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Microbiological Studies in Mice of Intestinal Decontamination Regimens

by

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Dedicated to:

My wife LAILA, parents, brothers, sisters, and my best friend OTHMAN.

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ABBREVIATIONS

AGNB	Aerobic gram negative bacilli
Amph B	Amphotericin B
ANGPS	Anaerobic gram positive spore bearing bacillus
BA	Blood agar
ß-aspgly	ß-aspartylglycine
BHIB	Brain heart infusion broth
CDC	Center for disease control
CFU	Colony forming unit
CR	Colonization resistance
CRF-mice	Colonization resistance factor mice
CTX	Cefotaxime
DCA	Desoxycholate citrate agar
e.g.	Exsample
GNB	Gram negative bacilli
HPLC	High performance liquid chromatography
ICU	Intensive care unit
i.p.	Intraperitoneal
MC	Mac Conkey agar
MIC	Minimal inhibitory concentration
MRSA	Man Rogosa and Sharpe agar
NA	Nalidixic acid
Nm	Neomycin
Nm^s	Neomycin sensitive
OPT	O-phthaldialdehyde
Pe	Polymyxin E
PPM	Potentially pathogenic microorganisms
PsSA	Pseudomonas selective agar

PTA	SDD antibiotics (polymyxin E, tobramycin, and amphotericin B)
RCW	Relative caecal weight
SE	Standard error of the mean
Sm	Streptomycin
Sm^R	Streptomycin resistant
SDD	Selective decontamination of the digestive tract
SPF-mice	Specific pathogen free-mice
TDD	Total decontamination of the digestive tract
TMP-SMZ	Trimethoprim-sulphamethoxazole (co-trimoxazole)
Tn	Tobramycin
TVC	Total viable count
VFA	Volatile fatty acids

SUMMARY

Selective decontamination of the digestive tract (SDD) uses orally administered antibiotics to eliminate or markedly reduce the number of aerobic gram-negative bacilli colonizing the digestive tract, thus preventing endogenous infection with these organisms. The selective activity of the drugs used allows retention of the normally predominant anaerobic flora of the tract, thus preventing overgrowth or colonization with organisms resistant to the decontaminating agents; this phenomenon is termed "colonization resistance" (CR). SDD has been increasingly applied to medico-surgical intensive care patients, a procedure that remains highly contentious.

The concept of colonization resistance is central to the use of SDD. However, the mechanisms underlying this phenomenon are both complex and disputed. Further, the experimental basis for the clinical use of SDD is dependent to a surprising degree on a restricted series of studies involving total decontamination of the digestive tract of mice carried out by van der Waaij and colleagues between 1971 and 1974.

This thesis described studies in two areas. First, repetition of the total decontamination studies of van der Waaij et al., (1971) in order to verify or modify their conclusions concerning colonization resistance. Second, the application of the same mouse model to the SDD regimen most commonly used in clinical ITU practice: orally administered mixture of polymyxin E, tobramycin, and amphotericin B (PTA) with or without the addition of systemically administered cefotaxime.

Treated and untreated Balb / c mice were subjected to direct oral challenge with bacteria resistant to the decontaminating agents, together with detailed microbiological monitoring of the aerobic and anaerobic faecal flora. Throughout these studies the results were greatly influenced by the details of

the technique employed.

Untreated, control mice showed a very high CR. A challenge dose of 10^{8-10} cfu of *E. coli* s-R21 was required to achieve colonization in 50 % of mice, and 6 other challenge strains (*Klebsiella pneumoniae* s-R10, *Pseudomonas aeruginosa* s-R321, *Proteus mirabilis* s-R9, *Pseudomonas cepacia* s-R13, *Providencia stuartii* s-R7 and *Candida albicans* s-R5) failed to colonize the control mice at a maximum test dose of 10^{8-10} cfu.

TDD rapidly reduced the aerobic faecal flora to undetectable levels. A marked reduction in the numbers and diversity of anaerobic flora was also seen, but this effect was more variable and often incomplete. These changes were associated with a striking reduction in the CR for *E.coli* s-R21. Values of < 2 were obtained, together with extensive multiplication of the challenge in the gut of mice. However, there was a marked variation in the CR results with different challenge organisms.

SDD rapidly eliminated indigenous *E.coli* from the faeces, but had little effect on the levels of enterococci. Effects on anaerobic flora were limited to some simplification in flora diversity. A small reduction in CR was noted in these mice, but this was much less than that seen with TDD and multiplication of the challenge organisms within the gut was not observed.

Treatment with systemic cefotaxime alone was associated with elimination of faecal *E.coli* and overgrowth with enterococci. There was also a major reduction in the diversity of anaerobic flora, although the total viable count was unchanged. These changes were not associated with a significant reduction in CR.

However, SDD supplemented with systemic cefotaxime produced striking and surprising results. As previously, *E.coli* was rapidly eliminated, but this was accopanied by a marked overgrowth with enterococci. In addition,

there was either apparent elimination of anaerobic flora or a marked reduction in both the numbers and the diversity of these organisms. These changes were associated with a marked reduction in CR (to value < 4, the smallest challenge tested). These results are surprising in view of the clinical experience with this widely used regimen. They also give rise to clinical concern and they warrant further investigation.

Chapter One

INTRODUCTION

1. INTRODUCTION

1.1 What is Selective Decontamination of the Digestive Tract?

Selective decontamination of the digestive tract (SDD) is a drug regimen designed to allow the use of antimicrobials for prophylaxis of infection in seriously ill patients, without incurring problems of drug resistance. Non-absorbed drugs are administered orally to selectively eliminate aerobic gram-negative bacilli, and yeasts from the digestive tract, thus removing a major source of endogenous infection. The highly selective activity of the drugs used allows retention of the normally predominant anaerobic flora and this (together with other factors) prevents outgrowth or colonisation with strains resistant to the decontamination agents (van der Waaij et al., 1971; van Saene and Stoutenbeek, 1987; Ledingham et al., 1988).

SDD has been employed in patients suffering from neutropenia, in the medico-surgical intensive care unit (ICU), and in patients undergoing liver transplantation (Tetteroo et al., 1990; Wiesner, 1990) and marrow transplantation (Heimdahl, 1984). For convenience these patients will be described hereafter as the SDD patient group. There is a very high incidence of infection in this patient group (Kunin et al., 1973; Thorp et al., 1979; Haley et al., 1981; Stevens et al., 1981; van der Waaij, 1982; Guglie et al., 1983; Cruse, 1986; Eikhoff, 1986; Kaiser, 1986; Milatovic and Braveny, 1987; Potgieter et al., 1987; Platt, et al., 1990); the incidence of infection may exceed 80 % in ICU patients admitted for more than five days (Thorp et al., 1979; Kerver et al., 1987). In addition, most of the infection episodes display a characteristic pattern of pathogenic mechanisms that are central to the application of SDD as a prophylactic measure.

1.2 Pattern of Pathogenesis in the SDD Patient Group:

These patients usually have significant and often very major impairment of host defences. Infections are managed in hospitals and they can be caused by both community, or hospital-acquired microorganisms. However hospital-acquired infections represent the major problem. The distinction between hospital and community acquired infection may be difficult. By convention, infections diagnosed on admission to the hospital and up to 48 hours thereafter are usually assumed to be caused by community microorganisms and subsequent infections are assumed to be hospital acquired.

A wide range of organisms is associated with infection. However, there is a marked predominance of coliform bacilli (particularly *Escherichia coli, Klebsiella, Proteus, Enterobacter, Serratia), Pseudomonas aeruginosa*, and, less commonly, *Acinetobacter* species (Northey et al., 1974; Dummer et al., 1983; Potgieter et al., 1987; Ledingham et al., 1988). In the present context, the term aerobic gram-negative bacilli (AGNB) is a useful collective term for this group of organisms (Alcock, 1990). In addition to AGNB, yeasts (usually *Candida albicans*) are also a significant cause of infection in these patients (Gaya et al., 1973; Thorp et al., 1979; Potgieter et al., 1987). This predominance of AGNB and yeasts is particularly true of hospital-acquired infection.

Infection in the SDD patient group may be further classified as exogenous or endogenous. This distinction is necessarily somewhat arbitrary, but it has considerable practical application.

An exogenous infection is caused by organisms that are acquired from outside the patient (Sanderson, 1989; Stoutenbeek, 1989: Stoutenbeek and

van Saene, 1990), and that are seeded directly to the site of infection (e.g. a wound). It was once assumed that hospital-acquired infections were mainly exogenous, acquired directly or indirectly from other patients, food (Shooter et al., 1969; Cooke et al., 1970; Pasch, 1974), attendants, staff, environment (Sanderson, 1989), or equipment (Stoutenbeek, 1989).

Endogenous infection is caused by the patients own body flora (normal or abnormal), and the most important sites have been identified as the oropharynx, stomach, intestine , and skin (van Saene et al., 1983; Kerver et al., 1987). Colonization of these sites with AGNB and yeasts are now believed to create a major source of infection in the SDD patient group (Ravin and Fine, 1962; Woodruff et al., 1973; van der Waaij, 1982; van Saene et al., 1983; Kerver et al., 1987; Deitch, 1988). Depending on the time occurrence, endogenous infections can be classified either as primary or secondary endogenous infections.

A primary endogenous infection is caused by organisms which the patient carries at the time of admission to the hospital. Overgrowth of these organisms, and colonization of abnormal sites may occur after admission (Stoutenbeek and van Saene, 1990; van Saene and Stoutenbeek, 1991). The major importance of the respiratory tract, and the digestive tract as a source of primary endogenous infections has been repeatedly demonstrated in liver transplantation (Tetteroo et al., 1990; Wiesner, 1990), marrow transplantation (Heimdahl et al., 1894), granulocytopenic patients with cancer or leukemia (Tancrede and Andermont, 1985), and ITU patients (Thorp et al., 1979; Pptgieter et al., 1987).

A secondary endogenous infection is caused by organisms that colonize the patient after hospital admission. They are thus of initially exogenous origin and frequently involve hospital strains (Stoutenbeek,

1989). However, after acquisition, proliferation of the these microorganisms (particularly in the oropharynx and gastrointestinal tract) is an essential prerequisite for endogenous infection elsewhere.

Secondary endogenous infections, usually develop after several days of admission (Stoutenbeek and van Saene, 1990; van Saene and Stoutenbeek, 1991). Aerobic gram negative bacilli are the principal organisms involved. These are hospital strains and frequently show resistance to many antibiotics (Abrams and Bishop, 1966; Schimpff et al., 1972; van der Waaij and de Vries-Berghuis 1974; van der Waaij, 1982; Mulligan et al., 1984; Nord, 1984; Stoutenbeek, 1989). This abnormal pattern of colonization with indigenous, and acquired organisms not only predispose to endogenous infections, but also provides a novel source for further exogenous spread.

Colonizing microorganisms may contaminate internal tissues (and hence initiate endogenous infection) by three main routes:-

I. Direct contamination of wounds, skins, catheters, and vascular lines.

II. Ventilated patients are particularly likely to suffer from lower respiratory tract infections. Most of these infections are now thought to be endogenous and involve AGNB. There is considerable evidence to indicate that these organisms are derived from the abnormal colonization of the stomach , and the oropharynx, and that in many cases this probably represents progression of colonization from the stomach to the oropharynx to the lower respiratory tract (Atherton and White, 1978; Chow et al., 1979; Daschner et al., 1982; Du Moulin et al., 1982; Ufflen et al., 1984; Maki, 1989)

III. Translocation: Under normal circumstances the intestinal mucosa functions as a major local defence barrier that prevents bacteria and

endotoxin contained within the intestinal lumen from reaching systemic organs and tissue (Deitch, 1990). However, under certain circumstances, as in neutropenic or in immunocompromised patients, intestinal bacteria can escape from the gut and cause infections (Deitch, 1988). This process of intestinal bacteria invading extraintestinal tissue has been termed " bacterial translocation " (Deitch, 1988). Transient bacterial translocation probably occurs in normality with no ill effects. It appears that one or more of three basic pathophysiological factors are necessary for bacterial translocation to initiate infection.

1) Disruption in the balance of the normal intestinal microbial flora resulting in bacterial overgrowth with certain bacteria (especially AGNB) (van der Waaij et al., 1971, 1977; van der Waaij, 1984).

2) Impaired host immune defences (Deitch, 1990).

3) Physical or physiological disruption of mucosal barrier function , as after treatment with cytotoxic drugs.

One or more of these factors frequently occur in the SDD Patients Group. It is hypothesized, therefore, that translocation may be an important factor in infections of endogenous origin in these patients (Deitch, 1990).

1.3 Therapeutic Response To Prevent Infection:

Considerable attempts have been made to prevent these types of hospital infections (Stoutenbeek et al., 1984; Unertl et al., 1987; Kerver et al., 1988; Konrad et al., 1988; Ledingham et al., 1988; Aerdts et al., 1989; Crockerill et al., 1989; Guillaume et al., 1989; Thulig et al., 1989).

1) Considerable control over exogenous infections can be exercised by strict implementation of the center for disease control (CDC) guidelines Maki et al., 1982). These guidelines concern the disinfection of wounds, and sterilization of equipment (e.g. catheter and line policies used around the

patients) and include measures to reduce the transmission from patient to patient by nursing techniques and discipline (CDC, 1985, Atlanta, Georgia, USA). These extensive procedures have shown appreciable success in preventing exogenous infection. However, in modern hospitals, and against the background of these practices, endogenous rather than exogenous transmission is now the major problem in the SDD Patient Group.

2) Systemic antibiotics have been used to prevent infection. These systemic antibiotics are active against organisms seeded internally from exogenous, and endogenous sources, and are administered to eliminate the infecting organisms at the site of infection (Stoutenbeek, 1989). However, this approach has enjoyed only limited success. This is because it does not primarily address the source of infection, and also because of major problems of drug resistance (McCoy, 1954; Hahn et al., 1978).

The major limitation of these approaches have provided the stimulus for other methods of infection prophylaxis that are focussed primarily on the major source of endogenous infection, that is AGNB and yeasts colonizing the digestive tract.

Two techniques have been described:

I. Total decontamination of the digestive tract (TDD).

II. Selective decontamination of the digestive tract (SDD).

1.4 Total Decontamination of the Digestive Tract:

TDD involves oral administration of broad spectrum antibiotics in an attempt to totally eliminate the aerobic, and the anaerobic flora of the digestive tract (gut sterilization) (Levine et al., 1973; Bodey and Rosenbaern, 1975).To achieve TDD a variety of antibiotic regimens have been used **(Table. 1)**. These regimens have proved surprisingly successful as a mean of sterilizing the intestinal tract and thus removing a major source of

Table 1 **Example of nonabsorbable antibiotic regimens used for total decontamination of the digestive tract.**

Antibiotics	Representative Reference
Gentamicin - Vancomycin - Nystatin	Preisler et al., (1970)
Framycetin - Colistin - Nystatin	Storring et al., (1977)
Neomycin - colistin - Nystatin	Storring et al., (1977)
Vancomycin - Kanamycin - Nystatin	King, (1980)

endogenous infections (Levine et al., 1973; Bodey and Rodriguez, 1975; Dankert et al., 1978; Rodriguez et al., 1978; Pizzo, 1989). However, the application of TDD has been limited because of problems of drug resistance, poor patient compliance (King, 1980), and the need for a protective environment (sterilized food, and reverse isolation) (Kurrle et al., 1881).

Drug resistance has proved particularly intractable. Following successful elimination of both aerobic, and anaerobic flora from the digestive tract, a substantial number of patients suffer overgrowth or colonization with organisms resistant to the TDD agents (particularly AGNB, yeasts and fungi) (Dankert et al., 1978). The digestive tract, in particular, may rapidly harbor a massive population of resistant strains. Endogenous transmission may then result in life threatening infection with drug resistant strains (Schimpff et al., 1975).

These problems may be maintained after TDD is discontinued in the period required for recovery of the normal intestinal flora (Schimpff et al., 1975; King, 1980).

1.5 The Concept of the Colonization Resistance and Selective Decontamination of the Digestive Tract:

The concept of colonization resistance (CR) was introduced by van der Waaij, and colleagues in 1971, in the Netherlands (van der Waaij et al., 1971). They have defined CR as " The resistance which a potentially pathogenic microorganisms (PPM) encounters when it tries to colonize a landing site on the mucosal of one of the three tracts that have an open communication with the exterior" (van der Waaij, 1987). In the present context, the oropharynx and the gastrointestinal tract are the major sources of endogenous infections.

There is considerable evidence to suggest that the indigenous anaerobic flora of the digestive tract plays a major role in the maintenance of CR at this site (van der Waaij et al., 1971; Welling et al., 1980). However, CR is a complex and multifactorial phenomenon which will be reviewed in subsequent section **(1.11-1.12)**.

The method of SDD is based on the concept of CR (van der Waaij et al., 1971; van der Waaij and Berghuis de Vries, 1974). Like TDD, SDD aims to achieve elimination of AGNB and yeasts from the digestive tract by the use of oral nonabsorbable antimicrobial agents. Unlike TDD, SDD uses antimicrobial agents with a selective action that has minimal effects on the overall numbers of anaerobic flora (Guiot and van Furth, 1977; van Saene, 1987; de vries-Hospers et al., 1981). This, in turn, allows retention of CR which (together with exposure to high concentrations of antimicrobial agents), prevents colonization or overgrowth of the digestive tract with strains resistant to the decontaminating drugs (the major problem in TDD regimen) (van der Waaij et al., 1971; van Saene et al., 1983).

Clinical SDD regimens are designed to produce three main effects:

1) To eliminate or markedly reduce the AGNB, and yeasts present in the oropharynx, and the gastrointestinal tract.

2) To prevent acquisition and secondary colonization by AGNB and yeasts from hospital environments.

3) To prevent overgrowth or colonisation with strains resistant to the decontaminating agents.

1.6 History of SDD:

The history of SDD can be traced back for more than 75 years. Nissle, (1916) was the first to suggest that the resident microflora of the gut may prevent colonization with exogenous organisms. Thereafter many attempts have been made to solve the problem of endogenous infections from the digestive tract by oral administration of antimicrobial agents. In 1939, Garlock and Seley, were the first to describe the use of antimicrobial agents for intestinal flora suppression, when they recommended the oral use of sulphonamides for wound infection prophylaxis in colorectal surgery. Suppression of tracheal flora was first described by Lepper et al., (1954), where they used antibiotics following tracheotomy in poliomyelitis. Poth, (1960) was the first to suggest that augmentation of topical drugs with systemic agents in order to close gaps in the antibacterial spectra of the antibiotics used, and to prevent the development of resistance. As discussed, Van der Waaij and his group formally introduced the concept of CR in 1971 (Van der Waaij et al., 1971), and from this finding the concept of SDD evolved.

The first clinical use of SDD was described by Guiot and van Furth (1977) who applied the technique to leukaemia patients in 1977. They used a mixture of neomycin or kanamycin, polymyxin E or colistin, nalidixic acid, and amphotericin B or miconazole. This was administered orally as regimen for decontamination of the gut (Guiot and van Furth, 1977), and was called " partial antibiotic decontamination" . The result showed a rapid elimination of potentially pathogenic organisms from the intestinal tract. Only one out of nine patients developed endogenous infection, and exogenous infection was not recorded. The anaerobic flora of the gut seemed to remain normal during partial decontamination, and this was assumed to have maintained the CR (Guiot and van Furth, 1977).

Since 1977, successful infection prophylaxis in leukopenia has been reported using a variety of SDD drugs. For example oral trimethoprim-sulphomethoxasole (TMP-SMZ) has been used alone, when the bacterial infection rate in leukopenic patients was reduced to between zero and 50 % of the infection rate in control patients (Hughes et al., 1977; Gurwith et al., 1979; Gualtieri et al., 1983; Kauffman et al., 1983; Riben et al., 1983; Estey et al., 1984; Henry et al., 1984). Colonization with AGNB susceptible to TMP-SMZ was generally found to be reduced, but not eliminated. Dekker et al., (1981) reported a serious problem due to colonization, and infection with TMP-SMZ resistant AGNB, but this problem was solved by the addition of oral colistin to the TMP-SMZ regimen (Kauffman et al., 1983). Other studies have employed oral TMP-SMZ plus erythromycin (Pizzo et al., 1983), TMP-SMZ plus nystatin (Hughes et al., 1977; Mulder et al., 1979; Wade et al., 1981), colistin plus neomycin (Storring et al., 1977; Rohatener and Lowes, 1981), and colistin plus both neomycin and nalidixic acid (Guiot et al., 1983). Nalidixic acid with TMP-SMZ or polymyxin, and amphotericin B for the elimination of the yeasts have also been employed (de vries-Hospers et al., 1981).

Clearly, successes have been recorded following the use of SDD in neutropaenic patients. However, this has proved a large and very complex field, and opinion is still divided of the precise value of SDD in this area.

Stoutenbeek et al.,(1983) described the use of SDD regimen in acute trauma patients admitted to an unspecialized medico-surgical intensive care unit. This was the first application of SDD regimen to non-neutropenic patients, and the result was strikingly successful. The SDD regimen consisted of three drugs; polymyxin E, tobramycin , and amphotericin B (PTA). These drugs were administered to the stomach and more distal

gastrointestinal tract by means of a nasogastric tube, and were also applied directly to the oropharynx in a sticky "orabase" vehicle. This SDD regimen showed a striking reduction in unit-acquired infection from 81 % in control patients to 16 % for SDD patients. Respiratory tract infections, urinary tract infections, and septicemia were the major unit-acquired infections in both groups.

Stoutenbeek et al., supplemented the topical SDD drugs with systemic cefotaxime, during the first few days of treatment. This was employed for three reasons: to provide additional cover against AGNB during the first few days of treatment whilst SDD was established, to provide additional cover against organisms resistant to SDD agents (particularly gram positive respiratory pathogens) during intubation and other invasive procedures in the period following ITU admission, and to treat the appreciable proportion of patients already developing a lower respiratory tract infection at the time of admission (Alcock, 1990). The antimicrobial spectrum of cefotaxime was compatible with these aims whilst exerting a minimum effect on the anaerobic flora of the intestinal tract (and hence on CR).

This study made a considerable impact and since 1984 there have been numerous further studies in an ITU environment, mainly using the PTA regimen (with or without cefotaxime) **(Table. 2)**. A summary of the acquired infections rates in 24 SDD trials is shown in **Table 3**. Results of these trials have been remarkably uniform . The incidence of oral and tracheal colonization with AGNB was reduced from 50 % to 80 % in control patients to 5 % to 15% in patients with SDD . Twenty of these SDD trials showed a statistically significant reduction in the rate of unit-acquired infection. Most of this benefit derived from a reduction in lower respiratory tract infections:

Table 2 **Prophylactic regimen based on a combination of topical and systemic antibiotics*.**

Topical Antimicrobials (PTA Regimen)

These are administered throughout the ICU stay

1) Oropharyngeal Cavity: A small volume of a 2 % mixture of polymyxin E, tobramycin and amphotericin B in paste with carboxy methyl cellulose (Orabase) is applied to the buccal mucosa with a gloved finger 4 times a day.

2) Gastrointestinal Canal: 9 ml of suspension of polymyxin E 100 mg, tobramycin 80 mg and amphotericin B 500 mg is administered via the gastric tube 4 times a day.

Systemic Antimicrobials

Administered for the first 4 days of the ICU stay.

Cefotaxime 50 - 100 mg / kg body weight/ day given intravenously.

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* From van Saene et al., (1983).

Table 3 **Summary of % Acquired Infection in SDD trials 1984 - 1991.**

Reference	ICU-Acquired-infection (%)	
	control	SDD
1. Stoutenbeek, et al. (1984)	81	16*
2. Unertl, K., et al. (1987)	70	21*
3. Van Uffelen, R., et al. (1987)	-	7
4. Ledingham, I., et al. (1988)	24	10*
5. Wiesner, R. H., et al. (1988)	-	23
6. Kerver, A. J. H., et al. (1988)	81	39*
7.Sydow, M.,et al. (1988)	75	7*
8. Brun-Buisson, C., et al. (1989)	33	32
9. Konrad, F.,et al. (1989)	42	6*
10.Ulrich, C., et al. (1989)	44	6*
11. Thulig, B., et al. (1989)	46	10*
12.Guillaume, C., et al. (1989)	21	3*
13. Cockerill, F. R., et al. (1989)	62	24*
14. Thulig, B., et al. (1989 abst.)	57	18*
15. Schardey, M., et al. (1989)	47	10*
16. McClelland, P., et al. (1989)	83	33*
17. Flaherty, J., et al. (1990)	27	12*
18. Aerdts, S. J. A., et al. (1990)	78/62&	6*
19. Tettero, G. W. M., et al. (1990)	55	21*
20. Wiesner, R. H., et al. (1990)	-	28
21. Rodriguez-Ronald, et al (1990)	73	0*
22. Hartenauer, U., et al. (1991)	69.5	26* (ICU 1)
	87.5	44.5* (ICU 2)
23. Pugin, J., et al. (1991)	78	16*
24. Blair, P., et al. (1991)	30.8	16.7*

* Statistically significant difference.
& Two control groups.

nosocomial pulmonary infection was reduced from a typical incidence of 60 % in control patients to about 10 % in patients with SDD. In contrast, the effect of SDD on mortality remains unclear, and further studies employing much larger patients numbers are required .

The use of SDD regimen in ICU remains highly contentious. Much of the criticism had centered on the inability to demonstrate a fall in ICU-associated mortality and on the possibility of major problems of drug resistance.

1.7 Emergence of Resistance During SDD:

SDD regimens apply mixtures of antimicrobial agents to vast microbial populations encompassing several hundred species. Intense selective pressure for resistance to the decontaminating agents is an inevitable consequence of this procedure. Against this factor are ranged the preservation of colonisation resistance and variable exposure to mixtures of potent bactericidal agents used at very high concentrations. In these circumstances the danger of major resistance problems has remained a justified concern.

However, significant problems have yet to be described. Patterns of drug resistance were closely monitored over a period of thirty months continuous SDD use by Stoutenbeek et al., (1987). No increase in resistance to PTA and cefotaxime was seen. This also has been the case in the study by Alcock et al., (1990) in Glasgow with five years of continuous use of the SDD regimen . It also has been reported that the emergence of AGNB was uncommon in patients during liver transplantation (Wiesner, 1991). From other published trials of SDD, significant problems involving drug-resistant gram negative organisms have yet to be reported. Several studies have noted an increase of enterococci and coagulase negative

staphylococci from the digestive tract of SDD patients (Poole et al., 1992) but this has not been associated with problems of infection.

In view of the rapidly extending clinically use and associated controversy in this area, it is pertinent to question the experimental bases for the concepts of colonization resistance, and selective decontamination of the digestive tract.

1.8 The Microbial Ecology of the Murine Intestinal Tract as A Model for that of Humans:

Mice have been extensively used to study human intestinal pathogens, the effects of antibiotics on intestinal flora (Meynell,1963; Meynell and Subbaian, 1963; Bohnhoff et al., 1964[a], 1964[b]; van der Waaij, 1968; van der Waaij et al., 1971, 1972; van der Waaij and Berghuis de vries, 1974), and the effect of enterotoxins produced by pathogenic microorganisms (Dubos and Schaedler, 1959, 1962; Schaedler et al., 1962). They have also been employed to study the influence of antimicrobial agents on colonization resistance (van der Waaij et al., 1971, 1972). The particular value of animal experimentation in this latter area is that it allows the administration of resistant microorganisms as a challenge strain, whereas a deliberate administration of resistant microorganisms to patients presents ethical difficulties and limits the possibilities for study of the fate of the challenge organisms.

There are many anatomical differences between the intestines of humans and mice e.g. the existence of a large caecum in mice. In addition, there are many differences in the intestinal microflora, which will be discussed in detail in the following section. However, in previous studies of colonization resistance, the intestinal microflora of mice and man appeared

to respond to antimicrobial agents in a similar fashion (van der Waaij, 1979; van der Waaij et al., 1982).

1.9 The Intestinal Microbial Flora:

In all mammals, including man and mice, the microbial colonisation of the new born infant is established immediately after birth. Infants are colonized by flora derived from the mother initially, and from other sources subsequently (Hurst, 1965). After only a few weeks in most cases, the representation of microbial species in these new born infants is remarkably similar to the adult pattern of colonization (Lee et al., 1971; Long and Swenson, 1977).

The intestine of adult man and animals harbours a complex mixed population of bacteria, and as the complexity of the flora increases, the ease with which new strains are able to become established is decreased (Sears, et al., 1956). The major microbial species represented in individual human or animal populations are usually characteristic, and difficult to change by means of diet or environment (Haenel, 1961).

There are two caveats associated with the study of the intestinal flora. Firstly, most of the studies especially those of the large intestine, have concerned themselves only with the microbial flora of the lumen, which may not reflect the microbial population of the mucosal surface. Secondly, in most studies, only a small proportion of the large number of species present (as many as 400 in human colon) have been examined, a deficiency that particularly applies to the anaerobic flora.

1.9.1 The intestinal microbial flora in man:

This thesis is mainly concerned with large intestinal flora, but for the sake of completeness a brief description of the proximal intestinal tract flora is included.

1.9.1.1 *Aerobic normal flora:*

The upper region of small intestine including the stomach, duodenum, jejunum, and upper ileum has a sparse microbial flora consisting predominantly of gram-positive facultative organisms. Streptococci, aerobic lactobacilli, diphtheroids, and fungi are major constituents, and their total concentration is generally less than 10^4 cells per ml of intestinal fluid (van der Reis, 1925; Gregan and Heyward, 1953; Goldstein et al., 1962; Shiner et al., 1963; Kaser et al., 1966; Donaldson et al., 1967; Cohen et al., 1969; Drasar et al., 1969; Gorbach et al., 1969; and Hamilton et al., 1970). Many of these microorganisms are derived from the oral cavity, colonizing the stomach and upper intestine in a wave-like fashion following meals (Drasar et al., 1969). Some investigators reported a low concentration of coliforms (less than 10^4 cell per ml) in the upper small intestine of normal subjects (Kalser et al., 1966; Donaldson et al., 1967;and Hamilton et al., 1970).

The lower small intestine, i.e. the distal and terminal ileum appears to contain a richer and more permanent flora than the upper. Although lactobacilli and streptococci are still predominant, *Enterobacteriaceae* occur more frequently, and the total count is in the region of 10^{5-7} cells per ml (Nicholes and Glenn, 1940; Cregan and Hayworth,1953; Bornside and Cohn,1965; and Kelser et al., 1966). Facultative aerobic organisms such as enterococci and coliforms are also present in the large intestine (Plaut et al., 1967; Nelson and Mata, 1970). Variation in concentration of faecal aerobic microorganisms is considerable: in 10 % of healthy people, *E.coli* has been found at a level of 10^9 organisms / gram of faeces, in 37 % at 10^{6-8}; and in 16 % at 10^{3-5} organisms / gram of faeces. Other gram-negative rods, such as *Pseudomonas , Proteus, Klebsiella, Enterobacter, Serratia,* and *Citrobacter*

species have been found in less than 5 % of healthy subjects, and the numbers were usually less than 10^5 organisms / gram of faeces (Koopman et al., 1978).

1.9.1.2 *Anaerobic normal flora:*

The colon contains the largest total population of microorganisms of any region of the human body. Microbial counts, which exceed 10^{11} cells per gram of colonic contents in healthy individuals, are represented by more than 400 species (Moore and Holdman, 1974). Anaerobes outnumber aerobes by a factor of 100 : 1 to 1000 : 1 (Finegold and George, 1989). Microorganisms commonly isolated from human faeces are listed in **(Table.4)**.

Numerically, *Bacteroides spp.* are usually the predominant genus in the human intestine, present at approximately 10^{11} cells per gram dry content (Finegold et al., 1983). The two species most frequently isolated are *Bacteroides vulgatus,* and *Bacteroides thetaiotaomicron.* Among the gram-positive rods *Bifidobacterium, Eubacterium,* and *Lactobacillus* are usually predominant. Finegold et al., (1974, 1977) reported that 100 % of specimens examined contained *Clostridium species* which were present at a mean count of $10^{9.8}$ cells per gram of faeces. *Peptostreptococcus, Ruminococcus, Veillonella,* and *Acidaminococcus* are also commonly present (Holdman et al., 1976; Finegold et al., 1983).

1.9.2 The intestinal microbial flora in mice:

The available studies on mouse intestinal tract flora overwhelmingly concern the caecum and large intestine flora. In addition, no explicit distinction is usually made between large and small intestinal flora. Lee et al., (1971) reported that the anaerobic flora of the colon is qualitatively and quantitatively similar to the anaerobic caecal flora.

Table 4 Colonic Microial Flora in Human*

Anaerobic bacteria	Aerobic and facultative anaerobic bacteria
Gram-positive rods	***Enterobacteriaceae***
Actinomyces	*Escherichia coli*
Arachnia	*Citrobacter*
Bifidobacterium	*Klebsiella*
Clostridium	*Enterobacter*
Eubacterium	*Proteus*
Lachnospira	
Propionibacterium	***Pseudomonas***
Gram-negative rods	***Lactobacillus***
Bacteroides	
Butyrivibrio	***Streptococcus***
Desulfomonas	***Staphylococcus***
Fusobacterium	
Leptotrichia	
Succinimonas	
Succinivibrio	
Wolinella	
Gram-positive cocci	
Coprococcus	
Gaffkya	
Peptococcus	
Peptostreptococcus	
Ruminococcus	
Sarcina	
Gram-negative cocci	
Acidominococcus	
Megasphaera	
Veillonella	

* From Finegold and George (1989) Drasar and Barrow (1985)

1.9.2.1 *Aerobic normal flora:*

The aerobic normal flora in mice is similar to that in humans, consisting predominantly of *E.coli*, and enterococci, both in concentration of (approximately) 10^5 organisms / gram of faeces (Clasener et al., 1987). Lee et al., (1971) reported that enterococci and coliform bacilli were present in the caecum of new born mice before the strict anaerobes, and that their numbers decreased shortly after the time when the population of strict anaerobes reached their maximum. Brown and Balish, (1978) reported that *Lactobacilli, Corynebacteria*, and *Enterococcus faecalis* were the most numerous facultative and aerobic bacteria isolated from the stomach at a range of 10^5 - 10^6 cells, whereas the facultative aerobicgram negative rods were present in lower numbers. However, *E.coli* was the major species of *Enterobacteriaceae* cultured from caeca of mice, and it reached a level of 10^6 - 10^7 cells in the ilea of 8 out of 16 different strains of mice (Brown and Balish, 1978). The diversity of facultative gram negative fermenting rods increased in the caeca (as reflected by the occurrence of detectable levels of *Proteus mirabilis, Enterobacter aerogenes, Klebsiella pneumonia*e, and *Acinetobacter* species), whereas *Ps. aeruginosa* was isolated only from small numbers of mice (Brown and Balish, 1978).

1.9.2.2 *Anaerobic normal flora:*

The gastrointestinal tract of normal healthy mice contains a total population of 10^{10} - 10^{11} anaerobic organisms per gram of intestinal contents (Moore et al., 1963; Savage et al., 1971) that consists of a large diversity of species. Direct microscopic examination of the intestinal contents showed that the predominant morphotypes of the anaerobic population were gram negative rods with pointed ends, although the gram positive rods, and cocci were also present in smaller numbers (Gordon and Dubos, 1970; Lee et al.,

1971; Harris et al., 1976). These organisms, and also gram negative non-sporulating rods with rounded ends, were found to be obligate anaerobes. They were assigned to the genera *Bacteroides* and *Fusobacterium* respectively (Lee et al., 1968; Gordon and Dubos, 1970; Harris et al., 1976;, Gordon and Dubos, 1970; Lee et al., 1971; Savage et al., 1971; Harris et al., 1976). These were present at a level of 10^{10} - 10^{11} organisms / gram of caecal content. *Eubacterium* species were also present at a level of 10^{10} - 10^{11} organisms / gram of caecal content (Gordon and Dubos, 1970), and this species was also isolated from a 10^{10} - 10^{11} dilution of the colon content (Harris et al., 1972). *Lactobacilli* (Moore et al., 1963; Dubos et al., 1965; Holdman and moore, 1972), *Propionibacterium*, and *Peptostreptococcus* (Harris et al., 1976) were present, but in lower numbers; for example Moore and Holdman, (1963) reported a concentration of 10^{6} - 10^{8} *Lactobacilli* per gram of small intestine contents, and Harris et al., (1976) reported that 10^{9} *Lactobacilli* were present per gram of colon contents. *Clostridium* species were found at 10^{7} and 10^{8} dilutions of the mouse caecal contents and of faeces respectively (Dubos et al., 1965; Gordon and Dubos, 1970). Brown and Balish, (1978) observed that the greatest diversity of species of one genus was seen in the *Lactobacilli* (19 species), and *Bacteroides* (18 species). This finding, together with a comparison of the colon flora of mice and humans, is summarized in **Table. 5**.

1.10 Mice as Models of Decontamination of the Digestive Tract in Humans:

In humans many different antibiotics and combination of antibiotics have been used for reducing the microbial flora of the intestines prior to operations (Cohn and Longacre, 1956; 1957; Klastersky et al., 1975; Stoutenbeek et al., 1986), and as a treatment of certain gastrointestinal

Table 5 **Organisms in order of prevalence in human and mice colon*.**

Human	Mice
Anaerobic bacteria	
Bacteroides	*Bacteroides*
Bifidobacterium	*Fusobacterium*
Eubacterium	*Eubacterium*
Clostridium	*Propionibacterium*
Peptostreptococcus	*Peptostreptococcus*
Ruminococcus	*Clostridium*
Veillonella	
Acidominicoccus	
Aerobic and facultative anaerobic bacteria	
Lactobacillus	*E.coli*
Streptococcus	*Enterococcus*
Staphylococcus	*Lactobacillus*
diphtheroids	*Corynebacterium*
Enterococcus	*Proteus*
E.coli	*Klebsiella*
Proteus	*Acinetobacter*
Pseudomonas	*Pseudomonas*
Enterobacter	
Serratia	
Citrobacter	

* From Lee et al., (1971); Harris et al., (1976); Brown and Balish (1978); Drasar and Barrow (1985) and Finegold and George (1989).

diseases such as enterocolitis (Stralford and Dixon, 1964). In most cases a marked reduction of the faecal flora was obtained.

