CHAPTER 1

INTRODUCTION

Poultry and poultry products are sometimes involved in human salmonella infection (Bhatia *et al* 1979; Nurmi 1980; Todd 1980) as they are frequently contaminated with salmonella (Bolder *et al* 1980). Salmonella potentially pathogenic for man are often carried asymptomatically in the intestinal tract of chickens (Williams 1978; Barnes *et al* 1980b). Based on the salmonella-host relationship, salmonella are generally grouped into three categories (Robinson 1970):

1. Strict human pathogen: e.g. *S. typhi*;

2. Predominantly host-adapted although spread to other species has been recorded: e.g. *S. paratyphi B* in man; *S. dublin* in cattle; *S. pullorum* and *S. gallinarum* in poultry;

3. Non-host adapted, having the potential to infect mammals, birds, reptiles and arthropods; e.g. *S. typhimurium*.

The host-adapted salmonella constitute less than 1% of approximately 1700 serovars (Pivnick and Nurmi 1982). Most serovars show little host specificity and can cause gastrointestinal disease, salmonellosis, when ingested by man (Fagerberg and Avens 1976).

Salmonella, other than S. gallinarum and S. pullorum, are designated paratyphoid organisms when they infect poultry, and the diseases they cause in chickens, paratyphoid infections (Hungerford 1969; Williams 1978).

Several factors make it extremely difficult to eradicate paratyphoid infections:

1. their wide host range (Williams 1978 and Davey 1979),
2. their ability to remain viable in the environment for long periods (Tucker 1967; Woodburn and Stadelman 1968; Zeccha et al 1977; Williams and Benson 1978),
3. their ability to be egg transmitted by both ovarian and shell contamination (Snoeyenbos et al 1970; Snoeyenbos et al 1971; Bullis 1977),
4. the difficulty to detect infection (Snoeyenbos et al 1969; Williams and Whittenmore 1976; Weinack et al 1979).

Paratyphoid infection is primarily a gut infection and as such often results in chickens which though carriers, show no clinical signs of the disease. Most chickens that have received an initially heavy infection with paratyphoid organisms rid themselves of infection within one or two months (Milner and Shaffer 1952; Sadler et al 1969; Lloyd et al 1977). Due to improved growth rates, broilers in Australia are now processed at an earlier age - 6 to 7 weeks - instead of the previous 10 to 14 weeks and as a result infected broilers do not have time to rid themselves of infection. These birds act as a significant source of contamination to other carcasses during processing (Morris et al 1968; Woodburn and Stadelman 1968). Dougherty (1974) indicated that the

number of broiler chickens entering a processing plant harbouring salmonella is generally low but that mechanical spread of the organisms may result in 90% infected carcasses.

Based on the success of the S. pullorum eradication program considerable effort has been directed towards methods for the elimination of salmonella carriers with strict hygiene and eradication techniques being suggested by many workers (Snoeyenbos et al 1970; Williams 1978). However such measures to date have been unsuccessful (Zeccha et al 1977) and the incidence of enteric salmonellosis in poultry flocks and salmonella food poisoning in humans is apparently still increasing (Taylor 1967; Green et al 1982). Todd (1980) considers that increased consumption of poultry has resulted in an increase of poultry associated foodborne disease, particularly, salmonellosis.

The use of chemotherapy in prevention of salmonellosis is of limited value. Some workers have shown that the use of certain antibiotics increases the sensitivity of chickens to infection by altering the normal microflora in the intestine (Olesiuk et al 1973; Rantala and Nurmi 1974; Smith and Tucker 1975a).

The development of the intestinal flora in young animals normally takes place in an environment where the maternal intestinal flora constitutes the main source of microbes. An exception is in the modern poultry industry where the natural transfer of microbes from hen to chicken is broken (Lloyd et al 1977). The Finnish workers, Nurmi and Rantala (1974) were the first to suggest that the introduction of an "adult" microflora to young chickens would aid in the control of salmonella infections. The principle they established was to inoculate day-old chickens with intestinal or caecal washings from healthy adult

birds. When challenged with salmonella 24 to 72 hours later, such pretreated chickens are relatively far more resistant to salmonella challenge than control birds. Since the first publication of Nurmi and Rantala (1973) their work has been confirmed and extended by themselves and other research groups around the world (Lloyd *et al* 1977; Snoeyenbos *et al* 1977, 1979; Barnes *et al* 1980b; Impey *et al* 1982).

The use of unidentified intestinal contents from adult birds may, however, be spreading bacterial, viral or mycoplasma pathogens, for example, *Arizona sp.*, infectious bronchitis, infectious bursal disease. The use of specific isolates would avoid this problem. Soerjadi (1979) isolated 2 caecal bacteria, *Streptococcus faecalis* and *Bacteroides fragilis*, that, when given to day-old chicks in monocultures, gave a high degree of protection from salmonella challenge.

The experiments reported in this thesis are an attempt to isolate other caecal bacteria from healthy adult chickens and to assess their potential in reducing salmonella infection in young chickens.

CHAPTER 2

REVIEW OF THE LITERATURE

With the advance of medicine and technology the majority of the serious infectious diseases of man have been brought under control. These diseases are now vaccinated against or have reached the point where they have been virtually eradicated in most advanced nations. Attention is now focused on those diseases that result in economic loss due to high medical care costs and lost productivity of those inflicted. One of these diseases is salmonellosis. Salmonella are among the most widely distributed pathogens associated with disease outbreaks in all types of poultry, as well as other animal species and man (Edwards and Miller 1967; Frost and Rieman 1967; Merson et al 1974; Bullis 1977). Although their role in causing disease has been recognised since the last century, their implications for public health have only become apparent in recent times. This increase in awareness of the salmonella problem has resulted in accelerated research activities and increasing numbers of publications, meetings and conferences. Public health agencies as well as food processors are searching for solutions to the salmonella problem.

This review covers the following aspects of the relationship between salmonella and the poultry industry:

1. Paratyphoid infection;
2. Industry and public health aspects of paratyphoid infection;
3. Transmission of paratyphoid within a poultry organisation;
4. Prevention and control of paratyphoid infections;
5. Competitive exclusion.

2.1 PARATYPHOID INFECTION

2.1.1 THE DISEASE

Though adult chickens may be carriers of salmonella the disease caused by paratyphoid infection is usually only exhibited by young chickens under 4 weeks of age. Even then it is usually precipitated by concomitant stresses such as the presence of other infections, or by food, water or heat deprivation (Williams 1978). Clinical symptoms of paratyphoid infection include the following: a progressive state of somnolence evidenced by a tendency to stand in one position with head lowered, eyes closed, wings drooping and feathers ruffled; a marked anorexia and increased water consumption; a profuse and watery diarrhoea with pasting of the vent; and a tendency of the birds to huddle together near the source of heat (Williams 1978). However, clinical signs are often not evident even in chickens which have necrotic foci in their livers, swollen kidneys and fibronous peritonitis (Sadler et al 1969). The infection may result in mortality which is encountered most frequently during the first 2 weeks after hatching and rarely after 4 weeks, surviving birds may be stunted (Williams 1978). The occurence of the disease in breeding stock may impair fertility, hatchability and egg production and because of its chronic nature and

difficulty of eradication may lead to the termination of breeding operations (Williams 1978).

2.1.2 THE CAUSAL ORGANISM

The genus Salmonella, within the family Enterobacteriaceae, is composed of more than 1700 serovars (Bergey's Manual 1974; Bullis 1977) with S. typhimurium the most common serovar reported (Taylor 1967; Smith 1967; Stevens 1971). Salmonellae are gram-negative, non-spore forming, aerobic rods, most of which are peritrichously flagellated and naturally inhabit the intestinal tract of humans and many other animals and have world wide distribution (Fagerberg and Avens 1976; Davey 1979).

Salmonellae can be readily cultivated on initial isolation from sources other than faeces on simple beef extract and beef infusion agars and broths (Williams 1978). Optimum growth temperature is 35°-37°C (Fagerberg and Avens 1976). Stock cultures can be maintained in paraffin corked plain agar stabs for many years without transplanting (Williams 1978).

Both antigenic and biochemical properties of salmonellae are required for the identification of the salmonellae serotypes. Most of the serovars of salmonellae possess both the 'O' (somatic) and 'H' (flagellar) antigens (Kaufffmann 1969). Based on the presence of these antigens, the salmonellae are classified using the Kauffmann White Scheme (Kauffmann 1969, Edwards and Ewing 1972; Cruickshank et al 1975).

The biochemical properties of salmonellae are listed in Table 2.1.

Table 2.1
The biochemical properties of Salmonellae
(modified from Williams, 1978)

Type of Tests		Results
Motility		Positive
BIOCHEMICAL		
Deaminase:	Phenylalanine	Positive
Decarboxylase:	Arginine	Positive, usually delayed
	Lysine	Positive
	Ornithine	Positive
Gelatin		Rarely liquefied
H_2S		Usually positive
Indole		Not produced
KCN		Negative
Malonate		Negative
Methyl Red		Positive
Nitrates		Reduced
Simmons citrate		Usually utilized
Urea		Not hydrolysed
Voges Proskauer		Negative
Fermentation:	Glucose	Fermented with gas
	Lactose	Not fermented
	Maltose	Fermented with gas
	Sucrose	Not fermented
	Adonitol	Not fermented
	Dulcitol	Usually fermented with gas
	Inositol	Fermented or not
	Mannitol	Fermented
	Sorbitol	Fermented with gas
	Salicin	Not fermented

2.1.3 FACTORS AFFECTING THE SUSCEPTIBILITY OF CHICKENS TO SALMONELLA CHALLENGE.

Several factors are known to affect the susceptibility of chickens to salmonella challenge. These are:

2.1.3.1 AGE -

Resistance to salmonella infection increases rapidly with age (Milner and Shaffer 1952; Perry *et al* 1972). Sadler *et al* (1969) reported that an inoculation dose of 10 cells of *S. typhimurium* induced infection in 100% of 2 day old chicks, 66% of 7 and 14 day old chicks and 0% in 4 and 8 week old birds. Rigby and Pettit (1979) observed that not only did the infection level drop with age but in addition fewer salmonella were detected in the caecae and faeces of infected chickens.

