error	12	155.6	79.3	202.8	132.5	149.8	96.2	153.2
Total protein content (g)								
Treatment	n				Mean ± SE			
Infected	4	125 ± 11	745 ± 87	704 ± 62	792 ± 66	657 ± 54	608 ± 45	523 ± 21
Control	4	128 ± 9	918 ± 97	834 ± 71	783 ± 57	647 ± 128	679 ± 99	548 ± 129
Control + Lf	4	158 ± 30	868 ± 135	807 ± 92	933 ± 148	806 ± 148	697 ± 113	467 ± 92
Infected + Lf	4	137 ± 15	824 ± 135	876 ± 134	841 ± 106	738 ± 72	651 ± 36	511 ± 80
Infected + BMF	4	125 ± 17	714 ± 75	771 ± 75	722 ± 49	628 ± 54	602 ± 91	516 ± 36
					Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	2056 NS	97242 NS	52813 NS	40315 NS	97608 *	47038 NS	19678 NS
Treatment	4	800 NS	28493 NS	16976 NS	24683 NS	22506 NS	6983 NS	3468 NS
Error	12	1105	34536	27922	33169	25069	22689	28552

NS $p > 0.05$; * $p < 0.05$ MS = Mean Square

Table 5.18. Ratios of tissue to DNA and of protein to DNA and total DNA content of the mucosa of the small intestine in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA

		Duodenum	Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Ratio of tissue weight to DNA								
Treatment	n				Mean ± SE			
Infected	4	167 ± 21	158 ± 13	186 ± 10	178 ± 16	194 ± 13	177 ± 20	141 ± 9
Control	4	221 ± 37	167 ± 24	176 ± 27	186 ± 18	195 ± 18	184 ± 13	150 ± 19
Control + Lf	4	179 ± 20	173 ± 24	176 ± 17	194 ± 27	206 ± 31	197 ± 13	146 ± 14
Infected + Lf	4	219 ± 30	188 ± 7	178 ± 7	192 ± 10	194 ± 30	175 ± 10	154 ± 11
Infected + BMF	4	167 ± 17	162 ± 9	166 ± 8	175 ± 19	176 ± 22	176 ± 20	145 ± 21
					Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	7607 *	3154 *	3174 **	2565 NS	5027 NS	1628 NS	2461 *
Treatment	4	2911 NS	542 NS	204 NS	281 NS	449 NS	337 NS	97 NS
Error	12	1505	649	452	1152	1547	852	565
Ratio of protein to DNA								
Treatment	n				Mean ± SE			
Infected	4	19.0 ± 2.6	18.1 ± 1.6	20.2 ± 2.0	19.6 ± 0.9	19.3 ± 0.9	18.9 ± 2.8	14.2 ± 1.0
Control	4	25.5 ± 4.6	20.0 ± 2.2	20.4 ± 2.5	21.2 ± 2.6	18.9 ± 2.3	20.3 ± 1.5	15.5 ± 2.3
Control + Lf	4	20.9 ± 2.3	20.8 ± 3.2	20.8 ± 2.7	21.6 ± 2.7	21.7 ± 3.4	19.6 ± 2.3	15.0 ± 1.9
Infected + Lf	4	22.8 ± 4.1	20.2 ± 1.2	20.3 ± 0.3	19.7 ± 1.0	19.4 ± 2.2	17.1 ± 0.8	16.3 ± 1.7
Infected + BMF	4	17.3 ± 0.7	17.4 ± 0.8	19.5 ± 0.4	18.2 ± 0.5	16.8 ± 1.0	17.6 ± 2.0	14.0 ± 1.0
					Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	88.5 NS	53.3 **	33.9 *	32.8 *	52.2 *	37.6 NS	35.4 **
Treatment	4	41.2 NS	8.5 NS	0.9 NS	7.4 NS	12.5 NS	7.0 NS	3.8 NS
Error	12	29.4	6.5	9.4	7.8	11.0	10.8	5.2
Total DNA content (mg)								
Treatment	n				Mean ± SE			
Infected	4	6.9 ± 1.1	42.3 ± 6.9	35.6 ± 3.5	40.9 ± 4.9	34.1 ± 2.7	35.3 ± 6.9	37.3 ± 2.5
Control	4	5.5 ± 0.8	47.2 ± 5.4	42.6 ± 5.5	38.9 ± 6.0	34.1 ± 4.9	33.7 ± 4.9	34.5 ± 6.4
Control + Lf	4	7.7 ± 1.2	42.6 ± 5.1	39.9 ± 4.6	43.6 ± 5.9	37.3 ± 4.7	35.6 ± 3.6	30.8 ± 3.7
Infected + Lf	4	6.3 ± 0.4	40.6 ± 6.3	42.9 ± 6.2	42.7 ± 4.1	38.9 ± 4.1	38.0 ± 1.3	31.0 ± 3.0
Infected + BMF	4	7.2 ± 0.8	40.9 ± 3.5	39.6 ± 4.2	39.6 ± 2.2	37.4 ± 2.4	34.1 ± 4.0	37.5 ± 3.9
					Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	5.45 NS	349.42 *	205.50 NS	171.30 NS	190.45 **	148.77 NS	23.15 NS
Treatment	4	3.02 NS	28.16 NS	34.64 NS	15.90 NS	19.06 NS	11.60 NS	42.78 NS
Error	12	2.85	68.38	67.62	74.14	28.33	65.53	79.71

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$ MS = Mean Square

Table 5.19. Ratio of RNA to DNA in the mucosa of the small intestine in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA with individual comparisons

		Duodenum	Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	n	Ratio of RNA/DNA (Mean ± SE)						
Infected	4	0.95 ± 0.15	0.98 ± 0.07	1.03 ± 0.18	0.94 ± 0.10	1.05 ± 0.08	1.03 ± 0.18	0.75 ± 0.08
Control	4	0.81 ± 0.22	0.80 ± 0.16	0.83 ± 0.15	0.89 ± 0.17	0.80 ± 0.15	0.85 ± 0.15	0.68 ± 0.13
Control + Lf	4	0.46 ± 0.10	0.61 ± 0.15	0.47 ± 0.08	0.31 ± 0.04	0.32 ± 0.05	0.41 ± 0.09	0.64 ± 0.12
Infected + Lf	4	0.91 ± 0.13	0.71 ± 0.11	0.86 ± 0.11	0.62 ± 0.18	0.70 ± 0.19	0.60 ± 0.15	0.70 ± 0.12
Infected + BMF	4	0.83 ± 0.12	0.84 ± 0.11	0.98 ± 0.15	0.87 ± 0.13	0.88 ± 0.13	0.92 ± 0.19	0.76 ± 0.15
		Analyses of Variance						
Sorce	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	0.2350 *	0.2001 **	0.1311 NS	0.1087 NS	0.1382 NS	0.2846 *	0.1410 *
Treatment	4	0.1518 NS⁺	0.0770 NS⁺	0.1933 NS⁺	0.2774 *	0.2970 **	0.2542 *	0.0106 NS
Error	12	0.0551	0.0284	0.0634	0.0596	0.0479	0.0488	0.0375
		Comparison of individual treatments						
Control vs Infected		NS	NS	NS	NS	NS	NS	NS
Control + Lf vs Control		NS⁺	NS	NS⁺	**	**	*	NS
Infected + Lf vs Infected		NS	*	NS	NS⁺	*	*	NS
Infected + BMF vs Infected		NS	NS	NS	NS	NS	NS	NS

NS $p > 0.05$; NS⁺ $0.1 > p > 0.05$; * $p < 0.05$; ** $p < 0.01$ MS = Mean Square

jejunum, and the proximal and mid-ileum ($p < 0.05$ or < 0.01), whereas supplementation with BMF had no effect (Table 5.14). When the proliferating cells were expressed per 0.01 mm^2 crypt area, the BMF-fed piglets showed a significant reduction in proliferating cells in the duodenum, the proximal jejunum and the distal ileum ($p < 0.05$ to < 0.01). In other segments, the difference in means was close to significant ($0.05 < p < 0.1$) except in the distal jejunum (Table 5.14). Feeding lactoferrin to non-infected piglets did not significantly affect crypt cell proliferation regardless of the method of expression of the data, but the group means for proliferating crypt cells in the lactoferrin-fed group were greater throughout the small intestine except in the mid-ileum (Tables 5.13 and 5.14).

5.3.6.3 Goblet cells

There was a significantly greater population of goblet cells on the villi in the distal ileum in *Y. enterocolitica*-infected piglets than in controls ($p < 0.05$) (Table 5.15), whereas the crypt goblet cells were significantly fewer in infected piglets than in controls in the mid-jejunum ($p < 0.05$). Both lactoferrin and BMF feeding reduced the number of crypt goblet cells in the duodenum of infected piglets ($p < 0.05$) (Table 5.16). Lactoferrin feeding did not significantly change the number of goblet cells either on the villi or in the crypts of non-infected piglets.

5.3.7 Biochemistry of the Small Intestine

5.3.7.1 Mucosal protein, DNA and RNA

Mucosal protein and DNA content of all segments of the small intestine showed no differences between the 5 groups, regardless of the method of expression of the data (Tables 5.17 and 5.18).

The ratio of RNA to DNA is shown in Table 5.19. This was not affected in any segments of the small intestine by *Y. enterocolitica* infection. Oral supplementation with lactoferrin significantly reduced the ratio of RNA to DNA from the distal jejunum to the mid-ileum in non-infected piglets and in the proximal jejunum and the proximal and mid-ileum in infected piglets ($p < 0.05$). In other segments of the small intestine, the mean RNA:DNA ratios were all lower in lactoferrin-supplemented groups, some p values being between 0.05 and 0.1 (Table 5.19). Oral BMF did not affect the ratio of

Table 5.20. Lactase activity per gram mucosal protein in the small intestine in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA with individual comparisons

		Duodenum	Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	n	Lactase activity, µmol/g protein/min, (Mean ± SE)						
Infected	4	46 ± 5	79 ± 17	104 ± 6	128 ± 44	149 ± 26	107 ± 24	24 ± 9
Control	4	100 ± 21	202 ± 19	199 ± 11	210 ± 9	160 ± 27	129 ± 26	28 ± 5
Control + Lf	4	89 ± 13	173 ± 20	180 ± 38	220 ± 24	197 ± 11	232 ± 41	51 ± 16
Infected + Lf	4	20 ± 11	44 ± 12	112 ± 17	128 ± 14	134 ± 19	87 ± 23	13 ± 6
Infected + BMF	4	72 ± 11	126 ± 19	171 ± 9	198 ± 14	149 ± 24	140 ± 23	23 ± 8
		Analyses of Variance						
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	711 NS	1081 NS	2988 NS	4325 NS	2620 NS	3311 NS	607 NS
Treatment	4	4197 **	16874 ***	7299 **	8154 *	2265 NS	12414 *	774 NS⁺
Error	12	693	1305	1197	1916	1783	3122	316
		Comparison of individual treatments						
Control vs Infected		*	***	**	*	NS	NS	NS
Control + Lf vs Control		NS	NS	NS	NS	NS	*	NS⁺
Infected + Lf vs Infected		NS	NS	NS	NS	NS	NS	NS
Infected + BMF vs Infected		NS	NS⁺	*	*	NS	NS	NS

NS $p > 0.05$; NS⁺ $0.1 > p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 5.21. Lactase activity per gram wet mucosa of the small intestine in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA with individual comparisons