These clinical applications have been successfully modelled in C57BL/Rij, CBA/Rij, C3H, RFO, and ND2[2] mice. A combination of bacitracin with either kanamycin, streptomycin, or neomycin was administered in the drinking water at 4 g l^{-1} (van der Waaij and Sturm, 1968). Decontamination was accomplished in 4 days in 94 % of groups of 30 - 100 mice, and maintained for as long as 12 weeks, without overt signs of intoxication due to antibiotics. Another study modelled the clinical use of TDD (kanamycin and chloramphenicol) in neutropenia using CBA/Rij, C57BL/Rij, and ND2 mice (van der Waaij, 1968). Effective TDD was achieved and after discontinuation of TDD treatment, the normal flora recovered in two weeks. This study also found that TDD mice could easily be colonized with gram negative rods before recovery of the normal flora, whereas after recovery of the normal flora, mice appeared to be more resistant to colonisation with gram negative rods.

Several studies have shown that following treatment with antimicrobial agents the phenomenon of overgrowth by resistant bacteria occurred in mouse gut as well as in humans (Bohnhoff et al.,1954; Freter, 1955; 1956; Clark, 1971). Most authors who reported gastrointestinal tract decontamination in mice or humans, mentioned cases in which colonization with yeasts occurred; Shidlovsky et al. (1955) tried to solve this problem by adding Kaprylex (caprylic acid combined with a polyamine) to the antibiotics, whereas others (Spaulding et al., 1955; Meyer, 1962) have employed the fungicidal drugs nystatin or Amphotericin B with success.

1.11 The Experimental Basis of the Concept of Colonization Resistance and Selective Decontamination of the Digestive Tract:

The experimental basis and the theory behind the concept of colonization resistance is largely dependent on a series of studies which were performed between 1971 - 1974 by van der Waaij and colleagues in the Netherlands (van der Waaij et al., 1971; Wensinck et al., 1971; van der Waaij et al., 1972; van der Waaij and Berghuis de Vries, 1974).

Perhaps the most important study involved total decontamination of the digestive tract of Swiss ND2 mice using a combination of broad-spectrum nonabsorbable antibiotics, namely streptomycin and neomycin, given *ad libitum* in drinking water for a period of two weeks (van der Waaij et al., 1971). The study was divided into 4 phases:

Phase I :The period before antibiotic treatment (control mice).

Phase II : The actual treatment period.

Phase III : The period in which organisms surviving the treatment repopulated the intestinal tract.

Phase IV : When repopulation was completed. Two weeks after discontinuation of antibiotic treatment.

Treated mice became free of detectable *Enterobacteriaceae,* enterococci, staphylococci and of yeasts and other fungi during phase II. Groups of 20 mice were challenged or deliberately contaminated with streptomycin-neomycin resistant strains of *E.coli, Kl. pneumoniae,* or *Ps. aeruginosa* using different doses at different phases of antibiotic treatment. The result showed that the number of phase I (control) and phase IV (recovery) mice excreting the three challenge strains gradually decreased, and that this decrease was more rapid with lower doses of the challenge organisms. In contrast, persistent colonization with the three challenge

organisms took place in all phase II and phase III mice. From these results they concluded that there was a process that was associated with the elimination of orally introduced organisms , and this mechanism was termed " Colonization Resistance".

Van der Waaij et al., (1971) suggested that colonization resistance could be determined semi-quantitatively by expressing colonization resistance as the log of the oral dose of challenge organisms resulting in colonization of 50 % of the animals for at least two weeks. The value of colonization resistance in control mice was then found to be slightly different for the three challenge strains tested: the CR of phase I mice to *E.coli* was 7, whereas the CR to *Pseudomonas aeruginosa* or *Klebsiella pneumoniae* was > 9. The statistical significance for this observation was not stated by the author, and these results may be within experimental error. After the onset of oral administration of antibiotics colonization resistance decreased, and the value remained low (less than 2) for the three organisms during the treatment period. There was a slow recovery of CR during phase III, which returned to normal levels at phase IV (recovery).

1.12 Mechanism Of Colonization Resistance:

1.12.1 Implication of the anaerobic bacteria in CR:

Wensinck et. al. (1971) studied the caecal flora of groups of 5 Swiss ND2 germ-free mice contaminated with the faecal flora of phase IV (recovery) ND2 mice from the TDD experiments of van der Waaij et. al.,(1971). Five *species* of anaerobes were detected in this simplified population. Anaerobic lactobacilli and *Bacteroides,* the predominant anaerobes of conventional mice **(see section 1.9.2)**, were absent; the major constituents were tapered rods belonging to the genus *Clostridium* (Wensinck et al., 1971). Germ-free mice colonized with the flora of phase IV

offspring were termed colonization resistance factor mice (CRF-mice) (van der Waaij et al., 1971) . These reconstituted germ-free mice and their offspring were found to be highly resistant to colonisation with *E. coli* as a challenge organism (higher than untreated normal mice). This study neither determined which of the CRF organisms was most associated with CR, nor a mechanism by which CR operated. In addition, the intestinal microbial flora was not characterized before treatment with TDD antibiotics (van der Waaij et al., 1971). It was therefore not possible to know whether the CRF flora was represented in the normal flora of mice. It was nevertheless suggested, that colonization resistance was due to anaerobic organisms which survived the TDD antibiotics, and subsequently repopulated the intestinal tract. Van der Waaij also reported that results from preliminary experiments with germ-free mice contaminated with various combinations of these five species strongly suggested that colonization resistance was due to the presence of a large gram-labile tapered *Clostridium* (van der Waaij et al., 1971).

Koopman et al., (1981) fundamentally disagreed with the conclusion of van der Waaij et al., (1971), namely that CR can be restored with a simple anaerobic microflora of only 5 strains. They attempted to normalize germ-free mice, and to obtain mice with a standardized gastrointestinal microbial flora. Five groups of germ -free Cpb:SE (Swiss) mice were colonized with different anaerobic cultures obtained from the faeces of CRF-mice, and of specific pathogen free-mice (SPF-mice), containing respectively 36 strains, 55 strains, 110 strains, or the complete microbial flora. Mice were then challenged with 10^7 *E.coli* resistant to streptomycin. Colonization resistance and other parameters were investigated to assess the normalization of germ-free mice. The results showed that the colonization resistance

against oral challenge with *E.coli* (10^7 cfu) was relatively low in germ-free mice, and relatively high in SPF-mice. Despite the numbers of strains with which the mice were colonized, the CR was found to be less than that of the SPF-mice, though it was greater than that of germ-free mice. These results suggested either that to normalize germ-free mice a very complex gastrointestinal microflora is needed, or that the strains associated with CR in normal mice were not represented in the flora with which the germ-free mice were colonized in this experiment (Koopman et al., 1981). In addition, the intestinal architecture and immunocompetence of germ-free mice differs from that of normal mice, and this may have had effects on the CR (Koopman et al., 1981).

Studies conducted by Syed et al., (1970), and Freter and Abrams, (1972) were able to normalize the CR of germ-free mice only with more than 100 bacterial strains, using the relative caecal weight (RCW) and other parameters. This supports the suggestion of Koopman et al., (1981) that normalization can only be reached with a complex microbial flora.

Other attempts have been made in mice, by the administration of cultures of anaerobic bacteria to germ-free mice, to analyze which species of the anaerobic flora are responsible for resistance to colonization with aerobic microorganisms. Administration of *Bacteroides* strains isolated from normal mice led to a massive colonization in groups of 20 germ-free mice, but did not produce recovery of resistance to colonization to aerobes. However, this did occur after administration of fresh faeces of normal NCS mice (Schaedler et al., 1965R-8-96). Apparently successful attempts were made to restore CR to germ-free mice using a large fusiform bacterial species (Wensinck et al., 1971; Hazenberg et al., 1981); however, in spite of

further study, the identity of this species remained vague (Hazenberg et al., 1976; 1977) and later it appeared that dozens of different species with this morphology live in the intestine of mice (Koopman and Kennis, 1983; Koopman et al., 1983). The above studies appear to indicate that the more species of anaerobes that were discovered, the more species appeared to be necessary to restore the colonization resistance of the intestine of the germfree mice (Koopman and kinnes, 1980; Welling et al., 1980). In general, it appears that complete normalization with a relatively simple microflora is probably not feasible.

There are further studies in mice and in humans that suggest that the anaerobes are part of first line defence in the gut, and a major factor in colonization resistance. Van der Waaij et al.,(1972), described the influence of systemic antibiotic treatment on colonization resistance after oral challenge of mice. Groups of 30 ND2 mice were treated with 5 mg of streptomycin given by i.p. injection twice a day, or with 10 mg of ampicillin given by i.p. injection; control mice were injected with saline. On the third day of treatment, mice were challenged with 10^8 or 10^{10} cells of *E.coli*, or enterococci. A strong negative influence of high dose of systemic antibiotics on colonization resistance was found. The challenge strain persisted much longer and in a much higher concentration in the caecum of systemically treated mice than in control mice. Also, translocation of the challenge strain into the mesenteric lymph nodes and the spleen was found much more frequently in the antibiotic treated group. From these results they suggested that colonization resistance is due to the anaerobic flora that live in close contact with the mucosa, although the status of mucosal anaerobes was not investigated in this study. In a further study by van der Waaij andBerghuis de Vries, (1974) mice were given 10 mg ml^{-1} nalidixic acid (NA) in drinking water

for 3 weeks, and monkeys were given 500 mg NA administered orally twice a day in 5 ml of water. The enterobacteria were successfully eliminated from the faecal flora of both mice and monkeys within 9 days of treatment. As the concentration of enterococci and *Candida albicans* remained unchanged during treatment, it was suggested that the colonization resistance was not affected. This study therefore used non-overgrowth of indigenous organisms rather than colonization by challenge strains as a criterion of CR. Again, the anaerobic flora was not studied directly, but assumed to be unaffected by the drugs used. As the nalidixic acid eliminated the enterobacteriaceae, the anaerobes which were assumed to remain were assumed to be responsible for the CR.

Vollaard et al., (1990) performed a study in humans , and they reported that it was not possible to colonize the gut with a *Klebsiella pneumoniae* challenge strain after eliminating the *E.coli* from the gut by a dose of 20 mg of pefloxacin. However, in the same study when mice were treated with clindamycin which eliminated the anaerobic flora from the gut, *Klebsiella pneumoniae* easily colonized the gut. The authors therefore suggested that the anaerobic flora are responsible for colonization resistance. However, clindamycin also does not affect only the anaerobic flora. Wells et al., (1988), colonized germ-free Balb/c mice with *E.coli* and other aerobic and anaerobic organisms obtained from the caecal content of mice, to study and to clarify the role of the intestinal anaerobic bacteria in CR. They also concluded that the anaerobic bacteria play a major role in colonisation resistance and also in controlling translocation of facultative anaerobic bacteria.

1.12.2 Suggested mechanisms by which anaerobes exert their effect on CR:

Strictly anaerobic bacteria are thought to exert this activity directly by producing toxic metabolites such as the volatile fatty acid (VFA) (Bohnhoff and Miller, 1962; Dubos and Schaedler, 1962; Meynell, 1963; Lee and Gemmell, 1972) which may inhibit the multiplication of non-indigenous organisms. It has been shown previously that the normal microbial flora destroys ingested yeasts (Montgomery et al., 1931) possibly because of the toxic effect of VFA on fungi (Bergien, 1940). This substance also prevents the conversion of yeasts forms into invasive mycelial forms (Barlish and Phillips, 1966; Phillips and Barlish, 1966). Similarly it has also been shown that VFA produced by the anaerobic colonic flora inhibits *Pseudomonas aeruginosa in vitro* (Buck and Cooke, 1969). However, VFA can not explain fully the requirement of a complex microflora for the restoration of CR, and the actual mechanisms by which anaerobes exert CR are unknown.

1.12.3 Implication of factors other than anaerobes in CR:

Although many studies suggested that the anaerobes are part of first line defence in the gut, and play an important part in colonization resistance and in prevention of overgrowth by AGNB (as described above) , other studies have indicated that the facultative anaerobic bacteria (Freter and Abrams, 1972; Koopman et al., 1981; Hentges et al., 1985) and aerobic bacteria (Freter and Abrams, 1972; Ducluzeau et al., 1977; Goren et al., 1984) are also involved in the protection of animals against overgrowth with potentially pathogenic bacteria, and that they therefore contribute toward the colonization resistance. Hentges et al., (1985) studied the effect of

selective antibiotic modulation on colonization resistance to orally administered drug resistant strains of *Ps. aeruginosa* in Swiss mice. These investigators noted that a larger oral dose of the challenge strain was required to colonize mice treated with metronidazole , ampicillin, or clindamycin (affecting both aerobes and anaerobes), than those treated with kanamycin or streptomycin (affecting aerobes only), suggesting that both aerobic and anaerobic organisms were involved with colonization resistance.

There are other factors, including competition between the intestinal normal flora and non-indigenous challenge organisms for limited nutrients or for adherence to sites of attachment on the intestinal mucosal cells and establishment by the normal flora of environmental conditions that adversely affect the non-indigenous organisms (Meynell, 1963; Bohnhoff et al., 1964; Lee and Gemmel, 1972; Mayer et al., 1972; Levison, 1973; Byrne and Dankert, 1979; Snoeyenbos, 1979; Bibel et al., 1983; Freter et al., 1983[a]; 1983[b]; Hentges, 1983; Nord et al., 1984; Louie et al., 1985).

Host factors probably also contribute to the colonization resistance of the digestive tract. These include four major mechanisms: saliva and mucin production; saliva swallowing and peristaltic activity of the intestines; the excretion of immunoglobulin A with saliva and mucin; and the desquamation of mucosal cells (van der Waaij et al., 1982). These host activities limit the adherence of potentially pathogenic bacteria with the result that the chance of their colonizing part of the digestive tract is reduced. Anaerobic normal flora may also indirectly exert an influence on these host factors by stimulating the secretion of mucin, cell desquamation, muscular tonus of the intestine and peristaltic activity (Gordon and Pesti, 1971).

1.13 Evaluation of Colonization Resistance:

The concentration of resistant challenge organisms in the faeces following experimental oral challenge as described by van der Waaij et al., (1971) is one of the most direct indicators of colonization resistance and allows the generation of quantitative results. In addition, the following variables have used to assess CR: the relative weight of the caecum (Koopman and Janssen, 1975; Koopman et al., 1977; Emmelot and van der Waaij, 1980; Rugi, 1985) and the concentration of ß-aspartylglycine (ß-aspgly) in the faeces (Welling, 1979; Emmelot and van der Waaij, 1980; Manson et al., 1981; van der Waaij et al., 1982).

The calculation of the relative weight of the caecum (RCW) (weight of caecum / weight of body) has been used because it has been shown that the RCW increases when the colonization resistance is impaired (Koopman and Janssen, 1975; Koopman et al., 1977; Ruijs and van der Waaij, 1985). This increase in the RCW is thought to be because mucus, normally digested by anaerobes accumulates in the caecum. This value appeared to be a good indicator for the colonization resistance in mice; Koopman et al., (1977) found a linear inverse correlation between the colonization resistance and the relative caecal weight. Other studies have also reported that the mean weight of the caecum in mice with a disturbed intestinal microbial flora and decreased colonization resistance due to antibiotic treatment is larger than that of control conventional mice; Thijm and van der Waaij, (1979) indicated that the colonization resistance was strongly decreased during systemic treatment with ampicillin or epicillin and there was an associated increase in the mean caecal weight of these mice compared to control conventional mice. Similar results have recorded by others (van der Waaij et al., 1982;

Ruijs and van der Waaij, 1985).

Beta-aspartylglycine is formed in the course of digestion of collagen and other proteins from preexistent anhydro-aspartyl-glycyl bonds (Pisano et al.,1966) by preferential ring opening on the alpha-carbonyl bond and due to the metabolic inertness of the ß-aspartyl bond. It is a *dipeptide* of molecular weight 190.2 **(Figure.1)**. It is continuously secreted into the intestine, and is present in the faeces and caecum of germ-free or totally decontaminated intestines of mice and humans (Welling and Groen, 1978; Welling, 1979) and in normal urine (Buchanan et al., 1962[a]; 1962[b]; Tanaka and Nakajima, 1979). In one study by Welling and Goren (1978), the concentration of ß-aspgly was observed to be 200 times greater in the caecum content of germ-free and totally decontaminated mice than in the caecum of normal mice. This difference was attributed to breakdown of ß-aspgly by the anaerobic normal flora (Pisano et al., 1966). Van der Waaij et al., (1982) found that the total count of anaerobic bacteria (tapered rods and fusiform bacteria) was approximately inversely proportional to the ß-aspgly concentration in the faeces. These observations also appear to be valid for man and other animal species (Welling et al., 1980).

As a consequence of these studies, ß-aspgly has been used as an indicator of the effect of different antibiotics on anaerobic flora of the gut and hence on colonization resistance (Welling, 1979; Emmelot and van der Waaij, 1980; Manson et al., 1981; van der Waaij et al., 1982). Cephradine led to the presence of ß-aspgly in the faeces of mice when given at doses of 280 mg / kg, and in monkeys when given when given at a dose of 90 mg / kg (Manson et al.,1981) and in humans when given a dose of 6 g / day (van Saene and Driessen, 1979).

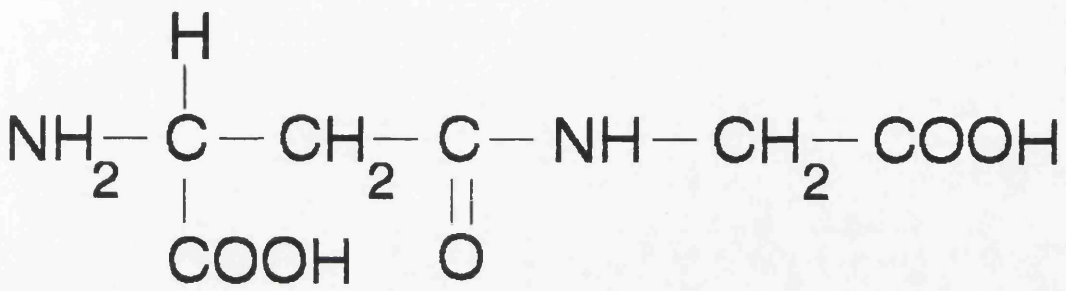


Figure 1 ß-aspartylglycine.

However, ß-aspgly was also found to have increased even with low doses during treatment with gentamicin or amikacin in the faeces of Swiss mice (van der Waaij et al., 1982). This is not consistent with ß-aspgly being inversely proportional to the anaerobes as these drugs have no direct activity against anaerobic populations. As aminoglycosides are frequently used in gut decontamination regimens, care is obviously required in the interpretation of ß-aspgly data obtained in this context.

In general there would appear to be no convincing substitute for direct challenge studies in the assessment of CR and other techniques should probably be regarded as a source of supporting data only.

1.14 Influence of Antimicrobial Agents on Colonization Resistance:

The differential effect of antimicrobial agents on CR is central to the concept of selective decontamination of the digestive tract. In the past twenty years this phenomenon has been studied in animal models either as a probe to alter the resident normal flora of the gut (Meynell, 1969; Meynell and Subaian, 1963; Bohnhoff et al., 1964[a]; 1964[b]; van der Waaij 1968; van der Waaij et al., 1971; 1972; van der Waaij and Berghuis de Vries, 1974) or to identify antimicrobial agents that would preserve the colonization resistance (Thijm and van der Waaij, 1979; van der Waaij et al., 1982).

From these studies it has been concluded that antibiotic drugs can be grouped in three major classes in term of their influence on the CR of the digestive tract (van der Waaij et al., 1979; Wiergersma et al., 1982).

1.14.1 Antimicrobial agents which do not affect the CR even when administered in very high oral doses.

In tests with tobramycin, amikicin, gentamicin, paromomycin and streptomycin, only tobramycin used in high doses (maximum dose 2.9 mg ml^{-1}) did not affect CR in mice (van der Waaij et al., 1982; Hentges et al., 1985). The CR was measured using 3 parameters; the concentration of ß-aspgly, the relative caecal weight and the mean faecal concentration following oral challenge with *E.coli,* or *Kl. pneumoniae* (van der Waaij et al., 1982).

Polymyxin B, a narrow-spectrum antibiotic did not influence the CR even after relatively high oral doses of 9.7 mg day^{-1} (Emmelot and van der Waaij , et al., 1980). This was determined using the concentration of the challenge organisms in the faeces, the RCW, and ß-aspgly concentration in the faeces (Emmelot and van der Waaij, et al., 1980).

Of the cephalosporins tested, cephradine in a dose of 3.4 mg $mouse^{-1}$ had no effect on CR (Thijm and van der Waaij, 1979). These experiments used the RCW and the concentration of the challenge organisms in the faeces as an indicator of CR. Other cephalosporins such as cephalexin and cefaclor both in doses of 4.6 mg $mouse^{-1}$ (Wiegersma et al., 1982) acted in the same way as cephradine. The CR was not affected as estimated using 3 parameters; the relative caecal weight, the concentration of the challenge organisms in the faeces and the concentration of the indigenous *Enterococci faecalis* in the faeces Wiegresma, 1982). The fact that these antibiotics does not affect CR may be due to the complete absorption of these drugs in the small intestine , even during high dose oral treatment.

Three of the quinolones were found not to affect CR at high doses. In study by van der Waaij et al., (1974) using mice and monkeys, CR was not affected by relatively high (but not toxic) doses of nalidixic acid. Mice were treated with 1 g k g^{-1} body weight, whereas monkeys were treated with 0.3 - 0.5 g kg^{-1} body weight. The lack of overgrowth of indigenous *Enterococcus faecalis* and yeasts was used as an indicator of CR. The same result was seen in a study by Wiegersma et al., (1982). They reported that CR was not affected when mice were treated with daily doses of 14 mg $mouse^{-1}$ of nalidixic acid, or 15.2 mg $mouse^{-1}$ of cinoxacin. CR was estimated as above. Rosoxacin did not affect the CR even during treatment with a daily dose of 6 mg $mouse^{-1}$ (Ruijs and van der Waaij, 1985). The relative caecal weight, the concentration of ß-aspgly in the faeces and the concentration of the indigenous *Enterococcus faecalis* in the faeces were used as a parameter for *CR* (Ruijs and van der Waaij, 1985).

Wiegersma et al., (1982) studied the effect of different antibiotics on CR measured by the relative caecal weight, the concentration of the challenge strain in the faeces and the concentration of the indigenous *Enterococcus faecalis* in the faeces. Antibiotics such as co-trimoxazole (1.1 trimethoprim + 5.8 mg sulphamethoxazole per mouse), pivmecillinam (> 2.5 mg per mouse) or doxycycline(1.2 mg per mouse) were found not to affect the CR (Wiegersma et al., 1982).

Amphotericin B or nystatin have been used to eliminate yeasts from the intestine. These agents have had no effect on CR, but it has been suggested that amphotericin B was superior to nystatin (van der Waaij, 1979).

1.14.2 Antimicrobial agents that do not affect CR during treatment with low doses, but produced a decrease in CR following high doses:

Of the aminoglycosides, neomycin decreased CR in mice when given in doses higher than 4 mgml^{-1} (Emmelot and van der Waaij, 1980). This decrease in CR was determined using the concentration of the challenge organisms in the faeces, the relative caecal weight and ß-aspgly concentration in the faeces (Emmelot and van der Waaij, 1980). Similar results were obtained with amoxycillin at doses > 1.6 mg mouse^{-1}: the relative caecal weight was increased and the concentration of the challenge organism was higher and persisted longer than in control mice (Wiegersma et al., 1982). A similar effect was seen with norfloxacin at a dose >3 mg mouse^{-1} (Ruijs and van der Waaij, 1985).

1.14.3 Antimicrobial agents which decrease the CR even when given at low doses:

In tests with aminoglycosides kanamycin (at a dose of 1 mg ml^{-1}) or streptomycin (at a dose of 5 mg ml^{-1}) severely decreased the CR in mice, as estimated by the increase of susceptibility of mice to colonization with a *Ps. aeruginosa* challenge strain (Hentges et al., 1985).

Most penicillins severely affected CR: penicillin V (at a dose of 0.5 mg mouse^{-1}), cloxacillin (at 0.6 mg mouse^{-1}), phenethicillin (at 0.7 mg mouse^{-1}) (Wiegersma et al., 1982), ampicillin (at 0.5 mg mouse^{-1}), epicillin (at 0.6 mg mouse^{-1}) (Thijm and van der Waaij, 1979; Hentges et al., 1982), azlocillin and mezlocillin (both at > 0.86 mg mouse^{-1}) (van der Waaij et al., 1982). In these studies, the concentration of the challenge organisms in the faeces, the relative caecal weight and the concentration of indigenous *Enterococcus*

faecalis were used to measure CR. The decrease in CR seen in these experiments was frequently associated with bacterial overgrowth in the digestive tract by resistant strains (Laura and Kaminski, 1962; Pollack et al., 1972).

In tests with cefuroxime, cephradine, cephalexin, cefaclor and moxalactam, only cefuroxime and moxalactam (both at doses of > 0.86 mg mouse^{-1}) were active against the anaerobic flora of the intestine, and decreased the CR in mice (van der Waaij et al., 1982).

Other drugs such as clindamycin, which has high activity against anaerobic bacteria (at a dose of 1 mg ml^{-1},) severely decreased the CR. This was estimated by the increase in susceptibility of mice to colonization with a *Ps. aeruginosa* challenge strain (Hentges et al., 1985).

In summary the effects of the drugs used in the present study are as follows:

1) *TDD antibiotics:* Neomycin in low doses did not affect CR, whereas when given in higher doses of (> 4 mg ml^{-1}) the CR was reduced. Similar results were seen with streptomycin which decreased CR only when given at doses of > 5 mg ml^{-1} to mice. When combined these two drugs were associated with total decontamination of the digestive tracts and severe impairment of CR in mice (van der Waaij et al., 1971).

2) *SDD antibiotics:* Tobramycin (at a maximum tested dose of 2.9 mg ml^{-1}) did not effect CR in mice. Similar results were seen with polymyxin at a maximum dose of 9.7 mg day^{-1}. A combination of the two drugs with the addition of amphotericin B was widely employed in clinical practice as a regimen for selective decontamination of the digestive tract (Stoutenbeek et al., 1984) with results that strongly suggest no significant impairment of CR.

Unfortunately, there have been no direct studies on the effect of this SDD regimen on CR as measured by oral challenge of experimental animals.

In clinical trials, cefotaxime was added to the SDD drugs in the first a few days to provide additional cover against AGNB during treatment whilst SDD was becoming established and to provide cover against gram-positive bacteria resistant to SDD agents (Alcock, 1990). There have been no previous studies on the effect of cefotaxime alone on CR. However, cefotaxime was chosen because when used alone it is thought to have only minimal effects on the TVC of anaerobic flora and is thus unlikely to impair intestinal CR (Todd and Brogden, 1990).

AIMS OF THESIS

Aims of Thesis

The previous review has described the long standing use of the techniques of total decontamination and of selective decontamination of the digestive tract in certain groups of patients. In particular, the last ten years has witnessed the extension of selective decontamination to medico-surgical intensive care patients, a procedure that remains highly contentious.

The rationale of selective decontamination depends crucially on the concept of colonization resistance. However, the mechanisms underlying this phenomenon are both complex and controversial. Further, the experimental work underpinning the clinical use of SDD depends to a great extent upon a restricted series of studies involving the total decontamination of mice carried out by van der Waaij and co-workers between 1971 and 1974.

Against this background the thesis has two main aims:-

1) To repeat the original total decontamination studies of van der Waaij and colleagues in order to verify or modify their conclusions.

2) To use the same mouse model to study the SDD regimen most commonly used in clinical ICU practice - an orally administered mixture of polymyxin E, tobramycin and amphotericin B (PTA) supplemented with systemic cefotaxime.

The techniques employed centred upon direct oral challenge of mice with bacterial species of defined characteristics, together with detailed microbiological monitoring of the aerobic and anaerobic flora of the gut.

Chapter Two

MATERIALS AND METHODS

2. Materials And Methods:

2.1 Animals:

Conventional inbred female Balb/c /OLa mice (*Harlen Olac, Shows Farm, Bicester, England*), varying in age between 8 - 16 weeks, and weighing between 18 - 24 gram were used.

2.1.1 Wild mice:

Wild mice were obtained by placing a number of small mammal steel traps overnight in a field at the University of Glasgow biology field station at Rowardennan. Three wild mice were captured, moved to the laboratory and killed using cervical dislocation on the same day. Faecal pellets , and the gut content after dissecting the mice, were assayed for aerobic flora, anaerobic flora, and ß-aspartylglycine.

2.1.2 Housing conditions:

Mice were housed individually in autoclaved polypropylene cages (size M 1: 33 x 15 x 13 cm), covered with stainless steel tops, with autoclaved wood shavings as bedding. For housing groups of 8 - 16 mice size MB1 (45 x 28 x 13 cm) autoclaved cages were used. The experiments were carried out in a normal animal room. Control, treatment, and post-treatment mice were kept in separate rooms. Cages, and wood shavings were changed daily.

2.1.3 Diet:

Food and water were given *ad libitum* . Control and post-treatment mice were given sterile water acidified after autoclaving to pH 3 with HCl. During antibiotic treatment, mice were given unacidified sterile water. Food consisted of sterile irradiated pellets (*GIP grade diet irradiated, Biosure Cambridgeshire, England*).

2.2 Antibiotic Treatment Of Mice:

Antibiotics were used singly and in combination' to study the effect on CR of TDD and of SDD.

2.2.1 Antibiotics used for TDD of mice:

For total elimination of the normal flora of the gut, a combination of streptomycin sulphate (Sm) (*Sigma chemical company Ltd., Dorset, England*). and neomycin sulphate (Nm) (*Sigma*), at 5 mg ml^{-1} of each antibiotic, was given in drinking water, to which pimaricin (*Sigma*) 0.1 mg. ml^{-1} was added as an antifungal agent (TDD regimen). The TDD regimen was continued *ad libitum* for a period of 2 weeks. During treatment the drinking water bottle was wrapped with aluminium foil to protect it from the light, since pimaricin is light-sensitive. The antibiotics and the drinking water were changed every 5 days, as neomycin is unstaible at room temperature **(see section 2.6.1)**.

For comparative purposes, Sm (10 mg) + Nm (10 mg) in 0.2 ml water were administered to mice by gavage in one experiment. In some TDD experiments 3 mg. ml^{-1} of bacitracin (*Sigma*) was added to the TDD regimen to eliminate any resistant gram positive organisms.

2.2.2 Antibiotics used for SDD of mice:

For selectively eliminating the AGNB from the digestive tract a combination of 0.51 mg. of polymyxin E sulphate (Pe), (*Sigma*), 0.42 mg of tobramycin (Tn) *(Lilly France S. A. Fegensheim, France, for Eli Lilly and company LTD, Basingstoke, England*), and 0.86 mg amphotericin B. (*E.R. Squibb & Sons LTD, Middlesex, England*) (SDD regimen) in aqueous suspension (PTA), were administered by gavage in a volume of 0.2 ml to each mouse once a day (Speekenbrink et al., 1987; 1988).

Cefotaxime (CTX) (*Roussel Laboratories LTD., Uxbridge, England*) was administered to mice with or without the PTA regimen at a dose of 3 mg $mouse^{-1}$ given by a single intraperitoneal (i.p.) injection each day for 4 days.

For selectively eliminating the anaerobic normal flora from the digestive tract clindamycin (*Sigma*) was administered to mice at a dose of 0.6 mg. $mouse^{-1}$ given by gavage in a volume of 0.2 ml once a day for 4 days.

2.3 Challenge Strains:

Seven challenge strains were used in the evaluation of CR **(Table 6)**. Strains were rendered Sm and Nm resistant by passage in the presence of antibiotics, except for *E.coli* s-R 21 which was made resistant to Nm by transfer of plasmid RP4 by Dr. D. Platt (*Department of Bacteriology, Royal Infirmary, Glasgow, U.K.*). *E.coli* s-R21was derived from mouse faecal flora. Human isolates of *Proteus mirabilis* s-R9 resistant to TDD and SDD regimen, and sensitive to CTX, *Ps. aeruginosa* s-R321, *Kl. pneumoniae* s-R10, and *Providencia stuartii* s-R7 were kindly donated by Dr. A. B. J. Speekenbrink (*Department of Bacteriology, Western Infirmary, Glasgow, U.K.*). Human isolates of clinically drug resistant *Ps. cepacia* s-R13, and *Candida albicans* s-R5 were kindly donated by Dr. S. R. Alcock (*Department of Bacteriology, Western Infirmary, Glasgow, U.K.*).

2.4 Culture Media:

Brain heart infusion broth (BHIB) (*Oxoid* ,Basingstoke, Hants, U.K.) was used for homogenizing the faecal pellets, and as a diluent in all dilution procedures. The aerobic flora and the challenge strains were cultured on Mac Conkey agar without salt (*Oxoid*) (MC), and on blood agar base No. 2 (*Oxoid*) plus 5 % defibrinated horse blood (*Oxoid*) (BA). Pseudomonas selective agar (*Oxoid*) (Ps.S A) was used for selecting and culturing *Pseudomonas* species, Desoxycholate Citrate Agar (DCA) (*Oxoid*) for culturing *Providentia stuartii* s-R7, and de Man, Rogosa, and Sharpe agar (*Oxoid*) (MRSA) for isolating *Lactobacillus* species. For culturing *Candida albicans* s-R5, Sabouraud broth (*Oxoid*), and Sabouraud agar (*Oxoid*) were used .

The anaerobic flora was cultured using Wilkins-Chalgren anaerobic

Table 6 **Challenge Strains**

Species	Strain	MIC level in mg ml^{-1}				Source
		Sm	Nm	Pe	Tn	
E. coli	s-R21	10	10			Mouse
Klebsiella pneumoniae	s-R10	0.22	0.1			Human
Pseudomonas aeruginosa	s-R321	10	10			Hunam
Proteus mirabilis	s-R9	5	2.5	1	0.5	Human
Pseudomonas cepacia	s-R13	10	10			Human
Providencia stuartii	s-R7					Human
Candida albicans	s-R5					Human

Sm: Streptomycin, Nm: Neomycin, Pe: Polymyxin E, Tn: Tobramycin.

agar (*Oxoid*) plus 5 % defibrinated horse blood(anaerobic BA), and M 10 agar (Caldwell and Bryant, 1966) **(see appendix 1)**.

2.5 Assay of Antibiotic Activity in Murine Faeces and Caecal Content:

Fresh faecal pellets were collected from mice treated with TDD, SDD and / or cefotaxime to estimate the concentration or the activity of these drugs in murine faeces or caecal contents. The faecal pellets or the caecal content were immediately weighed, and homogenized in 3 parts by weight of distilled water. The homogenates were centrifuged for 10 minutes at 13000 RPM using a micro-centrifuge (*M S E, England, U.K.*). The supernatant fluid was removed without disturbing the sediment using a syringe, and filtered using a 0.45 μm sterile acrodisc syringe filter (*Gelman science, Michigan, U.S.A.*). A standard solution of each antibiotic was prepared before starting the antibiotic assay.
The antibiotic activity was assayed by one of the following 3 procedures:
1) Antibiotic discs were prepared by adding 5 μl of the filtered supernatant, and of each dilution of the standard on autoclaved paper discs 5 mm in diameter. The discs were left to dry at room temparature in a sterile petri dish. Sensitivity agar (*Oxoid*) plates were lawned with an overnight culture of an indicator strain. The antibiotic discs were placed on the agar plates, and all plates were incubated overnight at 37°C . Four discs of each sample and control were used.
2) Sensitivity agar plates were lawned with an indicator organism, and 4 wells in each agar plate were made using a 5 mm well punch. The bottom of the wells were sealed by adding 8 μl of melted nutrient agar (*Oxoid*). Aliquots of (20 μl) of supernatant fluid or the standard were added to the wells. The plates were incubated overnight at 37°C.
3) The faecal pellets were pooled , homogenized, and compressed into 5 x 3 mm cylindrical pellets. An indicator strain was cultured overnight in BHIB. Blood agar base was mixed with an equal volume of BHIB, and cooled to 40°C. The

overnight culture of the indicator organisms was ten-fold serially diluted in BHIB, and 2 ml of the fourth dilution (2 x 10^5 cells ml^{-1}) was added to the agar, which was poured into 30 mm culture dishes (*Flow Laboratories*) . One standardized faecal pellet was placed in the centre of each culture dish, and the dishes were incubated overnight at 37°C.

An estimate of antimicrobial activity was determined in each procedure by squaring the diameter of the zone of inhibition of bacterial growth. Each experiment was performed 4 times.

2.6 Preliminary Experiments:

2.6.1 Stability of streptomycin and neomycin in drinking water:

For testing the stability of Sm and Nm in drinking water at room temperature, 1 ml of the drinking water containing Sm and Nm was collected daily in an Eppendorf tube, and stored at -70°C until assayed. A standard solution of Sm and Nm was prepared before starting the assay by dissolving 6 mg of each antibiotic in 1 ml of distilled water. Two fold serial dilutions were made in distilled water in 50 μl volumes to give a final concentrations of 6,3,1.5,0.75,0.38, and 0.19 g l^{-1} Antibiotic discs were prepared as described in section **(2.5)**. Sensitivity agar plates were lawned with an overnight culture of *E.coli* NCTC 10418 or *E.coli* Sm^R Nm^S to test the activity of the combination of Sm and Nm , or of neomycin alone respectively.

2.6.2 The level of resistance of the challenge organisms to Sm and Nm:

The activity of Sm and Nm in mouse faeces was monitored to indicate the minimum level of resistance necessary in a challenge strain during TDD. Fresh faecal pellets were collected at 4, 8, 12,16, and 24 hours after administration of antibiotics. The pellets were assayed and discs of standard solution and of the supernatant fluid were prepared in the same manner as in section **(2.5)**. *E. coli* NCTC No. 10418 was used as an indicator organism.

2.6.3 Creation of multiple resistant challenge organisms:

E.coli s-R21, *Kl. pneumoniae* s-R10, *Ps. aeruginosa* s-R321 and *Proteus mirabilis* s-R9 were passaged in BHIB containing increasing concentrations of Sm and Nm and PTA antibiotics. The MIC of these organisms was assayed to see if they could be used as challenge organisms for evaluation of CR during TDD and SDD treatment. MIC was measured using standard procedures

2.7 Evaluation of Colonization Resistance:

Groups of 8 conventional mice were used for each challenge experiment. Strains were cultured overnight in BHIB, and then serially ten-fold diluted in BHIB. Mice were given 0.2 ml of saturated aqueous sodium bicarbonate by gavage, followed by 0.2 ml of one of the dilutions. For estimating the total viable counts (TVC) of the culture 20 μl of each dilution was cultured on BA and incubated overnight at 37°C.

2.8 Quantification of Colonization Resistance:

CR was expressed as the log of that oral dose of an organism resulting in colonization in 50 % of animals for at least 2 weeks (van der Waaij et al., 1971) as estimated by presence of the organism in the faeces.