Age has to be considered in relation to competitive exclusion. Age plays an important role in the succession of microorganisms in the gut of chickens reared without mothers (Pivnick and Nurmi 1982) and therefore it may be the acquisition of organisms rather than age *per se* that is responsible for the increase in resistance as the chickens age. Studies with conventional and germ free chickens are indicated to clarify the relationship between age, microbial succession and resistance to salmonella challenge. Age, whether influenced by microbial succession or not, is a very important factor influencing the susceptibility of chickens to salmonella challenge.

2.1.3.2 SEROVAR OF INGESTED SALMONELLA -

Henderson et al (1960) administered 7 different serovars orally in graduated doses to inbred White Leghorn day-old chicks. All serovars produced mortality varying from 2% for S. anatum, 3% for S. reading, 55% for S. seftenberg, 7% for S. heidelberg, 13% for S. enteritidis, 17% for S. indiana and 80% for S. typhimurium. Vestal and Stephens (1966) reported that none of the 9 serovars that they studied for pathogenicity could be considered non-pathogenic.

In addition to differences in pathogenicity of serovars there are differences with the strain of serovar used. For example, Soerjadi (1979) inoculated 4-day-old chicks with 10^4 cells of 6 different isolates of S. typhimurium to determine the virulence of the isolates. He found that one isolate produced an infection rate of 57% while infection rates for the others varied from 10 to 24 percent.

Thus the serovar of the ingested salmonella is very important in influencing the susceptibility of challenged birds. Some serovars producing low infection rates whilst others produce high infection rates. Within the serovar different strains produce different infection levels.

2.1.3.3 DOSE LEVEL OF INGESTED SALMONELLA -

Milner and Shaffer (1952) inoculated day-old chicks orally with S. typhimurium and obtained positive cloacal swabs 1 to 3 days post-inoculation in 5% of those given 1 - 5 organisms, 51% for 10, 88% for 10^2, 95% for 10^3, 97% for 10^4, and 100% for 10^5. Several breeds and strains of chickens tested showed no significant differences in susceptibility to infection by the oral route. Sadler et al (1969)

found that the level of intestinal infection as evidenced by faecal shedding of viable salmonella was correlated with inoculum dose. Lloyd et al (1977) recorded 8% and 37% of chickens subsequently shedding S. typhimurium when orally dosed with either 10^3 or 10^6 cells of S. typhimurium respectively. Soerjadi (1979) reported a 13% infection rate when day-old chicks were dosed with 10^2 cells of S. typhimurium as compared with 75% infection rate when chicks were dosed with 10^6 cells.

Both Lloyd et al (1977) and Soerjadi (1979) showed that resistance to salmonella challenge could be overcome by a massive challenge (10^8 cells of S. typhimurium). It appears that resistance decreases as dose level of ingested salmonella increases until a threshold is reached at which the resistance of the chicken is overcome.

2.1.3.4 STRESS FACTORS -

Chickens are more susceptible to salmonella challenge when it is associated with a concomittant stress. For example, Stephens et al (1964) recovered S. typhimurium from the livers and spleens more frequently from chicks infected concurrently with coccidiosis than from chicks given S. typhimurium alone. In addition, salmonella was eliminated from the intestinal tract less readily when chicks were infected with coccidiosis (Stephens and Vestal 1966). Brownell et al (1969) found that water deprivation did not increase infection rate but increased the period over which the salmonella were detected. They also found that intramuscular injection of Escherichia coli increased infection rates and the period over which the birds shed S. typhimurium. Thaxton et al (1971 and 1974) found that lowered brooding temperatures significantly increased the level of mortality caused by S. worthington infection in newly hatched chicks.

Rigby and Pettit (1979) report that subjecting 9 and 13 week-old chickens to "transport stress" (crowding, chilling and food and water deprivation) did not increase shedding or detectable infection. It is likely that the stresses involved were not sufficient to actually stress birds of this age.

Soerjadi (1979) reported that 70% of chicks kept at 18°-22°C after being dosed with 10^3 cells of salmonella were detected as being carriers compared with 0% in chicks kept at 32°-36°C. When challenged with 10^8 cells of S. typhimurium 80% of the chicks were detected as being carriers in both groups (hot and cold). Bhatia and McNabb (1980) observed that feed and water deprivation of day-old chicks increased shedding of salmonella.

Hence stress or stresses such as concomittant diseases, low or high temperatures, food or water deprivation often make the chickens more susceptible to salmonella challenge. In addition, there is probably an association between age, stress and salmonella resistance but more work is needed to clarify this area.

2.2 THE INDUSTRY AND PUBLIC HEALTH ASPECTS OF POULTRY SALMONELLOSIS

The presence of salmonella in poultry products is regarded as a public health threat (Snoeyenbos 1971). Although occassionally the production efficiency of chickens may decline significantly as a result of paratyphoid infection exacerbated by a concomittant stress, this loss does not appear to be large enough to compensate for probable control costs (Snoeyenbos 1971; Williams 1978; Pivnick and Nurmi 1982). Any control effort must, in large measure, be justified by public health

considerations.

In spite of numerous publications on the salmonellae and their effects on various hosts, few countries can assess accurately the annual toll of human illness or economic losses in livestock as a result of these infections (Taylor 1967; Robinson 1970; Merson et al 1974). Attempts are being made at both international and national levels to remedy this deficiency, particularly as food-borne salmonellosis in countries with high standards of living and hygiene is becoming a very real problem (Robinson 1970). Increasing mass food preparation, inadequate storage facilities, a trend towards eating raw or insufficiently cooked foods, increasing international trade, increasing public awareness of salmonellosis, increasing human and animal populations, improved methods of isolating the organism and special research projects in the field of salmonellosis have all been cited as contributing factors to the apparent increase of salmonellosis (Robinson 1970; Brownlie and Davey 1979). In Britain the annual incidence recorded in 1970 was between 5,000 and 10,000 cases (Smith 1971). In USA the Centre for Disease Control (CDC) estimates that salmonella infects more than two million Americans each year resulting in 500,000 hospitalisations, thousands of deaths and an annual cost of $1.5 billion in medical expenses (CDC 1978). These figures were based on the assumption that only one in ten cases of salmonellosis are reported.

In Australia, Sutton (1973) calculated that the incidence of human salmonellosis was approximately 2,000 cases per year, and assuming the same degree of under-reporting as had been reported in the USA, it was suggested that about 20,000 to 2,000,000 cases occur each year. With a population of approximately 14 million, such a level of infection indicates a food-borne disease of considerable public health

significance (Brownlie and Davey 1979). Direct transmission of salmonella to man from live poultry has been reported occassionally (Williams 1978). Usually, however, transfer to man has been from poultry meat and poultry by-products. Salmonella which may be present on poultry meat are destroyed on cooking but the danger comes from reinfection of the cooked meat or introduction of the bacteria into the kitchen. This occurs if the water from the uncooked chicken is allowed to come into contact with kitchen implements or surfaces and then is transmitted back onto the cooked carcass or onto other foods in the kitchen. If the cooked meat carrying a salmonella is left at room temperature instead of being refrigerated, the salmonellae multiply rapidly to a food poisoning dose of hundreds of thousands of organisms (Bullis 1977; Barnes 1982). Simple precautions such as ensuring that the water from the uncooked chicken is disposed of, washing of implements and placing the cooked meat into the refrigerator are adequate to prevent contamination by salmonella.

Though simple hygiene procedures would ensure that salmonella contaminated poultry posed no problems in the domestic kitchen it would be adverse to the marketing of poultry to emphasise the danger of salmonella contamination. Control of salmonella in poultry must be sought either at the production level or at the processing plant .

2.3 TRANSMISSION OF SALMONELLA WITHIN A POULTRY UNIT

Salmonella are introduced or transmitted within a poultry unit in several ways. These are through:

2.3.1 EGG TRANSMISSION

In considering the spread of infections through the medium of eggs, it is important that a distinction be drawn between direct ovarian transmission, when the ovary itself is infected, and transmission through organisms penetrating the shell usually from intestinal sources of salmonella.

Several workers (see Williams 1978) have recovered S. typhimurium from the ovaries of ducks and from the yolk of their eggs, providing evidence that direct ovarian transmission is quite common in this species, the yolks being infected as a result of localisation of S. typhimurium in the ovary. Ovarian transmission also occurs in turkeys, but to a lesser extent than in ducks. It is a rare occurence in chickens as far as the paratyphoid organisms are concerned (Williams 1978).

Schalm (1937) demonstrated that S. typhimurium in faecal material smeared on the surface of chicken eggs was capable of penetrating the shell and multiplying within the egg. Buxton and Gordon (1947) found that the pores in the egg shell would permit shell penetration by salmonella organisms under favourable conditions. They also noted that S. thompson could readily penetrate the shell of eggs stored at 37°C but penetration was less common in eggs stored at room temperature. Mundt and Tugwell (1958) were unable to recover salmonella from the contents of eggs laid by White Leghorn pullets experimentally infected orally and intravenously with various types of paratyphoid organisms.

Using food artificially contaminated with S. menston as the starting point, Gordon and Tucker (1965) demonstrated the complete cycle of salmonella infection in that S. menston was shown to be present in

the adult fowls to which contaminated food was fed, in the eggs laid by these fowls, the chicks hatched from these eggs and the eggs laid by these progeny at maturity. A similar cycle has been demonstrated with S. thompson (Buxton and Gordon 1947). In both reports it was not demonstrated whether transmission was from ovarian infection or from organisms penetrating the shell.

Forsythe et al (1967) were unable to establish a localized infection of the reproductive tracts of hens by direct ovarian inoculation with S. anatum. None of the eggs produced by such hens were contaminated with S. anatum. Williams et al (1968) found that the penetration of S. typhimurium through all outer structures of chicken eggs could occur as early as 6 minutes using eggs incubated at 37.2°C. Defects in shell structure rather than shell thickness determined the degree to which salmonella could penetrate into eggs, while moisture aided the penetration process. Sauter and Peterson (1974) reported a progressive decrease in penetration of chicken eggshells by salmonella as eggshell quality increased from low to intermediate to high.

Snoeyenbos (1971) states that it seems clear that egg transmission, as a result of either transovarian infection or of shell penetration, occurs with sufficient frequency to require that control efforts start at the breeder and multiplier flock level.