		Duodenum	Jejunum			Ileum		
			proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	n	Lactase activity, µmol/g tissue/min, (Mean ± SE)						
Infected	4	5.2 ± 0.4	9.0 ± 1.9	11.3 ± 1.5	13.9 ± 5.0	14.4 ± 1.5	11.0 ± 2.0	2.4 ± 0.9
Control	4	11.8 ± 2.7	24.2 ± 1.7	23.2 ± 0.9	23.8 ± 1.8	15.8 ± 3.3	14.2 ± 2.7	3.0 ± 0.7
Control + Lf	4	10.3 ± 1.4	20.6 ± 2.3	21.2 ± 4.4	24.7 ± 3.0	20.7 ± 0.8	22.1 ± 2.7	5.3 ± 1.9
Infected + Lf	4	2.2 ± 1.3	4.9 ± 1.5	12.9 ± 2.0	13.2 ± 1.7	13.5 ± 1.7	8.8 ± 2.4	1.5 ± 0.8
Infected + BMF	4	7.7 ± 1.4	13.5 ± 1.9	20.3 ± 1.8	21.2 ± 2.6	14.9 ± 3.3	14.0 ± 1.9	2.4 ± 0.9
		Analyses of Variance						
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	10.65 NS	6.00 NS	38.92 NS	83.35 NS	26.45 NS	33.70 NS	8.00 NS
Treatment	4	59.56 **	254.78 ***	113.53 **	120.10 *	31.60 NS	101.84 *	8.42 NS
Error	12	10.65	16.38	20.13	26.38	20.66	19.78	4.10
		Comparison of individual treatments						
Control vs Infected		*	***	**	*	NS	NS	NS
Control + Lf vs Control		NS	NS	NS	NS	NS	*	NS
Infected + Lf vs Infected		NS	NS	NS	NS	NS	NS	NS
Infected + BMF vs Infected		NS	NS	*	NS*	NS	NS	NS

NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 5.22. Total lactase activity in the mucosa of seven segments of the small intestine in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA with individual comparisons

		Duodenum	Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Treatment	n	Total lactase activity, µmol (Mean ± SE)						
Infected	4	5.8 ± 0.7	59 ± 17	74 ± 10	108 ± 42	96 ± 14	66 ± 17	12.7 ± 4.9
Control	4	12.8 ± 2.7	185 ± 26	164 ± 7	163 ± 6	111 ± 32	95 ± 32	15.9 ± 4.7
Control + Lf	4	13.9 ± 3.5	146 ± 23	139 ± 20	195 ± 13	156 ± 24	148 ± 8	26.9 ± 12.9
Infected + Lf	4	3.1 ± 1.8	41 ± 13	104 ± 29	109 ± 23	101 ± 22	58 ± 16	7.6 ± 4.7
Infected + BMF	4	9.1 ± 1.7	93 ± 22	133 ± 18	144 ± 17	90 ± 7	89 ± 27	11.3 ± 3.6
		Analyses of Variance						
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	4.33 NS	2218 NS	2569 NS	418 NS	1631 NS	896 NS	268.7 NS
Treatment	4	84.00 *	14380 **	4834 *	5375 NS	2798 NS	4995 NS	217.2 NS
Error	12	25.01	1546	1070	2661	1955	2159	179.3
		Comparison of individual treatments						
Control vs Infected		NS+	***	**	NS	NS	NS	NS
Control + Lf vs Control		NS	NS	NS	NS	NS	NS	NS
Infected + Lf vs Infected		NS	NS	NS	NS	NS	NS	NS
Infected + BMF vs Infected		NS	NS	*	NS	NS	NS	NS

NS $p > 0.05$; NS+ $0.1 > p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ MS = Mean Square

Table 5.23. Maltase activity in the small intestine in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA

		Duodenum	Jejunum			Ileum		
			Proximal	Mid-	Distal	Proximal	Mid-	Distal
Maltase (µmol/g protein/min)								
Treatment	n				Mean ± SE			
Infected	4	8.4 ± 3.1	35.9 ± 4.7	57.1 ± 9.8	40.0 ± 12.7	19.2 ± 5.0	7.5 ± 2.7	6.1 ± 1.6
Control	4	22.0 ± 20.2	37.5 ± 21.9	44.0 ± 23.1	24.8 ± 21.4	20.8 ± 18.4	10.2 ± 8.8	11.8 ± 11.0
Control + Lf	4	1.1 ± 0.9	47.2 ± 9.4	66.9 ± 16.4	25.4 ± 2.8	10.4 ± 4.0	7.2 ± 1.7	6.4 ± 2.7
Infected + Lf	4	14.5 ± 3.7	35.2 ± 13.2	72.1 ± 17.7	62.2 ± 13.7	26.0 ± 6.7	11.1 ± 2.7	5.0 ± 1.5
Infected + BMF	4	17.3 ± 8.2	54.6 ± 4.1	80.0 ± 16.0	32.6 ± 9.8	10.9 ± 6.2	7.6 ± 2.9	5.9 ± 1.7
					Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	385.38 NS	610.45 NS	837.32 NS	835.30 NS	419.14 NS	64.24 NS	63.26 NS
Treatment	4	263.75 NS	289.23 NS	779.23 NS	946.78 NS	179.40 NS	13.32 NS	29.10 NS
Error	12	402.76	626.20	1258.30	701.25	356.66	87.55	120.94
Maltase (µmol/g tissue/min)								
Treatment	n				Mean ± SE			
Infected	4	1.0 ± 0.4	4.1 ± 0.5	6.0 ± 0.9	4.2 ± 1.2	2.0 ± 0.7	0.8 ± 0.3	0.6 ± 0.2
Control	4	2.7 ± 2.5	4.7 ± 2.8	5.8 ± 3.4	2.9 ± 2.6	2.0 ± 1.7	1.1 ± 1.0	1.5 ± 1.4
Control + Lf	4	0.1 ± 0.1	5.6 ± 1.0	8.0 ± 2.0	2.8 ± 0.3	1.2 ± 0.5	0.7 ± 0.2	0.7 ± 0.3
Infected + Lf	4	1.5 ± 0.4	3.8 ± 1.5	8.2 ± 2.0	6.5 ± 1.6	2.7 ± 0.7	1.1 ± 0.3	0.5 ± 0.2
Infected + BMF	4	1.8 ± 0.7	5.8 ± 0.3	9.4 ± 1.7	3.3 ± 0.7	1.0 ± 0.6	0.8 ± 0.3	0.6 ± 0.2
					Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	5.45 NS	8.34 NS	12.85 NS	12.93 NS	4.61 NS	0.85 NS	1.07 NS
Treatment	4	3.75 NS	3.21 NS	9.56 NS	9.30 NS	1.84 NS	0.14 NS	0.62 NS
Error	12	5.92	9.43	20.05	8.03	3.40	1.03	1.86
Total maltase (µmol)								
Treatment	n				Mean ± SE			
Infected	4	1.1 ± 0.5	26.6 ± 4.5	38.5 ± 4.3	32.2 ± 9.9	13.4 ± 4.8	4.8 ± 2.0	3.3 ± 1.0
Control	4	3.3 ± 3.1	33.8 ± 19.5	38.7 ± 22.2	17.7 ± 14.8	12.4 ± 10.6	7.3 ± 6.1	8.8 ± 8.3
Control + Lf	4	0.2 ± 0.1	37.4 ± 3.3	53.6 ± 15.3	22.7 ± 2.4	9.3 ± 4.1	5.2 ± 1.7	3.7 ± 1.9
Infected + Lf	4	2.0 ± 0.5	32.7 ± 13.1	66.1 ± 23.5	51.1 ± 10.9	19.0 ± 4.6	7.5 ± 2.0	2.8 ± 1.3
Infected + BMF	4	1.9 ± 0.8	39.7 ± 6.6	62.2 ± 12.7	24.7 ± 8.9	7.3 ± 4.1	5.2 ± 2.0	2.9 ± 0.7
					Analyses of Variance			
Source	DF	MS p	MS p	MS p	MS p	MS p	MS p	MS p
Litter	3	7.88 NS	276.61 NS	815.09 NS	293.50 NS	165.77 NS	30.12 NS	35.50 NS
Treatment	4	5.40 NS	100.76 NS	663.47 NS	683.72 NS	80.28 NS	6.79 NS	25.93 NS
Error	12	8.91	558.11	1251.75	447.00	149.86	44.75	66.68

NS $p > 0.05$ MS = Mean Square

Table 5.24. Sucrase activity in three segments of the jejunum in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) and in infected piglets with added bovine milk fraction (BMF) and ANOVA with individual comparisons

		Jejunum		
		Proximal	Mid-	Distal
Sucrase (µmol/g protein)				
Treatment	n		Mean ± SE	
Infected	4	3.9 ± 3.9	17.5 ± 3.7	7.3 ± 2.5
Control	4	21.4 ± 10.7	32.4 ± 13.9	10.2 ± 8.9
Control + Lf	4	34.8 ± 13.2	50.0 ± 9.5	18.1 ± 8.6
Infected + Lf	4	4.5 ± 3.1	23.9 ± 3.0	9.1 ± 2.0
Infected + BMF	4	8.3 ± 3.5	26.0 ± 6.1	8.3 ± 4.1
			Analyses of Variance	
Source	DF	MS p	MS p	MS p
Litter	3	279.85 NS	283.46 NS	66.84 NS
Treatment	4	711.56 NS	614.07 NS	75.37 NS
Error	12	256.80	271.46	164.51
Sucrase (µmol/g tissue)				
Treatment	n		Mean ± SE	
Infected	4	0.5 ± 0.5	1.9 ± 0.4	0.8 ± 0.3
Control	4	2.7 ± 1.4	4.1 ± 1.9	1.2 ± 1.1
Control + Lf	4	4.0 ± 1.5	5.7 ± 0.8	2.0 ± 1.0
Infected + Lf	4	0.5 ± 0.4	2.8 ± 0.5	0.9 ± 0.2
Infected + BMF	4	0.9 ± 0.4	3.1 ± 0.8	0.8 ± 0.3
			Analyses of Variance	
Source	DF	MS p	MS p	MS p
Litter	3	3.67 NS	3.70 NS	1.09 NS
Treatment	4	10.06 NS	8.55 NS	1.13 NS
Error	12	3.59	4.41	2.08
Total Sucrase (µmol)				
Treatment	n		Mean ± SE	
Infected	4	3.6 ± 3.6	12.2 ± 2.2	6.1 ± 2.2
Control	4	20.4 ± 9.9	29.5 ± 13.6	7.2 ± 6.2
Control + Lf	4	30.5 ± 14.8	39.8 ± 8.1	18.2 ± 11.2
Infected + Lf	4	4.3 ± 3.0	20.8 ± 3.6	7.2 ± 0.9
Infected + BMF	4	6.7 ± 2.9	21.4 ± 6.2	6.5 ± 3.4
			Analyses of Variance	
Source	DF	MS p	MS p	MS p
Litter	3	302.86 NS	176.56 NS	59.47 NS
Treatment	4	565.07 NS	434.38 NS	105.05 NS
Error	12	270.07	260.43	167.17

Analyses of Variance for logarithmically transformed data of sucrase activity (µmol/g protein)

Source	DF	MS	p	MS	p	MS	p
Litter	3	0.62	NS	0.19	NS	0.28	NS
Treatment	4	0.82	*	0.15	NS	0.18	NS
Error	12	0.25		0.16		0.33	
		Comparison of individual treatments					
Control vs Infected		NS+		NS		NS	
Control + Lf vs Control		NS		NS		NS	
Infected + Lf vs Infected		NS		NS		NS	
Infected + BMF vs Infected		NS		NS		NS	

NS $p > 0.05$; NS+ $0.1 > p > 0.05$; * $p < 0.05$ MS = Mean Square

Table 5.25. Na^+-K^+-ATPase activity in the mucosa of ten segments of the intestine in control and *Y. enterocolitica*-infected piglets fed milk formula with or without lactoferrin (Lf) or infected piglets with added bovine milk fraction (BMF) and ANOVA