2.8.1 Faecal culture:

For culturing the different challenge strains from mouse faeces, fresh faecal pellets were collected in a test tube from individual mice at intervals after challenge. The pellets were homogenized in 2 ml of BHIB containing 2 mg ml^{-1} of Sm and Nm , and incubated at 37°C . After 24 hours incubation subculture was made on to solid media. The media used were MC and BA agar for all challenge strains, plus the following selective media: DCA for culturing *Providencia stuartii* s-R7 ,and PsS A for *Ps. aeruginosa* s-R321, and *Ps. cepacia* s-R13 challenge strains. Sabouraud broth and agar was used to culture *Candida albi*cans challenge strains. Mice were removed from the experiment if 2 successive negative cultures for the challenge strain were

obtained. Aerobic normal flora was identified by the colony morphology, growth on selective media, gram appearance. For the identification of AGNB the API 20 E system (*API, Bio Merieux, France*) was used in some cases.

2.9 Estimation of Aerobic Faecal Flora:

For monitoring TVC of the aerobic faecal flora, fresh faecal pellets were collected in test tubes from individual mice. The pellets were immediately weighed, and homogenized in 9 parts by weight of BHIB to make a 1:10 dilution. Serial tenfold dilutions of the homogenate were made in the same broth , and the viable bacteria were estimated using the Miles and Misra droplet count (Miles et al., 1938) on duplicate plates of MC and BA. The viable count was reported as $\log_{10}$ of the CFU per gram of faeces.

2.10 Bacterial Culture of Different Regions of the Intestine:

Mice were sacrificed by cervical dislocation, and dissected on a dissecting board under aseptic conditions. The microflora of five parts of the gut; the upper jejunum, lower jejunum, ileum, caecum, and colon was estimated. Each part of the gut was weighed immediately , 9 parts by weight of BHIB were added , and the gut segments were homogenized in a blender. For quantitative estimation of aerobic flora, ten fold serial dilutions were made, and 20 μl of each dilution was placed on duplicate plates of BA, MC agar, and MRS agar. The same media were inoculated for single colonies of aerobic normal flora. For qualitative determination of organisms present in low numbers all plates and dilution tubes were incubated at 37° C. After 24 hours incubation, subculture was made from the tubes on BA, MC agar and MRS agar: the plates were incubated overnight at 37°C.

2.11 Estimation Of Anaerobic Faecal Flora:

Fresh faecal pellets were collected from individual mice in a test tube. The pellets were immediately weighed , and moved into an anaerobic chamber

(*MK3 anaerobic work station, Don Whitley Scientific Limited, Shipley, West Yorkshire, England*). The pellets were homogenized in 9 parts by weight of reduced BHIB to make a 1:10 dilution. Serial ten-fold dilutions of the homogenates were made in the same broth. Aliquots 0.1 ml of the relevant dilutions were transferred to the middle of reduced anaerobic BA plates, and spread evenly over the surface of the media using a bent Pasteur pipette. Four plates of anaerobic BA were used for each dilution, and the plates were incubated for 7 days in the anaerobic chamber under anaerobic conditions (5 % H_2, 10 % CO_2, and 85 % N). After incubation the plates were removed from the anaerobic cabinet, and different types of colonies were picked and inoculated on MC and BA, and incubated in air, and in 10 % CO_2 for 24 hours to separate the facultatively anaerobic from the obligate anaerobic colonies. The diversity of the anaerobic faecal flora was estimated semi-quantitatively by counting the different types of anaerobic colony. Colonies were categorized by circumference, elevation, edge, surface, translucency, colour, texture and their effect on media.

2.12 Estimation of the Concentration of ß-aspartylglycine:

The concentration of ß-aspgly in the faeces was studied using a High Performance Liquid Chromatograph (HPLC) (*Gilson Medical Electronics, Middleton. WI, U.S.A.*). Reverse-phase HPLC was carried out on a spherisorb microbore analytical S5ODS1 column 250 mm long x 4.6 mm internal diameter (id), and an analytical guard column 20 mm long x 2 mm id packed with spherisorb SGODS1. Faecal o-phthaldialdehyde (OPT) derivative (20 μl) were chromatographed on a 5 mM phosphate buffer pH 6.5: methanol gradient. The flow rate was variable between 1 - 2 ml minute^{-1} **(Figure. 2)**. No appropriate internal standards were available, and ß-aspgly was identified in faecal derivative by comparison with the mobility of ß-aspgly (*Sigma*) in water (100 n mole ml^{-1} stock solution). The OPT reagent solution in a concentration of 1 g l^{-1} (*Sigma*) derivatives were detected on an in-line fluorometer with excitation filter

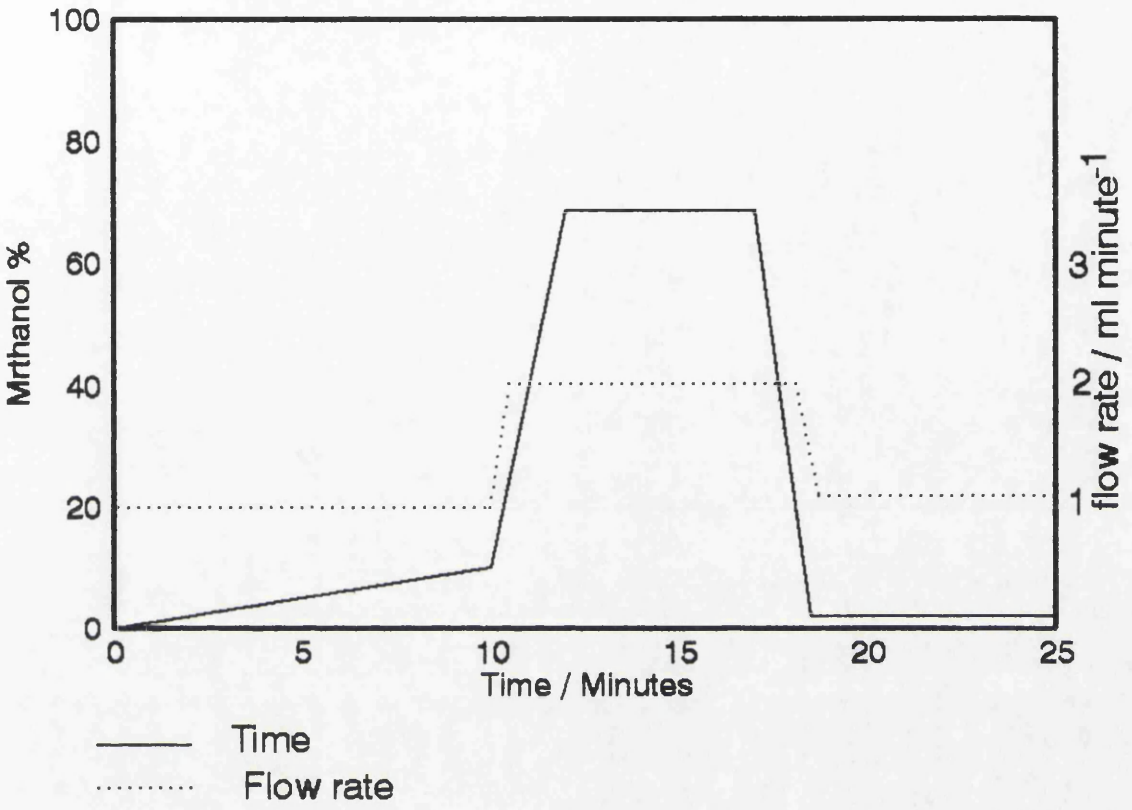


Figure 2 Methanol gradient used for detection of OPT derivative of ß-aspgly.

of 305-395 nm and emission filter of 430-470 nm. The output was recorded on a digital integrator (*Shimadzu model C-R3A, chromatopac, Dyson instruments LTD., Hetton, England*).

2.12.1 Preparation of ß-aspartylglycine standard:

ß-aspartylglycine (*Sigma*) was stored in small vials in a concentration of 100 n mole ml^{-1} at - 20°C. The standard was prepared by adding OPT to the standard in a ratio of 2 : 1 respectively (0.1 ml OPT : 0.05 ml standard), and diluted 1 : 5 in distilled water before use. The mixture was allowed to react for 2 minutes at room temperature.

2.12.2 Preparing the test sample from murine faeces:

Fresh faecal pellets were collected from individual mice in Eppendorf tubes before, during, and after treatment with different antibiotics. The pellets were immediately weighed, and homogenized in 9 parts by weight of distilled water to make 1 : 10 dilution. The homogenate was centrifuged for 10 minutes at 13000 RPM using a micro-centrifuge (*M S E, England , U.K.*). The supernatant fluid was removed using a syringe without disturbing the sediments, diluted 1 : 5 in distilled water, and filtered using 0.45 μm syringe filter *(Sterile Acrodisc, Gleman science, Michigan, U.S.A*). The filtered supernatant was then treated in the same manner as the ß-aspartylglycine standard.

2.12.3 Injection of samples into HPLC machine:

A Hamilton microliter syringe (*Hamilton Bonaduz AG, Bonaduz, Switzerland*) was used for injecting 20 μl of the samples into the HPLC. The ß-aspgly standard was injected before, and after injecting and testing a set of test samples. The ß-aspartylglycine peak in the test sample was identified by comparison with the retention time of the ß-aspartylglycine peak in the standard.

2.12.4 Estimating the concentration of ß-aspgly in test sample:

The concentration of the ß-aspartylglycine of test samples was calculated using the known concentration of the control sample, and the area of the peaks of the control and test samples. The area of the peak was estimated by the formula:

Area of the peak = Height x Width at 1/2 Height

2.13 Activity of Antibiotic in Murine Faeces or Caecal Content:

2.13.1 Mice treated with TDD regimen:

The activity of Sm and Nm in mouse faeces in mice treated by gavage or *ad libitum* in drinking water was compared. The faecal pellets were assayed, sensitivity agar was lawned with *E.coli* NCTC No.10418 and the supernatant fluid was added to the wells as described in section **(2.5)**.

2.13.1.1 Effect of TDD regimen in murine faeces on *E.coli* s-R21 challenge strain *in vitro*:

The activity of Sm and Nm in murine faeces on *E.coli s-R21* challenge strain was studied *in vitro*. Cylindrical pellets were prepared as described in section **(2.5)** Strains of *E.coli* NCTC No.10418 and *E.coli* s-R21 challenge strain were used to observe the zone of inhibition.

2.13.2 Mice treated with PTA regimen:

A standard solution of antibiotics was prepared by dissolving 1 mg each of Pe and Tn in 1 ml of distilled water. The supernatant fluid was prepared as described in section **(2.5)**, and antimicrobial activity in the faeces was estimated using *E.coli* NCTC 10418 as an indicator.

2.13.3 Mice treated with cefotaxime:

2.13.3.1 The concentration of cefotaxime in mouse caecal content:

The mice were dissected and the caecal contents were collected aseptically, and assayed for antibiotic activity as described in section **(2.5)**. The standard antibiotic solution contained 3 g l^{-1} CTX, and the indicator strain

was *E.coli* NCTC 10418.

2.13.3.2 *In vitro* effect of CTX in murine faeces on aerobic organisms:

The activity of CTX in murine faeces on the CTX sensitive organisms, *E.coli* s-R21 , *Ps. aeruginosa* s-R321, and *Proteus mirabilis* s-R9 was studied. Agar lawned with one of these strains prepared as described in section **(2.5).** Neomycin (1 mg ml^{-1}) was added to the agar. Cylindrical pellets were used to observe the activity of CTX as described in section **(2.5).**

2.13.3.3 *In vitro* effect of CTX in mouse caecal content on the anaerobic flora:

The activity of CTX in mouse caecal content on the anaerobic normal flora was tested *in vitro*. Supernatant fluid from mouse caecum was obtained as described in section **(2.5)**. Anaerobic BA was lawned in an anaerobic cabinet with 0.01 ml of homogenized faecal suspension. Wells were made in agar as described in section **(2.5)**, and 20 μl of the samples were added to the wells. One well contained CTX (20 μl) at 3 mg ml^{-1} as a positive control. All plates were incubated for 5 days at 37°C in the anaerobic cabinet.

2.13.3.4 The *in vitro* effect of CTX on the anaerobic faecal flora :

The lowest concentration of CTX which affected the anaerobic faecal flora was determined in vitro. Two fold serial dilutions of 3 g l^{-1} of CTX were made using the same procedure described as in section **(2.5)**. An aliquot (20 μl) of each dilution was added to wells in agar plates lawned with 0.01 ml of faecal suspension. The plates were incubated for 5 days at 37°C in the anaerobic cabinet, after which the zones of inhibition were estimated.

2.14 Statistics:

All statistics were calculated using an in-house computer program. the significance of the differance between means was estimated using a two-sample Student's t test.

Chapter Three

RESULTS

3. Results:

3.1 Results Of Preliminary Experiments:

3.1.1 Recovery of *E.coli* and enterococci after antibiotic treatment:

One group of 18 mice was treated for two weeks with TDD antibiotics (Sm, Nm) given *ad libitum* in drinking water. Mice were caged individually, and kept in a clean isolated room. No *E.coli* or enterococci were isolated from the faeces during the treatment period. However, in 2 / 18 mice small colonies of lactose fermenters were seen growing on MC agar and on BA during treatment. From a gram stain it was seen that these organisms were gram positive bacilli with terminal spores morphologically similar to clostridia.

One day after cessation of treatment only 3 / 18 mice showed growth of *Lactobacillus species* on MRS agar, and no other aerobic organisms were detected. By day 5 after cessation of treatment , all mice showed growth of lactobacilli, and 9 / 18 mice showed growth of *Bacillus* species on BA. However, no *E.coli* or enterococci were detected 4 weeks after cessation of treatment. Even when 8 of these mice were housed together in one cage and moved to a room with conventional mice for 10 days , they failed to colonize with *E.coli* or enterococci. However, when treated mice were co-housed with a group of 8 conventional mice in one cage, they became colonized with enterococci, but no *E.coli* was detected during the 2 weeks of co-housing.

Before treatment and on day 5, 7, 11, 14, and 28 after cessation of treatment, 2 mice were sacrificed , dissected , and the microflora of 5 parts of the gut (upper jejunum, lower jejunum, ileum, caecum, and colon) was estimated as described **(materials and methods 2.10)**. In control mice lactobacilli, and *Bacillus* species were isolated from all 5 parts of the gut, whereas *E.coli* and enterococci were isolated only in the ileum, caecum, and colon. In TDD treated mice no *E.coli* or enterococci were detected in any part of the gut

during the 4 weeks after cessation of treatment. However, *Lactobacillus species*, and *Bacillus species* were detected in the gut of all mice.

3.1.2 : The stability of streptomycin and neomycin in drinking water:

The stability of the combination of Sm and Nm as TDD antibiotics in drinking water at room temperature was estimated as described **(materials and methods 2.6.1)**. The activity of the combination of Sm and Nm in drinking water was stable for the first 5 days. It was observed that this activity was 90 % of the original activity after 5 days , and it had decreased to 38 % by day 6.

The same result was obtained when only Nm was tested using Sm^R Nm^S*E.coli* . The activity of Nm dropped from 82 % on day 5 to 31 % by day 6 and remained the same until day 14, which was equivalent to the last day of TDD treatment **(Figure. 3)**.

3.1.3 The level of resistance of the challenge organisms to Sm and Nm:

One group of 10 mice was treated with one dose of 0.24 mg each of SM and Nm given by gavage. Faecal pellets were collected from the mice 4, 8, 12, 16, and 24 hours after administration of antibiotics. The highest antibiotic activity of the combination of TDD antibiotics in faecal pellets was seen 4 hours after treatment. By comparing the zone of inhibition of *E.coli* NCTC 10418 by TDD antibiotics in faecal pellets at 4 hours after treatment to the zone of inhibition of standard concentration of Sm and Nm, it was observed that organisms had to be resistant to at least 10 mg ml^{-1} each of Sm and Nm to be used as a challenge strain during treatment with TDD antibiotics.

3.1.4 Effect of the mode of administration of TDD regimen on CR:

Four groups of 8 mice were treated for 2 weeks with Sm and Nm. Two of these groups were treated with 10 mg $mouse^{-1}$ each of Sm and Nm given by gavage, where the other two groups were treated with 5 $mgml^{-1}$ of each Sm and

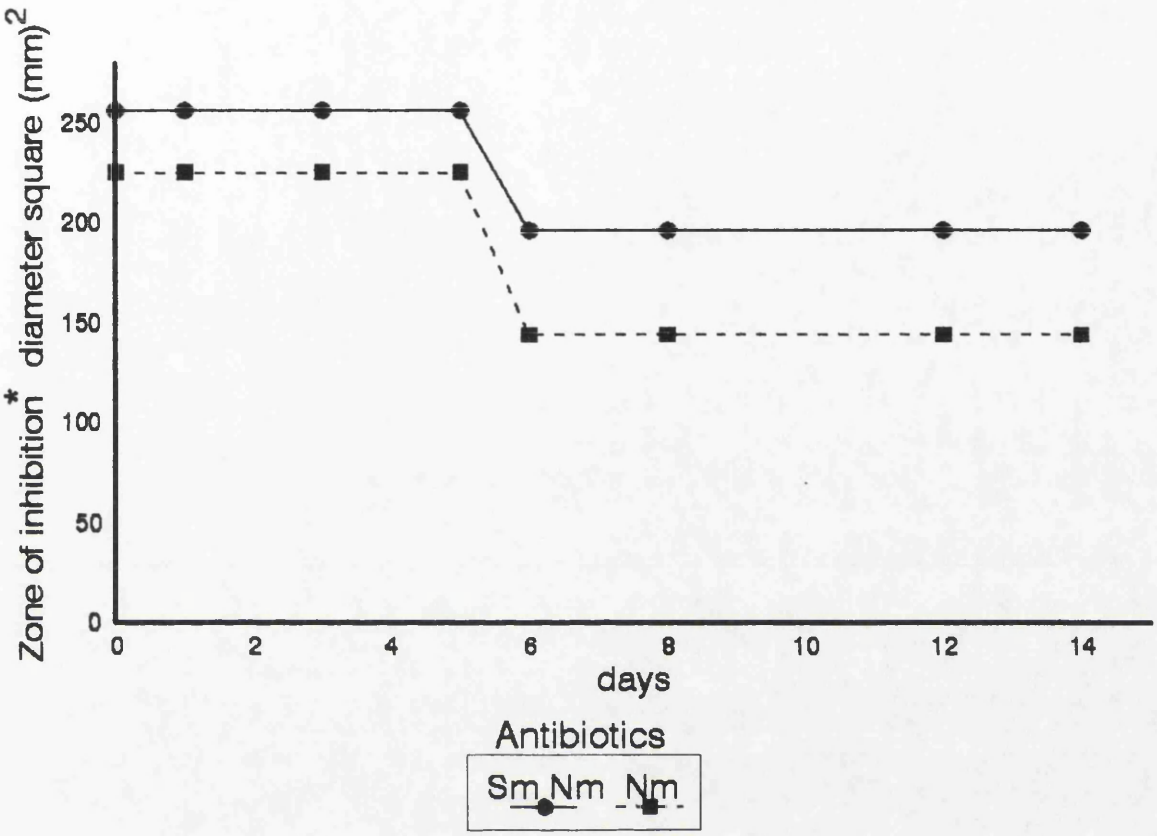


Figure 3 The activity of Sm and Nm in drinking water at room temprature during 14 days.

* of indicator strains : *E. coli* NCTC No. 10418 for testing the activity of the combination of Sm and Nm, and Sm^R Nm^S *E. coli* for testing the the activity of Nm alone.

Nm given *ad libitum* in drinking water. On the second day of treatment the two groups of each category were challenged with 10^2 and 10^3 cfu of *E.coli* s-R21 challenge strain respectively. In mice which were treated by gavage 3 / 8 mice of each group lost the challenge organisms during treatment **(Figure. 4)**. All mice were colonized for over 2 weeks after treatment when given antibiotics *ad libitum* in drinking water.

3.1.5 Comparison of two media for the enumeration of anaerobic faecal flora:

Anaerobic BA and M 10 agar were compared for the enumeration of anaerobic faecal flora. The TVC of anaerobic flora on ANO_2 BA was higher by 0.5$\pm$ 0.2 log cfu than on M 10 agar. On this basis, and the greater ease with which colony types could be distinguished on ANO_2 BA, it was decided to use this medium throughout this study.

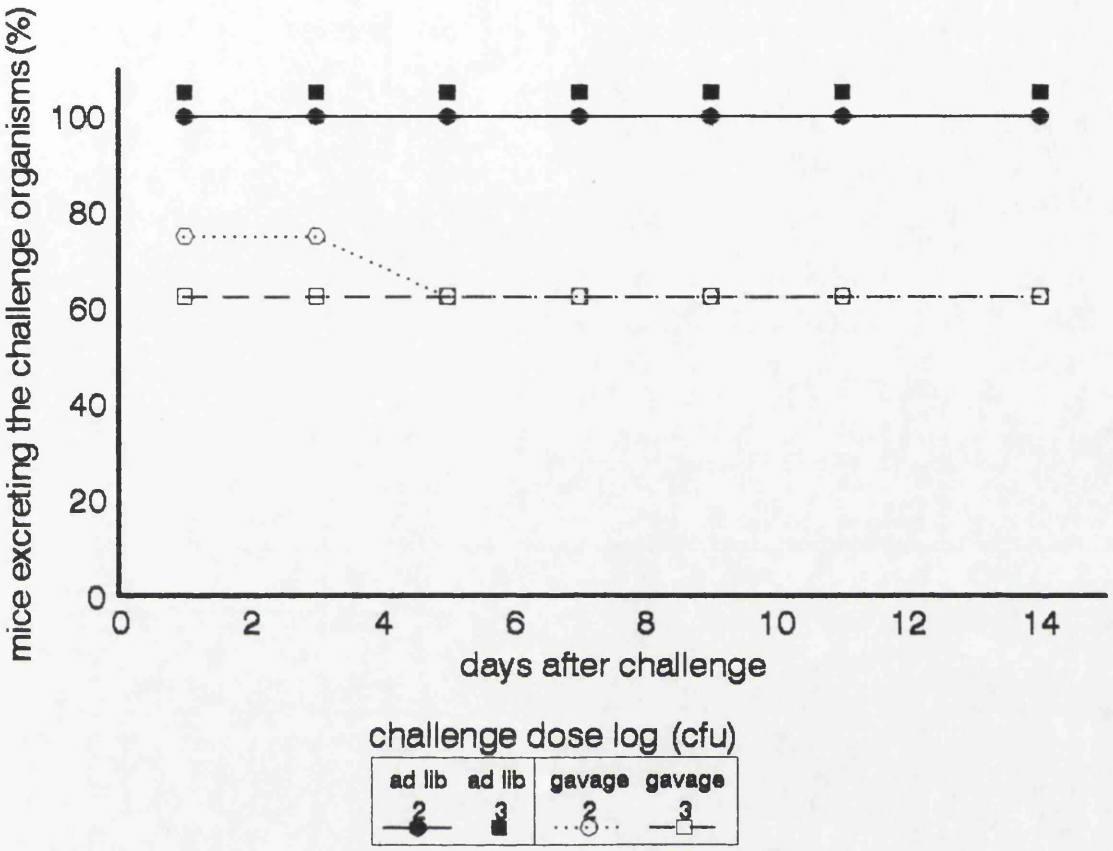


Figure 4 Excretion of *E.coli* s-R21 challenge strain by groups of 8 mice given TDD antibiotics by gavage or *ad libitum*.

3.2 Studies On Faecal Pellets:

Faecal pellets from batches of mice were subjected to direct gram film and microscopy, aerobic culture, anaerobic culture, and in some cases ß-aspartyglycine analysis **Table. 7.**

3.2.1 Faecal pellets / Direct Gram films:

3.2.1.1 Control mice: (256 mice)

The gram stain showed a complex mixture in the range of 16 - 20 different organisms. A representative gram film is shown in **Figure 5.**

3.2.1.2 Wild mice: (3 mice)

The gram stain showed a complex mixture in the range of 12 - 16 different organisms mainly short gram negative bacilli, short fat gram positive bacilli, tapered rods, fusiform, and gram negative cocci.

3.2.1.3 Mice treated with TDD regimen: (184 mice)

No organisms were seen on gram film of faeces during treatment of 7/23 groups of 8 mice treated with the TDD regimen . Sixteen other groups of mice showed two types of organisms; gram positive bacilli with terminal spores, and short fat gram positive bacilli. These organisms are probably the same as those seen on culture **(results section 3.3.2.3)**. By day 7 after treatment a more complex mixture of organisms was seen, but the gram film was not as complex as samples from control mice.

3.2.1.4 Mice treated with SDD regimen:(104 mice)

After one dose of treatment with PTA the large gram negative bacilli (GNB) seen in control mice **(Figure. 5)** were not detected **(Figure. 6)**. No further changes were seen after 5 days of treatment **(Figure. 7)**. Seven days after cessation of treatment, the diversity of the flora was similar to that of control mice **(Figure. 8)**.

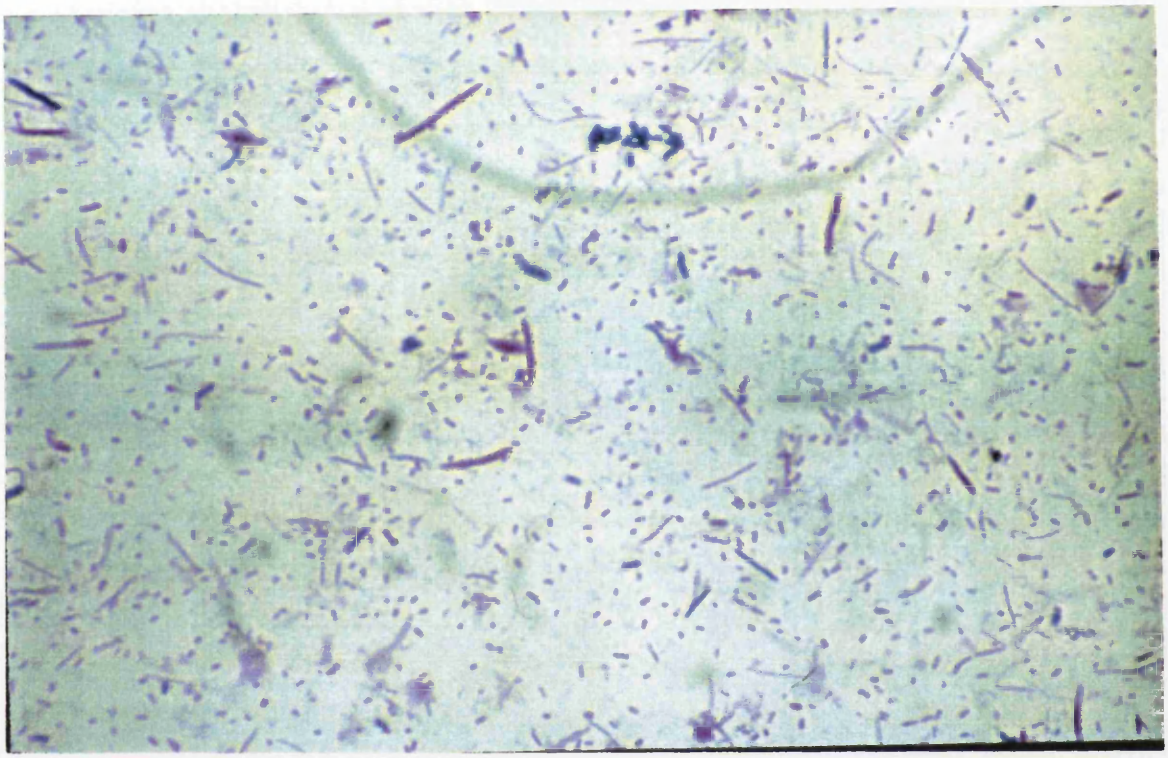


Figure 5 Gram film of faecal flora of untreated control mice.

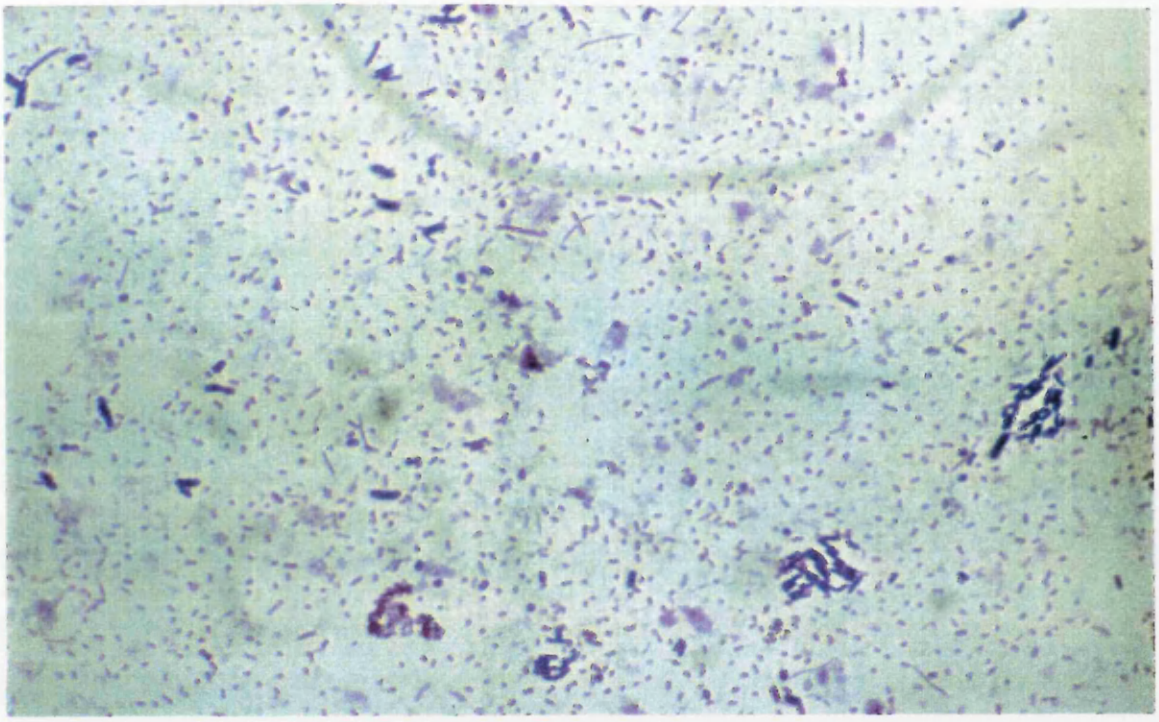


Figure 6 Gram film of faecal flora of mice after one dose of PTA antibiotics. (Notice the disappearance of the large gram negative bacilli shown in gram film of control mice **(Fig. 5)**)

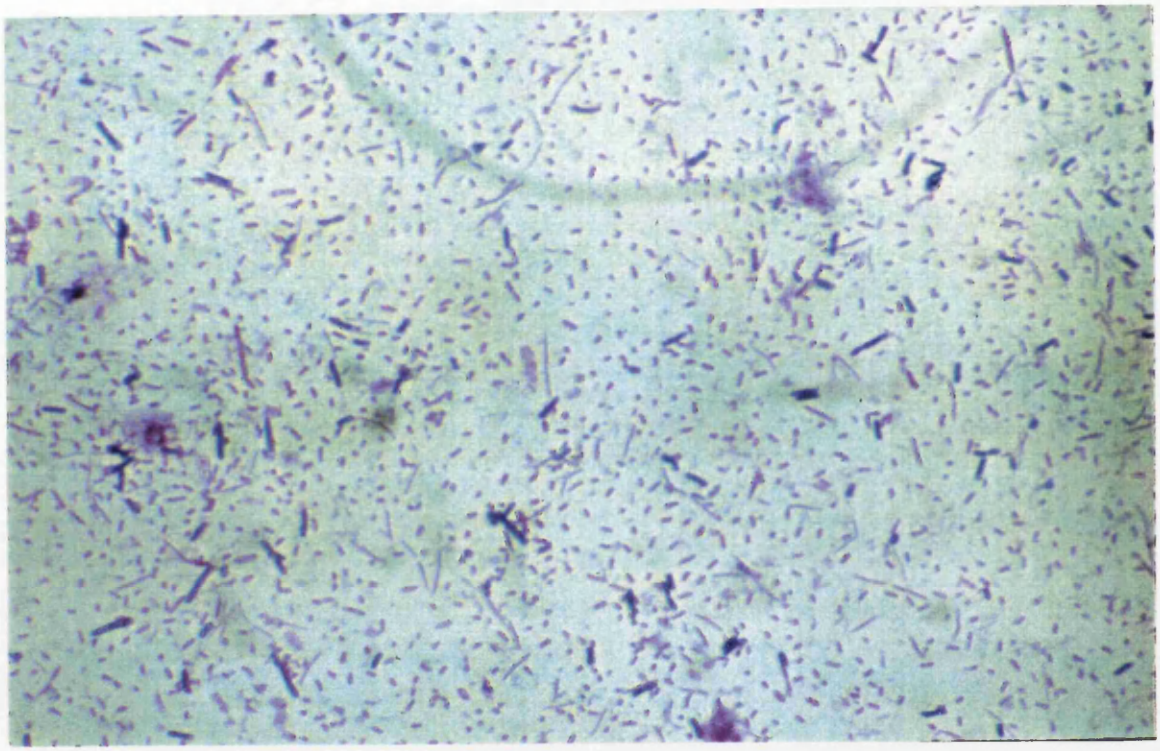


Figure 7 Gram film of faecal flora of mice on day 5 during PTA regimen.

Table 7 **Total number of mice used in each test analysis.**

Test Analysis	Total No. of Mice*	No. of Groups
Gram Stain	635	79 of 8
Aerobic Culture	451	56 of 8
Anaerobic Culture	427	53 of 8
ß-aspartylglycine	47	11 of 4

* Total number of mice including 3 wild mice.

3.2.1.5 Mice treated with cefotaxime: (32 mice)

A simplification of the normal flora was seen. Only gram positive cocci and 2 - 3 types of gram positive bacilli were present during treatment **(Figure. 9)**. A more complex mixture of organisms was seen 2 - 3 days after treatment, which is probably due to the recovery of large GNB, tapered rods, fusiforms, and other GNB.

3.2.1.6 Mice treated with PTA regimen with addition of systemic cefotaxime: (40 mice)

A simplification of the normal flora was seen. Only gram positive cocci, and three types of gram positive bacilli **(Figure. 10)** were present during treatment with PTA and cefotaxime. Some types of GNB did recover after stopping cefotaxime, during continued treatment with the SDD regimen **(Figure. 11)**. A gram film from one day after cessation of all treatment showed a more complex mixture of organisms (but less than control mice) **(Figure. 12)** than during treatment.

3.2.1.7 Mice treated with Clindamycin: (16 mice)

A simplification of the faecal flora occurred, and only gram negative bacilli were seen in large numbers during treatment **(Figure. 13)**. One day after cessation of treatment gram negative cocci, large GNB and short GNB were seen, whereas the GPB were first seen 3 days after cessation of treatment.

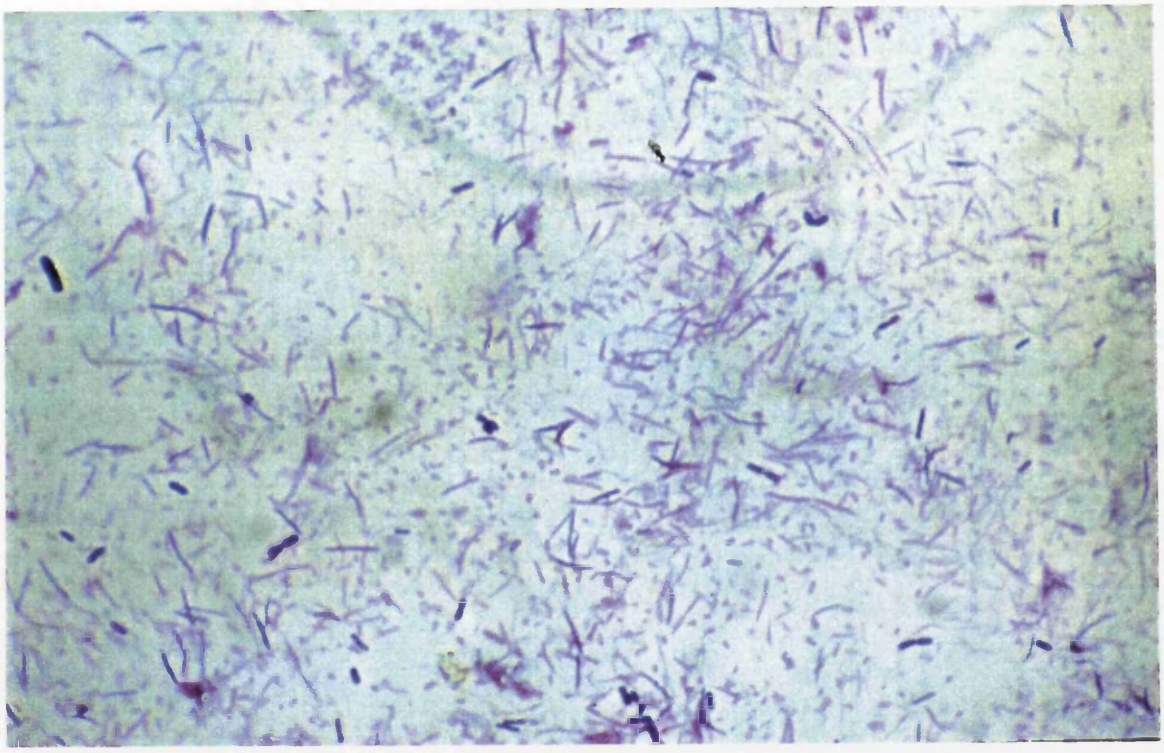


Figure 8 Gram film of faecal flora of mice 7 days after cessation of PTA regimen. (Notice the recovery of the fusiform bacteria, and other gram negative rods).