Williams (1978) who has given an informative review on the sanitization procedures for eggs, considers that faecal contamination of eggshells with paratyphoid organisms during the process of laying or from contaminated nests, floors or incubators after laying is of foremost importance in the spread of the disease.

Hence egg transmission, through contaminated material on the outside of the egg, happens with sufficient frequency to warrant special management procedures to prevent its occurence.

2.3.2 FEED TRANSMISSION

Feed for poultry is usually compounded from cereals, protein rich material and other nutrients in smaller amounts. The protein component is usually rendered from inedible material from slaughtered animals, animals or fish unfit for human consumption or it is the residue of seeds from which oil has been extracted. Although these materials are processed under conditions which destroy salmonella, sanitation and other elementary preventive measures are sometimes inadequate and the finished products may be recontaminated with salmonella (Pivnick and Nurmi 1982). Fish meal and meat and bone meal are considered to be the main sources of salmonella contamination of feed (Mulder 1980).

Though the level of salmonella contamination in poultry feeds is normally low, it has been shown that infection can result from one organism per gram of feed (Gordon and Tucker 1965). More recent investigations have shown that even one organism per 15 g of feed can produce infection (Harry and Brown 1974). Seuna (1979) suggested that in many cases a single organism could probably infect a chicken.

Morris et al (1969) traced serovars identified in broilers slaughtered at 5 - 7 weeks to similar serovars identified in the feed of these broilers. Zeccha et al (1977) reported the isolation of several serovars of salmonella from pelleted feed. Four of these serovars were subsequently found in the turkeys at slaughter suggesting the role of the feed in introducing infection.

Bolder et al (1980), Lindgren et al (1980) and Mulder (1980) all consider that feed is the most important factor in the infection of poultry with salmonella. However, legislation in their countries for poultry production requires very high standards of housing, management and specific procedures for monitoring for salmonella. Positive broiler flocks are destroyed and the premises cleaned and disinfected and checked to be negative before restocking. Under these conditions feed contamination may assume a greater importance than in other countries where other sources of environmental contamination are more frequent.

2.3.3 ENVIRONMENTAL TRANSMISSION

Rats and mice are frequent carriers of salmonella and their droppings may readily contaminate feed supplies (Goyal and Singh 1970; Williams 1978). Pigeons, sparrows and various other species of wild birds may also serve as sources of salmonella infection for domestic poultry flocks (Snoeyenbos et al 1967; Goyal and Singh 1970). These animals can serve as reservoirs of salmonella on farms and cause cleaning and sanitation procedures to fail because of immediate reinfection (Raevuori et al 1978).

Zeccha et al (1977) considered personnel and equipment as well as wild animals and birds to be significant sources of salmonella contamination. Fresh straw litter has been shown to be contaminated by salmonella (Kumar et al 1971; Simmons and Byrnes 1972; Zeccha et al 1977; Bhatia et al 1979; Bolder et al 1980).

Snoeyenbos et al (1970) reported that circumstantial evidence strongly indicated residual pen contamination was frequent following depopulation, cleaning and disinfection. In some pens identical serovars were isolated from litter samples from 3 consecutive

generations. Transporting boxes from processing plants may also spread salmonella from farm to farm (Raevuori et al 1978).

At the hatchery contaminated eggshells and other debris such as fluff, can also serve as a source of egg contamination in the incubator (Williams 1978). In the force draught incubator the organisms may be distributed by air currents throughout the hatcher and a high level of airborne infection created. Air and dust samples in the hatchery may be found to contain salmonella organisms for several weeks following an outbreak of infection, and as such, will be a source of infection for subsequent hatches. Bhatia and McNabb (1980) reported that when fluff and/or meconium were contaminated with salmonella at the hatchery, litter and carcasses were contaminated with the same serovars at the time when the chickens were processed.

Environmental transmission therefore occurs through litter, feed, wild animals, human attendants, residual pen contamination and at the hatchery. The extent of the contamination will depend on whether wild animals and birds have access to poultry units and whether previous batches of chickens were contaminated.

2.4 PREVENTION AND CONTROL OF SALMONELLOSIS IN A POULTRY ORGANIZATION

2.4.1 MANAGEMENT

Management will be considered under the same headings as in the section on transmission of paratyphoid organisms in a poultry organization.

2.4.1.1 Egg Transmission -

Salmonellosis, being an intestinal infection, results in the occassional shedding of salmonella in the faeces of infected birds, and as such will represent a considerable threat to the production of salmonella free eggs. Long used nest litter may be the most heavily contaminated area in a building, Snoeyenbos et al (1970) report that salmonella were isolated 7 times as often from unchanged nest litter as from unchanged floor litter. To decrease chances of salmonella transmission nest sites should be clean and litter changed frequently.

Snoeyenbos et al (1970) consider if hatching eggs are handled with appropriate sanitary precautions, including surface sanitizing within 30 minutes of laying, the percentage of egg transmission is usually so low that substantial sized groups of uninfected chicks can be secured from infected breeding flocks.

2.4.1.2 Feed Transmission -

The majority of researchers consider that heat applied during pelleting is usually sufficient to destroy bacteria in the feed but recontamination generally occurs during cooling or storage of the feed. However, Bolder et al (1980) consider that pelleting by means of water or steam does not produce enough heat to destroy all vegetative bacteria. Programmes to reduce salmonella contamination of feed have been effective in only a few countries, e.g. Denmark, Sweden, and more recently, Finland (Pivnick and Nurmi 1982). In most other countries there appears to be no economic benefit to renderers to produce a salmonella free product. Technically it is simple to decontaminate feed or individual feed ingredients by treatment with heat, radiation, gas or chemicals (Mulder 1980). During pelleting the pellets reach a

temperature of about 70°C and therefore cooling is necessary in salmonella free air - it is often difficult to have filtered air available and the feed is subject to recontamination with salmonella organisms (Mulder 1980).

Incentive to feed producers to ensure that feed is not recontaminated with salmonella is needed before salmonella-free feed will be produced.

2.4.1.3 Environmental Transmission -

Wild birds, vermin, personnel and equipment were all found to be possible vectors for introducing salmonella into poultry. Heard (1969) advised that poultry housing should be designed to be both rodent and bird proof. Snoeyenbos (1971) noted that eliminating salmonella from contaminated buildings and from infected stock in successive generations is the most challenging part of the salmonella control effort in poultry.

There is some evidence to suggest that rearing broilers on old litter results in a lower carrier rate at slaughter age. Botts et al (1952) were the first to note that used litter had an averse effect on salmonella survival. Tucker (1967) reported longer survival times of salmonella in new litter than old. There are several reports that chickens reared on old litter are less likely to become infected with salmonella (Tucker 1967; Olesiuk et al 1971, Duff et al 1973). This area needs further investigation and could prove to be another management tool to reduce salmonella infection in chickens.

Zeccha et al (1977) described a 5-year epizootiological study of salmonella infections in a new, quarantined commercial primary turkey breeding ranch (Dillon Beach Project). Salmonella were apparently introduced into the flock through feed and personnel traffic and could be readily detected in the birds by culture and serologic tests. Lindgren et al (1980) report from an 8 year study on a program for avian salmonella control in Sweden that it was not possible to discern any comprehensible pattern suggestive of the importance of environmental or managemental factors facilitating or counteracting salmonella organisms to gain foothold in poultry houses. The relatively few instances where true reinfections occured happened as frequently in shining new, purpose built poultry houses with all sorts of facilities for spotless hygiene, including first class management, as in old remodelled cowsheds offering ample facilities for salmonella survival.

Because of its wide host range and its ability to remain viable for long periods of time in the environment the application of hygiene and sanitation has reduced, but not eliminated the transmission of salmonellosis in poultry units. It still remains a significant problem.

2.4.2 CONTROL OF SALMONELLA AT THE PROCESSING PLANT

Methods evaluated to eliminate or control salmonella in the processing plants have included a wide range of physical, chemical and combined treatments applied during or after processing. Most methods have suffered one of four problems (Gwatkin and Murrel 1982):

1. The treatments are relatively ineffective. This is because they do not produce a significant reduction of the organisms hidden within the complex microtopography of the skin. The treatments do not penetrate the skin either because they are

prevented by physical forces e.g. hydrophobic repulsion, or, because the chemicals react rapidly with skin components and are hence rendered ineffective, e.g. chlorine.

2. Dubious or unknown safety of the proposed chemicals along with associated legal problems.

3. The deleterious effect the proposed treatments may have on the organoleptic properties of the chicken. This occurs where excessive heat treatments cause a yellowing of the skin particularly over fatty deposits. Some of the food acids also affect skin appearance and cause skin and flesh toughening.

4. The proposed process is too costly, either from the point of view of running costs, or the initial capital outlay required.

In-plant hygiene is sufficient to prevent build-up of salmonella from day to day but is inadequate to prevent spread of salmonella from one consignment to another during the processing day (Morris and Ayres Morris and Wells 1970; Timoney et al 1970), the salmonella serovars isolated can change from day to day. It is now recognised that salmonella must be eliminated from the live bird since processing cannot be relied upon to free the carcass from food poisoning organisms (Mead 1974; Watson and McQueen Brown 1975).

2.4.3 CONTROL OF SALMONELLA IN THE CHICKEN

2.4.3.1 Immunization -

As salmonellosis is primarily a gut infection vaccination is not likely to be of any significant benefit. Bacterins and attenuated live cultures for use as vaccines in the prevention of avian paratyphoid infections have been studied experimentally but have never had wide application under field conditions (Williams 1978). McCapes et al (1967) reported the use of 4 isolates of S. typhimurium in the preparation of an experimental whole broth aluminium hydroxide adsorbed bacterin for vaccination of turkey hens. Poults receiving the bacterin exhibited measurable resistance to yolk sac challenge with both S. typhimurium and S. schwarzengrund but not with S. anatum. Challenge of poults from S. typhimurium vaccinated dams produced 39.7% mortality compared with 85.6% in the controls. They concluded that further challenge trials were needed to be conducted to fully ascertain the range of poult protection obtained through the use of single serotype vaccination of dams. Knivett and Stevens (1971) found that when day-old chicks were orally vaccinated with a live attenuated strain of S. dublin and subsequently challenged with S. typhimurium, the growth of the challenge organism was considerably reduced or eliminated from the livers of the vaccinated chicks. Knivett and Tucker (1972) reported that once a salmonella pathogen is established in the intestine of a chicken neither vaccination with live salmonella cultures administered in the water nor furazolidone therapy is effective. This type of vaccination did not significantly reduce the number of salmonella carriers. Truscott (1981) reported that salmonella were cleared more rapidly from chicks treated with multivalent antigen derived from 6 strains of salmonellae, than from untreated control chickens.