	Treatment					ANOVA	
	Infected n=3	Control n=3	Control + Lf n=3	Infected + Lf n=3	Infected + BMF n=3	Litter DF=2	Treatment DF=4
	Mean ± SE						
ATPase activity (µmol/mg protein/h)							
Duodenum	5.71 ± 0.79	5.82 ± 1.02	6.58 ± 1.48	7.44 ± 1.35	4.35 ± 1.23	NS	NS
Proximal jejunum	6.17 ± 1.08	5.37 ± 1.14	6.41 ± 0.99	6.86 ± 3.54	6.97 ± 1.37	NS	NS
Mid-jejunum	5.63 ± 0.74	4.49 ± 1.40	3.77 ± 1.17	6.14 ± 1.50	7.10 ± 1.75	NS	NS
Distal jejunum	3.41 ± 1.28	3.20 ± 1.23	4.12 ± 0.66	5.61 ± 1.45	3.80 ± 1.80	NS	NS
Proximal ileum	5.83 ± 0.42	3.22 ± 1.74	3.42 ± 0.59	2.08 ± 1.12	3.67 ± 1.52	NS	NS
Mid-ileum	4.15 ± 0.10	3.40 ± 0.30	3.57 ± 0.62	3.46 ± 1.01	3.66 ± 0.97	NS	NS
Distal ileum	2.97 ± 0.31	2.54 ± 1.10	3.41 ± 1.09	3.10 ± 0.66	1.62 ± 0.52	NS	NS
Caecum	3.36 ± 0.13	2.38 ± 0.26	3.55 ± 0.49	3.27 ± 1.00	1.41 ± 0.73	NS	NS
Proximal colon	3.58 ± 0.26	2.67 ± 0.68	3.21 ± 0.71	1.97 ± 0.26	1.89 ± 0.90	NS	NS
Distal colon	3.09 ± 0.55	2.22 ± 0.48	4.93 ± 1.54	2.92 ± 0.47	2.31 ± 0.62	NS	NS
ATPase activity (µmol/g tissue/h)							
Duodenum	676 ± 93	651 ± 75	762 ± 144	800 ± 230	439 ± 139	NS	NS
Proximal jejunum	692 ± 99	642 ± 109	774 ± 90	783 ± 442	755 ± 139	NS	NS
Mid-jejunum	647 ± 135	508 ± 123	443 ± 143	716 ± 234	809 ± 193	NS	NS
Distal jejunum	354 ± 120	360 ± 144	460 ± 78	602 ± 176	441 ± 207	NS	NS
Proximal ileum	625 ± 80	331 ± 170	369 ± 53	227 ± 118	350 ± 144	NS	NS
Mid-ileum	467 ± 14	377 ± 39	362 ± 23	369 ± 129	362 ± 93	NS	NS
Distal ileum	303 ± 26	269 ± 104	350 ± 96	322 ± 58	162 ± 56	NS	NS
Caecum	331 ± 17	274 ± 23	330 ± 34	304 ± 101	132 ± 66	NS	NS
Proximal colon	370 ± 33	304 ± 80	339 ± 64	183 ± 18	197 ± 97	NS	NS
Distal colon	316 ± 59	241 ± 54	523 ± 134	266 ± 38	256 ± 70	NS	NS

NS $p > 0.05$

RNA to DNA in any region of the small intestine.

5.3.7.2 Enzyme activity

Lactase activity, expressed as per g protein, per g wet tissue and total lactase activity, is shown in Tables 5.20 - 5.22. Lactase activity, expressed both as per g tissue and per g protein, was significantly different between the 5 groups from the duodenum to the distal jejunum and in the mid-ileum (p from < 0.05 to 0.001). Infected animals had significantly lower lactase activity (per g protein and per g tissue) than controls from the duodenum to the distal jejunum (p from < 0.05 to < 0.001). Oral supplementation with lactoferrin significantly increased lactase activity (per g protein and per g wet tissue, but not total activity) in the mid-ileum in non-infected piglets ($p < 0.05$) and the group means for the proximal and distal ileum were also higher in these piglets, but not in infected piglets (Tables 5.20 and 5.21). Oral BMF significantly increased the lactase activity per g protein in the mid- and distal jejunum ($p < 0.05$) (Table 5.20) and lactase activity per g tissue and total activity in the mid-jejunum ($p < 0.05$) (Tables 5.21 and 5.22).

Maltase, sucrase and Na^+-K^+-ATPase activities are shown in Tables 5.23 - 5.25. Sucrase activity was undetectable in the duodenum and ileum of all piglets. There was no significant difference in maltase and Na^+-K^+-ATPase between treatments in all regions of the intestine regardless of the method of expression. When the data for sucrase activity (μmol/g protein) in the jejunum was logarithmically transformed, the ANOVA showed a significant difference between the 5 groups ($p < 0.05$), and the lower mean in the infected piglets was close to being significantly decreased below that in the controls ($p = 0.058$).

5.4 DISCUSSION

5.4.1 *Yersinia enterocolitica* Infection

These experiments were directed at examining the value of the bovine milk components lactoferrin and BMF in *Y. enterocolitica*-infected piglets. The infected piglets with which the two supplemented groups were compared have been included in the analysis in Chapters 3 and 4. In that analysis, the *Y. enterocolitica* infection has been identified as reducing the body weight gain, liver weight and plasma glucose and Cl^-

concentration. In the gastrointestinal tract, the caecal weight was increased but not the length or weight of other segments. Damage to the small intestine was demonstrated by severe injury to the mucosa, reduced villus height (proximal jejunum only), increased crypt cell proliferation, and diminished lactase and sucrase activities. More goblet cells were seen on the villi in the distal small intestine and fewer in the crypts in the proximal areas.

In the experiment described in this Chapter, the number of infected piglets was 4 compared with 8 in the group studied in Chapters 3 and 4. The different method of statistical analysis of the two groups of data and the different variances in the data have led to some parameters being statistically different from control values in one analysis but not in the other. In general, however this was not the case. Changes not demonstrable in the present infected group were in body weight gain, total muscle weight of the small intestine and in maltase activity. On the other hand, the muscle weight in some segments of the small intestine and plasma Na^+ concentration were statistically different in these piglets, whereas the difference in the same parameters did not reach significance in the larger group of piglets (Chapters 3 and 4, 3.3.3.2 and 4.3.1). The data reported here have demonstrated that plasma glucose and Na^+ as well as Cl^- were reduced by the infection, confirming similar differences in group means in Chapter 4. On the other hand, in the present group of infected piglets, the body weight gain was not demonstrably reduced although the group mean was 30% lower than that for the controls, a difference similar to that reported in Chapter 3 which was highly significant.

The caecal weight was not increased, whereas in the large group (Chapter 3, 3.3.3.2) there was a significant increase which occurred mainly from 5 to 14 days, but not earlier. No other changes in weight or length of the intestine were seen in either datum set. Mucosal damage was indicated by the marked crypt enlargement and reduced villus height/crypt depth ratio. The villus height was significantly reduced only in the jejunum but not in the ileum where the difference did not reach statistical significance. Crypt cell proliferation was increased as in Chapter 3 (3.3.6). Goblet cell numbers were increased on the villi of the mid-ileum and reduced in the crypts in the duodenum in the large group (Chapter 3, 3.3.7), but in the present group the differences were present in the distal ileum on the villi and in the mid-jejunum in the crypts. Both groups of piglets together showed that the infection increased the number of goblet cells on the distal small intestinal villi but reduced the numbers in the crypts in proximal segments.

Biochemical analyses of the mucosa of the small intestine for the larger group and for the present smaller group gave the same results. There were no significant differences between control and infected piglets in the concentration and content of protein and DNA, the ratio of RNA to DNA and Na^+-K^+-ATPase activity. Reduced lactase activity in infected piglets was demonstrable in both the large and the small groups. Sucrase activity tended to be reduced in the jejunum in infected piglets in both groups. There were no significant differences in maltase activity between control and infected piglets during the infection. A significant increase in maltase activity from the distal jejunum to the mid-ileum in the previously-infected piglets in the large group occurred mainly from days 5 to 14.

5.4.2 Effects of Oral Lactoferrin on *Y. enterocolitica*-infected Piglets

Oral lactoferrin supplementation in the milk formula fed to newborn piglets had no beneficial effects in protecting against *Y. enterocolitica* infection. Oral lactoferrin did not reduce the severity of diarrhoea and may even have accelerated the bacterial invasion of the mucosa in the duodenum and the proximal jejunum. In lactoferrin-fed piglets, the extent of mucosal injury, indicated by a larger group mean for the number of lesions, significantly more proliferating crypt cells and a higher intestinal muscle weight, was more severe than that in piglets fed milk formula alone. In the lactoferrin-fed group, mucosal lesions were present in almost every segment of the small intestine of each animal. Lactoferrin feeding did not increase the body weight gain, nor prevent the lowering of plasma glucose, Na^+ and Cl^- concentrations in *Y. enterocolitica*-infected piglets. Along with histological evidence of severe damage to the mucosa, lactoferrin-fed, infected piglets had lower mean lactase activity in the duodenum and the proximal jejunum (20 ± 11 and 44 ± 12 μmol/gram protein respectively) compared with those in non-lactoferrin-fed, infected animals (46 ± 5 and 79 ± 17 μmol/gram protein).

The significant effects of lactoferrin in *Y. enterocolitica*-infected piglets were the increased weight of the small intestinal muscle and the rate of proliferation of the crypt cells. These effects may be due mainly to more severe infection in the lactoferrin-fed, infected piglets than those fed milk formula alone. In infected piglets fed lactoferrin, a higher number of proliferating cells per crypt and a greater mean lesion number in the small intestine were accompanied by a lower lactase activity in the duodenum and the proximal jejunum. The weight of muscle was greater in infected piglets than in the controls, reaching statistical significance in two segments, but taken overall it does not

appear to be limited only to those segments but is a general feature of the small intestine. Overall, the infected piglets fed lactoferrin had even higher group means then those fed milk formula alone, which also supports the conclusion that lactoferrin may have exacerbated the effects of the *Y. enterocolitica* infection rather than reduced it, as might have been expected. A possible trophic effect of lactoferrin on crypt cells and on muscle growth cannot be discounted since the group means for proliferating crypt cells and crypt depth in all segments, and muscle weight in most segments of the small intestine, in lactoferrin-fed, control piglets were above those in animals fed milk formula alone.

5.4.3 Failure of Lactoferrin to Inhibit *Y. enterocolitica in vivo*

The preliminary *in vitro* study showed that lactoferrin inhibited *Y. enterocolitica* growth in two media, TSB and 1% peptone water. Bacterial growth was inhibited by 1% lactoferrin for at least 5 hours in 1% peptone water as well as by 5% lactoferrin in both TSB and 1% peptone water. However, oral lactoferrin supplementation in the milk formula fed to newborn piglets failed to show the effect seen *in vitro*.