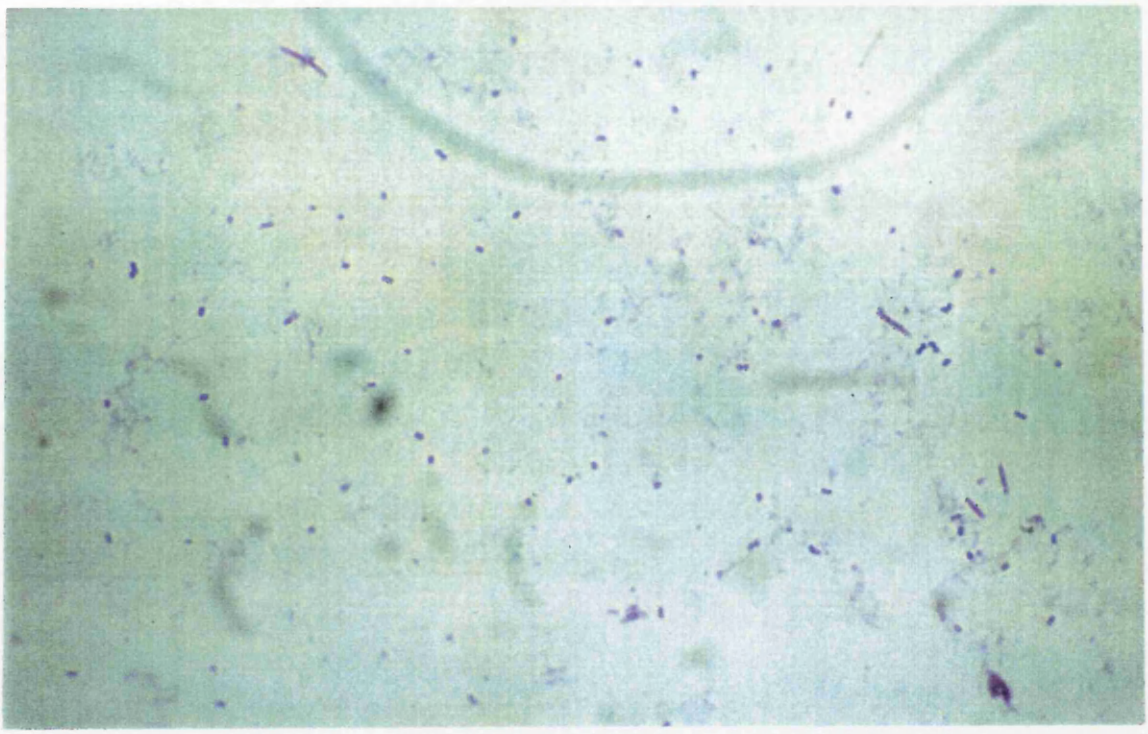


Figure 9 Gram film of faecal flora of mice after one dose of cefotaxime. (Notice the simplification of the normal flora compared to control mice **(Fig. 5)**).

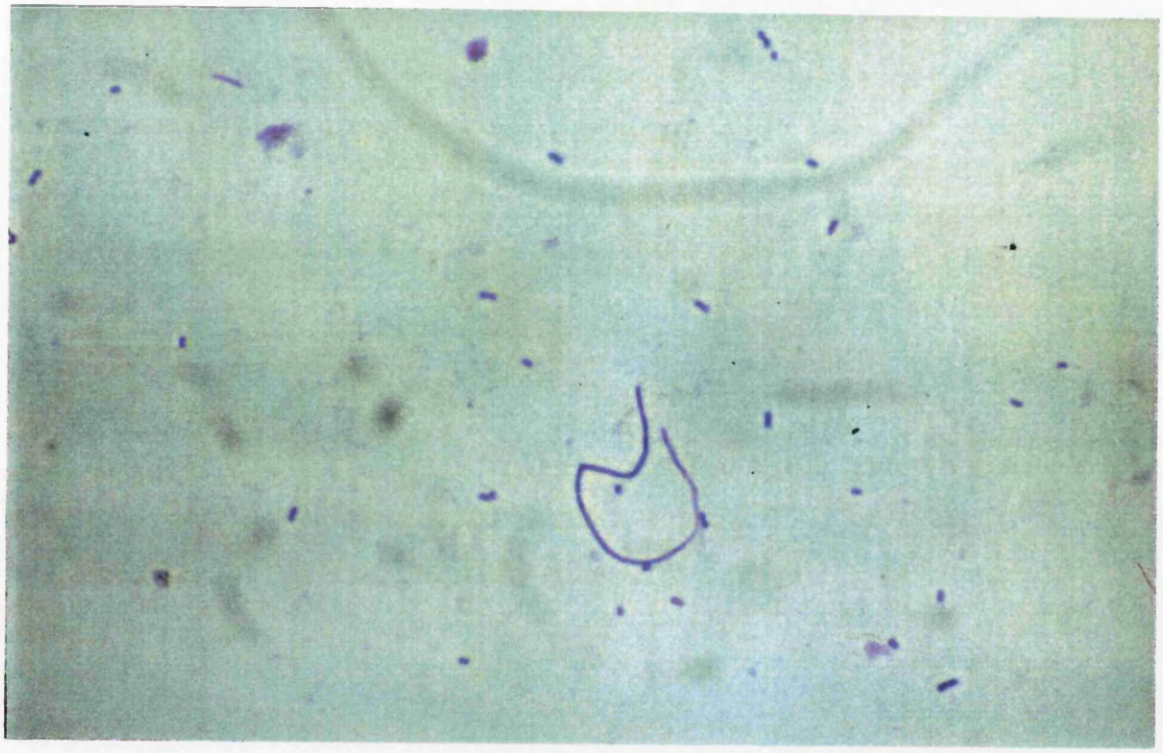


Figure 10 Gram film of faecal flora of mice after one dose of PTA antibiotics with addition of cefotaxime. (Notice the simplification of the flora compared to control mice **(Fig. 5)**).

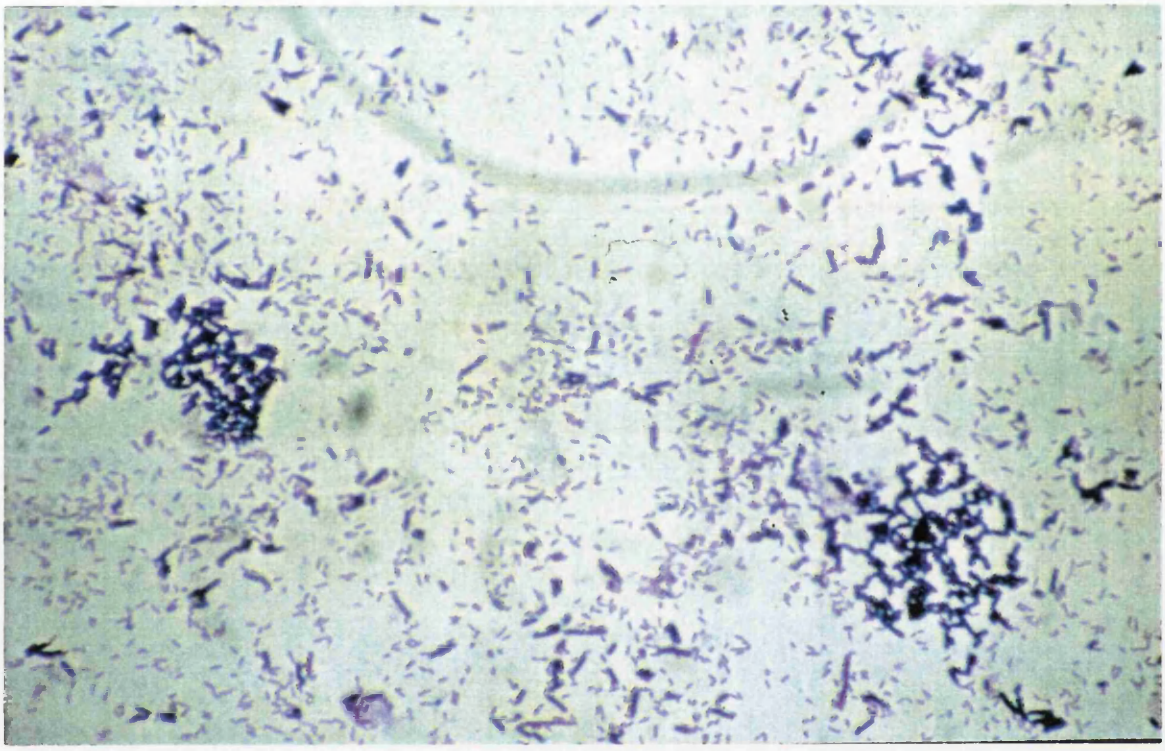


Figure 11 Gram film of faecal flora of mice after stopping cefotaxime, during 9 days of treatment with PTA regimen. (Notice the recovery of GNB).

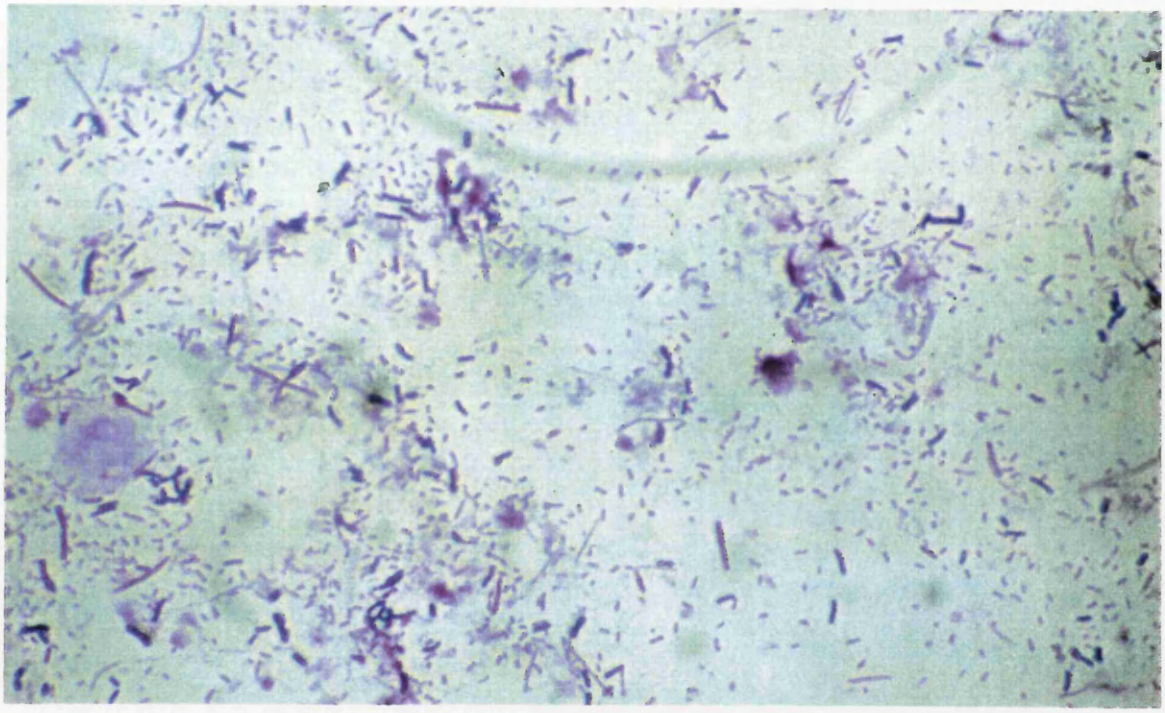


Figure 12 Gram film of faecal flora of mice one day after cessation of treatment with PTA antibiotics with addition of cefotaxime. (Notice the recovery of most of faecal flora compared to during treatment **(Fig. 10)**).

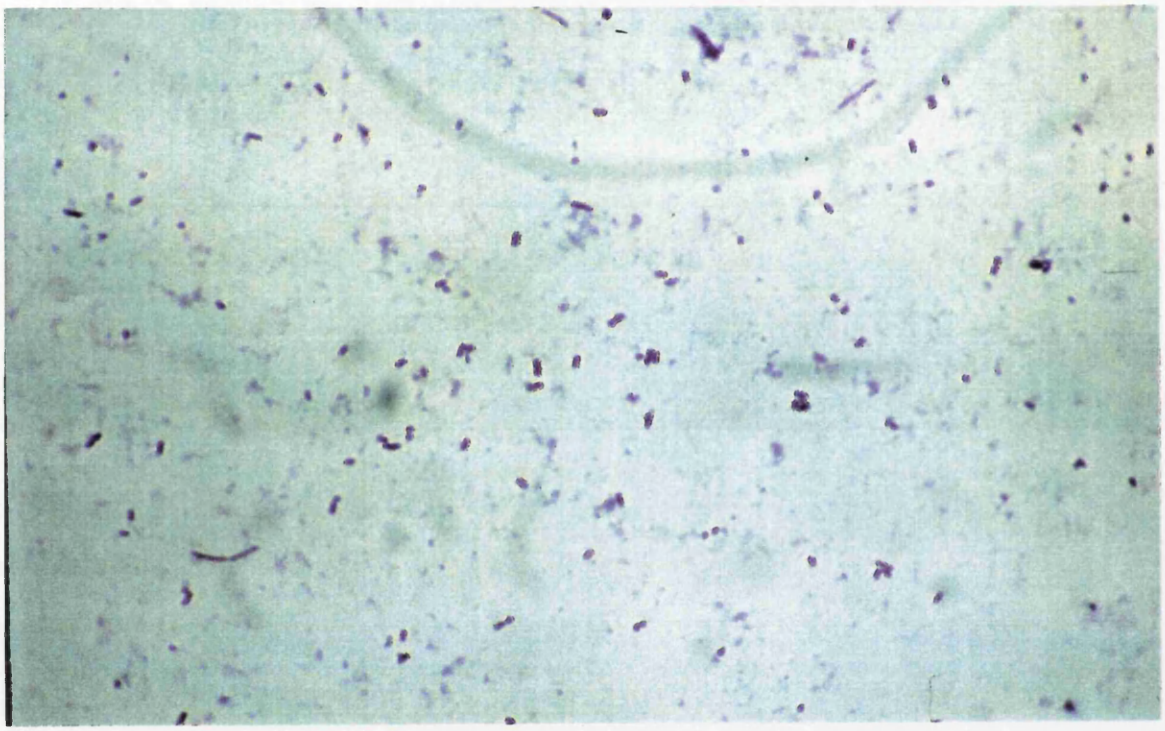


Figure 13 Gram film of faecal flora of mice 3 days during treatment with clindamycin. (Notice the simplification of the normal flora and the elimination of all gram positive organisms).

3.2.2 Faecal pellets / Aerobic culture:

3.2.2.1 Control mice: (9 groups of 8 untreated mice)

Faecal pellets from untreated mice of different batches were used to monitor the normal flora of the gut, as described in materials and methods. The total viable count, and colony types of aerobic faecal flora in control mice varied. The mean T V C was(7.3 $\pm$ 0.08 log cfu / g of faeces). The flora consisted mainly of *E.coli* (5.8 $\pm$ 0.2 log cfu / g of faecal pellets), enterococci (6.9 $\pm$ 0.1 log cfu / g of faeces), small numbers of lactose non-fermenting organisms, and *Bacillus sp.* **(Table. 8)**.

Swarming *Proteus sp.* and *Klebsiella oxytoca* were isolated from some mice in only 3 of the 9 groups of 8. It was not possible to isolate *Pseudomonas aeruginosa, Klebsiella pneumoniae,* or*Candida albicans* from the faecal flora of the 72 control mice.

3.2.2.2 Wild mice:

Only *E.coli* was isolated from the three wild mice. The total viable count was 6.7 log (cfu / g of faeces). No other aerobic organisms were isolated except lactose non-fermenting gram positive bacillus from one mouse **(Table. 8)**.

3.2.2.3 Mice treated with TDD regimens: (23 groups of 8 mice)

Mice from different batches were treated for two weeks with TDD antibiotics given *ad libitum* in drinking water, as described in materials and methods. After one day of treatment no aerobic flora was detected, and anaerobic bacteria did not reappear during the treatment period **(Table. 8)**. In 16 groups of 8 mice small colonies of lactose fermenters were seen growing on McConkey agar, and on blood agar throughout treatment at a TVC of (9 $\pm$ 0.2 log cfu / g of faeces). A gram stain showed these to be gram positive bacilli with terminal spores morphologically similar to clostridia.

Table 8 **The effect of different antibiotics on the aerobic flora in Balb / c mice.**

Treatment	No. of mice	*E.coli*		Enterococci	
		% of mice carrying	mean cfu*	% of mice carrying	mean cfu*
Control	72	100	5.8±0.2	100	6.9±0.1
Wild mice	3	100	6.7	0	--
TDD	184	0	--	0	--
PTA	104	0	--	100	6.5±.2
CTX	32	0	--	100	9±0.5
PTA + CTX	40	0	--	100	9.1±0.3
Clindamycin	16	100	9.4±0.2	0	--

*log ± SE.

The recovery of most aerobic bacteria after treatment with TDD regimens varied in mice from different batches. In 70 % of the cases the recovery of aerobic flora was slow. When kept in a clean environment to avoid the risk of contamination, mice were not recolonized with *E.coli* for three weeks or more after treatment. By contrast, enterococci recovered by 5 - 7 days after treatment.

3.2.2.4 mice treated with SDD : (13 groups of 8 mice)

Mice from different batches were treated for two weeks, with the PTA given by gavage. After one dose, *E.coli* and other aerobic gram negative bacilli could not be detected, and *E.coli* did not reappear for two weeks after stopping treatment **(Table. 8)**. No significant changes were seen in the total viable count of enterococci during treatment and after treatment.

3.2.2.5 Mice treated with cefotaxime: (4 groups of 8 mice)

Mice from different batches, were treated for four days with 3 mg day^{-1} of cefotaxime, given by i.p. injection. After one dose of cefotaxime, *E.coli* could not be detected, and it had not reappeared two weeks after stopping treatment **(Figure. 14)**. At the same time a significant ($P < 0.02$) increase of 1.1 ± 0.6 log (cfu) occurred in the total viable count of enterococci. In each case, the total viable count of enterococci returned within three days after treatment to that measured in the same mouse before treatment **(Figure. 15).**

3.2.2.6 Mice treated with SDD regimen with addition of systemic cefotaxime: (5 groups of 8 mice)

Mice from different batches were treated with PTA given by gavage, with the addition of 3 mg per day of cefotaxime given by i.p. injection. After one dose of treatment the *E.coli* could not be detected, and they had not reappeared two weeks after stopping the treatment. The total viable count of enterococci was significantly ($P < 0.009$) increased by 2.6 ± 0.4 log (cfu) during treatment with

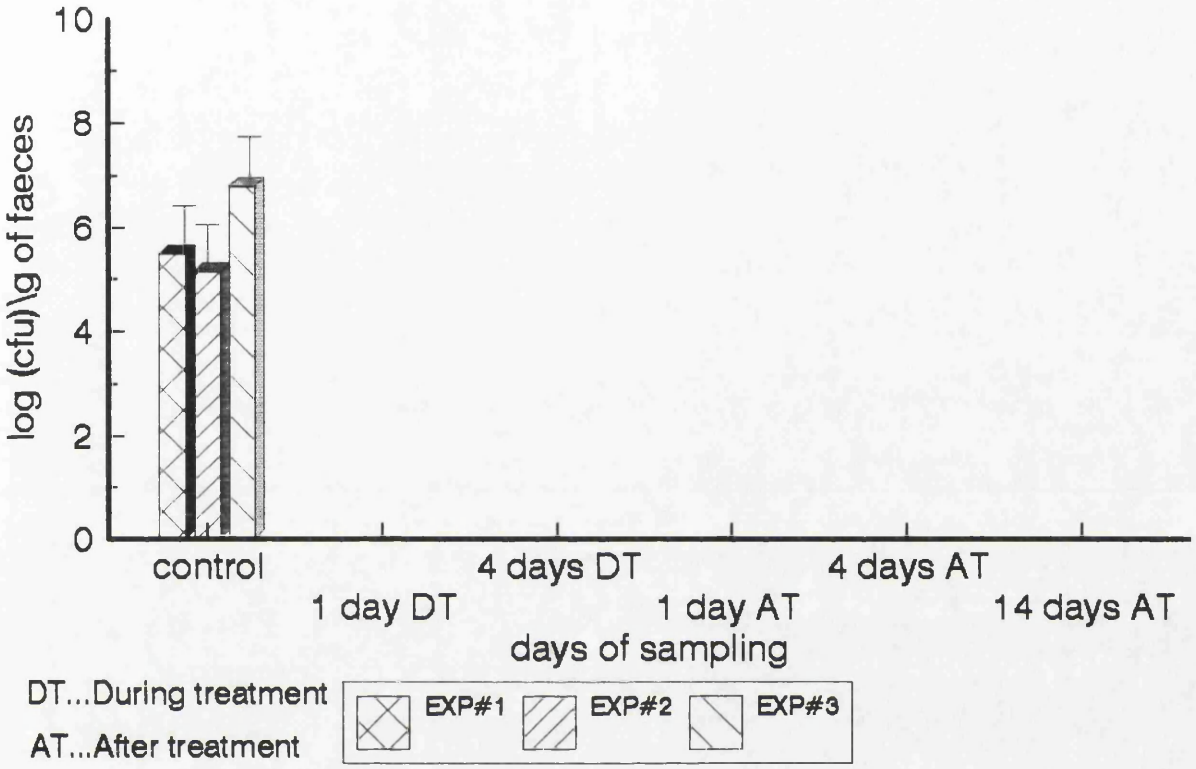


Figure 14 Effect of Cefotaxime on *E.coli* of the faecal flora in different groups of 8 mice. Result shown ± standard error of mean.

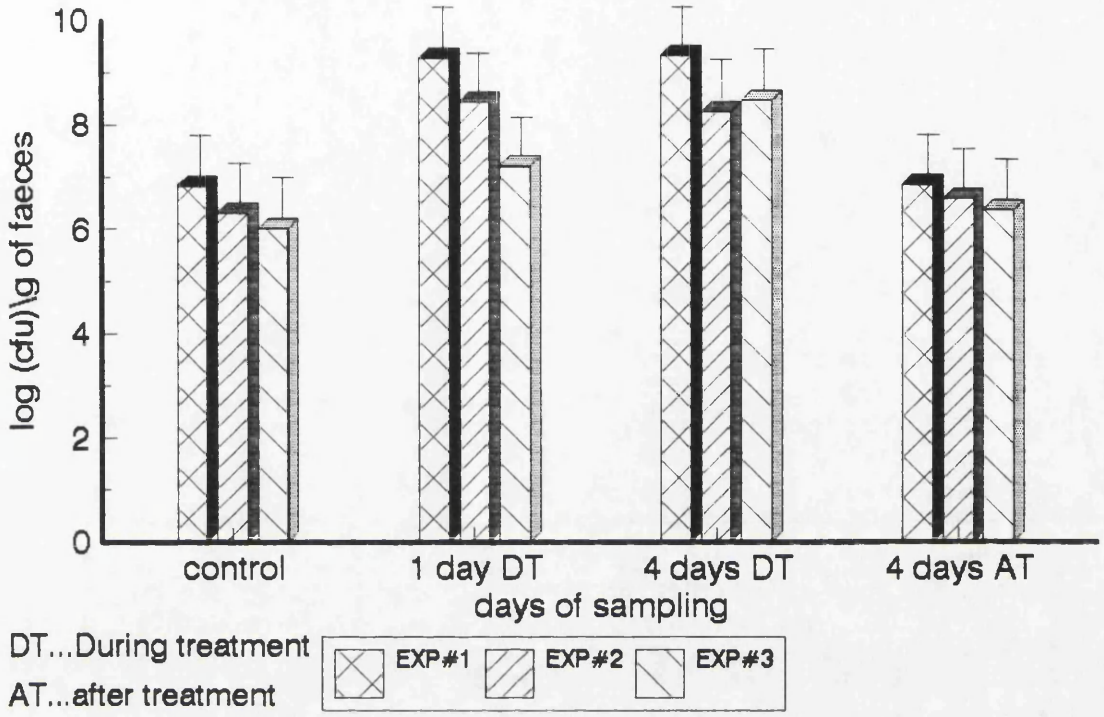


Figure 15 Effect of cefotaxime on enterococci of the faecal pellets in 3 groups of 8 mice .Result shown ± standard error of meam.

PTA plus cefotaxime **(Table. 8)**. In each case, the total viable count returned after treatment to that measured in the same mouse before treatment **(Figure. 16)**.

3.2.2.7 Mice treated with clindamycin: (2 groups of 8 mice)

Two groups of mice were treated for four days with 0.6 mg per day of clindamycin, given by gavage. After one dose of clindamycin the TVC of *E.coli* significantly ($P < 0.008$) increased by 3.5 ± 0.6 log (cfu), and remained at the same level during the treatment period. In 2 - 3 days after stopping treatment, the TVC of *E.coli* was similar to that measured in the same mice before treatment **(Figure.17)**.

The enterococci were reduced to a non detectable level during the treatment period, and they recovered to the normal level one day after treatment was stopped **(Table. 8)**.

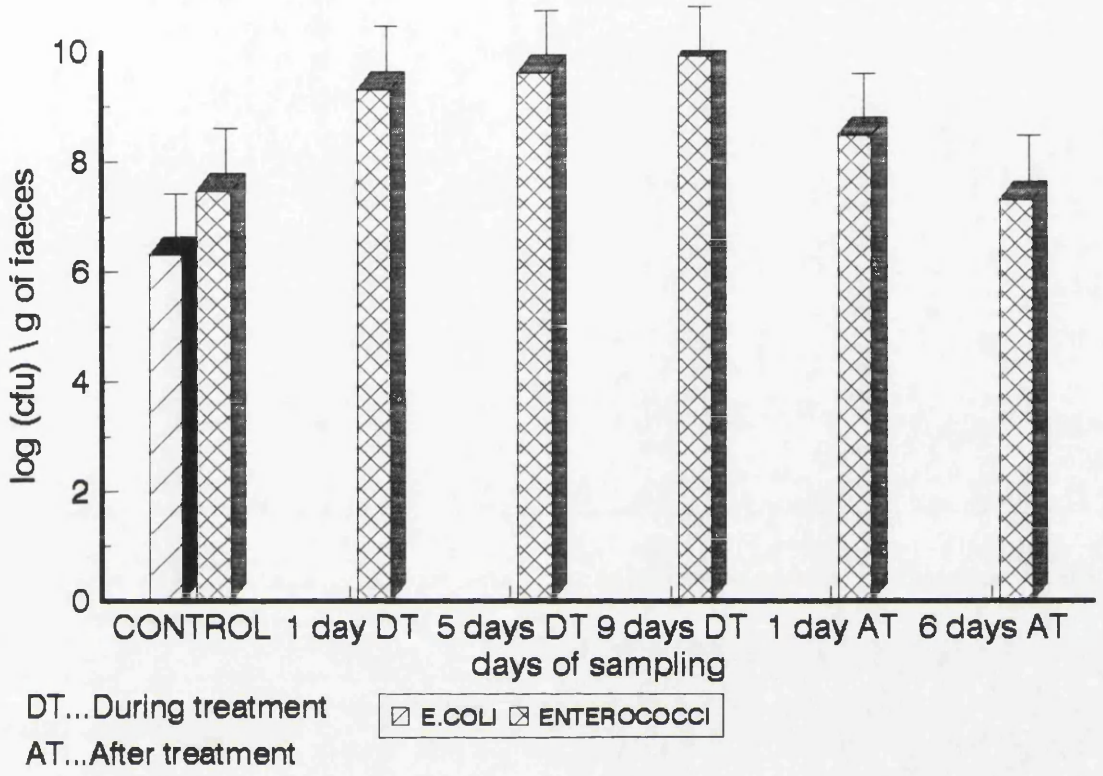


Figure 16 Effect of SDD regimen with addition of systemic cefotaxime on the aerobic flora of 16 Balb/c mice. Result shown ± standard error of mean.

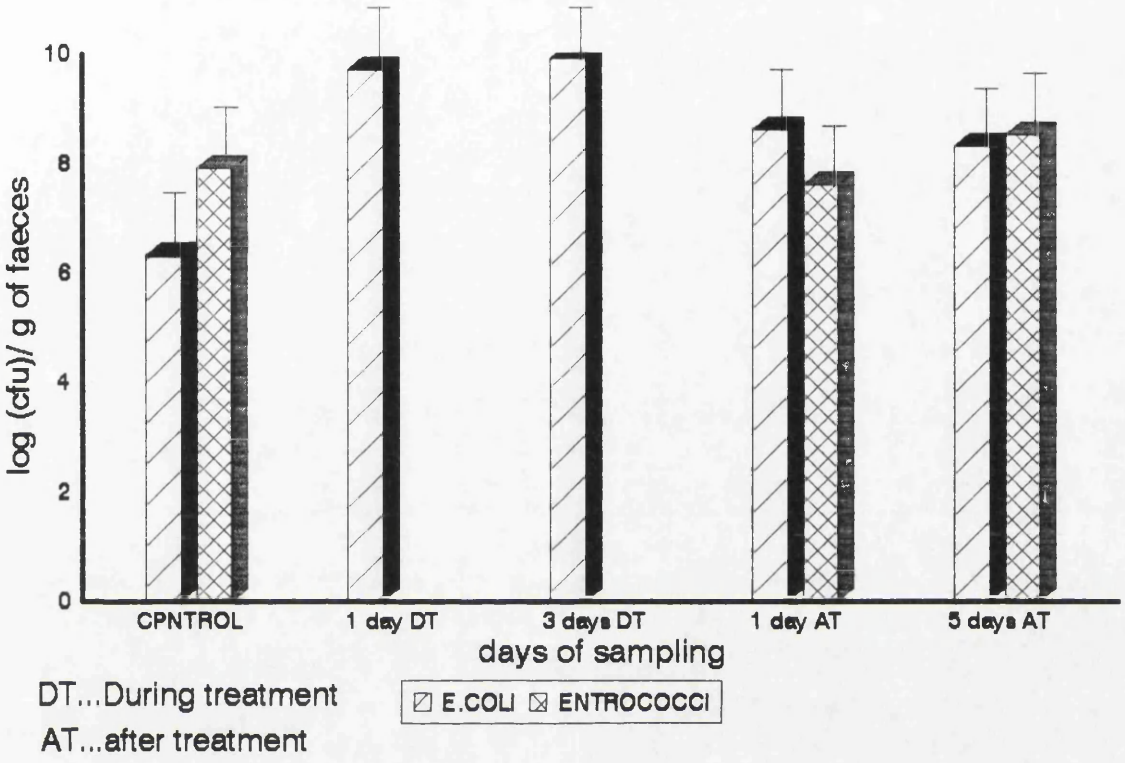


Figure 17 Effect of clindamycin on the aerobic faecal flora of 16 Balb / c mice. Result shown ± standard error of mean.

3.2.3 Faecal pellets / Anaerobic culture:

3.2.3.1 Control mice: (6 groups of 8 untreated mice)

The total viable count of anaerobic faecal flora varied in different groups of mice. The mean TVC was 10.5 $\pm$ 0.06 log (cfu / g of faecal pellets). The growth on anaerobic media showed 12 $\pm$ 1 different colony types **(Figure. 18)** at a dilution of 10^{-7}, which mainly consisted of spore-forming gram positive bacilli, fusiform bacteria, gram positive cocci, gram negative cocci, gram negative bacilli and non spore forming gram positive bacilli.

3.2.3.2 Wild mice: (3 mice)

The total viable count of the anaerobic flora in the wild mice was 9.8 log (cfu / g of cecum content), which consisted of 8 - 10 different colony types at a dilution of 10^{-7}. Gram morphology was almost similar to that of control mice.

3.1.3.3 Mice treated with the TDD regimen: (23 groups of 8 mice)

After one day of treatment with the TDD antibiotics no organisms were detected on anaerobic culture in 7 out of 23 groups of 8 mice, and they did not reappear during the 14 days of treatment. Residual anaerobes were detected in 16 / 23 other groups of 8 mice despite the TDD regimen: two types of colonies were observed on blood agar under anaerobic conditions. These were gram positive bacilli with terminal spores, and short gram positive bacilli. These probably were the aerotolerant clostridia observed earlier **(results section 3.2.2.3)**. The total viable number of these organisms was 9 $\pm$ 0.2 log (cfu / g of faeces) during treatment; this decreased to 6.9 $\pm$ 1.4 log (cfu / g of faeces) 2 - 3 days after stopping treatment **(Table. 9)**.

The recovery of anaerobic flora after stopping treatment was slow; seven days after treatment only 4 $\pm$ 1 types of colonies were seen in 18 groups of 8 mice. Two weeks after treatment the mean total viable count was (10.35 $\pm$ 0.2

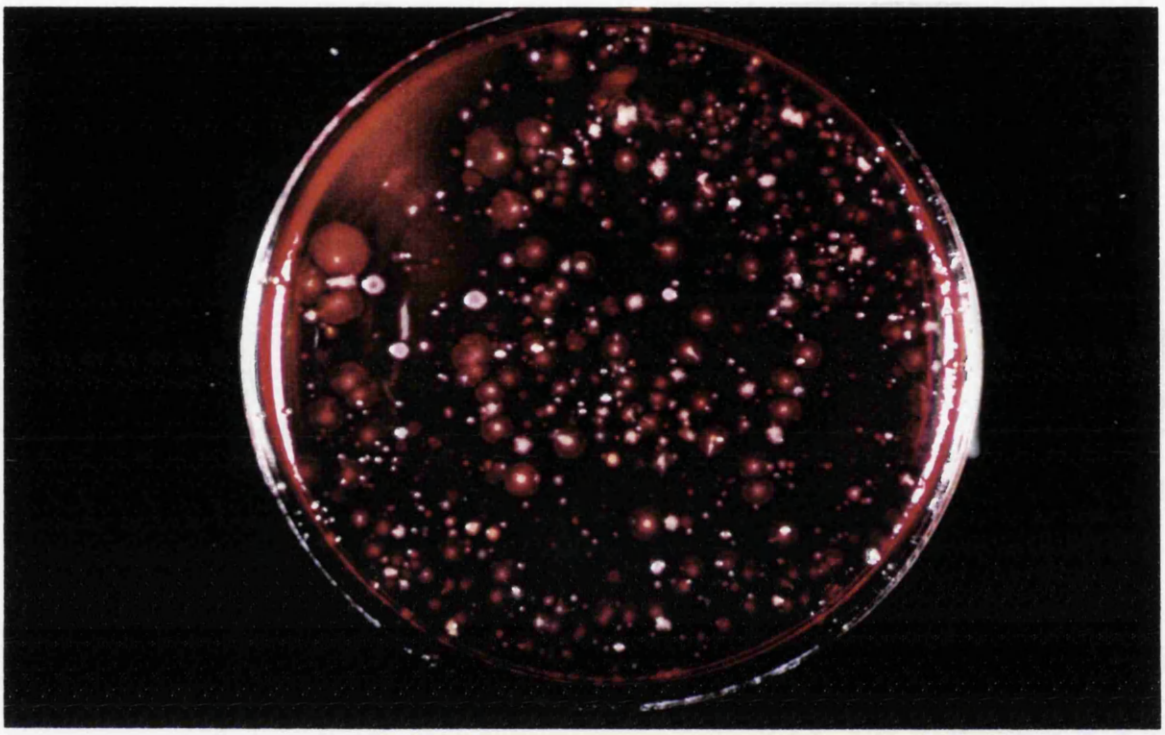


Figure 18 Anaerobic faecal flora of control mice at a dilution of 10^{-7} on anaerobic blood agar.

Table 9 **The effect of different antibiotics on the anaerobic total count and diversity of faecal flora of Balb / c mice.**

Treatment	No. of mice	% of mice carrying	No. of colony types at a 10^{-7} ditution of faeces	mean cfu*
Control	48	100	10-14[&]	10.5±0.1
Wild mice	3	100	8-10	9.8
TDD	184	70	2	9±0.2
PTA	104	100	8-12	10.9±0.2
CTX	32	100	2-4	10.8±0.13
PTA + CTX	40	50	1-2	9±0.8
Clindamycin	16	0	--	--

& Range.

* log ± SE.

log cfu / g of faecal pellets), and only 6 $\pm$ 1 types of colonies were recovered compared to 12 $\pm$ 1 types for control mice at a dilution of 10^{-7}.

3.2.3.4 Mice treated with SDD regimen: (13 groups of 8 mice)

The total viable number of anaerobic faecal flora was not affected significantly; an increase of 0.66 $\pm$ .2 log (cfu) was seen in 7 groups of 8 mice during treatment with the SDD regimen. One day after treatment, the total viable count of the anaerobic flora returned to the same level as control mice. Simplification of the anaerobic flora types was observed **(Figure. 19)**. This was associated with significant reduction in the colony types of anaerobic gram negative rods.

3.2.3.5 Mice treated with cefotaxime: (4 groups of 8)

After one dose of cefotaxime, a simplification of the anaerobic flora occurred in 4 groups of 8 mice. Growth on blood agar at a dilution of 10^{-7} showed a reduction from 12 $\pm$ 1 different colony types for mice before treatment, to only 3 $\pm$ 1 types for the same mice treated with cefotaxime. The total viable count of anaerobic flora did not change significantly **(Table. 9)**. This may be due to an increase in the numbers of the remaining anaerobic organisms in the gut, which consisted mainly of gram-positive spore-forming bacilli. Some types of anaerobic flora did recover during the treatment period; an increase of 5$\pm$1 colony types was observed. A full recovery of diversity was seen 2 - 3 days after treatment.

3.2.3.6 Mice treated with PTA with the addition of systemic cefotaxime: (5 groups of 8 mice)

After one dose of treatment a simplification in the anaerobic normal flora occurred; growth on blood agar at a dilution of 10^{-7} showed reduction from 12$\pm$1 different colony types, to only 1 or 2 types for the same mice treated with SDD regimen with addition of systemic cefotaxime **(Figure. 20)**. In 50% of total

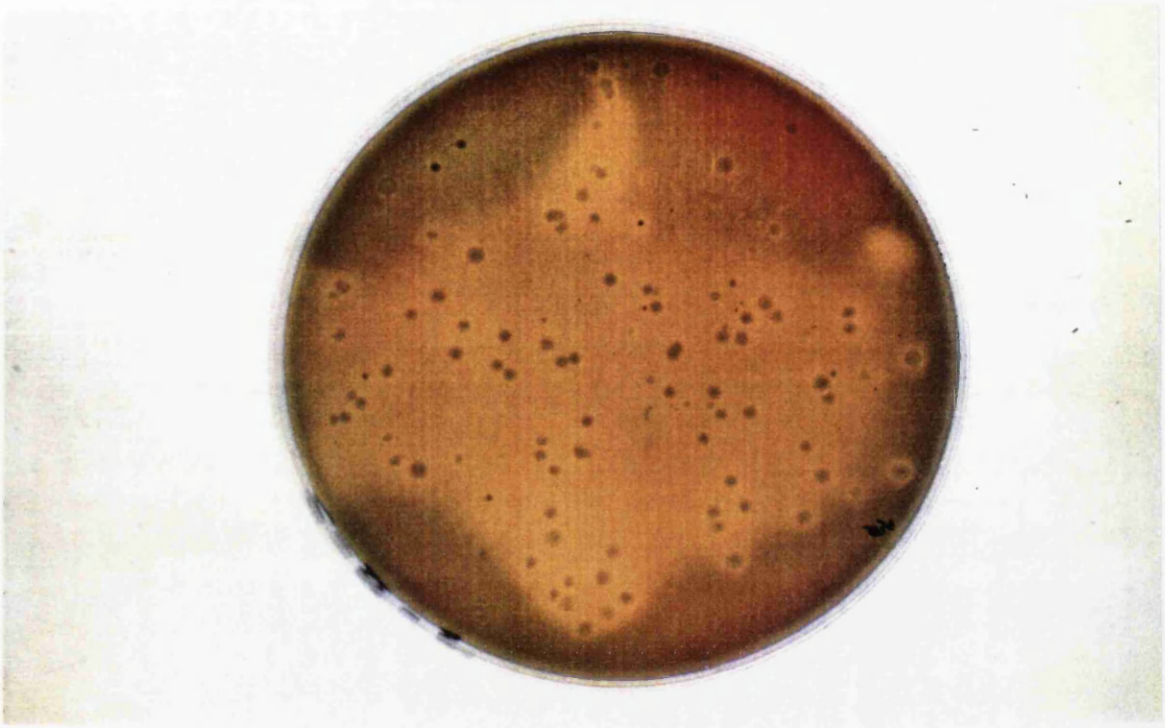


Figure 19 Simplification of colony types of anaerobic faecal flora on anaerobic blood agar after one dose of PTA antibiotics at a dilution of 10^{-7}.