While the trials cited indicate some resistance to paratyphoid infection can be stimulated by vaccination, as it is a gut and not a systemic infection it is unlikely that such practices will ever have a place in programs aimed at complete elimination of salmonella from poultry flocks.

2.4.3.2 Chemotherapy -

As salmonellosis is primarily an intestinal infection antibiotics have not been used extensively in the treatment of paratyphoid infection in poultry. High levels of some antibiotics given in the feed or water may suppress salmonella infections in flocks, but do not eliminate them and when the antibiotic treatment is terminated salmonella are again excreted (Smith 1955; Garside et al 1960; Barnes and Goldberg 1962; Nurmi 1980; Williams and Whittenmore 1980). Smith (1955) suggested that since salmonella were often found in large numbers in the alimentary tract and that very little furazolidone could be shown in the lower bowel of chickens this could be the reason for the high carrier rate after furazolidone withdrawal.

Some growth promotants, for example, avoparcin, have been shown to increase the salmonella infection rate (Smith and Tucker 1980). Several workers found an increase in antibiotic resistance in intestinal organisms with the use of certain antibiotics (Garside et al 1960; Barnes and Goldberg 1962; Smith and Tucker 1975a,b). In Britain, following the report on antibiotic resistance by the Swann Committee (Report 1969), legislation was introduced in March 1971 prohibiting the routine use of 'therapeutic' antibiotics, except sulphonamides, as feed additives for growth promotion in animals. Sulphonamides were exempted because it was considered that their use as a feed additive was

essential for prophylaxis of coccidiosis in poultry.

Therefore antibiotics or drugs, though useful in decreasing mortality if this should be occurring are not generally used for the control of salmonella in poultry. Disadvantages of use are the resulting high carrier rate and possible drug resistance.

2.4.3.3 COMPETITIVE EXCLUSION (CE) -

2.4.3.3.1 INTRODUCTION -

From the previous sections it was shown that though efforts are being made to reduce the incidence of salmonella contamination in the poultry industry these efforts are not particularly successful. It appears that irrespective of the standard of housing (Zeccha et al 1977; Lindgren et al 1980) salmonellae are able to gain access to the environment of both broiler and laying stock and from there infect future generations of birds and act as a serious threat to the quality of the finished product. In addition, it has now been accepted that salmonella must be eliminated from the live bird since processing cannot be relied upon to free the carcass from food poisoning organisms. Davey (1975) considers that in-plant chlorination can cope with an input of less than 20% of infected birds but levels above this may result in up to 100% contamination of the finished carcass.

Clearly efforts should be continued to decrease contamination in both the live bird and in the finished product by means of increased hygiene and prevention of salmonella transmission. Additionally, however, the live bird must be made more resistant to salmonella challenge so that if it does come into contact with salmonella then it has some form of resistance against it. Competitive exclusion provides

this resistance against salmonella challenge in the live bird.

The use of competitive exclusion for increasing the resistance of chicks to salmonella challenge has only been discovered in the last decade. It is the use of the conventional indigenous microflora of an animal or bird to inhibit the establishment of alien opportunistic organisms such as the enteric pathogen, S. typhimurium. It is not a complete answer to the problem of paratyphoid infection in poultry as resistance can be overwhelmed by a massive salmonella challenge but when used in conjunction with other management practices it should help decrease the number of infected birds arriving at processing plants to a level where the in-plant chlorination could cope. The mode of action of competitive exclusion is not fully known but the production of volatile fatty acids in the caecae and the occupation of sites on the caecal mucosa are considered to be important factors (Pivnick and Nurmi 1982).

The concept of competitive exclusion was appreciated first in mice when Bohnhoff et al (1954) reported that oral administration of 50 mg of streptomycin per mouse resulted in a lowering of resistance to salmonella challenge and concluded that this was due to changes in the components of the intestinal flora. Bohnhoff and Miller (1962) reported that resistance to enteric salmonellosis was quickly restored to streptomycin-treated mice by introducing faecal material, or fresh anaerobic cultures of faeces or colon content, from untreated mice. Miller and Bohnhoff (1963) found that isolates of Bacteroides spp. taken from the large bowel of untreated mice were partially effective in restoring resistance in streptomycin-treated mice.

Nurmi and Rantala (1973) applied the concept to newly hatched chicks. They found that by giving the newly hatched chicks diluted gut contents from adult cockerels they were able to not only decrease the number of chicks that were positive for salmonella compared with control chicks but also to decrease the severity of infection in those chicks that did become infected. They concluded that the abnormally hygienic conditions under which broilers are produced hampers the development of the intestinal flora which normally participates in defence against pathogenic bacteria.

Lloyd et al (1977) consider that such practices as incubating eggs away from adults and rearing the newly hatched chicks on fresh litter away from adult birds, disinfection of shell surfaces, rearing chickens on wire-floored brooders and feeding of heat-treated rations, all contribute to denying or delaying the newly-hatched chicken the necessary inoculum for the establishment of a conventional indigenous microflora.

Since the Finnish work was published the protective ability of adult flora has been confirmed by many other workers (Idziak and Caldwell 1977; Lloyd et al 1977; Snoeyenbos et al 1977 and 1979; Soerjadi et al 1978; Rigby and Pettit 1980; Barnes et al 1980a,b; Stersky et al 1981; Impey et al 1982).

2.4.3.3.2 EFFECT OF PRETREATMENT ON GROWTH RATE -

Snoeyenbos et al (1979) reported that in limited tests with 2 or 3 sources of protective microflora the growth rate of chicks in the absence of salmonella was significantly improved when compared with control chicks. Nurmi and Schneitz (1980) reported that under controlled conditions chickens given unidentified mixed cultures of

caecal flora showed significantly better growth rates than untreated chickens. In addition they report no adverse results on growth rate from the four years that the cultures have been used in grower flocks. Rigby and Pettit (1980) found no difference in growth rate when using protective cultures. Pivnick et al (1981) found no evidence of harmful effects or decreased weight gains or feed conversion due to use of faecal cultures.

Frazer (pers.comm.) from his work on S. faecalis found that 100 times the normal dose level (10^8 cells) of S. faecalis had no deleterious effect on bodyweight or food conversion ratio of broiler chickens. In fact, the pretreated chickens frequently outgrew the control birds.

From the work cited above it is evident that use of CE is not detrimental to the growth rate of chickens, often, it appears to be to the advantage.

2.4.3.3.3 SOURCES OF MATERIAL FOR PRETREATMENT -

Nurmi and Rantala (1973) originally used diluted gut contents from adult commercial cockerels for their pretreatment material but then found (Rantala and Nurmi 1973) that anaerobically cultured flora of the alimentary tract was as good. In addition they reported that culturing the flora in oxygen immeditately destroyed the protective effect of the culture. Bowman et al (1976) compared the protective value of intestinal material from bantams, hatched under hens for many generations in a 'backyard' environment, to that of commercially reared chickens and found the former considerably more effective than the latter. Snoeyenbos et al (1979) report the establishment of a flock of specific pathogen free chickens which has yielded a microflora of

consistently high protective value during several years of testing. Rigby and Pettit (1980) found that lyophilised extract of breeder litter or an anaerobic culture of this extract was protective.

An important consideration when using donor birds or unidentified cultures is that they should be free of other bacterial pathogens, parasites, viruses and mycoplasma organisms.

2.4.3.3.4 TIMING OF PROTECTION -

Timing of protection is important as the newly hatched chicks may come into contact with salmonella in the hatchery or when first placed in the growing sheds. In the original experiments by Nurmi and Rantala (1973) the chicks were challenged 24 hours after pretreatment. Seuna (1979) reported that chicks given an anaerobic culture of adult intestinal flora were nearly as resistant to infection one hour after receiving the pretreatment as two week old chicks. In addition he reported that treatment given several hours after challenge with salmonella was found to be ineffective. However Snoeyenbos et al (1979) in trials involving larger numbers of birds and sampling for longer periods found that treatment with intestinal microflora significantly abbreviated the period of infection when introduced after a salmonella infection was established in chicks.

Soerjadi et al (1981a) reported that protection following introduction of native gut microflora began within 2 hours post treatment, and optimum protection was established 32 hours post treatment.

2.4.3.3.5 METHOD OF APPLICATION OF PROTECTIVE BACTERIA -

In the original work by Nurmi and Rantala (1973) protective cultures were given to each bird. Several workers have since shown that protection was as good as per os inoculation when the protective bacteria was diluted in drinking water (Snoeyenbos et al 1977; Pivnick et al 1981 and Impey et al 1982). For commercial use mass application of protective microflora is necessary. Administration of live cultures in drinking water requires assurance that disinfected drinkers are free of disinfectant, and that tap water is free of chlorine and toxic metals that may inactivate the bacteria. One possible disadvantage of placing the protective culture in the drinking water is that newly hatched chicks do not drink immediately (Cumming pers.comm.) and thus the culture may be inactive before the chicks drink.

Snoeyenbos et al (1979) reported that the protective bacteria that they used readily transferred to penmates and apparently to birds in adjacent pens. Frazer (pers. comm.) from his work on the use of Streptococcus faecalis in salmonella control found that the organism will transmit horizontally very readily between chickens in wire floored pens or on litter. He suggested that 10% or less of day-olds need to be inoculated to produce the maximum resistance possible.

2.4.3.3.6 SAFETY MEASURES IN USING PRETREATMENT MATERIAL -

The use of suspensions or cultures of intestinal contents of adult chickens has created concern that pathogenic organisms might also be transferred. Pivnick et al (1981) report that after four serial subcultures and dilution in drinking water a faecal culture was still protective. This preparation ensured that less than 1×10^{-11} g of donor faeces was administered per chick and that protozoa, viruses and

mycoplasma were unlikely to be present. Numerous bacterial pathogens could, however, grow in the culture medium. The establishment of a specific pathogen free flock that were donors of microflora of consistently high protective value by Snoeyenbos et al (1979) appears to offer a solution to the problem of possible transference of pathogenic organisms. Such flocks are expensive to maintain but could be kept at a central location and utilised by many growers. However one disadvantage of this system is the difficulty in registering it with government bodies. Regulations pertaining to the use of vaccines etc are very strict and this type of 'black box' approach is unlikely to be allowed registration.