Iron deprivation has been established as the most important mechanism of lactoferrin inhibition of bacterial growth both *in vitro* and *in vivo*. The relative concentrations of iron (178 µM) and lactoferrin (463 µM) in the lactoferrin-supplemented milk formula are such that, on the theoretical basis that one lactoferrin molecule binds two iron, the lactoferrin content should be sufficient to bind all the iron and make it unavailable to the bacteria in the gut. However, the concentrations of calcium and magnesium in the milk formula (10.5 mM and 1.8 mM respectively) may be high enough to inhibit the bacteriostatic effect of lactoferrin, since an *in vitro* study by Ellison and Giehl (1991) showed that 8×10^{-4} M magnesium and 1.6×10^{-4} M calcium inhibited the lactoferrin-lysozyme bactericidal activity, even though lactoferrin does not chelate calcium. Their earlier study (Ellison *et al.*, 1988) demonstrated that the action of lactoferrin in releasing lipopolysaccharides from bacterial membranes was blocked by high concentrations of Ca^{++} and Mg^{++} (1.25 mM and 1 mM). The concentration of Ca^{++} (0.042%) was comparable to that in sow colostrum secreted during the first 24 hours (0.04-0.07%) and was lower than that in sow mature milk (0.25%). It is possible that the bactericidal effects of lactoferrin *in vivo* are not the same as its action *in vitro* since other factors may influence the bacteriostatic action of lactoferrin *in vivo*.

First, *Y. enterocolitica*, like other enteric pathogens, may be able to utilise iron-containing compounds derived from dead sloughed epithelial cells whilst growing in an iron-limited environment. Enteropathogenic *E. coli* (Law *et al.*, 1992) and *V. cholera* (Stoebner and Payne, 1988) are able to utilise haem or haemoglobin as an iron source during growth in an iron-limited environment. This may alleviate the iron-restriction induced by the iron-binding protein, lactoferrin, supplied in the milk formula. On the other hand, *Y. enterocolitica* may release siderophores, low-molecular-weight, high-affinity iron chelators, which are used to obtain iron by bacteria under conditions of iron starvation. These ligands bind and solubilize ferric iron before re-entering the bacterial cell via specific membrane receptors. Siderophores occur in two broad chemical classes, hydroxamates and catechols, at least one of which has been found in almost every aerobic or facultatively anaerobic bacterial species (Neilands, 1981). Since siderophores permit bacterial multiplication *in vivo*, they are regarded as virulence factors. Robins-Browne and Prpic (1985) demonstrated that desferrioxamine B, a trihydroxamate siderophore obtained from *Streptomyces pilosus*, markedly increased the susceptibility of animals to yersiniosis.

Secondly, the osmolarity in the gut lumen may be high enough to reduce the inhibition of bacterial growth by lactoferrin. The bactericidal effect of lactoferrin is correlated with the medium osmolarity. *Escherichia coli* cells exposed to lactoferrin at 40 milliosmoles/l (mOsm) become enlarged and hypodense, bacterial killing progressively decreased as the medium osmolarity was increased and was completely absent when the osmolarity was increased to 100 mOsm (Ellison and Giehl, 1991). Under normal conditions, the osmolarity of the gut contents (about 250-300 mOsm) is far higher than 100 mOsm and might be predicted to reduce the effectiveness of lactoferrin.

Thirdly, the *Y. enterocolitica* inoculum may have been too high to be inhibited by lactoferrin. The antibacterial activity of lactoferrin is influenced by the size of the bacterial inoculum *in vitro* since the antibacterial effect of lactoferrin was decreased when the bacterial inoculum was increased to 5×10^7 colony forming units/ml (Ellison and Giehl, 1991). According to previous studies (Robins-Browne *et al.*, 1985), a high dose of *Y. enterocolitica* is necessary for infection of neonatal piglets, so that the large numbers of *Y. enterocolitica* in the gut may reduced the bacterial inhibition by lactoferrin.

Fourthly, the bacteriostatic effect on enteropathgenic bacteria of the lactoferrin in milk

or colostrum may be due to synergism between lactoferrin and antibody or other agents present in the milk. *In vivo* studies have shown that the inhibitory effect of lactoferrin on enterobacterial growth was seen in colostrum (Nagy, 1975) and in mature milk (Teraguchi *et al.*, 1993) but not in milk formula with added lactoferrin (Hall *et al.*, 1988) or in water containing lactoferrin (Teraguchi *et al.*, 1993). The present study supported the observations of Hall *et al.* (1988) who failed to show bactericidal effects of bovine lactoferrin in neonatal pigs experimentally infected with a pathogenic strain of *E. coli* using purified lactoferrin administered orally in milk formula. *In vitro* studies showed that lactoferrin alone had only a slight inhibitory effect on the growth of *E. coli* 0111, but on adding specific antibody, bacteriostatis ensued (Bullen *et al.*, 1974), and that lactoferrin and lysozyme synergistically killed *E. coli* (Ellison and Giehl, 1991). The lack of effectiveness of lactoferrin in the piglets may have resulted from the absence of antibodies and lysozyme in the milk formula.

Finally, the lactoferrin in the milk formula may have been absorbed by epithelial cells in the small intestine via pinocytosis during the first 36-48 hours after birth, making the intraluminal lactoferrin concentration too low to inhibit bacterial growth and invasion, particularly in the distal portion of the small intestine. Therefore, during the first one or two days, *Y. enterocolitica* would have invaded the epithelium via the M cells in the distal small intestine. Once the bacteria have been transported into the Peyer's patch dome, the organisms multiply there and spread into the lamina propria (Hanski *et al.*, 1989) and lactoferrin in the luminal fluid may no longer be able to affect bacterial growth. Of all the suggested possible reasons for the failure of lactoferrin to inhibit *Y. enterocolitica in vivo*, the most likely are the absence of synergistic milk components and the likely absorption of lactoferrin by the newborn gut.

5.4.4 Lactoferrin Affects Development in Newborn, Non-infected Piglets

Although there may have been no bacteriostatic effect of lactoferrin in the infected piglets, quite distinct effects on the gut and other organs were seen in the newborn piglets. Lactoferrin feeding caused very marked vacuolation of the enterocytes in non-infected piglets but not in those infected with *Y. enterocolitica.* Protein synthetic capacity, indicated by the RNA:DNA was markedly reduced, whether the piglets were infected or not. There were indications of stimulation of growth by lactoferrin: a small effect on body weight gain, markedly heavier kidneys and greater crypt depth, crypt cell proliferation and lactase activity in the small intestine. The plasma glucose and liver

weight were reduced in both infected and non-infected piglets.

5.4.4.1 Vacuolation of the small intestinal epithelium

The presence of vacuoles in the villus enterocytes from the mid-jejunum to the distal ileum suggested that the epithelium of the small intestine remained capable of absorption of lactoferrin until day 5 in non-infected piglets fed lactoferrin. Newborn piglets show qualitatively nonselective absorption of macromolecules, including egg white, bovine and human serum protein, enzymes and polyvinylpyrrolidone (Lecce, 1966). Lactoferrin, a glycoprotein of molecular weight 80,000, would be expected to be absorbed by enterocytes causing vacuoles to form in the enterocytes of the small intestine in the neonatal piglets. Compared with piglets fed milk formula alone, piglets fed 5% lactoferrin had enterocytes with more vacuoles from the mid-jejunum to the mid-ileum.

Although the vacuoles were unstained, it is likely that the material contained in them was removed during fixation and dehydration of the tissues. The presence of the large vacuole is assumed to indicate increased quantities of lactoferrin in the cells resulting from increased uptake into the cells and/or reduced transmission from the enterocytes into the circulation. The small intestine is known to continue to take in macromolecules for up to 14 days in piglets (Clarke and Hardy, 1971), although these molecules do not pass into the circulation. It is possible that at 5 days of age, more proximal areas of the small intestine still contain lactoferrin which is unable to leave the enterocytes and remains in large vacuoles.

The higher protein content in the milk formula containing 5% lactoferrin may increase the uptake of macromolecules in the small intestine. Higher amounts of protein in the intestinal lumen increased the intestinal transmission of macromolecules in the preclosure piglet (Weström *et al.*, 1985). The milk formula itself contains readily digested protein (1.68%) of which 60% is lactalbumin and 40% casein. *In vitro*, both lactalbumin and casein are hydrolysed very quickly (Lindberg *et al.*, 1989) so that *in vivo* very little is likely to be available for absorption intact by the gut. The piglets fed milk formula alone might be expected to absorb lesser amounts of macromolecules than those fed lactoferrin, which has been shown in a number of studies to be hydrolysed only to a limited extent. Lactoferrin is known to be fairly resistant to endopeptidase in early human infancy (Lindberg *et al.*, 1989). A relatively large amount of lactoferrin is detected in the faeces of preterm and term infants (Schanler *et al.*, 1986). *In vitro*

bovine lactoferrin is hardly hydrolysed in gastric or small intestinal fluids from suckled rats (Britton and Koldovský, 1987). The extent of hydrolysis of bovine lactoferrin in newborn piglets does not seem to have been examined, although Hagemeister *et al.* (1987) have showed that ileal absorption of bovine lactoferrin in suckled piglets is significantly lower (less than 89%) than in adult pigs (more than 97%). Similarly, the rate of excretion of lactoferrin in the faeces of human infants was about 6.2% at one week of age but only 1.2% at 12 weeks of age (Davidson and Lönnerdal, 1987). The epithelial cells in the jejunum and ileum in suckled piglets still absorb the large macromolecule polyvinyl pyrrolidone (mean molecular weight 160,000) until 13 days of age (Clarke and Hardy, 1977), therefore, epithelial cells in 5-day-old piglets would have the ability to take up unhydrolyzed lactoferrin.

It was interesting that the very obvious vacuolation of the enterocytes in non-infected piglets fed lactoferrin did not occur when lactoferrin was fed to *Y. enterocolitica*-infected piglets. Less vacuolation of the cells in infected animals could be due to faster proliferation of crypt cells producing a new generation of villus epithelial cells which had lost much of their ability to absorb macromolecules. Alternatively, *Y. enterocolitica* may have attached to the brush borders of enterocytes reducing the absorption of lactoferrin. The malabsorption of whole horse serum, horse IgG and horse albumin in neonatal pigs, monocontaminated with *E. coli* within 46 hours after birth (Staley *et al.*, 1972), supports this hypothesis. Thus, in *Y. enterocolitica*-infected piglets, in spite of the large amount of lactoferrin available in the gut, lactoferrin may not be absorbed by the small intestinal mucosa.

5.4.4.2 Capacity for protein synthesis

Oral lactoferrin supplementation reduced the ratio of RNA to DNA in the small intestinal mucosa in both control and infected piglets, the lower ratio being dominated by the lower RNA content. Since protein synthesis is directly related to the tissue RNA content (Sarkar, 1975), the capacity for protein synthesis would likely be decreased in lactoferrin-fed piglets. The measurement of total mucosal protein content may not be sensitive enough to detect changes in enterocyte protein content, since the mucosal protein content in lactoferrin-fed pigs did not differ from that in animals fed with milk formula alone. On the other hand, any decrease in synthesized protein might be compensated for by protein absorbed into the vacuolated epithelial cells.

The RNA:DNA was reduced by oral lactoferrin in both infected and non-infected piglets, although the two groups had very different vacuolation of the enterocytes. The number and size of vacuoles probably depends on the amount of protein being taken up by the cells, as well as on the length of the life span of the enterocytes (Moon, 1972). Since the appearance of the cells did not correlate with the RNA:DNA ratio, there was no apparent explanation for the reduced protein synthetic capacity. Further, the high protein content of the diet containing lactoferrin is not likely to be the reason, since compared with 5% lactose, sow colostrum and mature milk, which contain 17% and 6.8% protein respectively, significantly increased the small intestinal RNA content of piglets (Simmen *et al.*, 1990). It is unclear whether the reduction in the RNA was due to lactoferrin itself, or to it being a foreign protein absorbed into the enterocytes, which may reduce the capacity for protein synthesis in the small intestine in some way.

5.4.4.3 Trophic effects of lactoferrin

Lactoferrin appeared to have trophic effects, which were more apparent in the non-infected piglets. The mean body weight was 9% heavier than in the control group and the kidneys were significantly increased in weight. The latter was also seen in *Y. enterocolitica*-infected piglets fed lactoferrin. It is unclear how trophic effects on the body or the kidneys are actually mediated in the animal, presumably after absorption into the circulation.