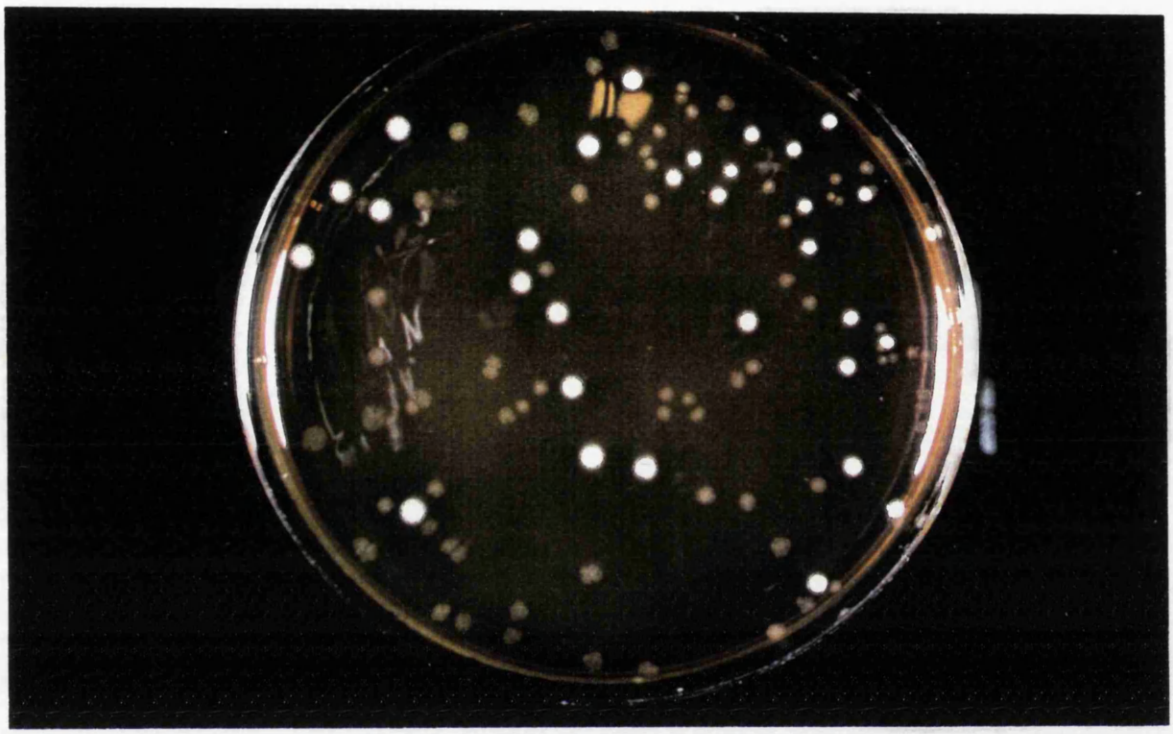


Figure 20 Simplification of colony types of anaerobic faecal flora of mice on anaerobic blood agar after one dose of PTA antibiotics with addition of cefotaxime, at a dilution of 10^{-7}.

treated mice, the anaerobic flora was reduced to a non-detectable level. The total viable count was reduced by 0.9 ± 0.08 log (cfu / g of faeces) **(Table. 9)**.

After discontinuation of cefotaxime treatment some components of the anaerobic flora began to recover and 5 ± 1 different types of colonies were isolated on blood agar. Seven days after treatment the anaerobic population had recovered and the total viable count had returned to the same level as before treatment.

3.2.3.7 mice treatment with clindamycin: (2 groups of 8 mice)

After one dose of clindamycin, the anaerobic flora could not be detected. One day after treatment some of the anaerobic flora had recovered. By five days after treatment the mean total viable count of anaerobes was 10 ± 0.2 log (cfu / g of faecal pellets), which was similar to that of control mice **(Figure. 21)**. A simplification of the anaerobic flora was observed; only 5 ± 1 colony types were seen on anaerobic blood agar at a dilution of 10^{-7} compared to 12 ± 1 types for the same mice before treatment.

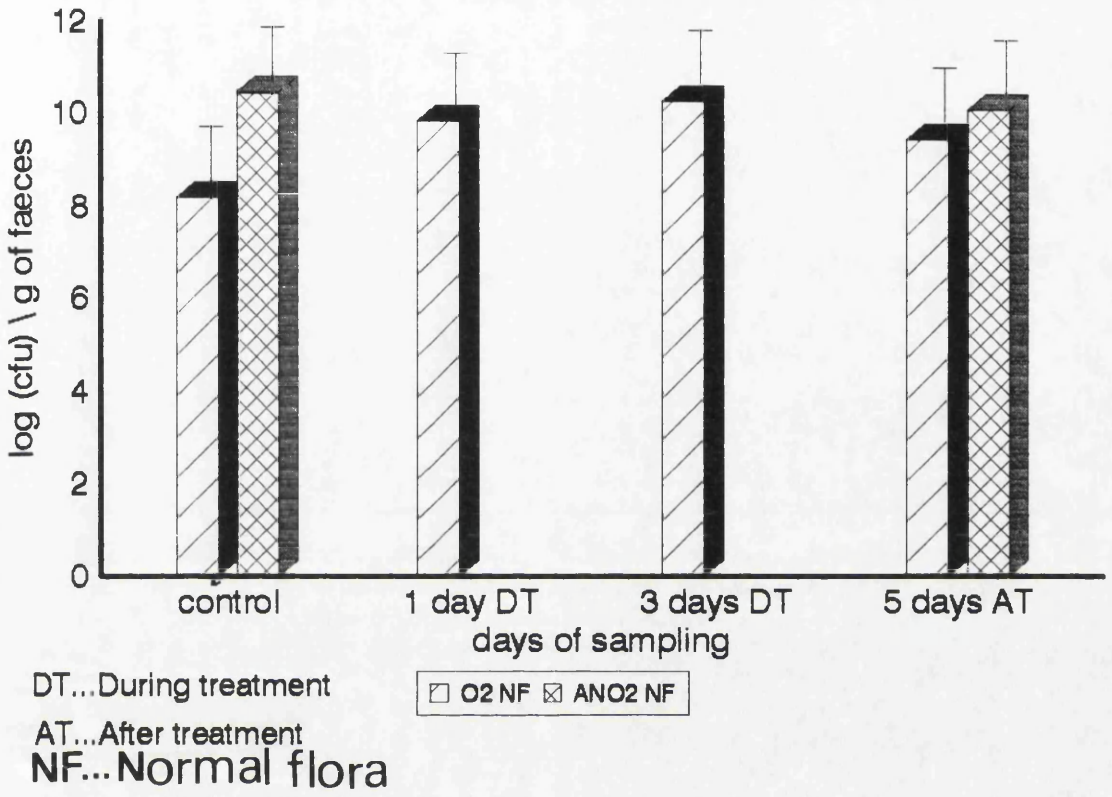


Figure 21 Effect of clindamycin on the faecal flora of 16 Balb / c mice. Result shown ± standard error of mean.

3.2.4 Faecal pellets / ß-aspartylglycine:

Faecal pellets from groups of four mice were collected, prepared, and assayed by H.P.L.C. for the presence of ß-aspgly. ß-aspgly was identified in faecal OPT-derivative by comparison with the mobility standard OPT-ß-aspgly in water as described in **materials and methods 2.12**.

3.2.4.1 Control mice: (6 groups of 4 untreated mice)

ß-aspgly was not detected in the faecal pellets of 24 control, untreated mice from different batches.

3.2.4.2 Wild mice: (3 mice)

No ß-aspgly was detected in the cecum content of 3 wild mice.

3.2.4.3 Mice treated with the TDD regimen: (4 mice)

The individual level of ß-aspgly on selected days of experiment is shown in **Figure. 22 .** ß-aspgly was not detected in the faeces before treatment. During treatment ß-aspgly was present, and by day fourteen of treatment the mean concentration in the 4 mice was 329±40 n mole per gram of faecal pellets. Two weeks after the end of treatment there was no significant reduction in ß-aspgly in 2 mice, a moderate reduction in 1 mouse, and no detectible levels in the fourth mouse. This finding coincided with the incomplete recovery of anaerobic bacteria at this time **(results section 3.2.3.3)**.

3.2.4.4 Mice treated with the SDD regimen: (4 mice)

The individual level of ß-aspgly in 4 mice before, during, and after treatment period is shown in **Figure. 23**. During the treatment period ß-aspgly was present, and the mean concentration was 226±28 n mole per gram of faecal pellets. This level started to decrease one day after the end of treatment and it could not be recovered thereafter from one mouse. By day seven after treatment it decreased to a non detectable level in another mouse, and an apreciable concentration, 24 n mole and 56 n mole, was detected in the other

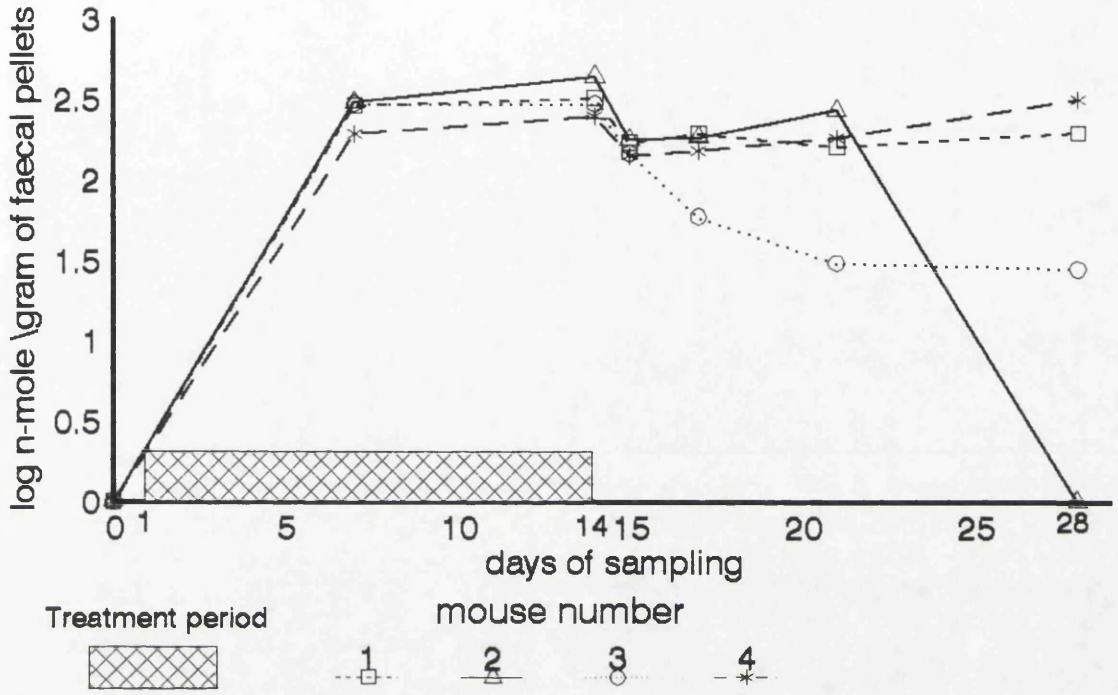


Figure 22 Effect of TDD antibiotics on the present of ß-aspartylglycine in Balb / c.

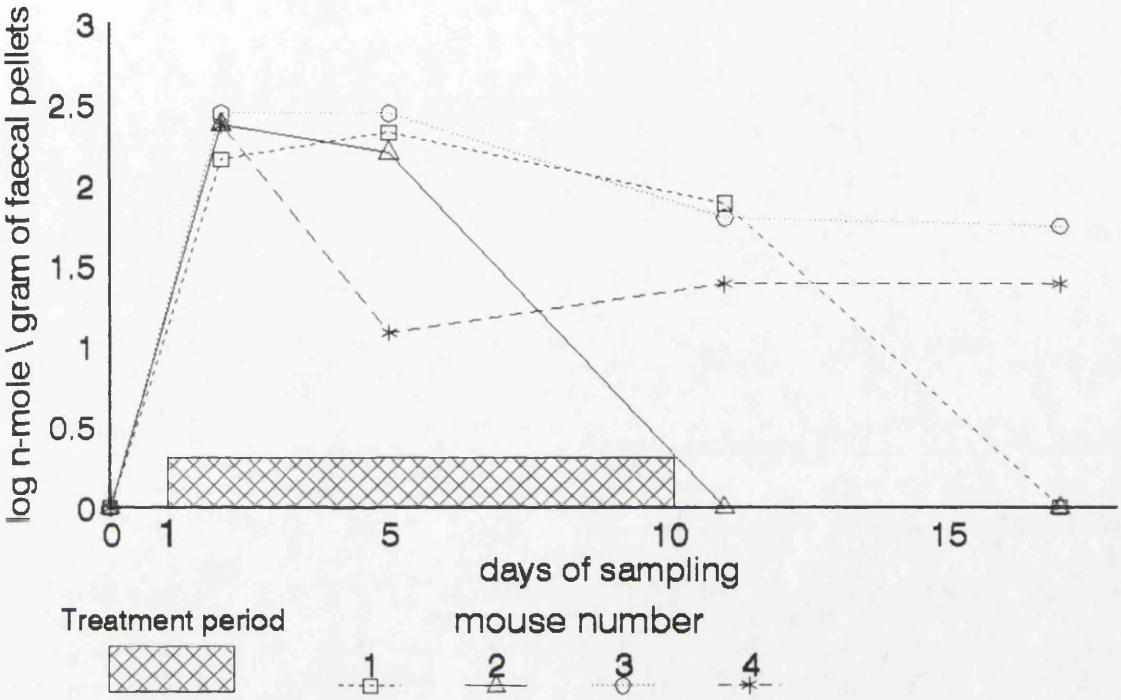


Figure 23 Effect of PTA antibiotics on the presence of ß-aspartylglycine in 4 Balb / c.

two mice respectively. This was associated with the recovery of the anaerobic tapered rods, fusiform, and other gram-negative bacilli by day 5 after the end of treatment **(Figure. 7 and 8)**.

3.2.4.5 Mice treated with cefotaxime: (4 mice)

The level of ß-aspgly on selected days of experiment is shown in **Figure. 24**. ß-aspgly was not detected in mice before treatment. During the treatment period ß-aspgly was present, and the mean concentration was 189±56 n mole per gram of faecal pellets. This level started to decrease after treatment, and in 2 out 4 mice it decreased to a non detectable level one week after treatment. This was associated with a recovery of anaerobic flora.

3.2.4.6 Mice treated with SDD regimen with addition of systemic cefotaxime: (4 mice)

The level of ß-aspgly on selected days of experiment is shown in **Figure. 25**. No ß-aspgly was detected in mice before treatment. During the treatment period ß-aspgly was present, and by day five during treatment the mean concentration was 233±29 n mole per gram of faecal pellets in different mice. By day nine of treatment ß-aspgly was not detected in 1 of 4 mice. One week after treatment ß-aspgly was still present, eventhough the diversity of anaerobic flora had recovered **(results section 3.2.3.6)**.

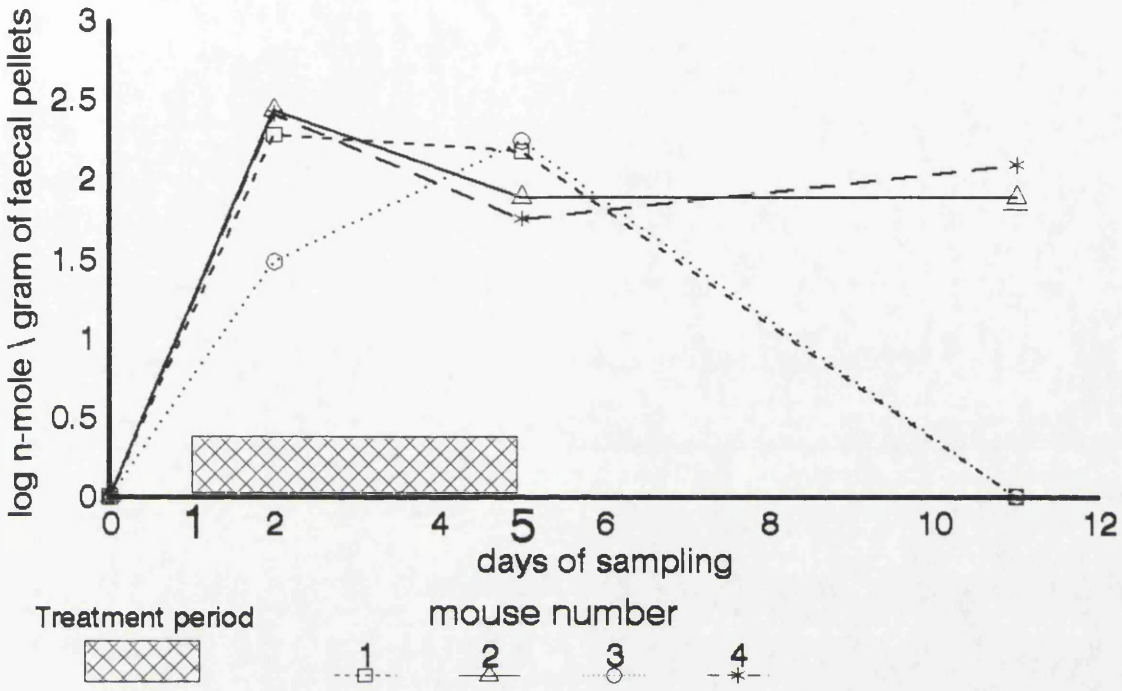


Figure 24 Effect of cefotaxime on the presence of ß-aspartylglycine in Balb / c.

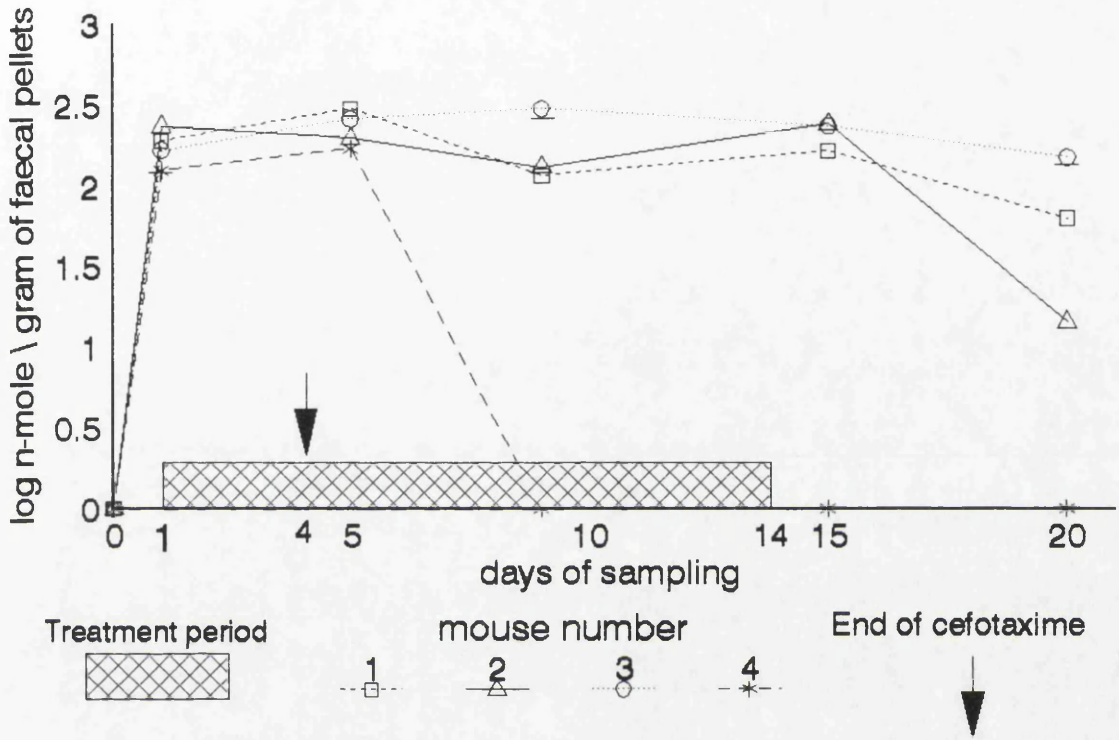


Figure 25 Effect of PTA regimen with the addition of systemic cefotaxime on the presence of ß-aspartylglycine in Balb / c mice.

3.3 Colonisation Resistance:

Groups of eight mice from different batches were used in 79 different challenge experiments. Colonization resistance was expressed as log of that oral dose of an organism resulting in colonization in 50 % of animals for at least 2 weeks (*Van der Waaij et. al., 1971)* as estimated by presence of the organism in the faeces.

3.3.1 Control mice: (32 groups of 8 mice)

Mice were challenged by gavage with different doses of each of 7 different challenge organisms. An oral challenge of 10^{8-9} cfu of the *E.coli* s-R21 challenge strain was required to achieve intestinal colonisation in over 50 % of control mice for over two weeks **(Figure. 26)** . The mean total viable count of *E.coli* s-R21 challenge strain in the colonized mice varied between 10^{6} and 10^{8} cfu / gram of faeces during the 14 days after succesful challenge **(Figure. 27)**. By contrast, the faeces of uncolonized mice contained no detectable challenge organisms three or more days after challenge.

Colonization of control mice with selected strains of *Proteus mirabilis* s-R9, *Pseudomonas aeruginosa* s-R321, *Pseudomonas cepacia* s-R13, *Klebsiella peumoniae* s-R10, *Providencia stuartii* s-R7, and *Candida albicans* s-R5 was not achieved at challenge doses of 10^{9-10} cfu **(Figures. 28, 29, 30, 31, 32, and 33)**. However, the clearance of challenge organisms did vary both between organisms and between different mice subjected almost to the same challenge **(Table. 10)**.

As expected the control mice had a high colonization resistance. The challenge organisms, and doses at which they failed to colonize the gut of control mice are shown in **(Table. 11)** .

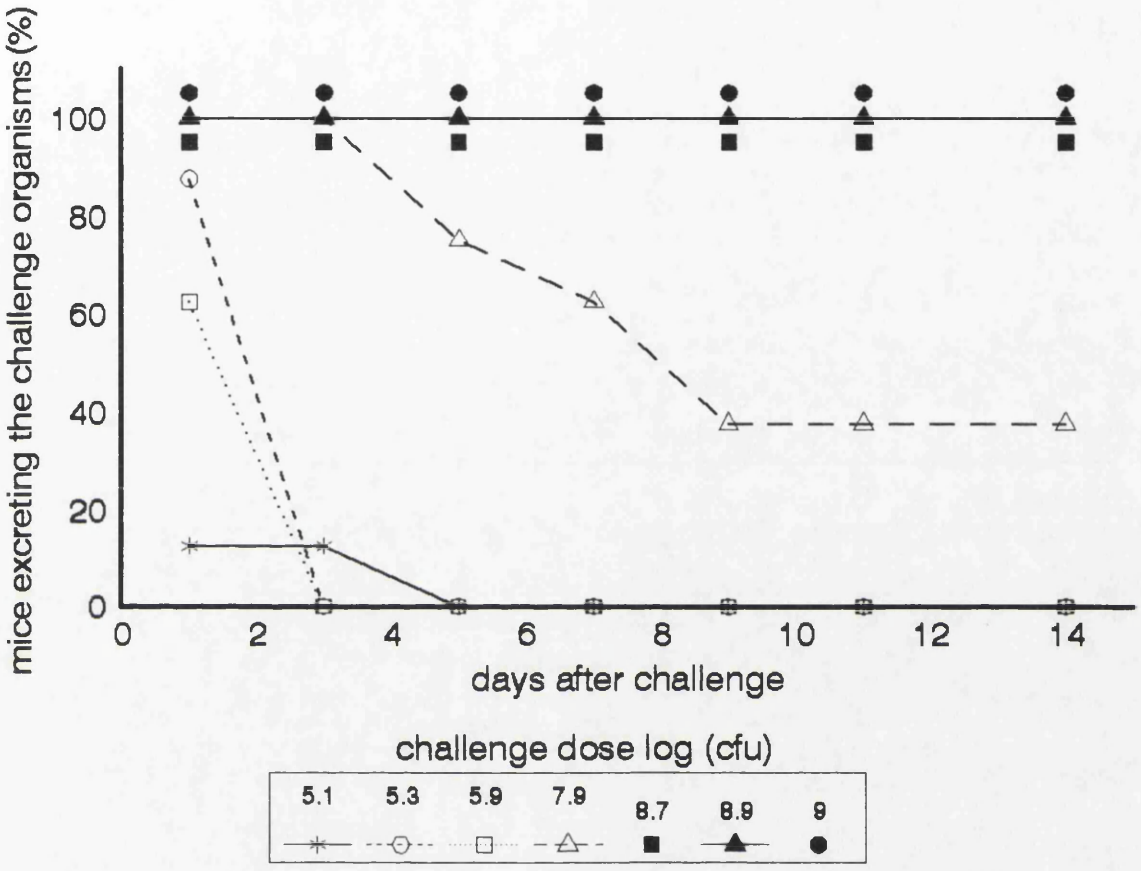


Figure 26 Excretion of *E.coli* s-R21 challenge strain by 7 groups of 8 control untreated mice.

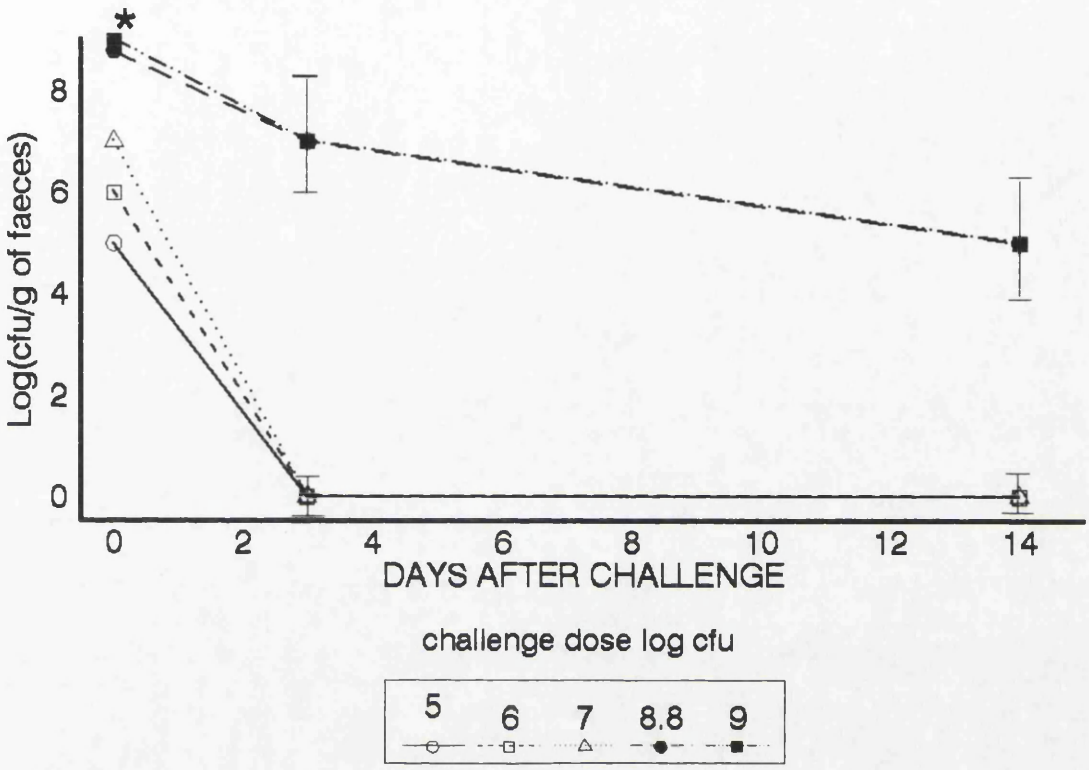


Figure 27 The total viable count of *E.coli* s-R21 challenge strain in faeces of control mice.

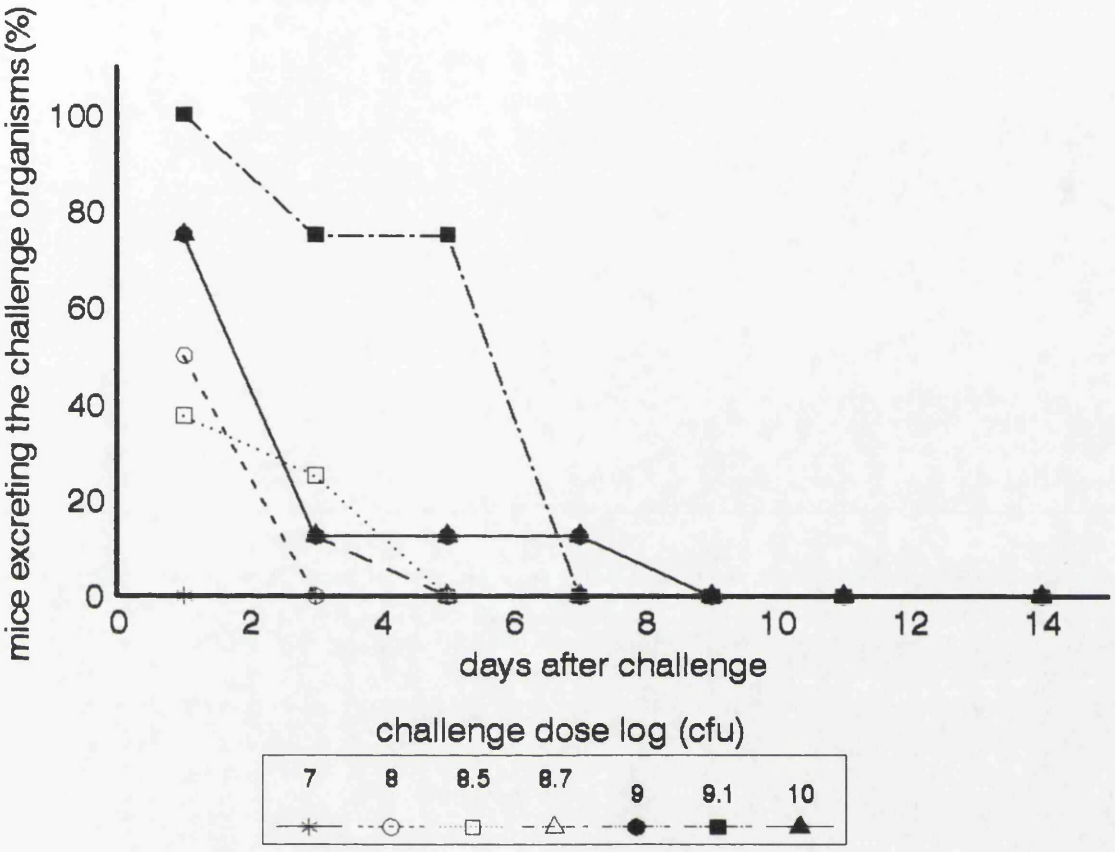


Figure 28 Excretion of *Proteus mirabilis* s-R9 challenge strain by group of 8 control untreated mice.

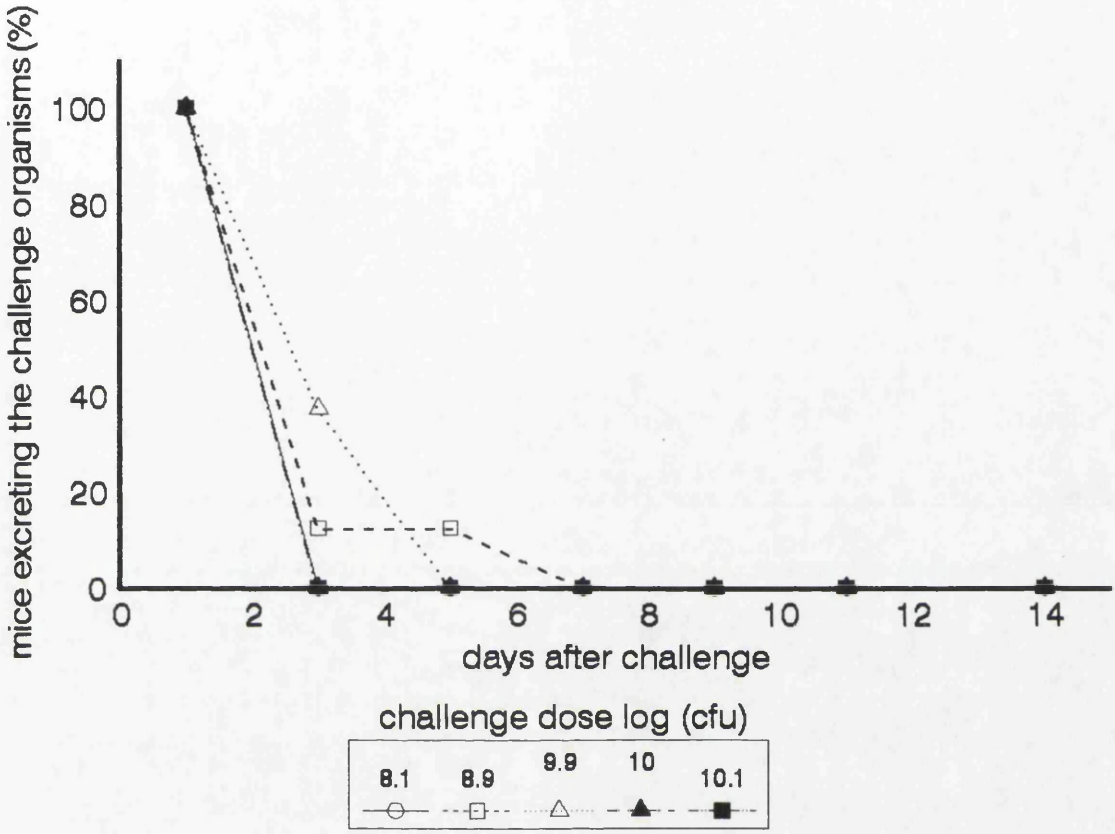


Figure 29 Excretion of *Pseudomonas aeruginosa* s-R321 challenge strain by 5 groups of 8 control untreated mice.

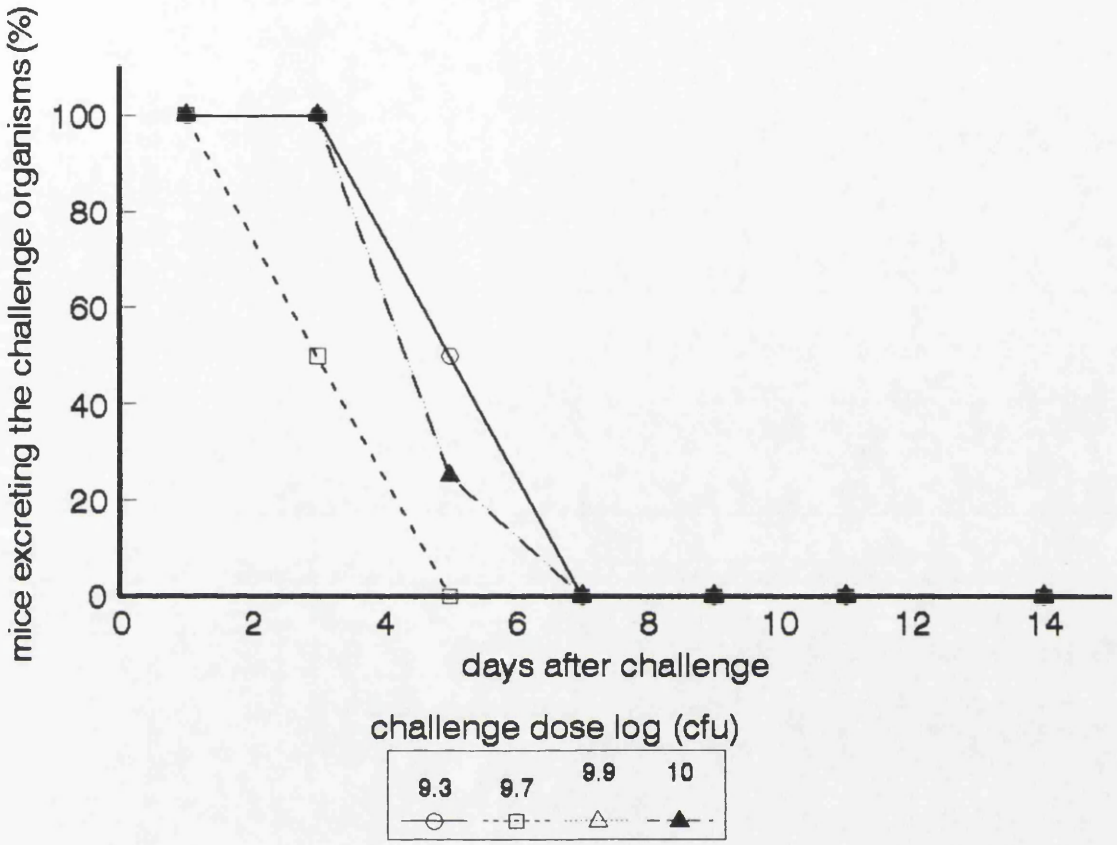


Figure 30 Excretion of *Klebsiella pneumoniae* s-R10 challenge strain by 4 groups of 8 control untreated mice.