The ultimate in safety would be the development of a known mixture of specific organisms that was fully protective. This mixture could be marketed in the same way as the present day vaccines and be reconstituted in disposable plastic packs made anaerobic by a disposable anaerobic kit. The reconstituted product could be inoculated per os to a small percentage of day-old chicks by normal hatchery staff. Impey et al (1982) reported that a mixture of 48 isolates from the caecal microflora of an adult bird conferred protection to the same degree as that obtained previously with a suspension of adult caecal contents. This large number of isolates appears unnecessary in the light of the report by Soerjadi et al (1978) and Soerjadi (1979) on the isolation of two caecal bacteria, Streptococcus faecalis and Bacteroides fragilis, that on their own were able to provide protection similar to fresh faecal material. Although statistically similar the level of protection afforded by these bacteria individually was never quite as good as that provided by faecal material.

A smaller number of organisms would allow much greater efficiency of handling at the hatchery and in addition be much more likely to pass strict registration requirements.

2.5 RESEARCH UNDERTAKEN IN THIS THESIS

The experiments in this thesis were aimed at isolating other caecal bacteria that when used alone or in conjunction with S. faecalis or B. fragilis would provide a level of protection to day-old chickens equal to that of fresh faecal material. It was envisaged that only 2 or 3 additional caecal bacteria need to be added to S. faecalis and B. fragilis for the protection to equal that of fresh faecal bacteria. Based on the hypothesis that birds brought up under a hen and having access to maternal intestinal bacteria would be more likely to have strains of bacteria adapted specifically to the environment of the chicken gut, feral birds were used as donors for bacterial isolates.

CHAPTER 3

MATERIALS AND METHODS

3.1 CHICKENS

In all experiments day-old White Leghorn-Black Australorp crossbred cockerels were used. Originally chickens were obtained from a commercial hatchery in Tamworth which was serologically negative for Salmonella pullorum, Mycoplasma gallisepticum and Newcastle disease. However due to contamination problems with Citrobacter freundii they were subsequently obtained from a Sydney hatchery that was serologically negative for Salmonella pullorum. Chickens from the Tamworth hatchery were debeaked prior to dispatch.

On arrival at the laboratory the chickens were either toe punched or wing-tagged for identification. Unless stated otherwise chickens were randomly divided into groups of 20 and 2 groups were allocated per treatment.

3.2 HOUSING AND MANAGEMENT

For the experiments in Chapters 4, 5 and 6 wooden isolation pens measuring 3.6 m x 1.8 m x 1.8 m high with solid floors and wire mesh covered open fronts were used as the basic units. The pens, ten in all,

were arranged in two lines of four and one line of two down a paddock facing north, with at least 20 m between pens. For experiments 4.1 and 4.2 the chickens were housed on litter on the floor of the pens. They were contained by a 25 cm high, 1.5 m diameter aluminium surround. Heat was supplied by a commercial brooding hood.

In Experiment 4.3 some of the birds were housed on the floor and the remainder suspended 45 cm from the floor in conventional wire carry-on cages. Each pen contained three cages, measuring 180 cm x 75 cm x 45 cm and divided into two equal compartments. A sheet of cardboard with litter on it was placed on the floor of each cage and removable aluminium partitions 20 cm high were fitted along the sides. Heat was supplied by a 100 watt light globe suspended in the middle of each compartment. Replicated treatment groups were placed in cages with one empty cage separating each replicate. The same cages were used in all pens to minimise positional effects.

From Experiment 7.1 one or two temperature controlled rooms, approximately 4.5 m x 5.0 m were used. The temperature was maintained at 17±2°C for the initial 6 experiments after which it was raised to 20±2°C. The chickens were housed in Ryan all-metal experimental brooders, each deck, measuring 150 cm x 90 cm x 25 cm, divided into four equal compartments of 75 cm x 45 cm fitted with individual feed and water troughs and with a central electrical heating area. Heaters were turned on to high for the first three days of an experiment after which they were turned off.

During all experiments chickens were given tap water and feed ad libitum. Unless specified a commercial starter crumble which was free from antibiotics was supplied. The following mixture was used when feed was mixed at the university:

Ingredient	Percentage
Crushed sorghum	70
Soybean meal	11
Sunflower meal	11
Meatmeal	8

A standard vitamin-mineral premix was included at recommended levels.

At the end of every experimental period in the isolation pens the chickens were killed and the cardboard and litter removed. The metal surrounds of the cages were removed and soaked in water overnight after which they were scrubbed, hosed then soaked overnight in a disinfectant solution (Deogen-Diversey, Pty Ltd). During this time the pens were swept and completely hosed out. Cleaning procedures for the temperature controlled rooms were similar. The brooders were dismantled, the feeders, drinkers and sides of the compartments soaked in water, scrubbed and then soaked overnight in a disinfectant solution. The wire floors were cleaned by steam while the frames of the brooders were wiped with a disinfectant solution. The rooms were swept and the floor and walls hosed.

One in ten of all experimental birds were vaccinated for Infectious Bronchitis (IB) by eyedrop if any of the groups were to go into a room or isolation pen that had held chickens involved in an IB experiment less than one week previously.

3.3 SALMONELLA TYPHIMURIUM CULTURES

A Salmonella typhimurium culture, originally obtained by Soerjadi (1979) from Dr A R B Jackson from the Regional Veterinary Laboratory, Armidale, was freeze dried in ampoules according to the method described by Annear (1962). To resuscitate a salmonella culture, a few drops of nutrient broth (NB) (Oxoid) were added to the opened ampoule and left for 30 minutes at 37°C (Annear 1962). A loopful of this culture was then plated onto 2 nutrient agar plates (Oxoid) and incubated for 24 hours at 37°C. The plates were then checked for purity and the colonies tested biochemically and serologicaly according to Cruickshank et al (1975), as described in section 3.9. A colony was then transferred to sterile cooked meat medium (Oxoid) in a McCartney bottle and further incubated for 24 hours at 37°C. These bottles were then stored at 4°C in the refrigerator for not longer than two months after which they were discarded and a new freeze-dried ampoule opened.

The day before infecting chickens, a loopful of the S. typhimurium culture from the cooked meat medium was transferred into sterile nutrient broth (Oxoid) in Bijou bottles and incubated at 37°C for 24 hours. To estimate the number of viable S. typhimurium cells per ml of broth a McFarlands standard (Campbell et al 1963) was used. This reading was periodically checked against a dilution plate count (Collins 1968). This material was diluted in NB to the required concentration and used to orally infect the chickens. Unless otherwise stated approximately 10^6 cells of S. typhimurium were given orally to each chicken.

To develop nalidixic acid resistance in the Armidale strain of S. typhimurium the following procedure was followed:

1. 5 ml amounts of selenite broth containing 100 mcg sodium naladixate per ml were inoculated with salmonella and incubated at 37°C for 24 hours. Cultures with visible turbidity were plated out onto brilliant green agar (BGA) containing 100 mcg sodium nalidixate per ml.

2. Isolated colonies from the BGA plates were selected and reinoculated into selenite broth containing 100 mcg of sodium nalidixate per ml and incubated at 37°C for 24 hours. Cultures with visible turbidity were again plated out onto BGA containing 100 mcg sodium nalidixate per ml.

3. Individual colonies from the BGA plates were then freeze dried for storage or inoculated into cooked meat medium (Oxoid) and kept for immediate use as described above.

3.4 ORIGIN OF DONOR BIRDS

The backyard birds were bantams that were reared in the backyard of a private house and were from several generations of such birds. The feral birds had been captured on North-West Island (latitude 24°S, longitude 150°E), a uninhabited subtropical island off the coast of Queensland, Australia. They are descended from birds that were brought onto the island more than 80 years ago (McBride et al 1969). Since their arrival at the university the feral birds were kept on litter in complete isolation from other adult birds. In order to preserve the original feral chickens fifty day-old White Leghorn-Black Australorp crossbred cockerels were obtained from a local hatchery and orally dosed

with a 4% solution of faeces from feral chickens (Section 3.4). These birds were then kept in the same shed as the ferals and used whenever feral caecal material was required.

3.5 PREPARATION OF FAECAL MATERIAL FOR USE AS A TREATMENT

In several experiments faecal material from either 'backyard' birds or feral birds was tested. To collect faeces the donor birds were placed overnight in a wire cage suspended above a layer of plastic sheeting. The next day the faeces were scraped from the sheet into a sterile plastic container and diluted 4% in saline. The mixture was then strained through sterile glass wool and the filtrate used to orally dose the experimental birds.

3.6 ORIGIN, ISOLATION AND STORAGE OF TEST ORGANISMS

Test organisms were isolated from the caecae of two to twelve month-old White Leghorn-Black Australorp crossbred cockerels that had been dosed at day-old with a 4% solution of faeces from feral chickens (See Section 3.5). These birds were reared on litter in the same isolation shed as the feral birds and fed on commercial starter crumbles.

The isolation method for the test organisms was that developed by Soerjadi (1979). When required, donor birds were sacrificed by cervical dislocation and the caecae aseptically removed, homogenised and diluted 4% in saline. This homogenate was then divided into 3 equal portions and either kanamycin, polymixin B or penicillin G at 100 mcg/ml added. These antibiotics were shown by Soerjadi (1979) not to destroy the protective effect of caecal material and were included in the isolation media to inhibit other non-protective bacteria. These mixtures were

left for two and a half hours at room temperature and continually stirred with magnetic fleas. Reinforced clostridial medium (RCM, Oxoid) agar plates, supplemented with 100 µg/ml of one of the above antibiotics was used as the plating medium. From each mixture 0.02 ml was spread on the appropriate antibiotic supplemented RCM agar plate.