Lactoferrin could be a growth factor for the gastrointestinal tract. Lactoferrin has been demonstrated to promote the incorporation of thymidine into the DNA of rat crypt enterocytes (Nichols *et al.*, 1990) and to stimulate myoblast proliferation (Byatt *et al.*, 1990). In the present study, oral lactoferrin significantly increased the mid-ileal lactase activity (µmol/g protein and µmol/g tissue). It is difficult to conclude from the statistical analyses that lactase was the only enzyme affected by oral lactoferrin. Sucrase activity (µmol/g protein) increased 63% for the proximal jejunum, 54% for the middle jejunum and 77% for the distal jejunum. The higher group means for both crypt depth and population of proliferating crypt cells in almost all segments of the small intestine suggest that lactoferrin acted as a growth factor in the crypts. There were large differences in the group means in several intestinal parameters which support this, however, the results here are inconclusive because of the small numbers of animals and the large variance, but further experimentation is warranted.

5.4.4.4 Plasma glucose concentration and liver weight

Feeding lactoferrin to non-infected piglets produced a decrease in plasma glucose concentration and in liver weight as was also seen in all infected piglets whether fed milk formula with lactoferrin, BMF or no added protein. This was attributed in the *Y. enterocolitica*-infected animals to reduced glucose absorption by the damaged intestine, which was supported by the concurrent reduction in lactase activity.

In the lactoferrin-fed non-infected piglets, both the plasma glucose concentration and the liver weight were significantly lower than in controls. This could result from either reduced absorption of glucose from the lumen of the small intestine or from increased utilization of glucose for metabolism. The lower plasma glucose was not associated with lower lactase or Na^+-K^+-ATPase activity in the small intestine, so there is no evidence to support impaired glucose uptake from the intestine. In the lactoferrin-fed piglets, the body weight gain was 9% higher, the small intestine increased 7% in length and 8.7% in weight and the kidney was 19% heavier than in the controls. The lower plasma glucose concentration was more likely to be the result of trophic effects of lactoferrin. The increased protein synthesis might require energy which could reduce plasma glucose concentration and mobilise liver glycogen, thereby leading to the lower liver weight. Another possible explanation for the lower plasma glucose, but not the lighter liver, is that the 5% lactoferrin in the milk formula increased insulin levels in the neonatal piglets, producing a reduction in the plasma glucose concentration. In sheep 10-11 months of age, feeding high crude protein pellets (220 g/kg) increased plasma insulin concentration (2.77 µg/l) compared with that (1.86 µg/l) in sheep fed a low protein diet (120 g/kg) (Waghorn *et al.*, 1987). Whether such an effect in older sheep might also occur in newborn piglets is unclear.

5.4.5 BMF Affects *Y. enterocolitica* Infection in Piglets

Although lactoferrin proved to have no benefits in reducing the severity of the *Y. enterocolitica* infection in newborn piglets, the BMF appeared to have some potential as an oral supplement. The body weight gain was 26% higher than in infected piglets fed milk formula alone and there was reduced mucosal injury in the small intestine and a higher ratio of villus height to crypt depth. The group means for the number of lesions in the proximal small intestine were lower in the BMF-fed piglets. Higher lactase activity in the distal jejunum, and a lower rate of crypt cell proliferation are

consistent with less damage during the *Y. enterocolitica* infection. Maltase and sucrase activities also showed increased mean activity, although not reaching significance, in some segments of the small intestine.

The BMF probably exerted its effect on the *Y. enterocolitica* infection by binding to the surface of the bacteria to prevent their adhesion to the intestinal mucosal surface. This hypothesis is supported by an *in vitro* study of inhibition of *E. coli* hemagglutination by the BMF (personal communication, Dr. L. Schollum). *Yersinia enterocolitica* has the ability to adhere to mucosal surfaces, which is regarded as an important property of microorganisms which cause disease by invasion of the intestinal mucosa. Surface properties of *Y. enterocolitica* which facilitate the initial adhesion to the intestinal mucosa are important for the virulence of this pathogen (Pærregaard *et al.*, 1990).

The evidence in the present experiments, although not conclusive, suggests that this milk protein should be examined further. All the available protein was used to supplement the diet of 4 piglets, which determined that the final concentration of the BMF in the milk was 0.15%. A more comprehensive study is needed to evaluate whether other concentrations of the BMF might prove it to be effective against *Y. enterocolitica* and possibly other enteric pathogens.

5.5 SUMMARY

Five per cent bovine lactoferrin has been used for oral supplementation in the milk formula of both control and *Y. enterocolitica*-infected piglets and 0.15% of a bovine milk fraction for *Y. enterocolitica*-infected piglets. In control animals, lactoferrin increased the vacuolation of the epithelium from the mid-jejunum to the mid-ileum, significantly increased kidney weight, and the mid-ileal lactase activity (µmol/g protein and µmol/g tissue). Although not reaching statistical significance, sucrase activity (µmol/g protein) increased 63%, 54% and 77% respectively for the proximal, mid- and distal jejunum, and body weight gain increased 9%. Lactoferrin also significantly reduced the plasma glucose concentration and liver weight.

In *Y. enterocolitica*-infected piglets, oral lactoferrin supplementation in the milk formula did not show any benefits judged by the clinical signs, histomorphological observations and morphometric and biochemical measurements. In the duodenum and the proximal jejunum, lactoferrin-fed piglets had more lesions (23 and 21/section respectively compared with 2 and 10/section), although the difference was not significant.

Oral BMF, however, significantly reduced the population of proliferating crypt cells, slightly reduced the lesion number in some segments of the small intestine, and significantly increased lactase activity (µmol/g protein) in the mid- and distal jejunum. Maltase and sucrase activities also showed increased group mean values in some segments of the small intestine. The body weight gain was 26% higher than in infected piglets fed milk formula alone.

Chapter 6

GENERAL DISCUSSION

A model of enterobacterial diarrhoea has been developed to investigate the effects of infectious diarrhoea on the growth of the neonate, particularly on the development of the gastrointestinal tract in the period of infection and during subsequent recovery after antibiotic therapy. Newborn, colostrum-deprived piglets were infected orogastrically with *Y. enterocolitica* biotype 4, serotype 0:3. Bovine lactoferrin and a bovine milk fraction were supplied orally in the milk formula to these *Y. enterocolitica*-infected piglets to observe whether there were any bacteriostatic effects of these two proteins in the neonatal piglet.

Yersinia enterocolitica infection decreased the gain in body weight and liver weight, caused severe damage to the mucosa of the small intestine, stimulated crypt cell proliferation resulting in longer crypts, altered disaccharidase activity and reduced the plasma glucose, Na^+ and Cl^- concentration. After antibiotic therapy, previously-infected animals had a similar body weight gain to controls, the mucosa no longer showed structural damage and plasma Na^+ and Cl^- concentration and crypt cell proliferation returned to the control level. The infection had lasting effects on disaccharidase activities: lactase activity remained lower while maltase and sucrase activities showed premature development. Oral supplementation with the bovine milk fraction, but not bovine lactoferrin, reduced the degree of severity of the *Y. enterocolitica* infection.

The principal reason that *Y. enterocolitica* was chosen as the pathogen was that *Y. enterocolitica* infection causes diarrhoea but is rarely fatal in humans (Mair and Fox, 1986) in contrast to some common enteropathogens e.g. *E. coli*, rotavirus and TGE virus. Since the aim of the project was to study the development of the animals, particularly of the small intestine, during and after a period of diarrhoea, it was anticipated that *Y. enterocolitica* would be a suitable organism to use. The second reason for choosing *Y. enterocolitica* was the increasing relevance of *Y. enterocolitica* gastroenteritis to human medicine. McCarthy and Fenwick (1991) have shown that the incidence of *Y. enterocolitica* infection in humans has recently risen in New Zealand, and that 92% of strains isolated from human patients are biotype 4, serotype 0:3. This

strain is also commonly isolated in most other countries (Asakawa *et al.*, 1973; Toma and Lafleur, 1974; Christensen, 1980; Marks *et al.*, 1980; Maruyama, 1987; Thompson and Gravel, 1986) and recently also in the USA (Lee *et al.*, 1991). There have been no reports of using New Zealand *Y. enterocolitica* isolates for experimental infection of animals to confirm the pathogenesis of the local isolate. Experimental infections with *Y. enterocolitica* in piglets in Australia (Robins-Browne *et al.*, 1985) and in rabbits in Canada (Pai *et al.*, 1980; O'Loughlin *et al.*, 1986) have provided information on the pathogenesis and pathophysiology of the human disease.

6.1 DIARRHOEA

Diarrhoea is one of the main clinical signs in human *Y. enterocolitica* infection (Vantrappen *et al.*, 1977; Marks *et al.*, 1980; Lee *et al.*, 1991) and in experimentally-infected animals (Pai *et al.*, 1980; O'Loughlin *et al.*, 1986; Robins-Browne *et al.*, 1985; Schiemann, 1988). In the present study, all piglets developed diarrhoea between 40 hours and 4 days after inoculation, except one which was killed on day 3. The severity of the diarrhoea was not reduced by supplementation of the milk formula fed to the *Y. enterocolitica*-infected piglets with either the bovine milk fraction or lactoferrin.

What was the mechanism by which *Y. enterocolitica* produced diarrhoea: by enterotoxin or by mucosal injury resulting in maldigestion and malabsorption? Most pathogenic strains of *Y. enterocolitica*, including serotype 0:3 biotype 4, produce, at 25 ^{0}C but not at 37 ^{0}C, an enterotoxin resembling *E. coli* heat-stable enterotoxin (Pai and Mors, 1978; Boyce *et al.*, 1979). The *Y. enterocolitica* cultures used for challenging the piglets in the present experiment would have contained some enterotoxin, although it may not have also been produced by these bacteria *in vivo*. Pai *et al.* (1980) have failed to show enterotoxin in the contents of the colon, ileum and jejunum of rabbits with diarrhoea after challenge with *Y. enterocolitica*. Unimpaired secretion of Cl^- in the small intestine in *Y. enterocolitica*-infected rabbits (O'Loughlin *et al.*, 1988) also supports the lack of enterotoxin production *in vivo*. Recently, Delor and Cornelis (1992) reached the opposite conclusion that enterotoxin was a major factor involved in *Y. enterocolitica*-associated diarrhoea in young rabbits. An enterotoxin positive strain of *Y. enterocolitica* produced diarrhoea whereas a mutant enterotoxin negative strain had reduced ability to cause diarrhoea. This was interpreted as evidence that the enterotoxin was essential for pathogenicity. These authors, however, did not confirm that the enterotoxin positive strain produced enterotoxin *in vitro*.

In the present study, several observations suggested that enterotoxin probably did not play an important role in producing the *Y. enterocolitica* diarrhoea. In general, the newborn is more susceptible to enterotoxigenic diarrhoea than the adult because enterotoxin can bind more readily to the microvillus surface of the newborn intestine (Bresson *et al.*, 1980). Typical enterotoxingenic diarrhoea due to *E. coli* infection caused a loss of 20.5-22.1% of the original body weight within 24 hours postinfection in colostrum-deprived piglets (Whipp *et al.*, 1975). The present piglets, which were inoculated within 1-3 hours of birth, should be very sensitive to enterotoxingenic diarrhoea if enterotoxin were a major factor in its production. Piglets infected with a high dose of *Y. enterocolitica* (approximately 3×10^{10}) did not develop diarrhoea for 40 hours and their body weight did not fall below the birth weight over the 5 days of the infection. The amount of enterotoxin in the *Y. enterocolitica* inoculum might not be sufficient to induce diarrhoea without further production of enterotoxin in the gut of the piglets.