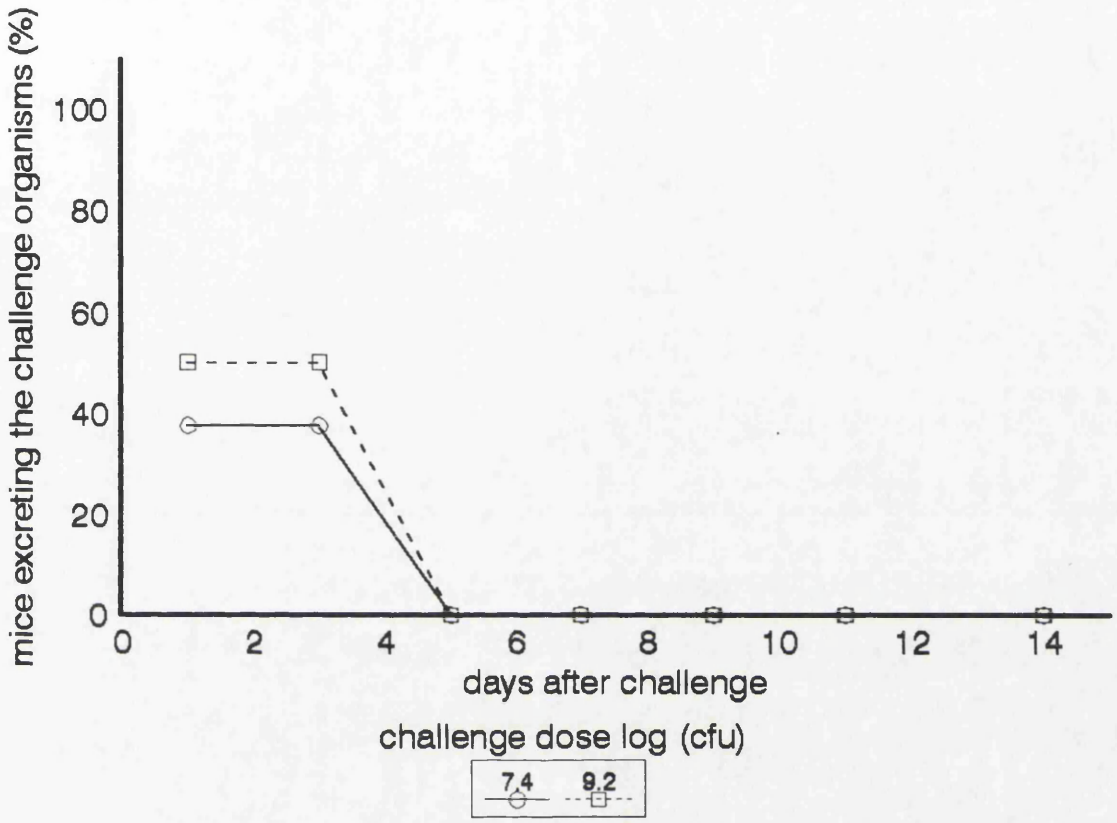


Figure 31 Excretion of *Pseudomonas cepacia* s-R13 challenge strain by 2 groups of 8 control untreated mice.

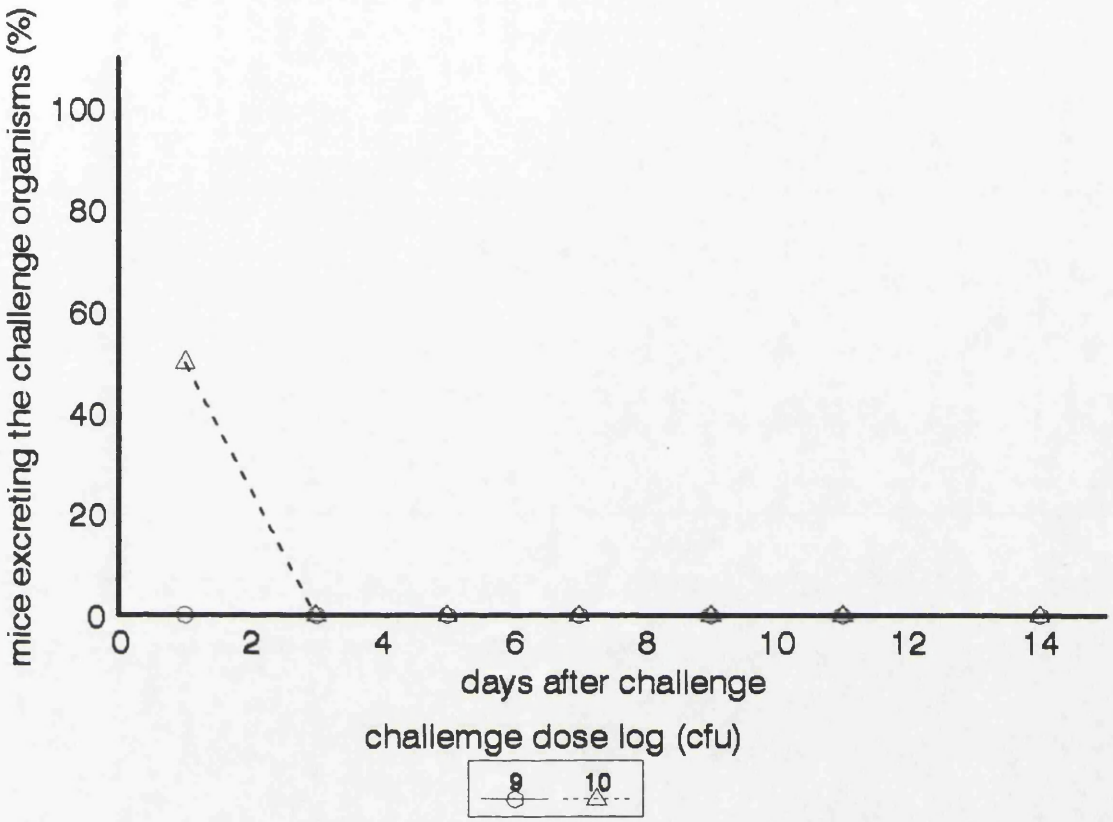


Figure 32 Excretion of *Provedincia stuartii* s-R7 challenge strain by 2 groups of 8 control untreated mice.

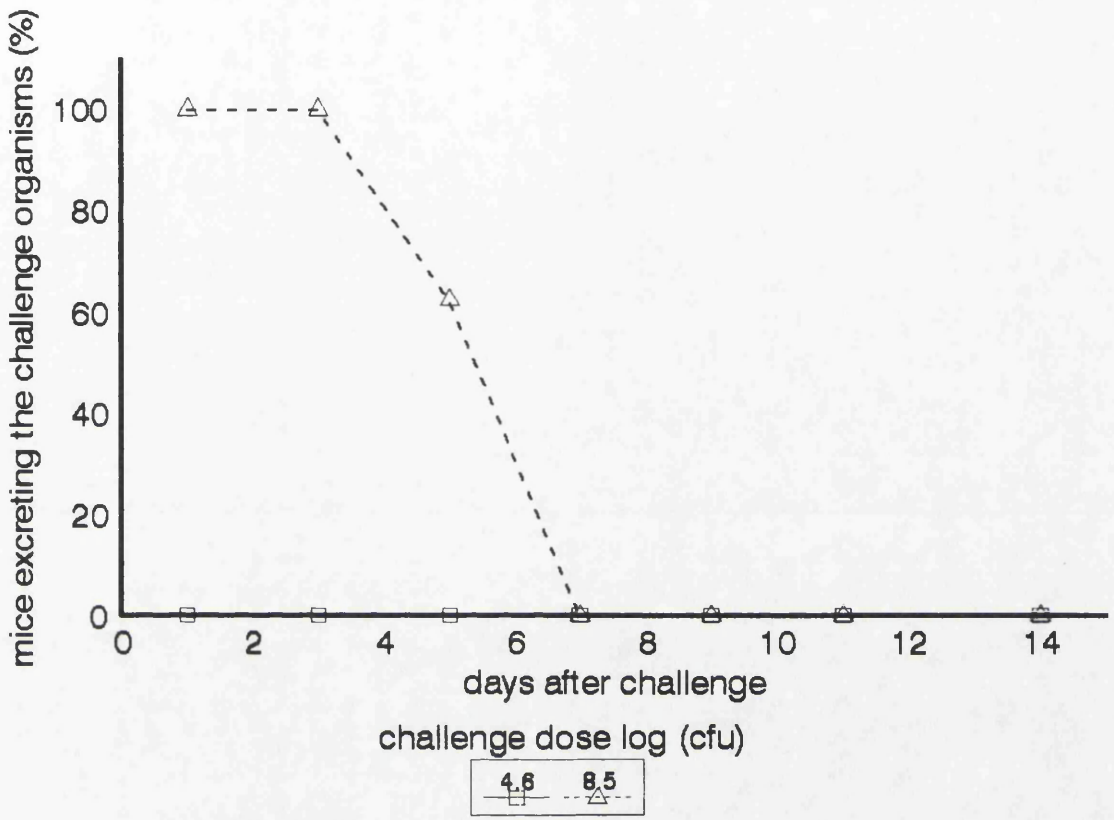


Figure 33 Excretion of *Candida albicans* s-R5 challenge strain by 2 groups of control untreated mice.

Table 10 **Examples of % of mice[&] excreting the challenge organism.**

Challenge Organism	Challenge Dose[*]	Days 1	3	5	7
Proteus mirabilis	9.1	100	77.5	77.5	0
Ps. aeruginosa	8.9	100	12.5	12.5	0
Kl. pneumoniae	9.3	100	100	0	0
Ps. cepacia	9.2	50	50	0	0
Providencia stuartii	9	0	0	0	0
Candida albicans	8.5	100	100	62.5	0

& Groups of 8 mice.

***** log (cfu).

Table 11 **Species that were not recovered from faeces two weeks after challenge.**

Challenge Organisms	Number of Experiments*	Highest Dose log (cfu / mouse)
Proteus mirabilis s-R9	7	10
Ps. aeruginosa s-R321	5	10
Kl. pneumoniae s-R10	4	10
Ps. cepacia s-R13	2	9.2
Providencia stuartii s-R7	2	10
Candida albicans s-R5	2	8.5

* Groups of 8 mice.

3.3.2 Mice treated with TDD regimens: (23 groups of 8 mice)

Mice from different batches were treated for two weeks with the TDD antibiotics given *ad libitum* in drinking water. Mice were challenged by gavage on the second day of treatment, and one day and 14 days after cessation of treatment.

3.3.2.1 Challenging mice during TDD treatment:

3.3.2.1.1 *E.coli* s-R21: (6 groups of 8 mice)

Residual anaerobic bacteria were detected in 4 / 6 groups of mice after one dose of the TDD antibiotics, and were present thereafter: two types of anaerobic gram positive bacilli were isolated from in the faeces. On the second day of treatment all six groups of mice were challenged by gavage with 1.6, 3.1, 3.8, 4.7, 6.1 and 8.1 log (cfu) respectively of *E.coli* s-R21.

All mice were colonized during treatment, and continued to be colonized up to the time when monitoring was stopped i.e. at least two weeks after treatment. The mean total viable count of the challenge organisms increased to 8.8 $\pm$ 0.3 log (cfu) during treatment, then dropped by 2 - 3 logs after stopping treatment, where the fall was variable between the different groups of mice **(Figure. 34)**.

3.3.2.1.2 *Pseudomonas aeruginosa* s-R321 : (5 groups of 8 mice)

Total decontamination was not achieved in 3 / 5 groups of mice during treatment with the TDD antibiotics: two types of residual anaerobic gram positive bacilli were present in the faeces. On the second day of treatment these 3 groups of mice were challenged with 3.1, 6.1, and 7.7 log (cfu) of *Pseudomonas aeruginosa* s-R321. All mice lost the challenge organisms in less than one week.

The 2 groups of totally decontaminated mice were challenged on day two of treatment with.3.7 and 7.7 log (cfu) of *Pseudomonas aeruginosa* s-R321.

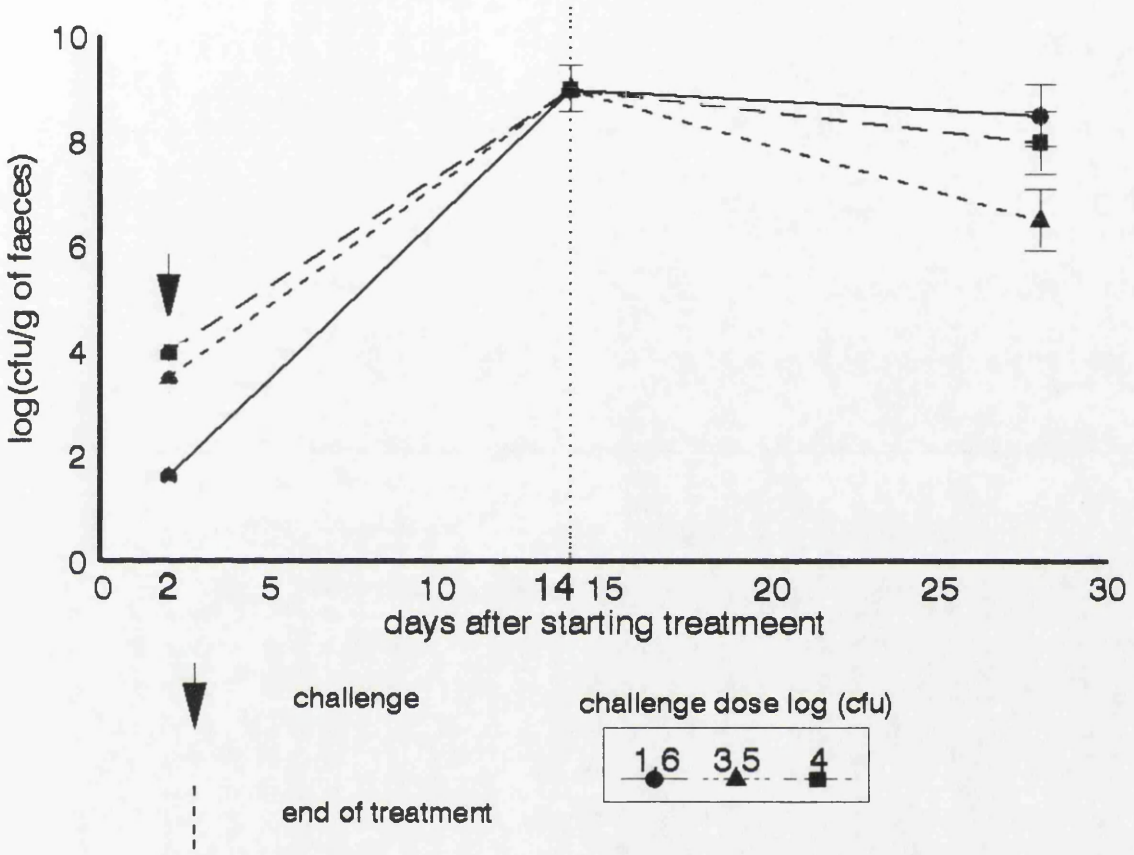


Figure 34 The total via ble count of *E. coli* s-R21 challenge strain in mouse faeces during and after treatment with TDD antibiotics. Result shown ± standard error of mean.

6 out of 8 mice lost the challenge organisms by day ten during treatment when given 3.7 log (cfu) of *Pseudomonas aeruginosa* s-R321 **(Figure. 35)**. Six out of eight mice were colonized for over two weeks when given 7.7 log (cfu). It was not possible to colonize control mice when these were given 10 log (cfu) of *Pseudomonas aeruginosa* s-R321 see **(section 3.3.1)**).

The loss of the challenge organisms from mice with residual anaerobic flora was thus faster than from totally decontaminated mice. In contrast, partial retention of anaerobic flora did not have any observed effect on colonization by *E.coli* of mice during treatment see **(section 3.3.2.1.2)**.

3.3.2.2 : Challenge of mice one day after the end of treatment:

3.3.2.2.1 *E.coli* s-R21: (3 groups of 8 mice)

One day after treatment with the TDD regimen three groups of mice were challenged with 1.3, 5, and 8.8 log (cfu) of *E.coli* s-R21 given by gavage. None of these mice were totally decontaminated during treatment **(see section 3.3.2.1.1)**. All mice were colonized with the challenge organisms for over two weeks, and no loss occurred **(Figure. 36)**. The total viable count of the challenge *E.coli* s-R21in the gut is shown in **(Figure. 37)**; they increased or decreased almost to the same level in all an all groups of mice .

3.3.2.2.2 *Pseudomonas aeruginosa* s-R321: (1 group of 8 mice)

One day after treatment one group of 8 totally decontaminated mice was challenged with 7.3 log (cfu) of *Pseudomonas aeruginosa* s-R321. All mice lost the challenge organisms in less than one week **(Figure. 36)**.

3.3.2.3 Challenge of mice two weeks after the end of treatment:

3.3.2.3.1 Treatment with TDD antibiotics only: (5 groups of 8 mice)

Total decontamination was not achieved in 3 / 5 groups of mice see **(section 3.3.2.1.1)**. To eliminate residual organisms from the gut, the partially decontaminated mice were treated for 3 days with 8 mg of bacitracin per day

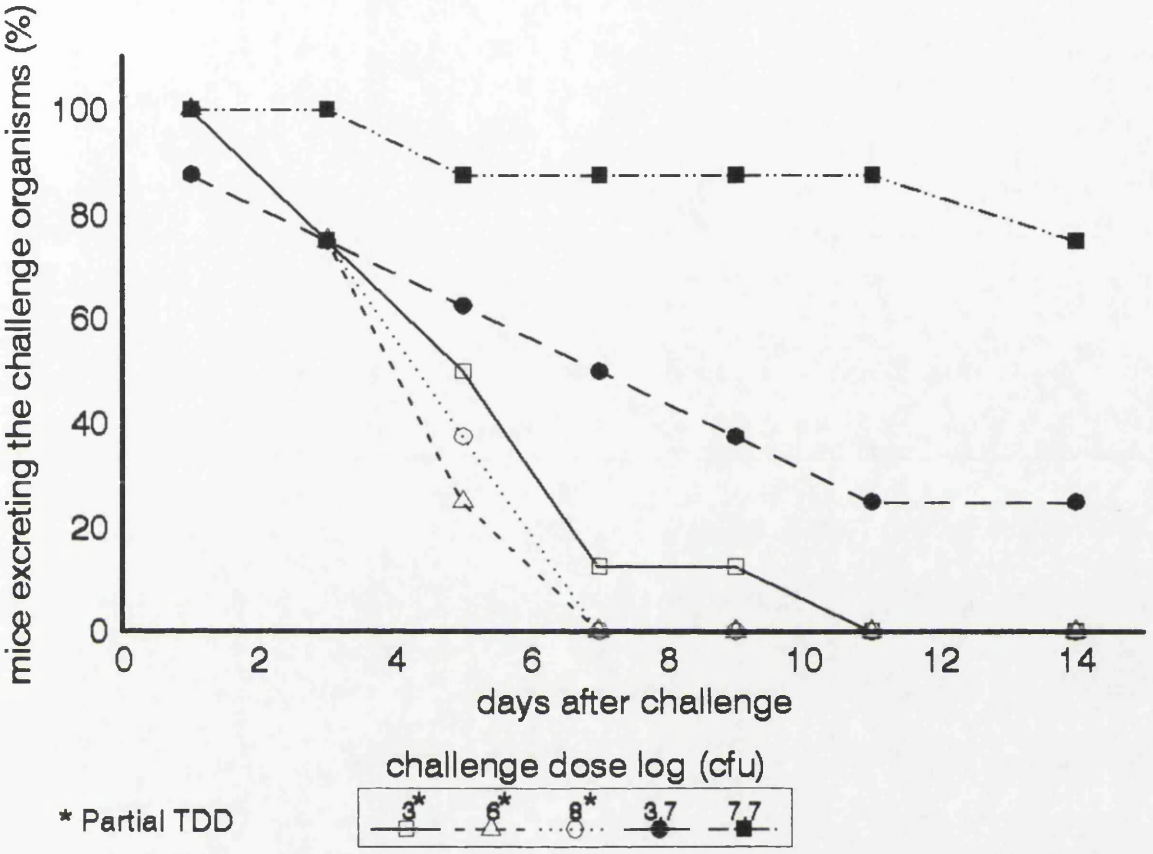


Figure 35 Excretion of *Pseudomonas aeruginosa* s-R321 challenge strain by groups of 8 mice challenged during treatment with the TDD antibiotics.

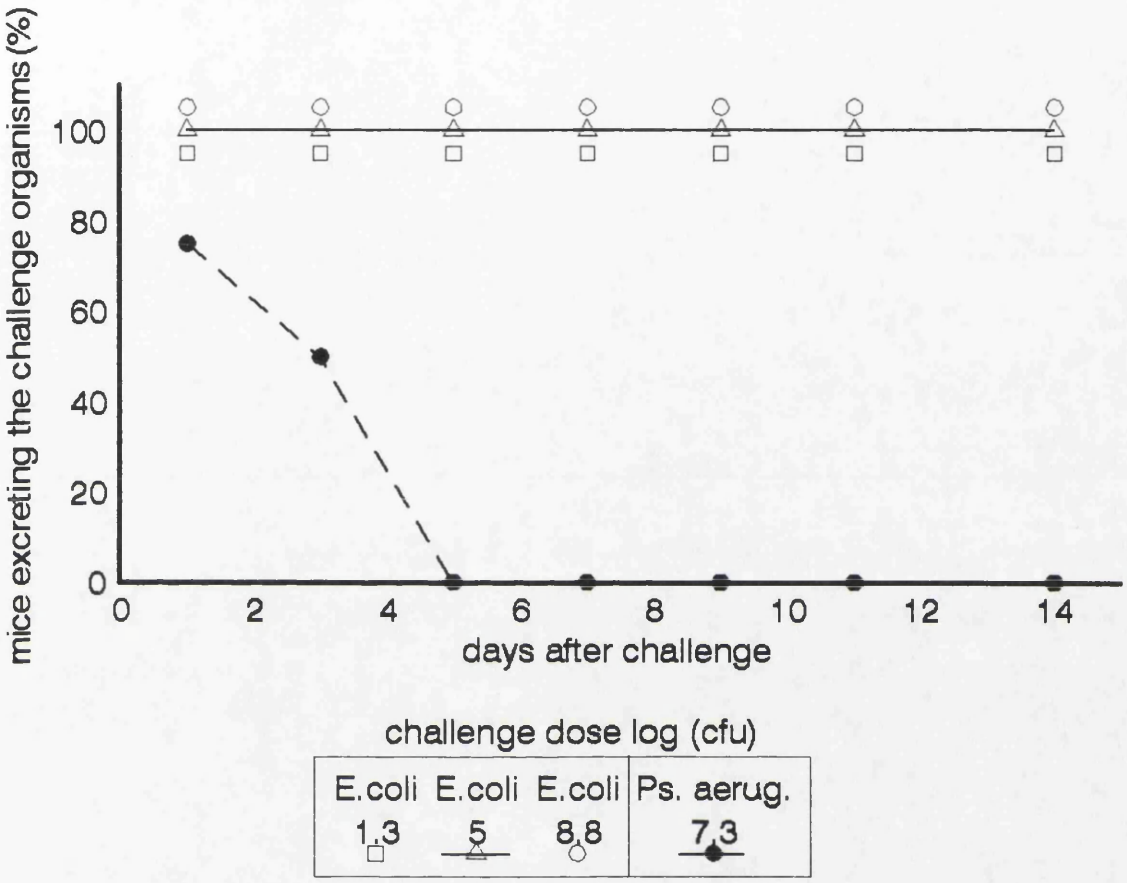


Figure 36 Excretion of *E.coli* s-R21 and *Pseudomonas aeruginosa* s-R321 challenge strain by 4 groups of 8 mice challenged one day after cessation of treatment with the TDD antibiotics.

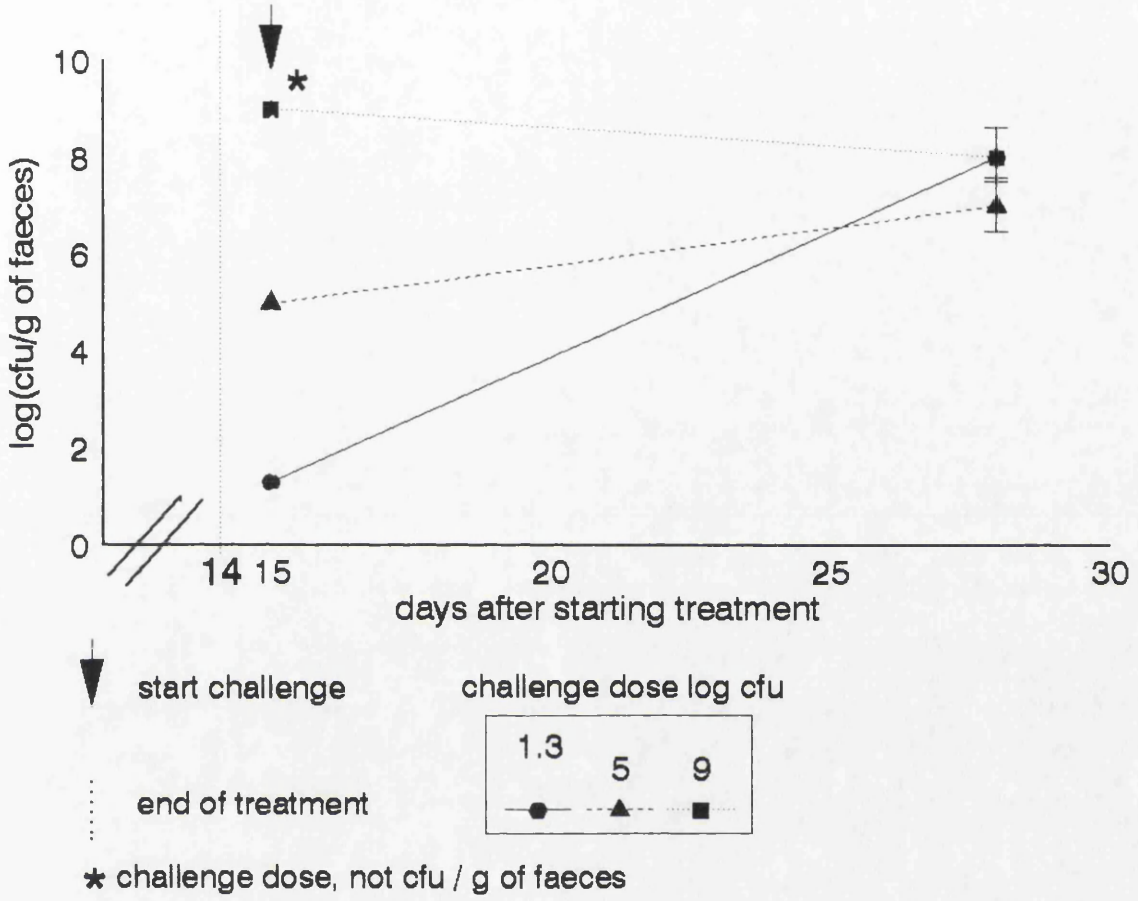


Figure 37 The total viable count of *E.coli* s-R21 challenge strain in mouse faeces following challenge 1 day after cessation of treatment with the TDD antibiotics. Results shown ± standard error of mean.

given by gavage started on day 5 of the TDD treatment. The residual organisms were then reduced to a non detectable levels, but reappeared three days after bacitracin treatment was stopped, even though treatment with TDD antibiotics was continued.

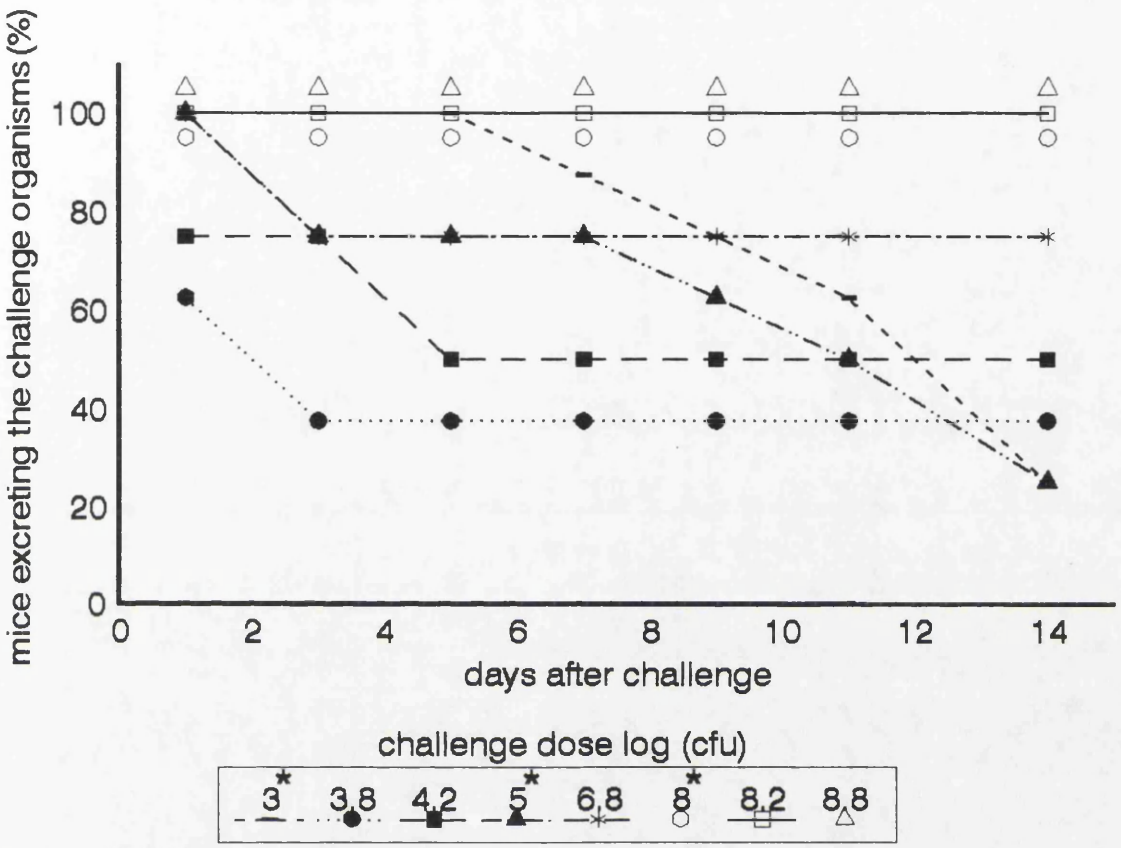
Two weeks after stopping all treatment all 5 groups of mice were challenged by gavage with 3.8, 4.2, 6.8, 8.2, and 8.8 log (cfu) of *E.coli* s-R21 respectively. Over 50 % of mice were colonized for over two weeks when challenged with 4 log (cfu) or more, and over 50 % lost the challenge organisms when challenged with less than 4 log (cfu) of *E.coli* s-R21 **(Figure. 38)**.

3.3.2.3.2 Treatment with TDD antibiotics with addition of bacitracin: (3 groups of 8 mice)

Mice were treated for two weeks with the TDD antibiotics and 3 mg ml^{-1} of bacitracin in drinking water. Mice were not totally decontaminated, Residual organisms see **(section 3.3.2.1.1)** were present during treatment in all mice.

Two weeks after treatment, mice were challenged with doses 3, 5, and 8 log (cfu) of *E.coli* s-R21. The colonization resistance was almost the same as in the other groups of mice which were treated only with the TDD regimens **(Figure. 38)**. Over 50 % of mice were colonized for over two weeks when challenged with 5 log (cfu) or more, and over 50% of mice lost the challenge strain of *E.coli* in less than two weeks when given less than 5 log (cfu) of *E.coli* s-R21.

Summary of the values of CR is shown in **Table 12 p.176.**



* Mice treated with TDD antibiotics with addition of bacitracin.

Figure 38 Excretion of *E.coli* s-R21 challenge strain by 8 groups of 8 mice challenged 2 weeks after cessation of treatment with the TDD antibiotics.

3.3.3 Mice treated with SDD regimens: (13 groups of 8 mice)

Mice from different batches were treated with the SDD regimen given by gavage. As it proved impossible to employ the same strain of *E.coli* s-R21 because of lack of appropriate resistance to PTA drugs, mice were challenged before, during and after treatment with the SDD regimen with various doses of *Proteus mirabilis* s-R9 resistant to PTA .

3.3.3.1 Mice challenged during SDD: (4 groups of 8 mice)

Mice were treated for two weeks with the SDD regimen. On day five of treatment, when SDD was fully established, mice were challenged with 5.7, 6.7, 7.7, 8.5 and 8.8 log (cfu) of *Proteus mirabilis* s-R9 challenge strain respectively. More than 50 % of mice lost the challenge organisms in less than two weeks after challenge with doses of less than 8 log (cfu) **(Figure. 39)**. Over 50 % of mice were colonized for two weeks following challenge with more than 8 log (cfu).

The total viable count of the challenge strain of *Proteus mirabilis* s-R9 was reduced from 6.8 $\pm$ 0.3 log (cfu) during treatment, to 4 $\pm$ 0.2 log (cfu) 5 days after treatment. Seventy five percent of colonized mice lost the challenge organisms by day 14 after cessation of treatment.

The colonisation resistance for mice treated with the SDD regimens was impaired, as was represented by a small but significant reduction in colonisation resistance compared to control mice challenged with *Proteus mirabilis* s-R9 **(section 3.3.1)**.

3.3.3.2 Mice challenged after SDD: (6 groups of 8 mice)

Mice were divided into 4 categories depending on length of treatment, and the challenge day after cessation of treatment.

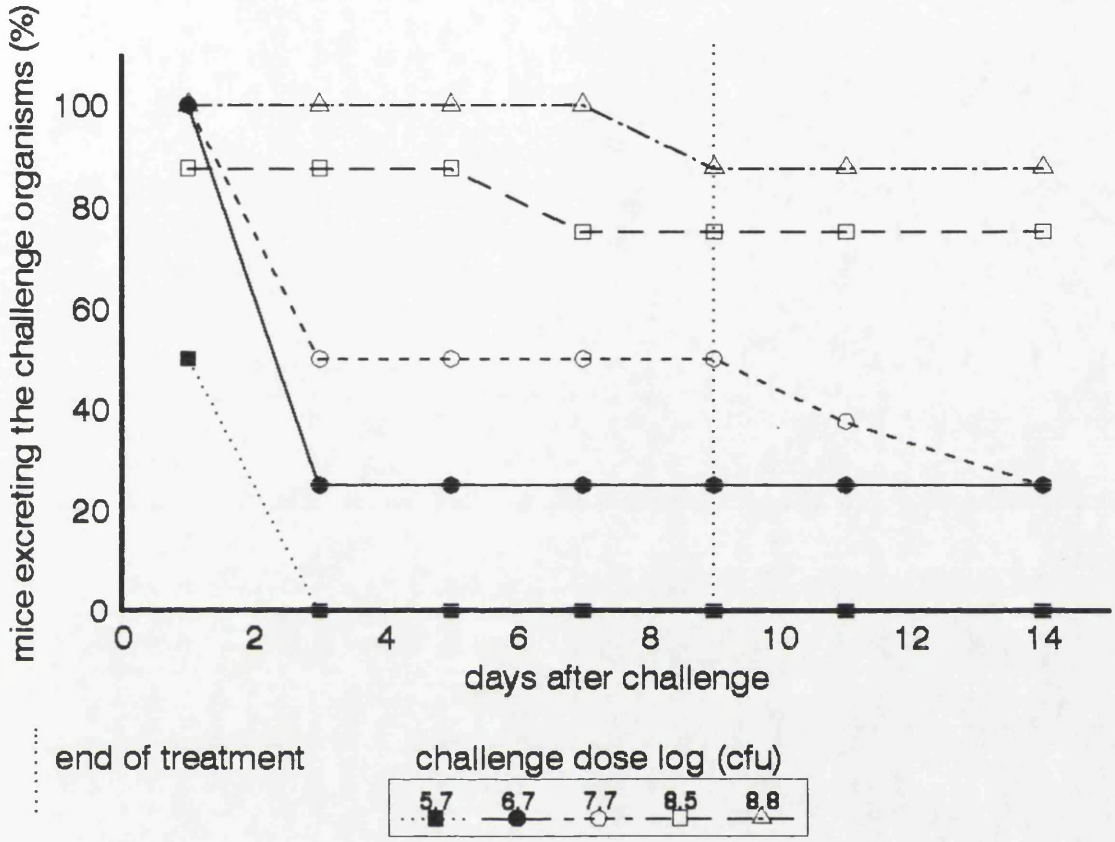


Figure 39 Excretion of *Proteus mirabilis* s-R9 challenge strain by 5 groups of 8 mice challenged on day 5 during 2 weeks treatment with the PTA regimen.

3.3.3.2.1 *Challenged one day after treatment:* (3 groups of 8 mice)

Mice were treated for 5 or 10 days with the SDD regimen, and challenged one day after the end of treatment. .

A) Five days Treatment: (2 groups of 8 mice)

Mice were treated for five days with the SDD regimen and challenged one day after treatment. All mice lost the challenge organisms when challenged with 5.9 and 7.9 log (cfu) of *Proteus mirabilis* s-R9 challenge strain **(Figure. 40)**.

B) Ten days treatment: (1 group of 8 mice)

Mice were treated for ten days with the SDD regimen, and challenged one day after treatment. Seven out eight mice were colonized for over two weeks after challenge when challenged with 7.5 log (cfu) of *Proteus mirabilis* s-R9.

This results suggest that the period of colonisation resistance impairment after cessation of treatment may be proportional to the length of treatment **(Figure. 40)**.

3.3.3.2.2 *Challenge seven days after treatment:* (3 groups of 8 mice)

Mice were treated for 5 or 10 days with SDD regimen, and challenged seven days after the end of treatment.

A) Five days treatment: (2 groups of 8 mice)

Mice were challenged 7 days after the end of 5 days treatment SDD regimen. Five out eight mice lost the challenge organisms in less than two weeks challenged with 6.2 log (cfu) of *Proteus mirabilis* s-R9, but when challenged with 8.2 log (cfu) 6 out of 8 mice were colonized for two weeks after challenge **(Figure. 41)**. This result is similar to that for challenge during treatment **(section 3.3.3.1)**.