These plates were incubated in an anaerobic jar, in an atmosphere of 90% H_2 and 10% CO_2, for 48 hours at 37°C. After incubation bacterial colonies which appeared to be morphologically different were selected from each plate. They were transferred using a VPI anaerobic unit (Holdeman and Moore 1975) into 25 ml tubes containing 20 ml of sterile RCM broth and fitted with butyl rubber stoppers. During transfer the tubes were gassed with medical grade carbon dioxide that was passed over a column of heated copper to remove any traces of oxygen (Hungate 1969). These cultures were incubated for 48 hours at 37°C. They were then freeze dried using a modification of the method used by Barnes and Goldberg (1962). In the modified method the tubes were centrifuged for 15 minutes at medium speed and then the supernatant poured off. One ml of a 16% (w/v) solution of glucose was added to the precipitate and this was then distributed rapidly to ampoules and freeze dried overnight. When required the cultures were resuscitated in 20 ml of gassed RCM broth and incubated for 48 hours at 37°C.

3.7 IDENTIFICATION OF TEST ORGANISMS

Test organisms were not identified further than gram stain and shape and these are given below:

TABLE 3.1
Identification of test organisms.

Number of culture	Gram Stain	Shape	Source
AC1	+	rods	backyard
AC2	+	rods	backyard
AC3	+	rods	backyard
AC4	+	rods	backyard
AC5	+	rods	backyard
AC6	+	rods	feral
AC7	+	rods	feral
AC8	-	rods	feral
AC9	+	rods	feral
AC10	+	rods	feral
AC11	+	rods	feral
AC12	+	cocci	feral
AC13	+	cocci	feral
AC14	+	rods	feral
AC15	+	cocci	feral
AC16	+	cocci	feral
AC17	+	cocci	feral
AC18	+	cocci	feral
AC19	+	rods	feral
AC20	-	rods	feral
AC21	-	rods	feral
AC22	+	rods	feral
AC23	+	rods	feral

3.8 ESTABLISHMENT OF CARRIER STATUS

The establishment of salmonella in the intestinal tract of each bird was assayed by either cloacal swabbing (Lloyd et al 1974) or caecal culture.

CLOACAL SWABBING

The cloaca of each chicken was swabbed with a cotton bud which had been boiled in distilled water for 2-3 minutes. Each swab was immediately placed into 10 ml of enrichment broth and incubated for 24 hours at 37°C. Selenite broth (Oxoid) which was supplemented with sulphapyridine at 1 g/l was used as the enrichment broth. After

incubation a loopful of culture from the selenite broth was then streaked onto a brilliant green agar (BGA) plate (Oxoid), similarly supplemented with sulphapyridine, and further incubated at 37°C for 24 hours. Non-lactose fermenting colonies which resembled colonies of salmonella were transferred onto triple-sugar iron agar slants (Oxoid) for detecting hydrogen sulphide production and into urease broth (Oxoid) for detecting hydrolization of urea. Colonies which produced H_2S but did not hydrolyse urea were subjected to further biochemical and serological tests for S. typhimurium (see Section 3.9). Four swabs were taken, one every second day after the chickens were challenged with salmonella. Any chicken which died after challenge with salmonella were post-mortemed and the caecae removed aseptically and cultured for salmonella. At the end of each experiment, all chickens from which S. typhimurium had been isolated only once were killed and the caecae cultured for salmonella.

CAECAL CULTURE

One replicate of each treatment group were killed by cervical dislocation on the tenth and eleventh day after challenge with salmonella. The caecae were removed from each chicken and surface sterilized by dipping in boiling water for 15 seconds, after which they were placed in 20 ml of selenite broth and macerated with sterile scissors. The procedure was then the same as for the swabs in selenite broth.

3.9 IDENTIFICATION OF S. TYPHIMURIUM

Apart from the gram stain and colony morphology, S. typhimurium was identified with slide agglutination tests using polyvalent O, polyvalent H and group specific antisera, i.e. 4.0 and iH (Cruickshank et al

1975).

In addition, randomly selected isolates were tested with biochemical media (Cruickshank et al 1975) as follows:

Citrate utilization	-	positive
Glucose	-	positive
Growth in KCN	-	negative
Lactose	-	negative
Urease	-	negative
Voges Proskauer	-	negative

The preparation of the biochemical media and the procedures of the tests were carried out as described by Cowan and Steel (1974).

3.10 CRITERIA FOR SALMONELLA CARRIER BIRDS

Birds were considered as salmonella carriers if they were positive for at least two swabs and/or positive by caecal culture.

3.11 STATISTICAL ANALYSIS

The results were analysed by the use of Chi-square (Snedecor and Cochran 1967).

CHAPTER 4

USE OF FERAL FAECAL MATERIAL AND CAECAL ISOLATES AS PRETREATMENTS

INTRODUCTION

Barnes (1982) states that though there is a general similarity in the alimentary flora between animals there are important strain differences which are characteristic of the host. Work done by Bowman et al (1976) and Snoeyenbos et al (1977) showed that in turkeys and chickens there was some sharing of intestinal flora in that when day-old chickens were dosed with intestinal contents from adult turkeys they became more resistant to salmonella challenge and vice versa. Within species there may be differences in alimentary flora as, for example, there is evidence to indicate that caecal flora of various populations of chickens varies in its ability to increase the resistance of chickens to subsequent challenge by salmonella (Bowman et al 1976; Snoeyenbos et al 1977; Soerjadi 1979). Commercial chickens that are hatched in incubators and then placed on clean litter have not access to the range of bacteria that have been adapted to the environment of the chicken gut. The break in generations has been important in the control of some diseases e.g. Mycoplasma gallisepticum, but there is increasing evidence to suggest that for diseases like paratyphoid it has not been so advantageous.

Backyard or feral chickens that have been hatched under a hen have access to a stable gut flora adapted to the chicken gut. This flora would be different from that of commercial birds which have not had the contact with older birds. This difference in the gut flora would account for the differences in chicken populations in their abilty to be donors of caecal material of high protective value.

Soerjadi (1979) used birds that had been reared in the backyard of a private home for his work on competitive exclusion (CE). In this work there was access to feral birds (see Chapter 3) whose ancestors had been living on an island off the north coast of Queensland for more than eighty years. Their alimentary flora was the result of a succession through generations without alteration from modern antibiotics or drugs. It would have attained a very stable relationship with the host. The following experiments evaluated the ability of the faecal microflora of the feral chickens to increase the resistance of day-old chickens to subsequent challenge by S. typhimurium. Faeces from a backyard bird was included for comparison. In addition to faecal flora, different bacterial isolates from caecal contents were tested and two methods of housing investigated for their suitability for CE work.

Experiment 4.1a

Thirty day-old cockerels were divided into 3 groups of 10 and orally dosed with one of the following treatments:

1. .2ml sterile saline (control)
2. .2 ml of diluted fresh faecal material from a backyard bird (BYM)
3. .2ml of diluted fresh faecal material from a feral bird (FFM)

Each group was then placed on the floor in a separate isolation pen as described in Chapter 3. On day 4 each chicken was orally dosed with 10^5 cells of S. typhimurium.

Challenging, sampling (by cloacal swabbing) and identification of S. typhimurium were carried out as described in chapter 3.

Experiment 4.1b

The first experiment (4.1a) was repeated with 30 chickens per group.

Experiment 4.2

This experiment was designed to repeat the above experiments and to compare two systems of housing and five anaerobic bacterial cultures which had been isolated from a 'backyard' bird. The cultures were AC1, AC2, AC3, AC4 and AC5 (see Chapter 3).

Three hundred and thirty day-old cockerels were randomly divided into groups of 30 and orally dosed with one of the following treatments:

1. Sterile RCM broth (Control) -housed on floor (floor)
2. Sterile RCM broth - housed in a cage (cage)
3. BYM - floor
4. BYM - cage
5. FFM - floor
6. FFM - cage
7. Anaerobic Culture No. 1 (AC1) - floor
8. Anaerobic Culture No. 2 (AC2) - floor
9. Anaerobic Culture No. 3 (AC3) - floor
10. Anaerobic Culture No. 4 (AC4) - floor
11. Anaerobic Culture No. 5 (AC5) - floor

On day 4 each chicken was orally dosed with 10^5 cells of S. typhimurium. Challenging, sampling (by cloacal swabbing) and identification of salmonella were carried out as described in Chapter 3.

RESULTS

Experiment 4.1a

These are presented in Table 4.1 and Figure 4.1. Treatment of day-old chicks with faecal material from the backyard or feral donors significantly ($P < 0.001$) reduced the number of salmonella carriers from 100% to 0% and 13% respectively. The protection afforded by either the feral or the backyard birds was not significantly different.

Experiment 4.1b

These are presented in Table 4.1 and Figure 4.2. Treatment of day-old chicks with faecal material from the backyard or feral donor significantly ($P < 0.001$) reduced the number of salmonella carrier chickens from 77% to 3% and 0% respectively. The protection afforded by either the feral or backyard birds was not significantly different.

Experiment 4.2

These are presented in Table 4.2 and Figure 4.3. Treatment of day-old chicks with either BYM or FFM significantly ($P < 0.001$) reduced the number of salmonella carrier chickens. For floor control birds this reduction was from 57% to 4% and 0% respectively and for caged control birds it was from 83% to 3% and 3% respectively. Caged control birds had an significantly ($P < 0.05$) higher infection rate than did floor control birds. Cultures AC1 and AC2 had no significant effect on the number of salmonella carriers while AC3 and AC5 significantly ($P < 0.001$) decreased carrier rate and AC4 significantly ($P > 0.05$) increased it.