Since there was mucosal injury in all *Y. enterocolitica*-infected piglets and diarrhoea in 18 of the 19 infected piglets, the diarrhoea probably resulted mainly from mucosal injury. In the present study, the small intestinal mucosa was not examined in infected piglets until 3 days postinfection, however, in *Y. enterocolitica*-infected mice, histological evidence of infection could be observed as early as 24 hours postinfection (Carter, 1975). Therefore, the mucosal injury may develop before the onset of diarrhoea in the infected piglets. Severe injury to the mucosa of the small intestine and a marked loss of lactase activity may alter digestive and absorptive functions resulting in an overload of nutrients, electrolytes and water being retained in the gut and stimulating increased motor activity.

6.2 *YERSINIA ENTEROCOLITICA* INFECTION ACCELERATED MATURATION OF THE SMALL INTESTINE

Physiological maturation of the gut in the newborn involves a changing capacity for digestion and absorption. Particularly apparent are "closure" so that macromolecules can no longer be transferred into the circulation and the altered activity of some enzymes particularly disaccharidase. Gall and Chung (1982) suggested that gut maturation is not age-dependent but that the biological signal for ontogenic development depends on the level of nutrient intake and the rate of body growth. Bacterial enteritis may also stimulate early maturation of the gut. *Yersinia enterocolitica* invaded the mucosa of the

small intestine resulting in villus loss, crypt hyperplasia, accelerated maturation of the epithelium, reduced ability to take up macromolecules and altered ability to digest specific disaccharides thus a possible decline in absorption of glucose.

Crypt hyperplasia was most marked at 5 days postinfection when the mucosal injury was most severe. Supplementation with the bovine milk fraction for 5 days reduced the degree of crypt hyperplasia as well as the mucosal injury, whereas oral lactoferrin stimulated greater crypt hyperplasia in infected piglets although the difference was not statistically significant.

Enterocytes in the crypts are different from those on the villi in both structure and function. The enterocytes in the crypts are undifferentiated cells which contain different enzymes from cells on the villi e.g. crypt cells are rich in thymidine kinase activity whereas lactase activity is mainly in the villi. At different times during the period of maturation, the activity of an enzyme is located at different levels along the crypt-villus axis e.g. maltase and sucrase activities are initiated in the crypts in rats before weaning (Simon *et al.*, 1979; Collins *et al.*, 1990) whereas, at an older age, high activities are located in the villi (Simon *et al.*, 1979). During enteritis, shedding of villus cells containing high lactase activity, thereby leaving crypt cells which have a lower ability to express lactase, would reduce the total activity.

One of the striking effects of *Y. enterocolitica* infection on the intestine was the accelerated maturation of the disaccharidase profiles, probably because of increased crypt cell proliferation. In infected piglets, crypt hyperplasia may alter the normal developmental pattern of disaccharidases. Loss of some villi due to *Y. enterocolitica* invasion would reduce lactase activity, but the increased number of crypt cells, which are less able to develop lactase activity, would result in markedly lower lactase activity at 5 days postinfection. When animals had clinically recovered following antibiotic therapy, the rate of proliferation of crypt cells had returned to that seen in control animals, but the crypts were still longer. The newly differentiated enterocytes on the villi could be physiologically more mature than those in controls of the same age, containing less lactase activity and more maltase and sucrase activity.

The accelerated gut maturation resulting from the *Y. enterocolitica* infection also changed the vacuolation of the small intestinal enterocytes, in that there was an early reduction in the number of vacuoles in the epithelium. Any effects of bacterial enteritis

on the vacuolation of the epithelium of the small intestine do not appear to have been reported, although *E. coli* decreased the amount of protein absorption in neonatal piglets (Staley *et al.*, 1972). The present study demonstrated that *Y. enterocolitica* infection reduced the extent of vacuolation of the epithelium of the small intestine during all periods of the experiment. Vacuoles were almost completely absent at 5 days postinfection regardless of whether or not there were oral supplementation with either lactoferrin or BMF.

During the infection period, the absence of vacuoles in infected piglets could be due to two factors: *Y. enterocolitica* attachment to the surface of the mucosa (Pærregaard *et al.*, 1990) resulting in reduced uptake of protein by the epithelium, and increased cell proliferation in the crypts leading to a shortened life span of the enterocytes. First, *E. coli* has been found to reduce the absorption of macromolecules by the small intestine in newborn piglets (Staley *et al.*, 1972). *Yersinia enterocolitica* adheres to the surface of the mucosa of the small intestine (Pærregaard *et al.*, 1990), so that the high dose given to the piglets may have interfered with the absorption of macromolecules. Secondly, the hypothesis of Moon (1972) that formation of vacuoles requires a long life span of the enterocytes, may explain the reduced number of vacuoles in infected piglets. Infected piglets fed with or without lactoferrin, had a larger population of proliferating crypt cells and a longer crypt. The faster proliferation may have shortened the life span of the enterocytes so that vacuoles did not fully develop in spite of high protein content in the gut lumen of the lactoferrin-supplemented piglets.

After antibiotic therapy, the rate of proliferation of crypt cells in the previously-infected piglets had returned to that in controls, which may indicate that the life span of the enterocytes had lengthened enough to allow again the development of vacuoles. The vacuoles, however, were fewer in number and smaller in size than those in non-infected piglets, suggesting that the enterocytes were more mature in the previously-infected piglets.

Yersinia enterocolitica infection reduced the plasma glucose concentration throughout the period of the experiment. During the infection period, the lower plasma glucose concentration might be due mainly to decreased capacity for digestion and absorption because of the loss of villi. When the mucosa of the small intestine was injured by the invasion of *Y. enterocolitica*, lactase activity dramatically declined, probably causing maldigestion of lactose. In rabbits, malnutrition resulting from mucosal injury in

Y. enterocolitica diarrhoea was not only due to maldigestion of disaccharides but also to malabsorption of glucose (O'Loughlin *et al.*, 1988). In the present study, the reduction in plasma Na^+ and Cl^- concentration supported the hypothesis that malabsorption had occurred during the 5 days of infection. Glucose would be mobilised from the liver glycogen to maintain a normal level of blood glucose, resulting in a decrease in liver weight. When the animals given antibiotic therapy had recovered from the diarrhoea by day 14, the persistence of a lower glucose concentration than in the controls could be due to the epithelium of the small intestine being physiologically more mature and absorbing less glucose than in non-infected animals, because glucose absorption is reported to decline with age in neonatal piglets (Puchal and Buddington, 1992).

6.3 NUTRITION AND RECOVERY FROM DIARRHOEA

There is a well-established link between the level of nutrition and the susceptibility to infectious diseases of the gastrointestinal tract. Diarrhoea leads to malnutrition which, in turn, could increase the risk of diarrhoea in the young (Scrimshaw *et al.*, 1968). Malnourished children have diarrhoeal episodes of longer duration than better nourished children (Black *et al.*, 1984). The combination of malnutrition and infectious diarrhoea results in a more serious illness for the child and a greater risk of death than if either were present alone (Scrimshaw, 1970).

During the period of recovery from gastroenteritis, it is important to provide a nourishing diet to animals or children. The present study demonstrated that acute bacterial diarrhoea of short duration may not significantly affect the later growth of neonates if they are treated with an effective antibiotic and are well supplied with nutrients. During the 9 day recovery period, previously-infected piglets gained 390 g in body weight compared with 377 g in controls, although the absolute body weight in the previously-infected animals remained lower than in the controls. The piglets appeared to be hungrier than the controls. It was unknown whether or not the body weight would have reached that of the controls some time after the 14 day experimental period if they had been studied for a longer time and continued to be provided with adequate nutrition. When killed at 14 days, the previously-infected piglets had a higher activity of maltase and sucrase in the small intestine than the controls, which may have increased the ability of the animals to utilize maltose and sucrose.

In children, the effects of diarrhoea may be greater than in the piglets, since mucosal injury decreases not only the lactase activity but also maltase and sucrase activity (Lebenthal and Lee, 1980). This may be because at birth all disaccharidases are fully developed in humans (Chapter 1, 1.2.1). Whether or not there is a higher than normal disaccharidase activity in human infants recovering from diarrhoea as was seen in the piglets does not appear to have been reported, although "catch-up-growth" has been described (Black, 1991).

6.4 ORAL SUPPLEMENTATION WITH A BOVINE MILK FRACTION AND LACTOFERRIN

Two bovine milk components which have *in vitro* antibacterial actions were used as oral supplements in *Y. enterocolitica*-infected piglets. Lactoferrin has been studied extensively *in vitro* and is frequently proposed as a potentially useful food supplement, whereas the bovine milk fraction prepared by the New Zealand Dairy Research Institute has not previously been examined *in vivo* and was available only in limited amounts. The latter was used only in a low concentration (0.15%), but even then some benefits were seen in the *Y. enterocolitica*-infected piglets. Damage to the small intestinal mucosa was less severe than in piglets fed milk formula alone, particularly in the proximal small intestine, indicated by the shorter crypt length, fewer proliferating crypt cells and lesser reduction in lactase activity. These observations suggest that further studies with this protein are warranted to evaluate its potential.

In contrast, there were no benefits when bovine lactoferrin was added to the milk formula of *Y. enterocolitica*-infected piglets for 5 days. The effectiveness of the bacteriostases against *Y. enterocolitica* shown by lactoferrin *in vitro* encouraged further investigation *in vivo* in piglets with infectious diarrhoea, but the results were negative. There were a number of reasons why lactoferrin may not be effective *in vivo*, including the availability of iron from other sources, the osmolarity of the gut contents, absorption of lactoferrin by the enterocytes or lack of synergism with other milk constituents. Lactoferrin is known to act synergistically with specific antibodies and with lysozyme. Further study of the conditions necessary for lactoferrin inhibition of bacteria *in vivo* might examine this synergism by adding lactoferrin to whole milk instead of milk formula, or adding lactoferrin to milk formula along with lysozyme or antibodies.

The ability of piglets to absorb macromolecules may reduce the efficiency of lactoferrin

in the preclosure period. However, lactoferrin may be more effective in older animals, so that the conclusions drawn from experiments on newborn piglets may not be valid for older animals or for human infants. Lactoferrin may also be useful as an oral supplement if antibiotics are administered at the same time, because lactoferrin may increase the effectiveness of the antibiotic against bacteria by releasing lipopolysaccharides from the membranes of Gram-negative bacteria, thus facilitating the entry of some antibiotics into the bacteria (Nikaido, 1989; Ellison and Giehl, 1991).

6.5 SIMILARITY OF PIGLETS AND INFANTS

The present study showed that *Y. enterocolitica*-infected, newborn, colostrum-deprived piglets were a good model for the human disease. The main clinical signs of anorexia, vomiting and diarrhoea (Marks *et al.*, 1980; Lee *et al.*, 1991), and also the histopathological changes, microabscesses in the intestine (Vantrappen *et al.*, 1977) and the liver (Ryan *et al.*, 1979), observed in human *Y. enterocolitica* infections were all present at both 3 and 5 days postinfection. One cannot be sure that the effects in the piglets of the enteritis on gut development, organ growth and on plasma glucose and electrolytes would occur to the same extent in human infants.