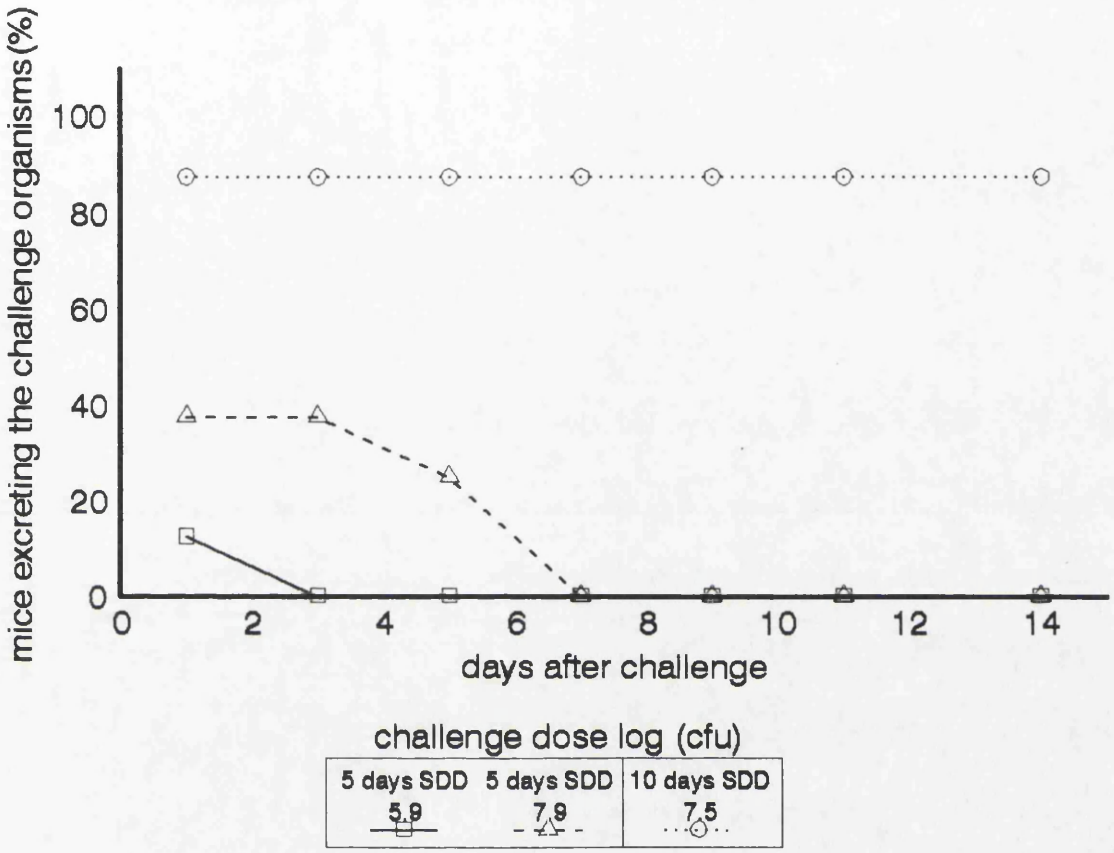


Figure 40 Excretion of *Proteus mirabilis* s-R9 challenge strain by 3 groups of 8 mice challenged one day after the end of 5 and 10 days treatment with PTA regimen.

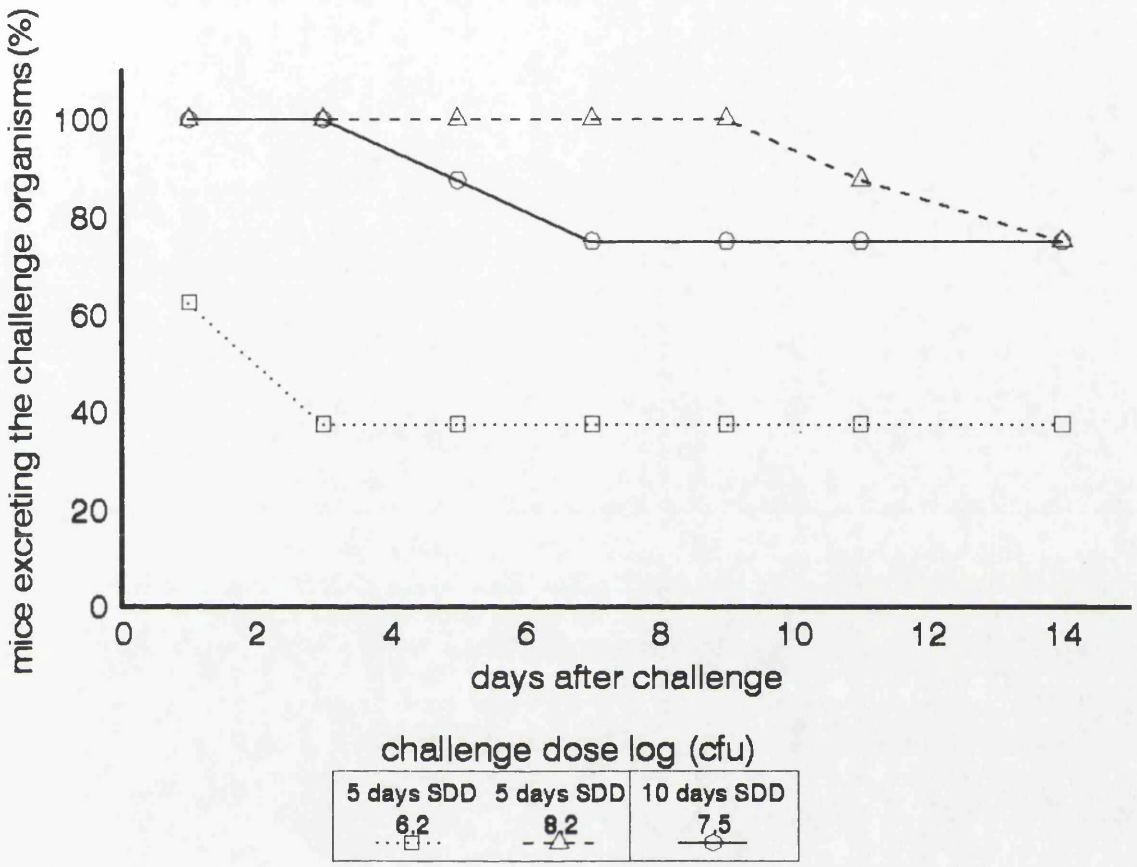


Figure 41 Excretion of *Proteus mirabilis* s-R9 challenge strain by 3 groups of 8 mice challenged 7 days after the end of 5 and 10 days treatment with PTA regimen.

B) Ten days treatment: (1 group of 8 mice)

Mice were treated for ten days with the SDD regimen, and challenged seven days after treatment. Six out of eight mice were colonized for two weeks after challenge when challenged with 7.5 log (cfu) of *Proteus mirabilis* s-R9 **(Figure. 41)**.

3.3.3.3 Challenged mice one day before treatment: (2 groups of 8 mice)

Mice were challenged with 9.3 and 10 log (cfu) of *Proteus mirabilis* s-R9 challenge strain respectively. One day after challenge they started treatment with the SDD regimen for ten days. Thirteen out sixteen mice remained colonized for two weeks, whereas in control mice no colonisation occurred **(Figure. 42).**

3.3.4 Mice treated with cefotaxime: (4 groups of 8 mice)

Mice were treated for four days with 3 mg per day of cefotaxime given by i.p. injection. As the challenge organism was sensitive to CTX, one day after the end of treatment mice were challenged by gavage with 6, 8, 8.6, and 9.17 log (cfu) of *Proteus mirabilis* s-R9 challenge strain respectively. Twenty two out twenty four mice lost the challenge organisms in less than two weeks **(Figure. 43)**.

The colonisation resistance for mice treated with cefotaxime is higher than 9 log (cfu), and it appears to be similar to the colonisation resistance for control mice **(Figure. 28)**.

3.3.5 Mice treated with SDD regimen with addition of systemic cefotaxime: (4 groups of 8 mice)

Mice were divided into 2 categories depending on the length of treatment with the SDD regimen.

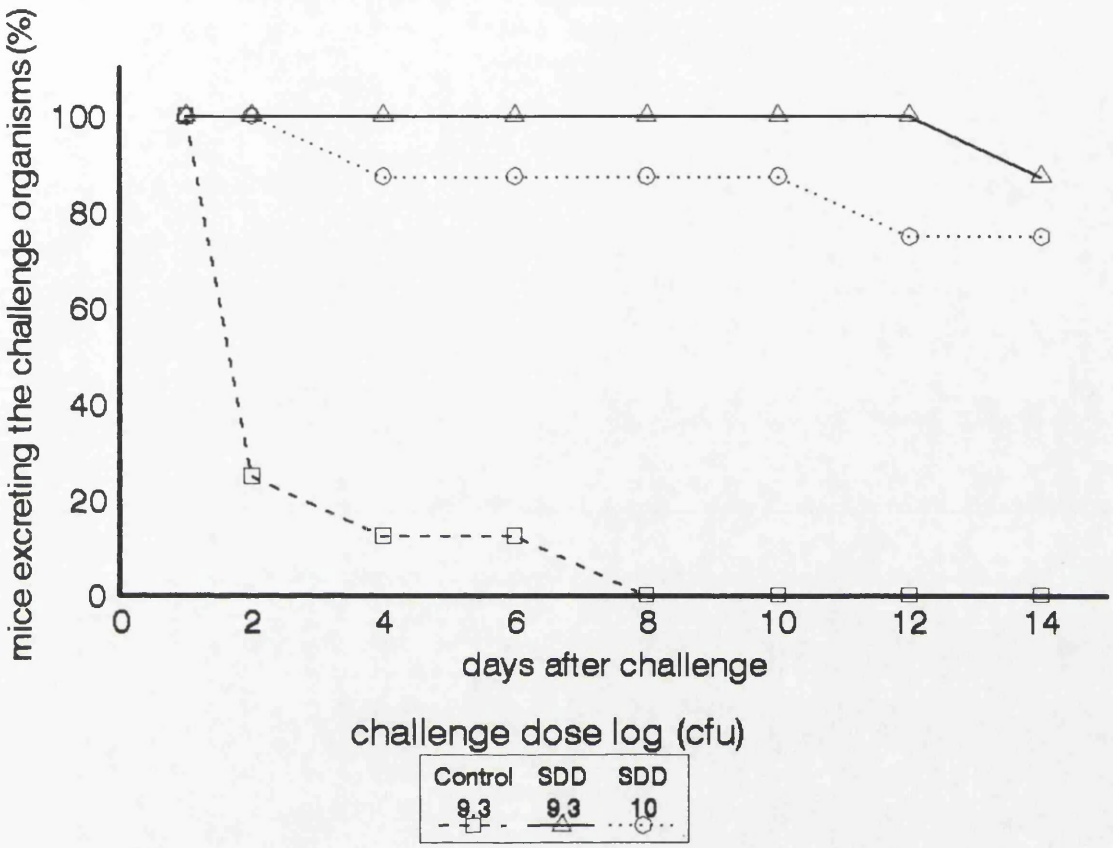


Figure 42 Excretion of *Proteus mirabilis* s-R9 challenge strain by 2 groups of 8 mice challenged one day before treatment for 10 days with PTA regimen.

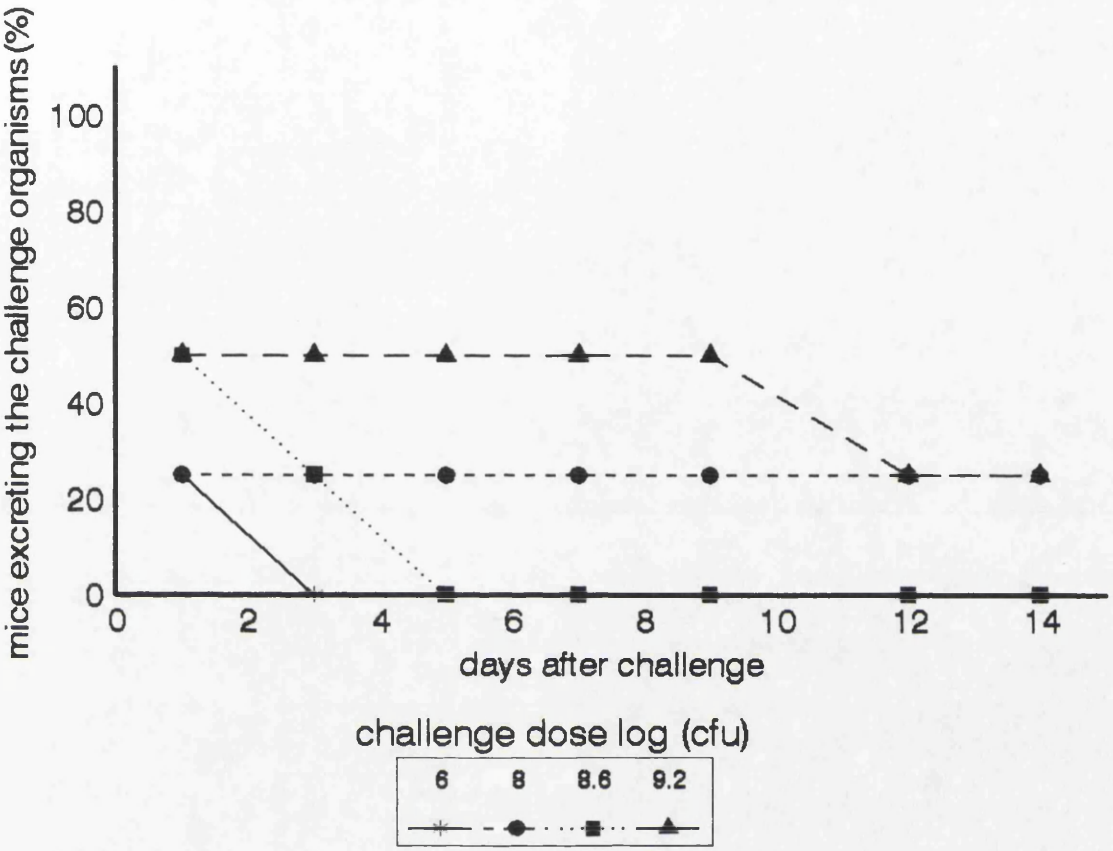
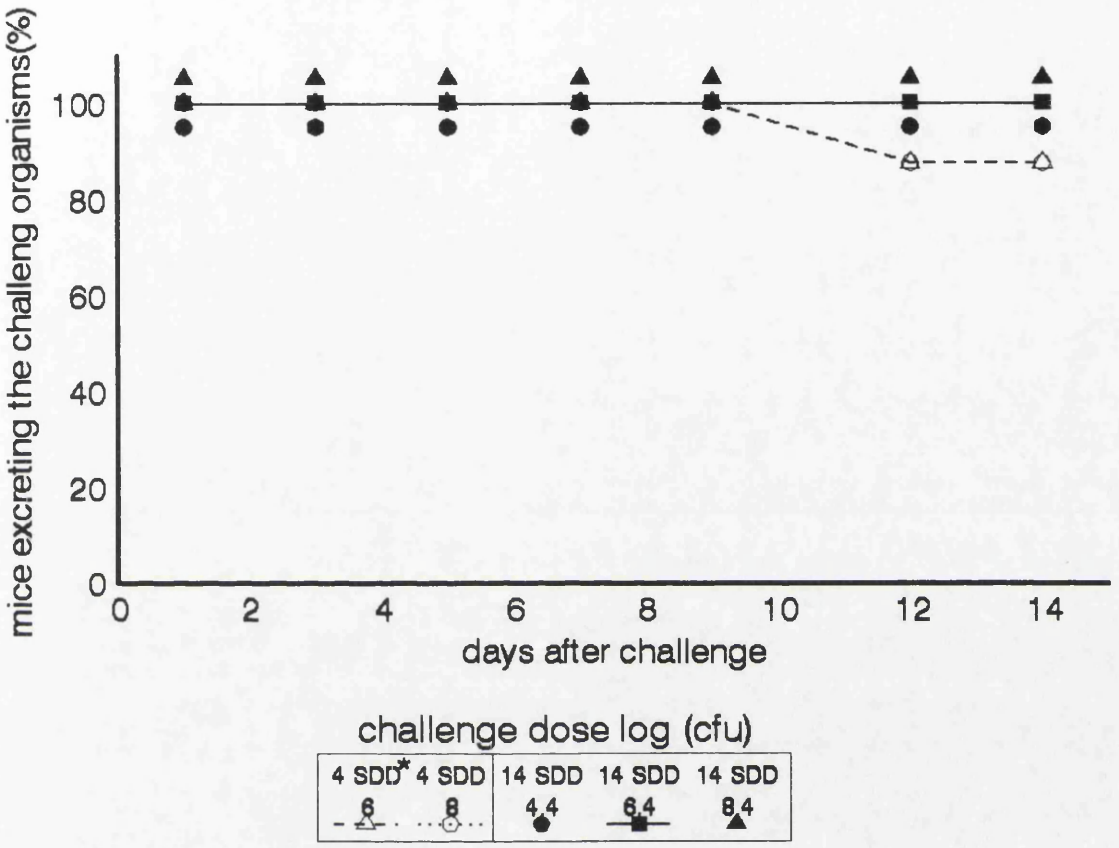


Figure 43 Excretion of *Proteus mirabilis* s-R9 challenge strain by 4 groups of 8 mice challenged one day after cessation of treatment with cefotaxime.



* Duration of treatment with SDD antibiotics in days.

Figure 44 Excretion of *Proteus mirabilis* s-R9 challenge strain by 5 group of 8 mice treated with PTA regimen for 4 or 14 days, with addition of systemic cfotaxime for 4 days, challenged one day after cessation of cefotaxime, during PTA regimen.

3.3.5.1 Four days treatment with SDD regimen: (2 groups of 8 mice)

Mice were treated for four days with the SDD regimen given by gavage, and 3 mg of cefotaxime given by i.p. injection. One day after treatment the mice were challenged by gavage with 6 and 8 log (cfu) of *Proteus mirabilis* s-R9 challenge strain. Seven out eight mice were colonized for two weeks after challenge **(Figure. 44)**.

3.3.5.2 Two weeks treatment with SDD regimen: (3 groups of 8 mice)

Mice were treated with for two weeks with the SDD regimen given by gavage, and four days with 3 mg per day of systemic cefotaxime. Three groups of mice were challenged with 4.4, 6.4, and 8.4 log (cfu) of *proteus mirabilis* s-R9 challenge strain respectively. All mice were colonized for two weeks after challenge **(Figure. 44)**.

The SDD regimen with the addition of systemic cefotaxime, affected the anaerobic flora **(section 3.2.3.5)**, and impaired colonisation resistance, almost to the same level of colonisation resistance for mice treated with TDD regimen **(section 3.3.2.1).**

3.3.6 Mice treated with clindamycin: (2 groups of 8 mice)

Mice were treated for four days with 0.6 mg per day with clindamycin given by gavage. One day after treatment mice were challenged with 6.3 and 8.3 log (cfu) of *Proteus mirabilis* s-R9 challenge strain. All mice were colonized for two weeks **(Figure. 45)**.

Clindamycin reduced the anaerobic flora to a non detectable level **(section 3.2.3.7)**, and impaired colonisation resistance.

Summary of the values of CR is shown in **Table 12 p.176.**

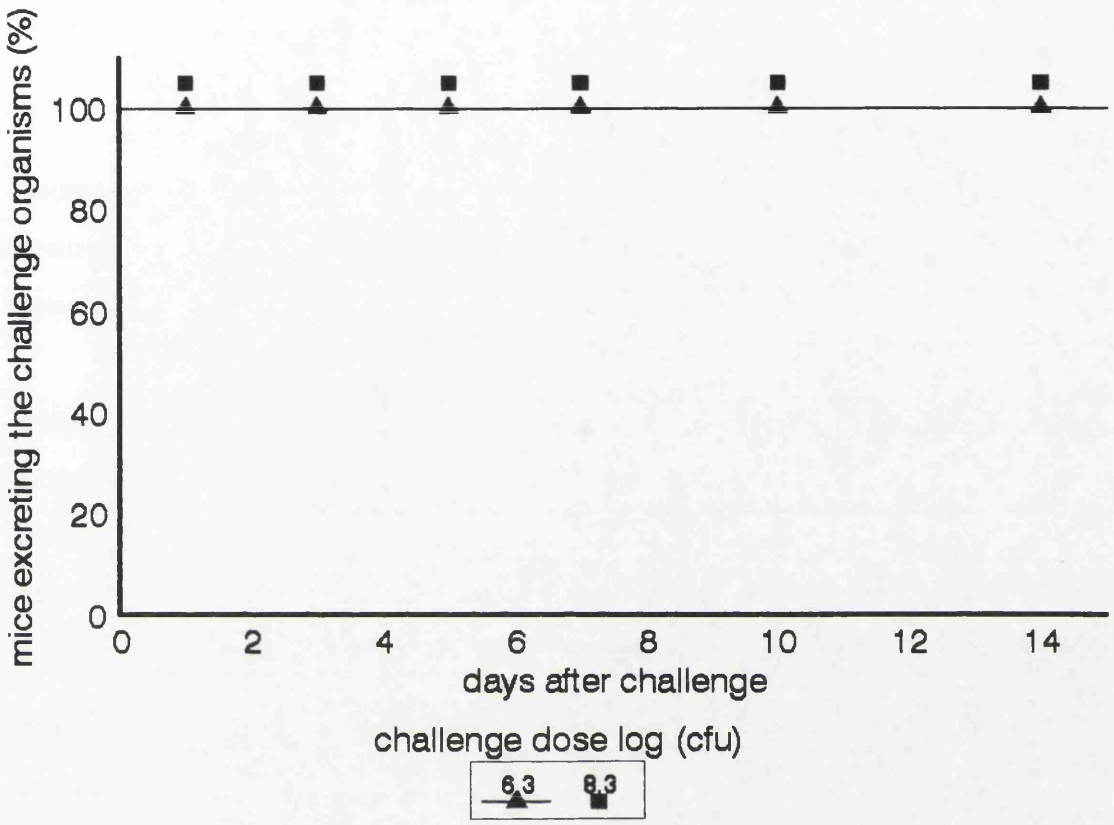


Figure 45 Excretion of *Proteus mirabilis* s-R9 challenge strain by 2 groups of 8 mice challenged one day after cessation of 4 days treatment with clindamycin.

3.4 Antibiotic Activity In Murine Faeces:

The antibiotic activity in the murine faeces or in the caecal content was determined in mice treated with TDD regimen, SDD regimen, or CTX.

3.4.1 Mice treated with TDD regimen:

Two groups of 4 mice were treated for two weeks with Streptomycin and neomycin (TDD antibiotics) given by:

group I : gavage 10 mg mouse^{-1} each of Sm and Nm given twice a day

group II : *ad libitum* in drinking water 5 mg ml^{-1} of each Sm and Nm.

In group I (gavage treated mice), faecal pellets were collected from four mice 4, 8, 12, 16, and 24 hours after the end of treatment. The highest antibiotic activity was seen 8 hours after the last dose (this results is different from single dose in results section 3.1.3). 24 hours after treatment antibiotic activity was detected in only 2 / 4 mice **(Figure. 46)**.

In group II, faecal pellets were collected at five different times during 24 hours during treatment. The antibiotic activity was not observed to rise or fall consistently over the sampling period of 24 hours **(Figure. 47)**.

3.4.2 Mice treated with SDD antibiotics:

Four mice were treated for two days with SDD regimens given by gavage. Faecal pellets were collected 4, 8, 12, 20, and 24 hours on the second day of treatment. Samples were prepared and assayed as described in **(materials and methods 2.11.2)**. Zones of inhibition were seen only 4, 8, and 20 hours after treatment, and no inhibition was seen at 12 and 24 hours after treatment. The highest antimicrobial activity was observed 8 hours after treatment **(Figure. 48)**.

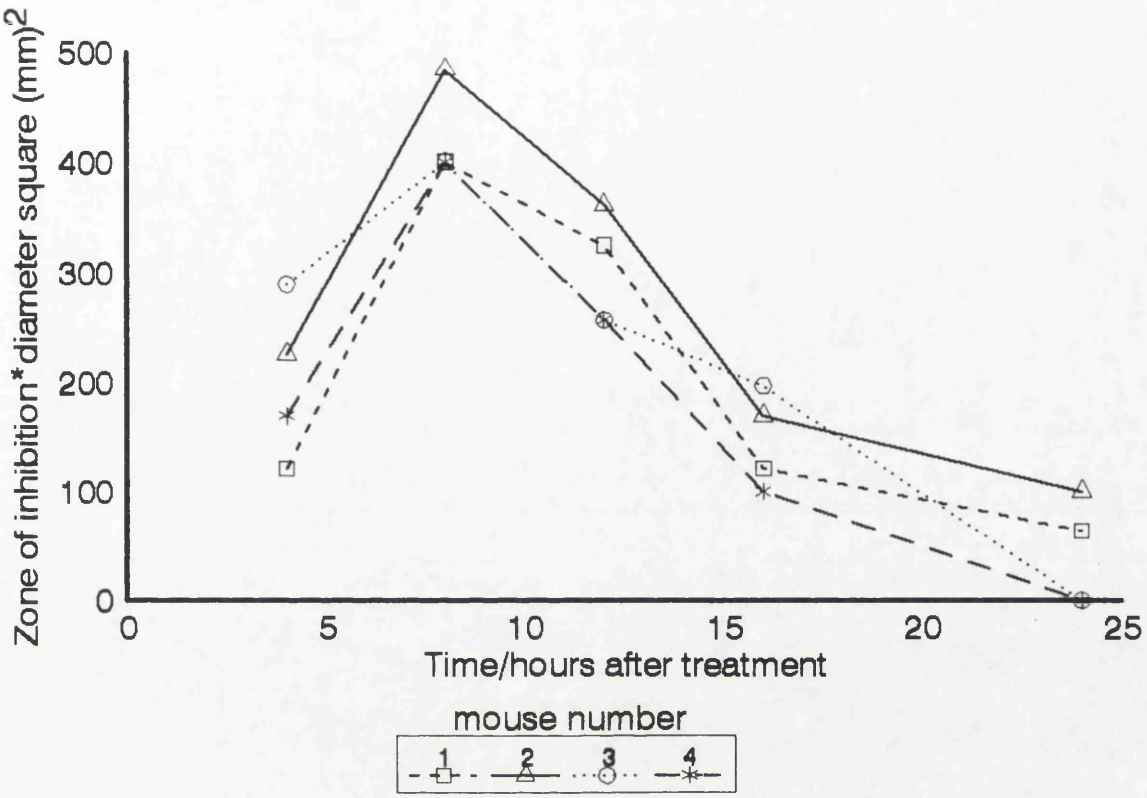


Figure 46 Antibiotic activity in the faeces of four mice treated with TDD antibiotics given by gavage 24 hours after end of treatment.

* of indicator strain : *E. coli* NCTC No. 10418.

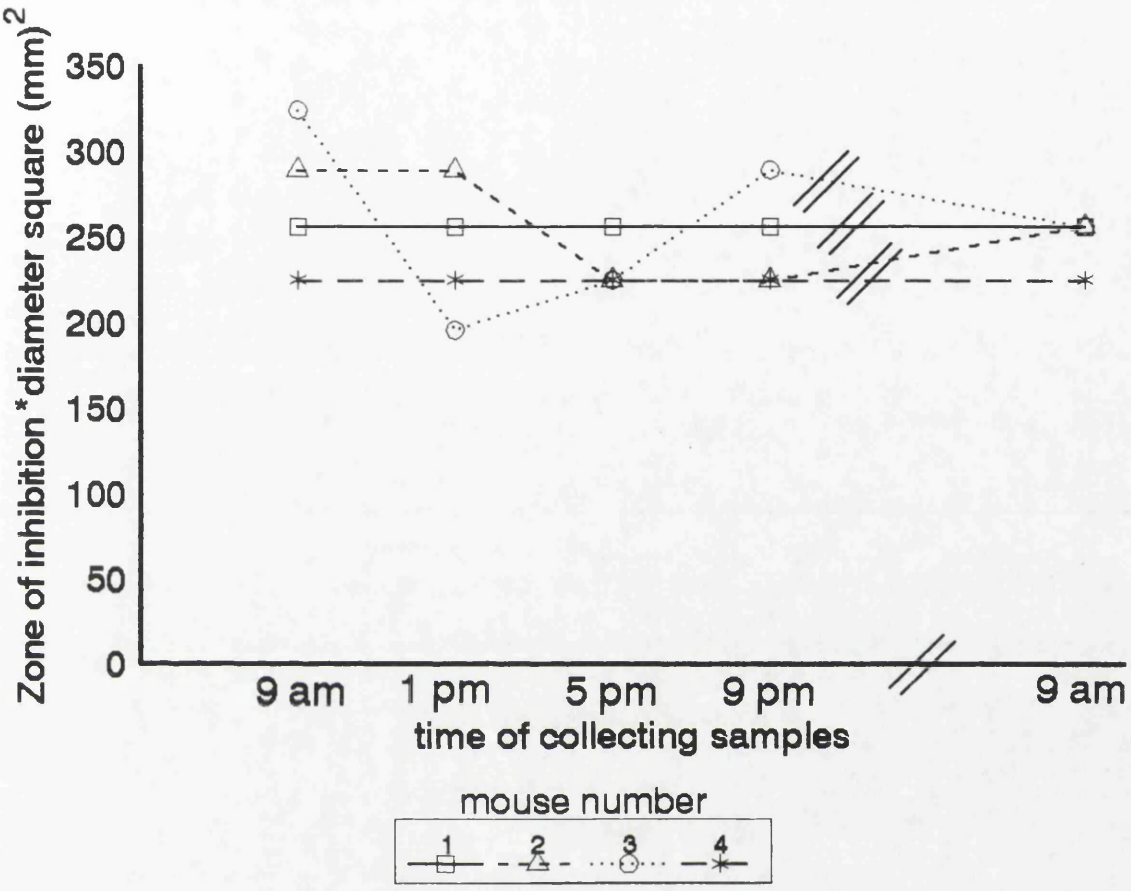


Figure 47 Antibiotic activity in the faeces of four mice treated with TDD antibiotics *ad libitum* in drinking water during 24 hours of treatment.

* of indicator strain: *E. coli* NCTC No. 10418.

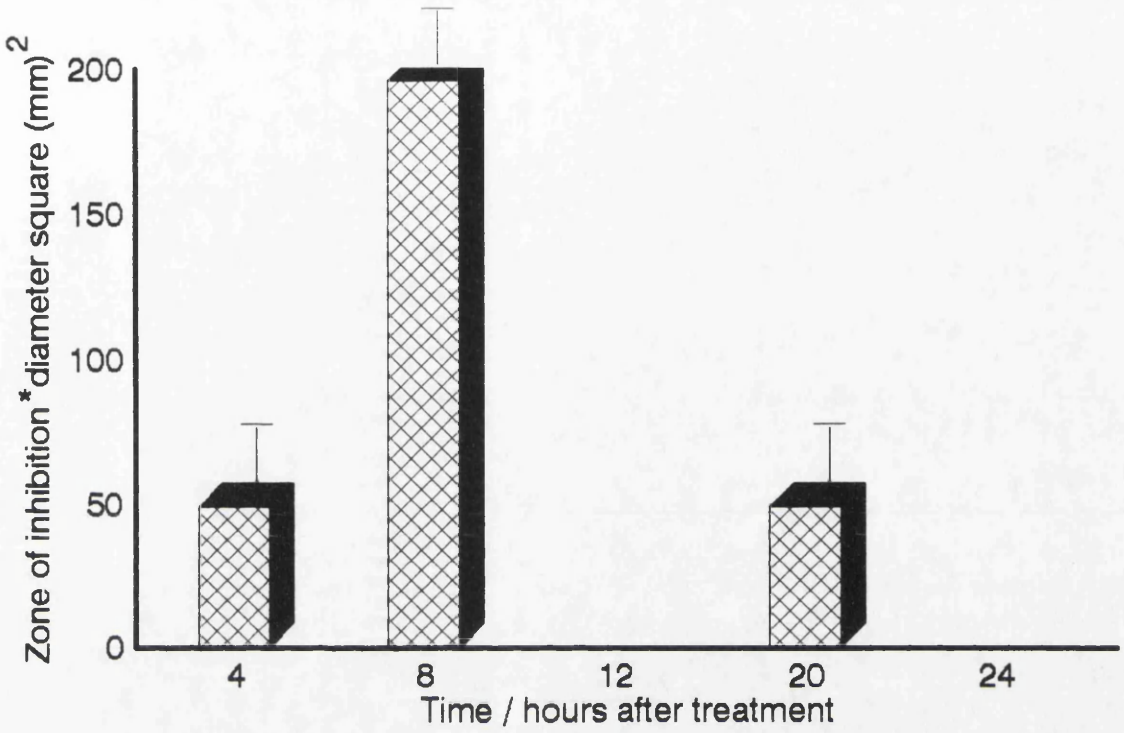


Figure 48 Faecal antibiotic activity in four mice given SDD antibiotics by gavage during 24 hours after treatment. Results shown ± SE of the mean.

* of indicator strain: *E. coli* NCTC No. 10418.

3.4.3 Mice treated with cefotaxime:

3.4.3.1 Concentration of cefotaxime in mouse caecum:

Two groups of ten mice were given an i.p. injection of 3 mg of cefotaxime.

Group I : treated for 1 day.

Group II : treated for 4 days.

One mouse from each group was dissected 2, 4, 6, 8, 10, 12, 16, 18, 20, and 24 hours after treatment, caecum contents were collected , prepared, and assayed as described in **(materials and methods 2.5)**. In group I, zones of inhibition were seen only 2, 6, 8, 16, 18, and 20 hours after treatment. The highest concentration was 20 $\mu g\ g^{-1}$ of caecum contents 10 hours after treatment **(Figure. 49)**. No further inhibition was observed 24 hours after treatment.

In group II, where mice were treated for four days, zones of inhibition were seen only at 2 hours after treatment, where the concentration was 1 $\mu g\ g^{-1}$ of caecum content, and no zone of inhibition was seen at any other time.

The experiment was repeated (figures not shown), and only at 2 hours after treatment was a zone of inhibition seen in either group. The concentration of cefotaxime in the cecum of mice given cefotaxime for one day, and for four days were 8.2 and 1 $\mu g.\ g^{-1}$ of caecal content respectively.

3.4.3.2 *In vitro* effect of Cefotaxime in murine faecal pellets :

3.4.3.2.1 *Aerobic organisms*:

Faecal pellets were collected 4 and 8 hours after treatment from four mice treated for two days with 3 mg. of cefotaxime, and the pellets were assayed for antimicrobial activity against *Proteus mirabilis* s-R9, *E.coli* s-R21, and *Pseudomonas aeruginosa* s-R321.

No antimicrobial activity against any of the challenge strains was observed.

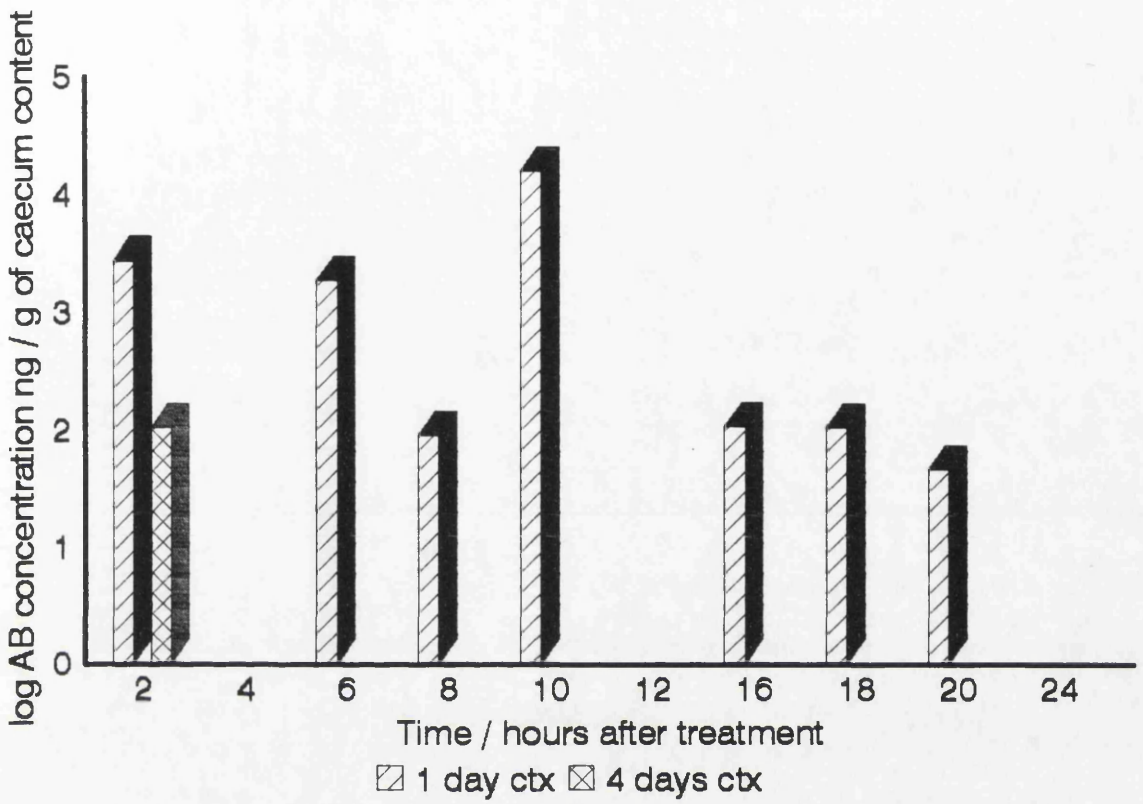


Figure 49 Concentration of cefotaxime in the caecum content of mouse during 24 hours after end of treatment with cefotaxime.

3.4.3.2.2 *Anaerobic organisms*:

The Cefotaxime in faeces did not have any visible effect on the anaerobic flora *in vitro*, as measured by inhibition of normal flora of murine faeces cultured on anaerobic blood agar plates and by direct microscopy of faeces. By contrast, an antibiotic disc containing 60 μg of cefotaxime inhibited the cultured anaerobic faecal flora *in vitro* (result not shown).

3.4.3.2.3 Concentration of cefotaxime which effects the anaerobic mormal flora:

Serial twofold dilutions of 3 mg ml^{-1} of cefotaxime were made and assayed for activity against murine faecal anaerobes. The lowest concentration of cefotaxime to show activity was 0.5 μg ml^{-1}. At this concentration the effect of cefotaxime was not seen on all types of colonies of anaerobic flora growing on anaerobic BA from murine faeces, whereas at higher concentrations all types of the anaerobic flora were affected (results not shown).