Table 4.1
Effect of orally treating day-old chickens with either sterile saline (control), faecal material from backyard (BYM) birds or faecal material from feral (FM) birds and subsequently challenging with 10^5 cells of S. typhimurium on day 4

Treatment	Salmonella carriers[a] Numbers	Overall(%)	Significance BYMvFM	BYMvFMvC
Experiment 4.1[a]				
Control	10	100		
BYM	0	0		
FM	1[c]	13	NS	***
Experiment 4.2[b]				
Control	23	77		
BYM	1	3		
FM	0	0	NS	***

a - 10 birds per treatment
b - 30 birds per treatment
c - 2 birds died before challenge

Table 4.2
Effect of orally treating day-old chickens with either saline (control), faecal material from backyard or feral birds or different bacterial isolates and subsequently challenging with 10^5 cells of S. typhimurium on day 4

Treatment	Salmonella carriers[a] Numbers	Overall(%)	Significance[b]
Control - floor	17	57	
Control - cage	25	83	*
BYM - floor	1[c]	4	***
BYM - cage	1	3	***
FM - floor	0	0	***
FM - cage	1	3	***
AC1 - floor	18[d]	64	NS
AC2 - floor	12	40	NS
AC3 - floor	4	13	***
AC4 - floor	24	80	*
AC5 - floor	5	17	***

a - 30 birds per treatment
b - All treatments were compared with control - floor
c - 1 bird died before challenge
d - 2 birds died before challenge

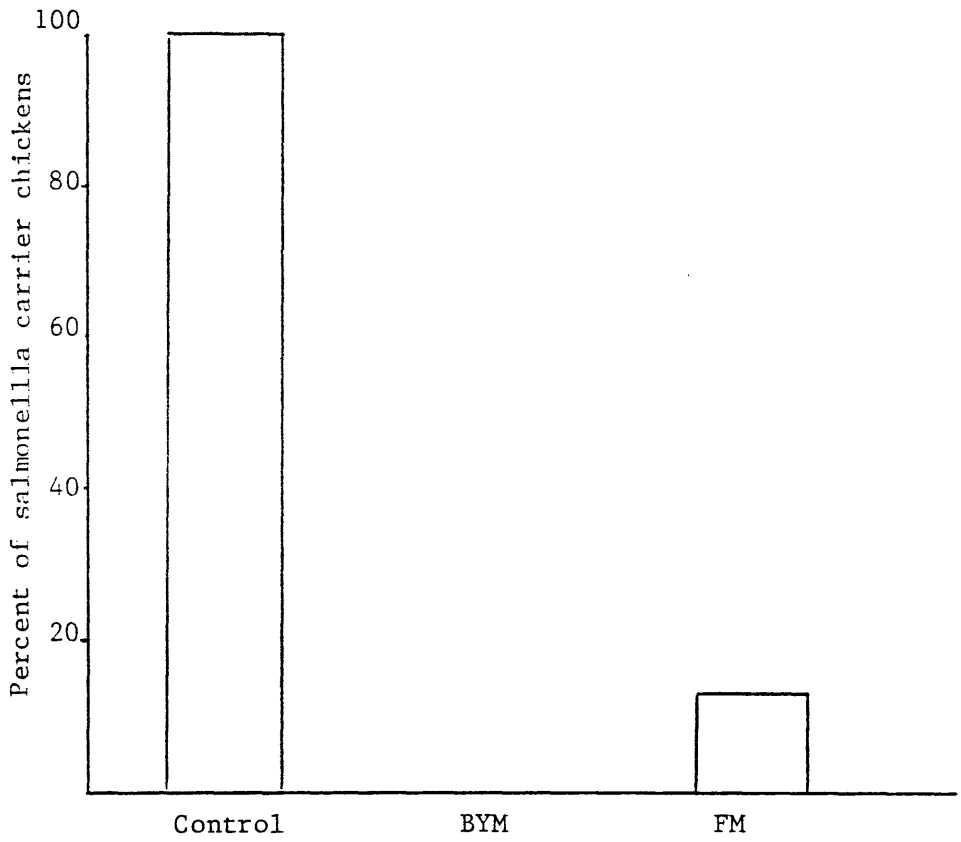

Figure 4.1

Effect of orally treating day-old chickens with either sterile saline (control), faecal material from backyard (BYM) birds or faecal material from feral (FM) birds and subsequently challenging with 10^5 cells of S. typhimurium

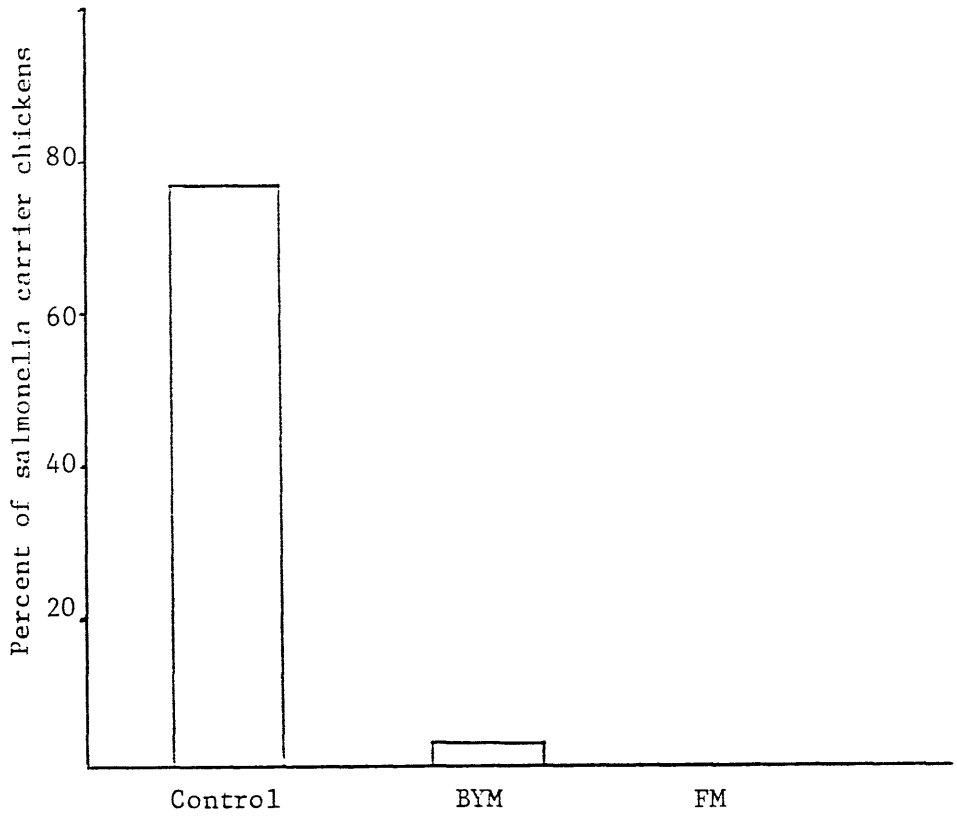

Figure 4.2

Effect of orally treating day-old chickens with either sterile saline (control), faecal material from backyard (BYM) birds or faecal material from feral (FM) birds and subsequently challenging with 10^5 cells of S. typhimurium

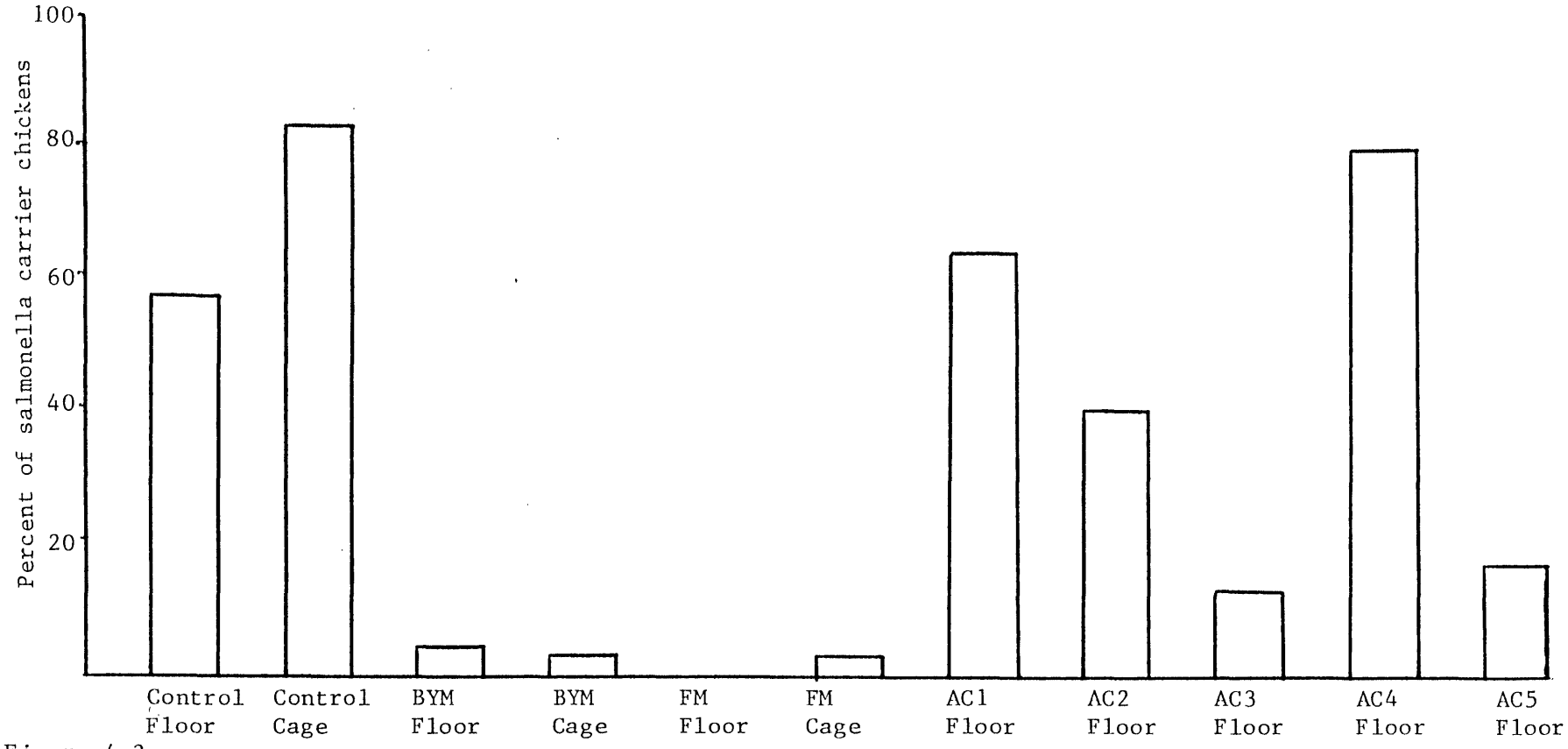

Figure 4.3

Effect of housing day-old chickens on the floor or in cages and orally treating them with either sterile saline (control), faecal material from bayckyard (BYM) or feral (FM) birds or different bacterial isolates and subsequently challenging with 10^5 cells of S. typhimurium on day 4

DISCUSSION

The results obtained confirmed that treatment of day-old chicks with faecal material from adult chickens increased their resistance to salmonella challenge. In all experiments the decrease in infection level of chicks dosed with faeces was significant at the 0.1% level. This demonstrated that the challenge system worked and that work could commence on isolating different bacteria from caecal or faecal material and testing them for their effect on infection levels.