The gastrointestinal tract of the pig, after all, is different from that in humans in several important ways, particularly in the development of sucrase and maltase. In humans, the levels of both enzymes at birth are comparable to those at maturity (Grant *et al.*, 1976), whereas in newborn piglets they are present at very low levels and rise during neonatal life. The activity of sucrase and maltase in the small intestine remained low during the first three days after birth in neonatal piglets, sucrase activity was undetectable in both the duodenum and the ileum by 14 days of age, and maltase activity in the duodenum was only 1.3 μmol/g protein/min by day 3 in these piglets. In comparison, the activities of sucrase and maltase in the duodenum of children under one year of age were 53.5 and 210.4 μmol/g protein/min respectively (Lebenthal and Lee, 1980). The maximum activity of sucrase and maltase in the mid-jejunum (17.8 and 38.3 μmol/g protein) in 3-day-old piglets was also much lower than that (71.1 and 224.3 μmol/g protein) in infants aged about 2.5 months with protracted diarrhoea (Shulman *et al.*, 1991). Because of the different patterns of development in α-glucosidase activity in neonatal piglets and infants, gastroenteritis in human infants may have more serious consequences than in neonatal piglets. In human infants with enteritis, lactase, maltase and sucrase all decrease (Lebenthal and Lee, 1980), which would severely limit sugar digestion.

6.6 CONCLUSIONS

The present study has demonstrated that newborn, colostrum-deprived piglets can be used to study the development of the gastrointestinal tract during *Y. enterocolitica* enteritis, which is not possible in human infants. *Yersinia enterocolitica* infection reduced the body weight gain during 5 day infection period, but at 14 days of age, although they had clinically recovered, the previously-infected piglets still had a lighter body weight. Crypt hyperplasia was very marked in *Y. enterocolitica*-infected piglets. Acute bacterial infection did not affect the growth of organs except the liver. The growth and development of the gastrointestinal tract was not altered significantly relative to body weight, the only marked difference being in the disaccharidase profile: reduced lactase activity and premature development of maltase and sucrase. The present study did not show any benefits from oral lactoferrin supplementation, however, another bovine milk protein slightly reduced the extent of mucosal injury and caused a lesser decline in lactase activity in *Y. enterocolitica*-infected piglets.

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Appendix 1

Daily Milk Intake (ml) of Control and *Y. enterocolitica*-infected Piglets from Birth to Day 14

Pig No.	Treatment	Day 1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Control	405	325	305											
2	Infected	430	317	326											
3	Control	365	260	358											
4	Infected	340	220	343											
5	Control	360	340	350											
6	Infected	365	270	255											
7	Control	360	325	375											
8	Infected	200	290	290											
9	Control	470	344	387											
10	Infected	485	373	380											
11	Control	356	322	305											
12	Infected	315	238	280											
13	Control	415	408	457	437	245									
14	Infected	421	342	369	294	154									
15	Control	465	424	434	348	240									
16	Infected	380	318	352	314	182									
17	Control	395	325	360	280	180									
18	Infected	380	325	335	215	140									
19	Control	450	420	450	260	237									
20	Infected	450	435	445	310	305									
21	Control	413	358	420	405	255									
22	Infected	311	175	282	304	158									
23*	Control	320	208												
24	Infected	290	290	339	305	213									
25	Control	350	405	400	450	340									
26	Infected	375	305	319	360	250									
27	Control	328	320	315	345	200									
28	Infected	335	310	315	345	232									
29	Infected	340	180	303	320	289	455	425	395	425	505	525	600	545	465
30	Infected	334	164	303	287	309	426	440	395	430	505	512	505	535	420
31	Infected	370	161	313	388	405	489	548	470	510	565	605	689	780	515
32	Control	380	367	335	410	440	475	463	400	470	525	545	605	617	385
33	Control	330	335	330	400	400	438	400	341	448	513	520	585	510	295
34	Control	390	355	330	410	425	470	473	410	480	555	575	646	715	370
35	Infected	415	360	349	332	344	400	430	435	465	175	200	395	465	265
36	Infected	420	263	344	305	332	365	352	126	220	338	415	370	445	350
37	Control	430	415	400	373	450	420	395	460	520	510	505	540	575	410
38	Control	400	400	400	410	445	420	395	464	495	520	505	540	575	395

* the piglet died on day 2

Appendix 2

MICROBIOLOGY

2.1. Cefsulodin-Irgasan-Novobiocin (CIN) Agar Preparation (Schiemann, 1979)

Cefsulodin-Irgasan-Novobiocin (CIN) Agar is a combination of *Yersinia* Selective Agar Base and *Yersinia* Antimicrobic Supplement CN.

1. Suspend 59.5 g of *Yersinia* Selective Agar Base (Difco Laboratories, Detroit, Michigan, USA) in 1 litre distilled water and heat to boiling to dissolve completely.
2. Sterilise in the autoclave for 15 minutes at 15 lbs pressure (121^{0}C).
3. Cool to 45-50^{0}C.
4. Aseptically add 10 ml of rehydrated *Yersinia* Antimicrobic Supplement CN (Difco Laboratories, Detroit, Michigan, USA).
5. Mix thoroughly, avoiding the formation of air bubbles, and dispense into sterile 90-100 mm Petri dishes, approximately 20 ml per dish.

2.2 Identification of *Y. enterocolitica*

Serotyping: carried out by slide agglutination with *Y. enterocolitica* typing serum 0:3 (Eco-Bio N.V./S.A. Woudstraat, Belgium) according to the manufacturer's instructions.

1. Place 1 drop of antiserum 0:3 on a clean slide
2. Pick up with a loop a few colonies on a blood agar plate (which has been incubated at 29 ^{0}C for 44 hours), add to the antiserum and mix thoroughly with the loop until the mixture is cloudy. As a control, suspend bacterial colonies in a drop of distilled water on the slide.
3. After one minute, examine the mixture for agglutination, which constitutes a positive reaction, and a cloudy drop is negative.

Biotyping: determined according to the method of Mair and Fox (1986). The bacteria are tested on the following media: triple sugar iron (TSI) slope, lysine iron agar (LIA) slope, urea, ornithine decarboxylase, sucrose, trehalose, rhamnose, indole, salicin, esculin hydrolysis, D-xylose and Voges-Proskauer (VP).

1. Place a colony of the isolate from a blood agar plate in a vial of tryptone water (TW) and incubate at 29 ^{0}C for 24 hours.
2. Add 3 to 5 drops of TW bacterial suspension to each of the above media, and incubate at 29 ^{0}C for 48 hours.
   (a) Add about 0.5 ml liquid paraffin to the ornithine bacterial culture before incubation. Purple: positive. No colour: negative
   (b) Set up 2 vials of VP broth, one to be incubated at 37 ^{0}C and the other at 29 ^{0}C for 48 hours. For the VP test, add 0.6 ml of α-Naphthol and 0.4 ml of 40% KOH to 1 ml of VP broth culture. Mix very well and allow to stand for 5 minutes. Bright orange-red colour: positive. Yellow: negative.
   (c) For the indole test, add 3 to 5 drops of Kovac's reagent to the TW bacterial suspension. Deep red: positive. Yellow: negative.
   (d) For both TSI and LIA, yellow: the bacteria produce acid; red: the bacteria produce alkali and no colour: negative.
   (e) For other media, after incubation red colour - positive, no colour change - negative.

Appendix 3

HISTOLOGY

3.1. Alcian Blue/Periodic acid-Schiff Stain (Culling, 1974)

REAGENTS

1. 0.3% Alcian blue (George T. Gurr Ltd., London, England) in 3% acetic acid
2. 1% periodic acid (BDH Ltd., Poole, England)
3. Schiffs reagent:
   Add 2 g of pararosaniline chloride (BDH Ltd., Poole, England) to 400 ml of distilled water (37 ^{0}C), agitate until dissolved, then add 7.6 g of sodium metabisulphite (May & Baker Ltd., Dagenham, England) and 2.0 ml of concentrated HCl. Shake well for 10 seconds. Store overnight in the dark. The solution should then be straw coloured. Add 2 g of activated charcoal, shake well and filter.

PROCEDURE:

1. De-wax
   xylene 5 min twice
   absolute alcohol 1 min
   70% alcohol 1 min
   Rinse in tap water
2. Alcian blue for 10 min, rinse in tap water
3. React with 1% periodic-acid for 7 min, wash in tap water for 5 min, and rinse in distilled water 3 times, 1 min each time
4. React with Schiffs reagent for 10 min, wash in running tap water for 10 min
5. Mayers Haemalum for 2 min, rinse in tap water
6. Scott's water for 2 min, rinse in tap water
7. Dehydrate: 70%, 100%, 100% alcohol, xylene twice and mount in DPX (BDH Ltd., Poole, England)

3.2. Immunocytochemistry (Based on Holle and Birtles, 1990)

REAGENTS

1. Phosphate buffered saline (PBS) pH 7.2, 0.01 M
2. 1% bovine serum albumin (BSA) in PBS (pH 7.2, 0.01M)
3. Primary antibody: anti-bromodeoxyuridine mouse monoclonal antibody + nuclease (Amersham international, Plc, Amersham, UK), 1:100 dilution in 1% BSA (10 µl of antibody in 1 ml of 1% BSA)
4. Secondary antibody: Anti-mouse IG, biotinylated species-specific from sheep (Amersham International, Plc, Amersham, UK), 1:200 dilution in 1% BSA (5 µl of antibody in 1 ml of 1% BSA)
5. Third layer: Streptavidin-biotinylated horseradish peroxidase complex (Amersham

international, Plc, Amersham, UK) 1:200 dilution in 1% BSA (5 µl of complex in 1 ml of 1% BSA)

6. 0.05% 3,3'-Diaminobenzidine tetrahydrochloride dihydrate (Aldrich Chemical Company, Inc. USA) (DAB): 5 mg DAB in 10 ml of PBS, 12 µl H_2O_2, one drop of 1% cobalt chloride solution

PROCEDURE (at room temperature)

1. De-wax
   xylene 5 min twice
   absolute alcohol 1 min
   70% alcohol 1 min
   rinse in tap water
2. Wash in PBS for 5 min
3. Treat with 1% BSA for 5 min. This serves to block non-specific tissue binding sites. Incubate in the primary antibody (1:100 in 1% BSA) solution for 1 h in a moist chamber. To ensure the specificity of the monoclonal antibody reaction, two controls are applied. First, sections from the same tissue samples remain covered with the 1% BSA while omitting the primary antibody; second, tissue sections of untreated with BrDU small intestine samples are covered with the primary antibody.
5. Wash all slides in 3 changes of PBS for 1 min each.
6. Apply second antibody (1:200 in 1% BSA) solution for 30 min and keep in a moist chamber.
7. Wash in 3 changes of PBS for 1 min each.
8. Apply third layer (1:200 in 1% BSA) solution for 15 min
9. Wash in 3 changes of PBS for 1 min each.
10. React with 0.05 % DAB for 3-5 min. The labelled cells become dark brown or black, control sections should have no colour (Check under microscope).
11. Wash in PBS and in tap water.
12. Counterstain with 1% Eosin for 40 seconds.
13. Dehydrate in 70% alcohol, absolute alcohol twice, xylene twice and mount in DPX (DBH Ltd., Poole, England).