Lloyd et al (1977) and Snoeyenbos et al (1977) considered that when dealing with biological control systems resistance could be overcome by a substantial challenge. In the experiments reported here the resistance afforded by treatment with either the feral or backyard faeces was substantial against challenge doses that resulted in very high infection levels in the control treatments. This effect emphasized the very high protective value of the faecal flora of both the feral and backyard chickens.

In the experiment comparing 2 systems of housing there was a significantly higher infection rate in the caged control group as compared with the floor control group. This may have been due to a slight temperature stress on the caged birds. The advantages of housing the birds in cages were:-

1. Use of cages allowed replication of treatments within isolation pens. Only one treatment group was able to be housed per pen when the birds were on the floor.

2. Birds housed on the floor were exposed to old litter that had lodged between the wooden floor boards. Use of cages prevented this.

3. Use of cages facilitated cleaning. All parts of the birds immediate environment could be cleaned thoroughly and soaked in a disinfectant solution.

Due to these advantages cages were used in subsequent experiments.

The results from the anaerobic cultures demonstrated the range of effects that can occur with the use of pure cultures of intestinal organisms. Firstly, AC1 and AC2 had no significant effect on the carrier rate indicating that on their own these organisms were not able to inhibit the salmonellae. Secondly, AC3 and AC4 were able to significantly reduce the carrier rate. Soerjadi et al (1978) and Soerjadi (1979) showed that when Streptococcus faecalis or Bacteroides fragilis were given orally to day-old chicks they significantly increased the chick's resistance to infection by salmonella. In a number of experiments level of protection induced by either S. faecalis or B. fragilis although statistically as effective as caecal contents was generally not as effective as that induced by caecal contents (Soerjadi 1979). Thirdly, the culture, AC5, significantly increased the carrier rate. Barnes et al (1980) reported that use of some pure bacterial cultures caused the most severe infections with numbers of salmonella 10 or 100 fold higher than those in the caecae of control chicks.

In subsequent experiments isolation of salmonella became very difficult due to an overgrowth on the brilliant green agar (BGA) plates of a gram negative organism, Citrobacter freundii. This organism, a member of the genus Enterobacteriaceae, covered the BGA plates in dense, green colonies and prevented and/or obscured the growth of salmonella. Subsequent work was concentrated on overcoming this obstacle.

CHAPTER 5

CONTAMINATION BY CITROBACTER FREUNDII

The appearance of C. freundii in the bacteriological media was relatively sudden. After several unsuccessful experiments due to its presence it was apparent that either the source must be located and eliminated or a means must be found to inhibit the organism in the bacteriological media if the work was to proceed satisfactorily.

Experiment 5.1

Location of Source of Citrobacter

To determine the origin of the citrobacter contamination the following steps were taken:

1. Three 5 g samples of the fresh woodshavings that were used for litter were cultured in 20 ml of selenite broth and plated onto BGA after 24 hours incubation at 37°C. After 24 hours incubation at 37°C the plates were examined for growth of citrobacter.

2. Ten BGA plates were left exposed to the atmosphere around the laboratory for 2 hours. They were then closed and incubated for 24 hours at 37°C after which they were examined for growth of citrobacter.

3. Swabs were taken of the following areas in three isolation pens:

1. 3 different positions on the floor
2. 3 different points on the cages
3. ceiling
4. 3 different points on horizontal timbers (noggins) inside the pens

The swabs were immediately placed in 20 ml of enrichment broth (selenite supplemented with sulphapyridine), incubated for 24 hours at 37°C and then streaked onto the surface of BGA plates. The plates were incubated for 24 hours after which they were examined for growth of citrobacter.

4. Twenty-five day-old chickens were obtained from the local hatchery (Tamworth) to determine if it was the origin of the contamination. They were sacrificed on arrival at the laboratory when 24 hours old and their caecae aseptically removed. These were cultured as described in Chapter 3 and the plates examined for growth of citrobacter. In addition three 5 g samples of the travelling box litter was cultured as described above.

RESULTS

The results are presented in Table 5.1. No citrobacter were detected in the woodshavings or from the laboratory. Six of the nine floor swabs and one surface swab taken in the pens were positive for citrobacter. Twenty-three of the twenty-five chickens were positive for citrobacter as was the travelling box litter.

DISCUSSION

From the above results it was evident that the chicks from the local hatchery introduced the citrobacter into the isolation pens. Though some citrobacter was detected in the isolation pens it was considered that this was due to a failure by the disinfection procedures applied to kill residual organisms rather than a source of the organism. The wooden floors of the isolations pens were difficult to clean due to the presence of cracks where litter could lodge. The presence of small numbers of citrobacter in the crevices of the isolation pens was not considered to be a problem as the chance of significantly contaminating the caged chickens was low.

As it was convenient to obtain chickens from the local hatchery means of inhibiting growth of citrobacter in the bacteriological media were investigated.

Table 5.1
Determination of the presence of C. freundii

Sample	Presence of Citrobacter[a]		
Litter			
1	ND		
2	ND		
3	ND		
Agar plates	ND		
	Pen a	Pen b	Pen c
Floor			
1	ND	++	++
2	ND	++	++
3	+++	+	ND
Light switch			
1	ND	ND	ND
2	ND	ND	ND
3	ND	ND	ND
Surface			
1	ND	ND	+
2	ND	ND	ND
3	ND	ND	ND
Ceiling	ND	ND	ND
Chickens	23/25[b]		
Travelling litter	3/3		

a. ND - not detected
+ - light growth of citrobacter
++ - moderate growth of citrobacter
+++ - heavy growth of citrobacter
b. No. positive/total tested

Experiment 5.2

This experiment was conducted to investigate whether by altering the enrichment broth, incubation temperature or plating media a means could be found to select against the citrobacter.

A loopful of culture from:

1. an overnight culture of C. freundii in nutrient broth (NB) (Oxoid)
2. an overnight culture of S. typhimurium in NB

were transferred into eight McCartney bottles containing either selenite broth supplemented with lg/l of sulphapyridine or tetrathionate broth (Oxoid). Half of the selenite and tetrathionate containing bottles were placed in an incubator set at 43°C, and the other half were placed in an incubator set at 37°. After overnight incubation these cultures were transferred onto 3 plates of the following agars:

1. Bismith Sulphide Agar (BS) (Oxoid)
2. Salmonella-Shigella Agar (SS) (Oxoid)
3. Desoxycholate Citrate Agar (XLD) (Oxoid)
4. Brilliant Green Agar (BGA) (Oxoid) containing either 1, 2, 3 or 4 g/l sulphapyridine.

Pure cultures of both C. freundii and S. typhimurium were streaked onto the different agars and incubated with the above as controls.

After overnight incubation at 37°C the plates were examined for growth.

RESULTS

Both the citrobacter and the salmonella grew on all the agars tested. No apparent competitive advantage was given to salmonella by incubating the enrichment broths at 43°C. No obvious differences were detectable in plates streaked from broth incubated at this temperature compared with that incubated at 37°C. In all mixed cultures it was difficult to isolate the salmonella from the citrobacter. The SS, BS and XLD agars appeared to offer no advantage over the BGA as the citrobacter grew profusely on them. Altering the level of sulphapyridine supplementation had no effect on citrobacter growth.

DISCUSSION

From the results of Experiment 5.2 it was decided that, though there were advantages in ease, economy and time in obtaining chickens locally, due to the contamination by citrobacter it was necessary to investigate other sources of chickens. It appeared that the media for isolation of salmonella also favoured the isolation of citrobacter. Additionally, even if a medium could be found that would inhibit the citrobacter, its presence in large numbers in the chicken gut could possibly interfere with the investigations as one of the mechanisms thought to be responsible for CE is the competition for attachment on the mucosa of the chicken gut. If the citrobacter is already attached then both the 'protective' cultures and the salmonella might be excluded from the gut.

Experiment 5.3

Different Sources of Chickens

Twenty day-old crossbred cockerels from 3 Sydney hatcheries were obtained. On arrival at the laboratory the chickens were sacrificed, their caecae removed and cultured as described in Chapter 3. After incubation the plates were examined for growth.

Experiment 5.4

Forty day-old crossbred cockerels from hatchery 1 were placed in an isolation pen in cages as described in Chapter 3. On day 4 they were orally challenged with 10^5 cells of S. typhimurium.

Sampling (by cloacal swabbing) and identification of S. typhimurium was carried out as described in Chapter 3. In addition plates were examined for the presence of citrobacter.

RESULTS

Experiment 5.3

These are presented in Table 5.2. No citrobacter were detected from hatchery 1 or 3. Twenty percent of the chickens from hatchery 2 were contaminated with citrobacter.

Table 5.2
Determination of a source of chickens that were free of citrobacter contamination

Source of Chickens	Citrobacter Carriers[a]
Hatchery 1	0
Hatchery 2	4
Hatchery 3	0

a - 20 birds tested per hatchery

Experiment 5.4

Twenty-five of the chickens were detected to be salmonella carriers. Though citrobacter was detected on 10 occassions in the 4 swabbings it was not detected in any one bird twice.

DISCUSSION

From the results of Experiment 5.3 it was found that either Hatchery 1 or 3 could be used for the supply of chickens. There was some contamination by citrobacter in hatchery 2 though the level of contamination was low. Experiment 5.4 revealed that there was some residual pen contamination by citrobacter in that it was detected on each swabbing. However as citrobacter was not detected twice in any one bird it was decided that this level of contamination was acceptable.

Soerjadi (pers. comm.) recommended the use of a nalidixic acid resistant strain of S. typhimurium to facilitate its isolation and as a possible means of inhibiting citrobacter and other contaminants. The method that Soerjadi supplied (see Chapter 3) of inducing nalidixic acid resistance was followed and the subsequent nalidixic acid resistant strain of the Armidale S. typhimurium was used in future experiments.