Appendix 4

Comparison of Two Body Weight Curves for the Control and *Y. enterocolitica*-infected Piglets#

Group	Regression coefficient	SE	Regression comparison	p
Inflection on day 4				
1 (Control, days 0-4)	0.0620	0.0106	Group 1 vs 3	$p > 0.1$
2 (Control, days 5-14)	0.0411	0.0020		
3 (Infected, days 0-4)	0.0450	0.0127	Group 2 vs 4	$p > 0.1$
4 (Infected, days 5-14)	0.0451	0.0023		
Inflection on day 5				
1 (Control, days 0-5)	0.0597	0.0071	Group 1 vs 3	$p < 0.05$
2 (Control, days 6-14)	0.0403	0.0025		
3 (Infected, days 0-5)	0.0360	0.0098	Group 2 vs 4	$p > 0.1$
4 (Infected, days 6-14)	0.0455	0.0029		
Inflection on day 6				
1 (Control, days 0-6)	0.05893	0.00501	Group 1 vs 3	$p < 0.05$
2 (Control, days 7-14)	0.04095	0.00319		
3 (Infected, days 0-6)	0.03535	0.00694	Group 2 vs 4	$p > 0.1$
4 (Infected, days 7-14)	0.04786	0.00334		

The body weight curves for the control and *Y. enterocolitica*-infected piglets are shown in Figure 3.2. The data for body weight were from 5 control and 5 infected piglets which were continuously studied from birth to day 14. The linear regression was calculated for each group for the different assumed points of inflection (days 4, 5 and 6). The slopes of the regression lines for control and infected groups for each point of inflection were tested using a *t*-test.

Appendix 5

BIOCHEMICAL ASSAYS

5.1. Protein
(Based on Lowry *et al.*, 1951)

REAGENTS:

A: 2% Na_2CO_3 in 0.1 N NaOH

0.1 N NaOH	
NaOH	4 g
Distilled water	1 l
2% Na_2CO_3	
Na_2CO_3	20 g
0.1 N NaOH	1 l

B: 0.5% $CuSO_4.5H_2O$ in 1% Na-K-Tartrate

1% Na-K-Tartrate	
Na-K-Tartrate (BDH Chemicals Ltd., Poole, England)	1 g
Distilled water	100 ml
0.5% $CuSO_4.5H_2O$	
$CuSO_4.5H_2O$	0.5 g
1% Na-K-Tartrate	100 ml

C: Mix 50 ml (A) and 1 ml (B) before use

D: Dilute Folin & Ciocalteu's Phenol reagent (BDH Chemicals Ltd., Poole, England) 1:1 in distilled water

PROCEDURE:

1. Make standard protein solutions, concentrations 0, 125, 250, 500, 750 and 1000 µg/ml with bovine serum albumen (Fraction V, Boehringer Manheim, Germany).
2. Take 0.2 ml aliquots of standards or mucosal homogenate (1:200)
3. Add 2 ml reagent C, vortex and allow to stand for 10 minutes
4. Add 0.2 ml reagent D, vortex and allow to stand for 30 min
5. Read the absorbance against the blank at 500 nm for the standards and samples using a Spectrophotometer (SP 6-550 UV/Vis, PYE nicam Ltd., Cambridge, England)
6. Calculate the protein concentration from the standard curve.

5.2. DNA and RNA
(Based on Burton, 1956; Flech and Begg, 1965; Giles and Myers, 1965)

REAGENTS:

1. 0.9 % NaCl
2. Perchloric acid (BDH Chemicals Ltd., Poole, England) solutions

Perchloric acid concentration	0.2 N	0.6 N	1.0 N	1.2 N
60% Perchloric acid (g)	33.5	100.5	167.4	200.9
Distilled water (ml)	966.5	899.5	832.6	799.1

3. Standard DNA stock solution containing 0.2 mg/ml
   DNA (Herring Sperm, Boehringer Mannheim, Smith-Biolab Lt., Auckland, NZ) 0.2 g
   1.0 N perchloric acid 100 ml
4. 4% diphenylamine in glacial acetic acid
   Diphenylamine (Sigma Chemical Company, USA) 4 g
   Glacial acetic acid (BDH Chemicals Ltd., Poole, England) 100 ml
5. 0.16% Acetaldehyde
   Acetaldehyde (BDH Chemicals Ltd, Poole, England) 160 mg
   Distilled water 100 ml
6. 0.3 N KOH
   KOH 16.8 g
   Distilled water 1 l

EXTRACTION of DNA and RNA:

1. Place 1 ml of 1:50 homogenate in a tube, mix with 1 ml of cold 0.6 N perchloric acid, stand in ice for 10 min, then centrifuge at 2000 rpm for 10 min.
2. Discard the supernatant and wash the precipitate twice with 2 ml of 0.2 N perchloric acid, centrifuge at 2000 rpm for 10 min, discard the supernatant.
3. Dissolve the precipitate in 2 ml of 0.3 N KOH, incubate at 37 ^{0}C for 60 min.
4. Add 1 ml of 1.2 N perchloric acid and stand the tubes in ice for 10 min, then centrifuge at 2000 rpm for 15 min.
5. Collect the supernatant, wash the precipitate once with 2 ml of 0.2 N perchloric acid, then centrifuge at 2000 rpm for 15 min. Collect the supernatant (total about 5 ml) for RNA measurement.
6. Dissolve the pellet in 4 ml of 1.0 N perchloric acid and heat in a boiling water bath for 10 min, then centrifuge at 2000 rpm for 20 min. Collect the supernatant for DNA measurement.

Determination of RNA:

1. Read the optical density (OD) of the RNA extracts at OD_{232} and OD_{260} with a Spectrophotometer (SP 6-550 UV/Vis, PYE Unicam Ltd., Cambridge, England).

2. Calculated the RNA concentration using the formula (Fleck and Begg, 1965):
   C_{RNA} (µg/ml)=(3.4 OD_{260} - 1.44 OD_{232}) / 0.068
   C_{RNA} (mg/g tissue)= C_{RNA} (µg/ml) / 4 (mg tissue/ml)

Determination of DNA:

1. Prepare a series of standard solutions containing 0, 10, 20, and 40 µg/ml of DNA in 1.0 N perchloric acid.
2. Add 1 ml of 4% diphenylamine in glacial acetic acid to 1.0 ml standard or sample solution, then add 0.1 ml of acetaldehyde aqueous solution and incubate in a water-bath at 30 °C for 17 hours.
3. Read the OD of the standard and the samples at OD_{595} with Spectrophotometer (SP 6-550 UV/Vis, PYE Unicam Ltd., Cambridge, England).
4. Calculate the DNA concentration:
   DNA concentration (mg/g tissue)=DNA (µg/ml)/ 5 (mg tissue/ml)

Any tissue DNA extracts with a concentration over 40 µg/ml should be further diluted with 1.0 N perchloric acid and re-assayed.

5.3. Disaccharidase
(Based on Dahlqvist, 1964, 1984)

REAGENTS

1. Phosphate buffer (0.1 M, pH 6.0)
   A: $NaH_2PO_4.2H_2O$ 31.2 g
   Distilled water 2 l
   B: $Na_2HPO_4.12H_2O$ 35.8 g
   Distilled water to 1 l
   mix A, 87.7 ml with B, 12.3 ml.
2. Standard glucose stock solution (10 mM)
   0.27% benzoic acid
   Benzoic acid (May & Baker, Australia Pty Ltd., Victoria) 0.27 g
   Distilled water 100 ml
   10 mM glucose stock solution
   D(+)-Glucose (May & Baker, Australia Pty Ltd., Victoria) 0.18 g
   0.27% benzoic acid 100 ml
   Store the solution at -20 °C in 2 ml aliquots. Thaw completely and mix well, then dilute 1:10 in phosphate buffer (0.1 M, pH 6.0) to give a 1 mM glucose solution before use (Dahlqvist, 1984). Make a series of glucose standard solutions of 0, 125, 250, 500, 750 and 1000 µM.
3. Disaccharide solutions
   A. 0.1 M lactose:
   α-Lactose (Sigma Chemical Company, USA) 10.8 g
   0.1 M phosphate buffer 300 ml
   B. 0.1 M sucrose:
   Sucrose (May & Baker Ltd., Dagenham, England) 10.3 g
   0.1 M phosphate buffer 300 ml

C. 0.05 M maltose:

Maltose (Sigma Chemical Company, USA)	5.1 g
0.1 M phosphate buffer	300 ml

Store the solutions at -20 °C in 50 ml aliquots. Thaw completely and mix very well before use.

4. Tris-glucose-oxidase (TGO): This solution is stable for several days in the refrigerator.

A. Tris buffer (0.5 M, PH 7.0)

Tris (hydroxymethyl)-aminomethane (Serva, Feinbiochemica Gmbh & Co.)		61 g
5N HCl		85 ml
Distilled water	to	1 l

B. 0.1% peroxidase solution (stored at -20 °C)

Peroxidase (Serva, Feinbiochemica, Heidelberg, New York)	20 mg
Distilled water	20 ml

C. 1% o-Dianisidine solution (kept in the dark. After a time it becomes dark brown because of oxidation, when it should be discarded).

o-Dianisidine (Sigma Chemical Company, USA)	1 g
95% ethanol	100 ml

D. 20% Triton X-100 (BDH Chemicals Ltd., Poole, England)

Triton X-100	20 ml
95% ethanol	80 ml

TGO: dissolve glucose oxidase in Tris buffer first, then add the other solutions.

Glucose oxidase (No.G-6125, Sigma Chemical Company, USA)		56 mg
or		
Glucose oxidase (236 U/mg solid, United States Biochem. Co.)		2.3 mg
0.1% peroxidase solution		5 ml
1% o-Dianisidine solution		10 ml
20% Triton X-100		10 ml
Tris buffer	to	1 l

PROCEDURE

1. Mucosal homogenates 1:50, 1:100 or 1:200, depending on the specific enzyme activity in the homogenate, and disaccharides (0.1 M lactose or sucrose, 0.05 M maltose) for lactase, maltase and sucrase assays. Set up two tubes per sample, one for the test and another for the blank.
2. To 0.2 ml of PBS (0.1 M, pH 6.0), add 0.1 ml of the homogenate and 0.2 ml of disaccharide solution.
3. Incubate the test tubes in a water bath at 37 °C for 20 min and place the blank tubes, after mixing, in boiling water for 2 min.
4. Make a glucose standard series containing 0, 125, 250, 500, 750, and 1000 µmol D(+)-glucose.
5. Add 2 ml of TGO to both standards and samples, incubate in the water bath at 37 °C for 1 hour.
6. Read the OD at 436 nm using a spectrophotometer.
7. The amount of glucose liberated by a specific disaccharidase is calculated from the regression formula of the glucose standard curve.
8. The enzyme activity is expressed as micromoles (µmol) disaccharidase per gram wet mucosal tissue per min, and obtained from the following formula:

1% metol

Metol	1 g
3% sodium bisulphite	100 ml

PROCEDURE

1. Set up 2 tubes for each homogenate, one with 0.98 ml of incubation medium, the other with medium containing in addition 0.2 mM ouabain. Add 20 µl of tissue homogenate (1:20) to each tube and incubate in a 37 ^{0}C water bath for 15 min.
2. Plunge tubes into an ice-water bath, and then add 0.5 ml of 8% cold perchloric acid in distilled water (w/w) to stop the reaction. Keep tubes in ice for at least 10 min.
3. Make a standard phosphorus (KH_2PO_4) series containing 0, 125, 250, 500, 750, and 1000 µmol phosphorus.
4. Assay inorganic phosphate (P_i) produced from samples during the reactions:
   a) Place 0.5 ml aliquots of the reaction mixture or standard solutions in tubes
   b) Add 0.5 ml of molybdate-sulphuric reagent to the standard or test tubes or inhibited tubes (from incubation medium with ouabain) and thoroughly mix
   c) Add 0.1 ml of 1% of metol solution to each tube
   d) After 45 min at room temperature read the absorbance at 700 nm.
5. Calculate Na^+-K^+-ATPase activity from the formula:
   Na^+-K^+-ATPase activity = 6 (A-B) (µmol/h.g tissue)
   or 6 (A-B)/C_p (µmol/h.mg protein)

where:

A = P_i concentration in the medium without ouabain tubes (µmol/l)

B = P_i concentration in the medium with ouabain tubes (µmol/l)

C_p = protein concentration (mg/g tissue)