Chapter Four

DISCUSSION

4. Discussion:

4.1. Preliminary Experiments

The main stimulus for this study was the rapidly increasing use of SDD in ITU patients following the impressive results described for this technique in the prevention of ITU-aquired infection (Stoutenbeek et al., 1987; Ledingham et al., 1988; Konrad et al., 1989). The concept of CR is central to the use of SDD. However, CR remains a highly contentious hypothesis: the existence of the phenomenon has been denied (Sanderson, 1989; Nau et al., 1990), and when detected it has been variously ascribed to physical factors (Gordon and Pesti, 1971), to aerobic gut flora (Freter and Abrams, 1972; Ducluzeau et al., 1977; Goren et al., 1984)), to anaerobic gut flora (van der Waaij et al., 1971; Wells, 1988) or to various combinations of these factors (Hentges et al., 1985; Wells, 1988). Further, the experimental work in animals that forms the basis of the concept is dependent to a surprising degree on a restricted series of studies carried out by van der Waaij and co-workers between 1971- 1974 (van der Waaij et al., 1971; 1972; 1974; Wensinck and Ruseler-van Embden, 1971).

I therefore determined first to confirm the presence of CR as described by van der Waaij in mice treated with TDD (van der Waaij et al., 1971). The same mouse model could be then used to study the widely employed SDD regimen first described by Stoutenbeek et al., (1984).

The first part of the thesis is essentially an attempt to repeat the experiments described by van der Waaij et al., in (1971). His procedures were followed as far as possible, but some changes were made. Using *E.coli* and enterococci as indicator organisms for aerobic faecal flora we were able to maintain TDD mice free of these bacteria for four weeks after cessation of treatment with TDD antibiotics. This was achieved by housing the mice individually in autoclaved cage in a normal room **(see materials and methods section 2.1.2)** without the need for transferring the autoclaved cages

to the peracetic acid-sterilized laminar cross-flow benches used by van der Waaij.

Van der Waaij used Swiss ND2 mice challenged with *E.coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (van der Waaij et al., 1971). These mouse-derived strains were resistant to streptomycin and to neomycin, the TDD agents used. We used Balb/c mice because our animal laboratory did not breed Swiss ND2 mice. Also, the diversity of mice used in previous experiments in this field; NIH, OT, Balb / c and C3H mice (Speekenbrink et al., 1987, 1988), C57BL / Rij, CBA / Rij, C3H, ROF, and ND2[2] strains (van der Waaij and Strum, 1968), and Swiss Cpb: SE mice (Koopman et al., 1981) suggested that the particular strain of mouse would not significantly affect the results obtained.

In order to obtain suitable mouse-derived strains of challenge organisms, we used a range of selective media for isolation of different aerobic gram negative bacteria and *Candida albicans* from mouse faecal pellets and from different parts of the gut **(materials and methods section 2.3 and 2.6.3)**. Despite considerable efforts, it proved impossible to isolate mouse-derived strains of *Pseudomonas aeruginosa* or *Klebsiella pneumoniae.* This suggests that these experimental laboratory mice do not carry these organisms as part of their gut flora.

Preliminary experiments established that challenge organisms required to be resistant to at least 10 g l^{-1} each of streptomycin and neomycin in order to resist the level of these drugs found in faeces during treatment. We attempted to enhance the resistance of promising mouse and human strains to the TDD antibiotics used. Again, considerable difficulties were experienced despite the use of a variety of procedures **(materials and methods section 2.3 and 2.6.3)**.

To challenge mice during TDD and SDD treatment fully resistant challenge strains were required. We tested murine isolates of *E.coli* s-R21 and *Proteus mirabilis* s-R9, and human isolates of *Klebsiella pneumoniae* s-R10, *Pseudomonas aeruginosa* s-R321, *Providencia stuartii* s-R7 and *Pseudomonas cepacia* s-R13. Potentially resistant strains were selected by passage in BHIB containing increasing concentration of one of the TDD drugs (Sm, Nm) or SDD drugs (Tn, Pe).

Eventually, the challenge strains employed were:-

- *E.coli* s-R21: this is a moused-derived strain made resistant to 10 gl^{-1} streptomycin by multiple passage and made resistant to 10 gl^{-1} neomycin by transfer of plasmid RP4.

- *Pseudomonas aeruginosa* s-R321: this strain is of human origin made resistant to 10 gl^{-1} each of streptomycin and neomycin by multiple passage in increasing concentrations of Sm and Nm.

Unfortunately, we were unable to repeat van der Waaij's experiment with *Klebsiella pneumoniae* in totally decontaminated mice. We failed to isolate this organism from mice and it proved impossible to make human-derived strains that were sufficiently resistant to the TDD antibiotics. Also, strains of this organisms failed to colonize totally decontaminated mice, even in the absence of the decontaminating drugs.

The problems encountered with the TDD challenge strains were even greater when we tried to find suitable challenge strains for the SDD studies. The original plan was to obtain multiple challenge strains to be used in both TDD and SDD experiments. Unfortunately, we were unable to make *E.coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* resistant to the PTA drugs. We managed to obtain one challenge strain (*Proteus mirabilis* s-R9) resistant to 0.5 g l^{-1} of tobramycin and resistant to > 1 g l^{-1} of polymyxin. This

strain was resistant to both PTA and TDD drugs; however, it remained sensitive to cefotaxime.

Although in principle it may be possible to obtain many strains which have the required resistance pattern, the combined requirements of both the ability to colonize mice and resistance appear to be much less easy to achieve. The reason is probably that organisms with a high level of intrinsic resistance to antibiotics tend to be opportunistic pathogens e. g. *Pseudomonas cepacia*, but these organisms rarely successfully colonize uncompromised hosts. Endogenous organisms of the intestine, not intrinsically resistant to PTA or TDD antibiotics, would require independent mutation to resistance for each antibiotic. This is a highly improbable event and may also affect the organisms ability to colonize.

Passaging resistant organisms through the intended host may overcome the latter problem. This procedure was not attempted in this study. Additionally, plasmids bearing antibiotic resistance markers may be introduced into suitable endogenous organisms; although this approach is likely to require much effort, it may be a more certain and reproducible way of obtaining suitably resistant organisms without affecting their ability to colonize.

Van der Waaij gave no information on the stability of the antibiotics used in drinking water or about how often the drugs were changed. We found that the drinking water containing the TDD antibiotics given to mice during the 14 days of treatment had to be changed every 5 days, as the combination of Sm and Nm in water was unstable after 6 days. The activity of the combination of Sm and Nm in water had decreased by 52 % after 6 days, and by testing the activity of Nm alone it was observed that its activity had decreased by 51 % after 6 days. This finding suggested that this instability of the TDD antibiotics in drinking water was due to the deterioration of Nm in water at room temperature.

This may have implications for the interpretation of van der Waaij's data as it suggests that his mice were treated with reduced active concentration of drugs after about 5 days.

Having determined the experimental conditions, it was then necessary to determine the normal flora present in the intestine of the experimental mice. We chose to study the normal flora of the gut as present in faecal pellets which represent the large bowel flora, rather than using caecal contents which have been used in previous studies (Lee, 1971; Brown and Balish, 1978). This was done because van der Waaij et al., (1971) used faecal pellets in his study and because they are a convenient source of the large intestinal flora. Also, this sampling technique does not require the sacrifice of mice, a procedure that would have been impossible in the present study as we studied the flora in mice before, during and after treatment with TDD antibiotics.

The results of aerobic culture were consistently compatible with the microscopy findings. It was therefore assumed that the aerobic culture technique allowed an acceptably accurate estimate of at least the total viable bacterial count of the faecal pellets. Aerobic culture yielded 4 - 5 different colony types. There was overwhelming preponderance of *E.coli* (5.8 ± 0.2 log cfu / g of faecal pellets) and enterococci (6.9 ± 0.1 log cfu / g of faeces). These organisms were also assumed to reflect the changes seen as a result of antibiotic treatment on other aerobes that were either rarely present or usually present in very low numbers. However, the media used always included a non-selective medium and the culture conditions employed were not restrictive to certain types of organisms. In terms of the literature (Brown and Balish, 1978), we expected that the media and aerobic techniques used would grow all aerobic organisms present in the faecal samples.

Technical considerations are accepted to impose major constraints on the study of anaerobic gut flora (Lee, 1971). Since 1960, however, the application of improved anaerobic technique, and improved media, has allowed bacteriologists to culture up to 85 % of the cells present, and make some progress in characterizing the flora (Moore and Holdeman, 1974). In this study we only investigated the total viable count of anaerobic flora. Subpopulations were then identified only on the basis of colonial morphology and gram morphology. We did not attempt to further characterize these organisms for the following reasons:

1) In the context of colonization resistance, we considered that the overall count of the anaerobic bacteria was probably more important than the distribution of individual species (van der Waaij et al., (1971).

2) A wide range of cultural conditions would be required to meet the *in vitro* growth requirements of different strains. The conditions needed to isolate subpopulations of the anaerobic flora have been found to be more stringent than those needed for a TVC. The reason for this are unclear but probably include satellitism and the provision of a reducing environment.

3) The great range of different kinds of bacteria which must be cultured, characterized, and catalogued in order to gain an understanding of there ecological role and inter relationships.

4) The technical complexity of such a studies and the difficulty in relating very complex patterns of species to changes in CR.

In these circumstances, the interpretative limitations associated with TVC data were regarded as acceptable, particularly as van der Waaij et al., (1971) described no anaerobic data of any description in their studies.

Caldwell and Bryant, (1966) reported that the medium M10 agar is highly effective for nonselective enumeration and isolation of rumen bacteria,

and that it showed a higher TVC when compared to other media. However, in our hands anaerobic blood agar produced higher anaerobic TVC values than M10 agar for faecal extracts and the colony types, shape and color were easier to distinguish. Anaerobic BA was therefore used throughout the study. In addition, we followed the methods for isolating fastidiously anaerobic bacteria i.e.- minimal exposure of faecal pellets or gut contents to air, inoculation onto pre-reduced media, and incubation under anoxic conditions as described by Drasar, (1967); Aranki et al., (1969); Moore et al., (1969); and Gordon and Dubos (1970).

The mean T.V.C. was 10.5 $\pm$ 0.06 log (cfu / g) of faecal pellets for control mice. This was almost identical to other published studies where the total cultivable flora of the mouse intestinal content was found to be 10^{10} - 10^{11} cells / gram of intestinal content (Moore et al., 1969; Savage et al., 1971). Only 12 $\pm$ 1 different colony types were seen which mainly consisted of spore-forming gram positive bacilli and fusiform bacteria. The large numbers of strains known to be present, presumably present in lower numbers, were not detected as selective media were not used **(results section 3.2.3.1)**.

These anaerobic culture studies were supplemented by the assay of ß-aspgly in mouse faecal pellets, as an additional indicator of anaerobic flora (Welling, 1979; Emmelot and van der Waaij, 1980; Manson et al., 1981; van der Waaij et al., 1982). The identification of ß-aspgly in HPLC profiles depended upon the identification in experimental samples of a single discrete peak with the same retention characteristics as the peak obtained with ß-aspgly control assayed in parallel. The indicator used, O-phthaldialdehyde, only labels amino acids and short peptides, and a study of standard amino acids revealed none with a retention time similar to that seen with the ß-aspgly standard (result not shown). However, no appropriate internal standard for ß-aspgly was available in the system we used **(material and methods section 2.12)**.

In faeces from control mice, no peak with a retention time similar to that of ß-aspgly was detected. By contrast, such a peak was consistently present in mice treated with TDD antibiotics and showed to have a marked reduction in the levels of faecal anaerobes. These results agree with the behavior of ß-aspgly as detected by paper chromatography by Welling et al., (1978) and as described by others (Welling and Groen, 1978; Welling, 1979; Emmelot and van der Waaij, 1980; Manson et al., 1981; van der Waaij et al., 1982). However, some caution in their interpretation is required. Entirely contradictory results were obtained in one isolated experiment involving clindamycin when no ß-aspgly were detected following the apparent elimination of the anaerobic flora (result not shown). However, this was probably due to inappropriate storage of the faecal samples before ß-aspgly assay. Overall I think that this technique provided useful auxiliary data when considering the effects of decontamination regimens on faecal anaerobic flora.

The choice of Balb / c mice can be criticized in that they represent a markedly abnormal model. The animals are inbred laboratory strains raised in a grossly abnormal environment designed to minimize problems of infection and subjected to a carefully standardized, sterilized diet. Their normal faecal flora (and response to microbial challenge) may differ markedly from wild mice as has been commented by other workers. Limited studies were therefore carried out with wild mice in an attempt to clarify this problem (and as a possible source of challenge strains).

The aerobic normal flora of wild mice was found to be simpler than that obtained from Balb / c control mice: the wild mouse aerobic flora consisted only of 2 different strains of *E.coli*, and no other organism was seen. As the intestinal flora of the laboratory mouse is generally described as simplified, this result is surprising. This variation in the normal flora between the 2 strains of mice could be due to many factors including the environment and diet (Aries et

al., 1969). There is a paucity of references concerning effects of diet on aerobic flora, however, numerous studies with anaerobic flora have shown that diet may have material effects. For example Americans have lower counts of clostridia in their faeces than people living in Japan (Akama and Otani, 1970). Ueno and colleagues (Ueno et al., 1974) also pointed out certain differences with regard to gram negative anaerobic bacilli in people on traditional Japanese diet, and those on a western diet. Aries et al. (1969) noted significantly more *Bacteroides* and *Bifidobacterium species* in faecal specimens from English subjects than in specimens from Ugandans. However, within these populations, the intestinal bacterial populations are remarkably stable.

In contrast there was little difference in the anaerobic normal flora of Balb / c and wild mice by the criteria employed. Although the TVC in wild mice was lower by 1 log (cfu), the gram and cultural morphology was almost identical to that of control mice.

It is of interest to note that we failed to isolate a mouse-derived *Ps. aeruginosa* and *Kl. pneumoniae* from the 3 wild mice for use in challenge experiments. The wild mice were delicate and difficult to handle making them unsuitable for challenge experiments even if this had been legal.

4.2. Studies With Control Mice:

Van der Waaij's method for quantification of colonization resistance was employed throughout this study (van der Waaij et al., 1971). Colonization resistance was expressed as the $\log_{10}$ of the minimum oral (gavage) dose of an organism that was associated with isolation of the challenge strain from at least 50 % of treated mice for a period of at least two weeks. This approach generated both a numerical assessment of the strength of CR and auxiliary data concerning the pattern of excretion of the challenge strain.

The results of this study confirmed van der Waaij's finding of a very high CR in untreated control mice. However, there were significant differences of detail between the two studies.

The failure to colonize conventional control mice for two weeks when given 9-10 log (cfu) of *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* challenge strains **(results section. Table. 11)**, supports the finding of van der Waaij. However, there is a variation in the values of CR for *E.coli* challenge strains between the two studies. In the present study the value of CR for *E.coli* s-R21 challenge strain was 8 whereas van der Waaij reported that the CR for his *E.coli* challenge strain in control mice was 7.

Another variation between the two studies isthe clearance of the challenge strain after challenge. Van der Waaij only recorded the presence or absence of the challenge strain in the faeces after challenge. We additionally followed the total viable count of the challenge organism over the experimental period. The mean TVC of *E.coli* s-R21 challenge strain in the colonized mice varied between 10^6 and 10^8 cfu / gram of faeces during the 14 days after challenge. (Van der Waaij presented no data of any kind in this area). This pattern was not seen when mice were challenged with doses between 5 log - 7 log (cfu), where the *E.coli* s-R21 could no longer be detected 3 - 5 days after challenge. In these circumstances, the clearance of *E.coli* challenge strain in van der Waaij's study was slower: between 30 % - 40% of mice were colonized for two weeks when challenged with 5 log (cfu).

The CR for *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in control mice were similar in the two studies, but there was a difference in the clearance rate of these challenge strains. In van der Waaij's study, between 10% - 20% of the challenged mice were still colonized two weeks after challenge when challenged with 9 log (cfu). However, in the present study the

7 challenge strains disappeared completely when given at high challenge doses between 8.5 log - 10 log (cfu) between day 1 - 7 after challenge (the clearance of the challenge organisms from the faeces was proportional to the challenge dose). The faecal clearance also varied both between challenge strains, and between different mice subjected almost to the same challenge. For example, control mice reproducibly lost all the *Proteus mirabilis* s-R9 challenge strain in 7 days after challenge with 9 log (cfu), whereas a complete clearance of *Providencia stuartii* s-R7 challenge strain from all of the challenged mice occurred one day after challenge with the same dose.

These variations between the two studies could be due to numerous factors, the use of different mouse strains housed under different conditions, the different diet given to the mice, the use of different challenge strains, and inevitable minor differences in technique. Nevertheless, the studies with control mice broadly supported van der Waaij's finding of a very high CR that is materially affected by the genus and species species of the challenge challenge strain employed.

4.3 Studies With Totally Decontaminated Mice:

In these studies van der Waaij and colleagues published only limited data concerning the aerobic and the anaerobic faecal flora before, during and after treatment. They unequivocally reported that all mice which were treated with TDD antibiotics became free of all of the aerobic flora, but no data of the anaerobic flora were given (van der Waaij et al., 1971; 1972; van der Waaij and Berghuis-de vries, 1974).

In contrast, fairly extensive monitoring was undertaken in the present study. TDD antibiotics were associated with a reduction in all of the aerobic flora to a non detectable level only one day after starting treatment. This flora did not recover during the two weeks of the treatment period, and recovery after stopping treatment was very slow when mice were kept in clean environment **(Materials and methods section 2.1.2)** to avoid the risk of contamination. In seventy percent of mice enterococci reappeared by day 5 - 7 after stopping treatment, but *E.coli* had not reappeared 3 - 4 weeks after stopping treatment. These faecal findings were extended by the study of the microbial flora in 5 parts of the mouse gut; upper jejunum, lower jejunum, ileum , caecum, and colon (a single experiment). These sites all yielded enterococci and *E.coli* before TDD, but these organisms were not isolated during TDD. In contrast to the previous studies enterococci were also absent both from these sites and from faeces for 4 weeks after stopping TDD treatment. These results illustrate the difficulties of obtaining reproducible results from experiment to experiment, but support the use of faeces as an index of mouse intestinal aerobic flora.

Although the TDD antibiotics were clearly very active against faecal aerobes, the effect on the anaerobic flora was less marked. Seventy percent of treated mice, whilst showing a reduction and simplification of anaerobic flora,

still showed appreciable anaerobic flora during TDD (**results section 3.2.3.3)**. Gram films of these organisms showed gram positive rods with terminal spores, and gram positive fat rods. I think that these organisms may have been of the genus clostridium.. In the present study we will refer to these organisms as " anaerobic gram positive spore bearer" (ANGPS).

As I mentioned earlier, in this study we did not characterize and study the TVC of each individual organism of the normal anaerobic flora before, during or after treatment. However, we did estimate the TVC of the ANGPS because of their resistance to van der Waaij's TDD regimen. During treatment, they were present at a concentration of 9 $\pm$ 0.2 log (cfu / gram of faeces), and this decreased to 6.9 $\pm$ 1.4 log(cfu / gram) of faeces 2 - 3 days after treatment (which is similar to the normal level of *Clostridium spp.* in conventional mice). This decrease in the TVC of ANGPS after stopping treatment might be due to the recovery of other anaerobic species and some of the aerobic flora. However, overall recovery of the anaerobes took much longer than the changes in ANGPS.

More generally, only a fraction of the anaerobic flora was recovered after treatment. The mean total viable count was 10.35 $\pm$ 0.2 log (cfu / gram of faeces) 7 days after stopping treatment and this result was maintained at 14 days after stopping treatment. In contrast, only 4 $\pm$ 1 types of different colonies were seen 7 days after stopping treatment and only 6 $\pm$ 1 colony types were seen 14 days after stopping treatment compared to 12 $\pm$ 1 types of colonies for control mice. Wensinck and Ruseler-van Embden (1971) described studies of the caecal flora of the offspring of germ-free mice contaminated with the anaerobes of CRF-mice which were originally obtained from one mouse two weeks after stopping antibiotics: only five species were present, and the major constituents were tapered rods belonging to the genus *Clostridium.* This supports the pattern of results obtained in the present study. Slow recovery of

simplified anaerobic flora after stopping TDD treatment is also described by van der Waaij et al., (1971) who stated that anaerobes in mouse faeces obtained 14 days after stopping treatment represented the fraction of conventional intestinal flora which survived treatment with TDD antibiotics. However, van der Waaij did not include in his work a study of the anaerobic flora before, during, and immediately after treatment in order to compare directly these data with the flora of mice two weeks after stopping treatment.

The ß-aspgly findings also suggests an impairment of the anaerobic flora with TDD antibiotics. As expected ß-aspgly was not seen in the faeces of control untreated mice. However, the concentration of ß-aspgly increased sharply in mouse faeces during treatment with TDD antibiotics. After stopping TDD, a variable decrease, but not a sharp fall in the concentration of ß-aspgly was seen. This variation in the concentration of ß-aspgly between different mice matched the rate of recovery of the anaerobic normal flora in each individual mouse as revealed by bacteriological studies. These results therefore support the slow recovery of anaerobic flora described earlier and the utility of ß-aspgly as an auxiliary index of intestinal anaerobic flora.

In summary, my findings suggest that the TDD antibiotics used by van der Waaij et al. (1971) are very active against the aerobic flora but only partially active against gut anaerobic flora. In other words, van der Waaij's TDD regimen was only partially effective. Van der Waaij's comments in this area are contradictory, on the one hand he implied that TDD was established during treatment (van der Waaij et al., 1971) and on the other he reported in his conclusion that reconstitution of CR was due to those anaerobic organisms which survived the antibiotics and subsequently repopulated the intestinal tract.

During treatment with TDD antibiotics a marked impairment of CR was

observed. Two days after the onset of oral administration of TDD antibiotics the value of CR for *E.coli* s-R21 challenge strain decreased to < 2, and remained at this level during the 14 days of treatment. This value of CR is very low compared to conventional control mice where the CR was approximately 8. This means that totally decontaminated mice can easily be colonized with a very low dose (40 cells) of *E.coli* s-R21 challenge strain during treatment. These results agree with van der Waaij's group, who also reported that the CR for *E.coli* during treatment with TDD antibiotics was < 2 (van der Waaij et al., 1971).

Bohnhoff and Miller (1962) described a similar difference in response between conventional mice, and streptomycin treated mice. In this study the untreated mice required approximately 6 log (cfu) of *Salmonella enteritidis* cells by stomach tube to colonize the gut of 50 % of mice. With lower doses fewer mice, and with higher doses (between 6 log cfu - 9 log (cfu)) more mice became colonized. In contrast, in streptomycin treated mice, doses as low as 10 cells or less colonized 50 % of mice. Dubos et al., (1963), and Savage and Dubos, (1968) also reported abnormal colonization with *E.coli* and evidence of overgrowth during the entire period of suppression of the anaerobic flora responsible for CR by oral treatment with penicillin or oxytetracycline, even though TDD was not fully established. Unfortunately, none of these studies provided detailed information on the bacteriological status of the experimental mice.

During TDD treatment, the challenge organism, *E.coli* s-R21, multiplied extensively and persisted in the gut of the mice, and the TVC increased to almost 9 log cfu / gram of faeces **(results Fig. 34)**. This level was almost the same in all the colonized mice , and exceeded the concentration of *E.coli* seen in control mice.

The level dropped by 2 - 3 logs during the 14 days after stopping treatment. This fall in numbers varied between the different groups of mice in the range of 6.5 - 8 log (cfu), but in general the TVC for *E.coli* in control and TDD mice was similar 14 days after stopping treatment. At this time the anaerobic TVC was 10.35 $\pm$ 0.2 log (cfu / gram of faeces) with 6 $\pm$ 1 colony types, and enterococci were also seen. Similar results were seen in mice treated with streptomycin (Bohnhoff & Miller, 1962): the TVC of *Salmonella enteritidis* challenge strain was 6 log (cfu / single pellet of faeces) during treatment, where as in control untreated mice the TVC was 4 log (cfu/ single pellet of faeces).

These findings suggest that the fall in the TVC of the challenge organism in decontaminated mice after stopping treatment may have been due to the partial recovery of the normal flora. Schaedler et al. (1965) reported that after oral challenge of germ-free mice the coliform bacilli multiplied extensively and persisted in all parts of the gastrointestinal tract. Their numbers then fell precipitously when the mice were fed the intestinal contents of healthy pathogen-free mice. Taken together these data clearly demonstrate the important role of the resident intestinal flora in host resistance to orally introduced organisms.

During treatment with TDD antibiotics 70 % of mice were not totally decontaminated due to the presence of ANGPS. However, the CR for *E.coli* s-R21 of completely and partially decontaminated mice was the same. Therefore, partial retention of the anaerobic flora (ANGPS) did not have any observed effect on the colonization by *E.coli* s-R21. Although van der Waaij et al., (1971) implied that TDD was complete, the flora reconstitution data indicated survival of at least some anaerobic flora.

Van der Waaij et al., (1971) reported that during treatment the CR for all of the 3 challenge organisms they tested (*E.coli, Kl. pneumoniae,* and *Ps. aeruginosa*) was < 2. In contrast, the present study revealed a marked variation in CR with different challenge organisms: during treatment with fully established TDD the CR for *Ps. aeruginosa* s-R321 was approximately 8 **(Table. 12)**. Thus the CR for *Ps. aeruginosa* s-R321 in TDD mice was almost identical to that of *E.coli* s-R21 in control mice. However, the CR for *Ps. aeruginosa* s-R321 was still reduced by TDD - because control mice were not colonized by a challenge of 10 log (cfu) of *Ps. aeruginosa* s-R321.

When TDD partially failed due to the presence of ANGPS the CR was higher (6 / 8 mice lost the challenge organisms when challenged with a dose of 7.7 log (cfu) of *Ps. aeruginosa* s-R321 by day 7 after challenge). The loss of this challenge organism from mice with residual anaerobic flora was thus faster than from totally decontaminated mice. In contrast, partial retention of anaerobic flora did not have any observed effect on colonization by *E.coli* s-R21 in mice during treatment.

This variation between the two studies in the value of CR for *Ps. aeruginosa* challenge strain during treatment might be due to the origin of the challenge strain. Van der Waaij used a mouse-derived strain, whereas our challenge strain was of human origin and thus might not have the same ability to colonize mouse gut. It also might be due to the presence of the residual anaerobic flora (ANGPS) during challenge. However, when TDD was fully established mice were not colonized when challenged with 3.7 log (cfu) of *Ps. aeruginosa* s-R321 which is still greater the value of 2 log (cfu) reported by van der Waaij.

Table 12 **Summary of CR and anaerobic flora in different groups of 8 mice.**

Treatment	Challenge strain	CR*	Diversity of anaerobic flora
Control	*E.coli*	8.2	12 - 16 colony types at 10^{-8}
	Ps. aeruginosa	> 10^{8}[a]	
	Pr. mirabilis	> 10^{8}[a]	
TDD antibiotics	*E.coli*	1.6	occasional ANGPS 1 - 2 colony types
	Ps. aeruginosa	8	
PTA	*Pr. mirabilis*	8	not affected significantly
Cefotaxime[b]	*Pr. mirabilis*	> 9.2[a]	Simplification occurred, reduction from 12 - 16 colony types to only 2 - 4 colony types
PTA + cefotaxime[b]	*Pr. mirabilis*	< 4.4[c]	Simplification occurred, reduction from 12 - 16 colony types to only 1 - 2 colony types

* Log of oral dose which resulted in colonisation of 50 % of mice for at least 14 days.

a. Colonization criteria were not met by the highest dose employed, in this case 10^{8} cfu.

b. Assayed 1 day after stopping treatment.

c. The lowest challenge employed.

Immediately after stopping treatment van der Waaij et al., (1971), found that the CR for *E. coli* was very low (< 2) . Fourteen days after stopping treatment the CR had recovered to 9 which was the same or higher than the CR for control, untreated mice. As elsewhere, essentially similar results were obtained for *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* at this time.

There was a minimum of detailed bacteriological data throughout his study. However, he stated that the recovery in the CR during the 14 days after stopping treatment, was coincident with profound changes in the intestinal flora, although he did not show any data for the recovery of the normal flora (van der Waaij et. al., 1971). These experiments were then extended by studies of reconstitution of germ-free mice (van der Waaij et. al., 1971). It is not clear from the text whether the original reconstituted germ-free mice, the offspring of those reconstituted mice, or both were used for further challenge studies, therefore, the data in this particular study are difficult to interpret and this is a major weakness of this study. They stated that flora from mouse faeces taken two weeks after stopping TDD treatment (consisting of anaerobes only), were given to germ-free mice. The flora of offspring (CRF-mice) of these reconstituted germ-free mice consisted of only 5 different species mainly of the genus *Clostridium* (Wensinck et. al., 1971), presumably the same as those of the reconstituted parents. They reported that the pattern of elimination of the challenge *E.coli* by CRF-mice (which have a simplified gut flora) closely resembled elimination by mice two weeks after stopping TDD treatment, and the CR was about 9, which is similar to that of conventional control mice. The author did not state the CR for germ free mice, but it was implied that this grossly reduced. It is unfortunate that this important study did

not contain more explicit information in this area; this experiment ought to be repeated.

From these studies van der Waaij concluded that the recovery in CR after impairment by TDD was due to the anaerobic subpopulations surviving TDD that gradually repopulated the intestine after stopping treatment.

In the present study the CR for *E.coli* s-R21 immediately after stopping treatment with TDD antibiotics was also < 2 (approximately 1.3) and the CR for *E.coli* s-R21 two weeks after stopping treatment had increased to 4. However, as described, the recovery of the normal flora was slow and not fully complete during the period of 14 days after stopping treatment when only 6 $\pm$ 1 types of colonies were recovered. Thus partial recovery of normal flora after stopping treatment was matched by a partial recovery of CR for *E.coli.*

As explained, I was unable to colonize either control or totally decontaminated mice with *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* during TDD treatment. There are therefore no results involving these organismsrelated to post TDD period.

The CR results obtained with *E.coli* s-R21 totally disagree with van der Waaij et. al., (1971) and their observation that CR had returned to normal values at 14 days. This discrepancy is the more striking in that both the "normalized" mice described by van der Waaij, et. al., (1971) and the mice used in our experiments were colonized with a simplified anaerobic flora consisting of 6 $\pm$ 1 colony types. Koopman et al., (1981) also disagreed with the contention that CR can be restored with a simplified anaerobic mouse flora of 5 strains. They suggested that a very complex gastrointestinal microflora was needed to normalize germ-free mice. Other studies including those conducted by Sayed et al., (1970), and Freter and Abrams (1972) also

disagreed, in that they were only able to normalize the CR of germ-free mice with mixed population of more than 100 bacterial strains. These data, including our findings, suggests that the normalization of CR can only be obtained with a complex microflora, and it appears that complete normalization with relatively simple flora is unlikely to be successful.

As a footnote to these studies we demonstrated that the mode of administration of decontaminating drugs had a major effect on the result obtained. When mice were treated by gavage with TDD antibiotics 3 / 8 mice lost the challenge organism *E.coli* s-R21 during treatment. This was ascribed to a peak of faecal antibiotic activity observed 8 hours after the last dose of antibiotics **(result Fig. 46)**, whereas in mice treated *ad libitum* in drinking water all mice were colonized for 2 weeks **(result Fig. 4)** and the faecal antibiotic activity was almost stable over the sampling period of 24 hours **(result Fig. 47)**.

In summary, our studies with TDD and control mice support the concept of CR, confirm that it can be reproducibly quantified, confirm that it can be markedly reduced by TDD regimens, and that the recovery of CR after termination of TDD antibiotics is associated with recovery of faecal flora. However, it is clear that these phenomenon reflect more complex mechanisms than those described by van der Waaij et al., (1971) and, also, that experimental detail may have a major influence on the outcome of results.

4.4. Effect Of SDD Regimen On Intestinal Normal Flora:

The mouse model used for the TDD studies was employed to study the PTA (polymyxin, tobramycin, and amphotericin B) SDD regimen described by Stoutenbeek et al., (1984) and subsequently widely employed in clinical practice. There have been very few previous studies of the effects of PTA on mouse faecal flora (Speekenbrink et al., 1987) and none concerning the effect on CR as measured by oral challenge of experimental animals.

It was unfortunate that we had to use a different challenge strain (*Proteus mirabilis* s-R9 resistant to SDD antibiotics) from that employed in the TDD studies, as it is well recognized this complicates the comparison of the TDD and SDD data. However, despite the extensive and detailed studies described in **(material and methods section. 2.6.3)** it proved impossible to obtain a challenge strain with the requisite pattern of antibiotic resistance. This whole area proved to be much more difficult than expected, and deserves major attention for future studies.

The PTA regimen proved to have a highly selective action on faecal flora and this selectivity extended to different species of aerobic bacteria. *E.coli* was reduced to a non detectable level after one dose of treatment and had not recovered two weeks after cessation of treatment **(results section. 3.2.2.4)**. By contrast, no significant changes were seen in the TVC of enterococci during or after treatment. This elimination of *E.coli* and maintenance of enterococci was also reported by Speekenbrink et al., (1987) when they used the PTA regimen described by Stoutenbeek et al., (1984).

Recovery of the eliminated aerobic flora was not seen within 10 days after stopping PTA. Recovery was seen by Speekenbrink et al., (1987), but they housed treated with untreated mice, a procedure not employed in the present study. Recovery of Enterobacteria in mice after stopping treatment was also seen in study by Rogers et. al., (1985) when co-housed with

untreated mice.

In previous studies of PTA in patients by de Vries-Hospers et al., (1981) and Sleigfer et al., (1983) have shown the same pattern of effective removal of AGNB whilst retaining pre-existing population levels of enterococci.

In previous investigations using polymyxin or tobramycin as a single drug, a similarly rapid effect on AGNB was reported compared to other drugs used. De Vries-Hospers et al., (1981), noted that the faecal culture became free of AGNB one week after the start of nalidixic acid or co-trimoxazole, but with polymyxin this was achieved within a few days. Van der Waaij et al., (1982) reported that tobramycin was very effective against the endogenous *Enterobacteriaceae*, and it's effects on enterococci was relatively small compared to other drugs.

The effect of PTA antibiotics on the anaerobic flora was very limited. A very small increase by (0.66 $\pm$ 0.2 log cfu) in the TVC of anaerobic flora was seen during treatment, and this returned to normal level one day after cessation of treatment. However, gram films indicated a simplification in the anaerobic flora. This was associated with a reduction in colony types of anaerobic gram negative rods **(results Fig. 5 and 6)**. An increase in the concentration of ß-aspgly **(results Fig. 23)** was also seen in these circumstances. This can not readily be explained in terms of TVC, and it has never previously been suggested that simplification of anaerobic flora is associated with elevated ß-aspgly levels. However, Welling et al., (1988) reported that a number of different strains of anaerobic bacteria may produce the enzyme ß-aspartylpeptidases.

Taken together, these results suggests that the PTA antibiotics did affect the composition of anaerobic parts of the intestinal flora, even though the anaerobic TVC remained unchanged. There have been no previous

studies of the effect of the of PTA regimen on anaerobic faecal flora in mice. However, the activity of polymyxin or tobramycin as a single drug on the intestinal flora has been extensively studied in both man (Guiot and Janssen, 1975; de Gast and van Saene, 1979; de Vries-Hospers et al., 1981) and mice (Emmelot and van der Waaij, 1980; van der Waaij et al., 1982). No direct bacteriological data of the effect of these drugs on the anaerobic flora have been reported, but indirect studies involving measurement of CR and ß-aspgly were interpreted in terms of a reduction in anaerobic flora. Van der Waaij et al., (1982), reported that during treatment of mice with oral tobramycin at doses between 0.9 - 2.9 mg mouse^{-1} daily, the CR was not affected according to the parameters used. However, higher doses of tobramycin did decrease the CR. Mulder et al., (1984) reported a similar decrease in the CR in human studies. By contrast, changes in CR have not been recorded following treatment with polymyxin (van der Waaij et al., 1982).

Treatment with cefotaxime alone also had a highly selective effect on mouse faecal flora. *E.coli* was reduced to a non detectable level after one i.p. dose and had not recovered two weeks after stopping treatment. However, this effect was associated with a significant increase in the TVC of enterococci (by 1.1 $\pm$ 0.6 log (cfu)), which returned to normal levels within 3 days after stopping treatment.

Treatment with CTX alone also showed a significant effect on the anaerobic flora. Although there was no significant difference in the total anaerobic TVC, there was a major simplification of the morphological colony types (a reduction from 12 $\pm$ 1 different colony types before treatment to only 3 $\pm$ 1 colony types for the same mice after 3 days treatment). The stability of the TVC may have been due to an increase in the numbers of the remaining anaerobic flora in the gut. An increase in the concentration of ß-aspgly

(results Fig. 24) was also seen, but the concentration was lower than in faecal pellets of mice treated with TDD antibiotics or PTA. Again this can not be explained in terms of TVC as described above.

Surprisingly, there was some recovery of some of the anaerobic faecal flora (mainly gram negative rods) during treatment with CTX alone: an increase to 5 $\pm$1 colony types was observed by day 3 of treatment. A full recovery of diversity was seen 2 - 3 days after treatment. The recovery of the anaerobic faecal flora during treatment may have been due to an inactivation of CTX by enzymes, possibly induced by the drug. The results from a preliminary experiment **(results section. 3.4.3.1)** which involved the study of the concentration of CTX in mice caecum treated with 3 mg of CTX for one day or for four days supported this view. Following one dose, a zone of antimicrobial activity was seen around faecal pellets up to 20 hours after treatment. In a second experiment the highest concentration of CTX in the pellets was estimated as 20 μg g^{-1} of caecum contents 10 hours after treatment **(materials and methods. 2.13.3)**. However, in mice treated for four days (when an increased concentration was expected due to the accumulation of the drug) zones of inhibition were only seen 2 hours after treatment and the maximum estimated concentration was 1 μg g^{-1} of caecum contents.

Early studies demonstrated that CTX is resistant to a wide range of plasmid and chromosome-mediated ß-lactamases (Carmine et al., 1983). However, subsequent studies showed that CTX is hydrolyzed by *Klebsiella kl* enzyme (type IVc) and the type Ic enzyme from *Proteus vulgaris* (Schrinner et al.,1984; Neu, 1987). Also CTX is hydrolyzed by ß-lactamases TEM-3, 4 and 5, and SHV-2,3,4 and 5 (Jacoby and Carreras, 1990). In an *in vivo* study performed by Soriano et al., (1989) it was reported that ß-lactamase activity of *Bacteroides fragilis* may be responsible for the diminished activity of CTX in

mice with mixed *E.coli* and *Bacteroides fragilis* infections.

Although, CTX is regarded as a drug of minimal value in the treatment of anaerobic infections, the drug has appreciable activity against certain species of anaerobes, particularly the anaerobic cocci (Carmine et al., 1983; Pollock et al.,1983; Jones et al., 1984; Conawati, 1989; Friis, 1989). This activity may be summarized as follows: The inhibitory values against *Bacteroides fragilis* as well as other *Bacteroides* varied widely and although most isolates are susceptible, the MIC_{50} value is usually < 4 gl^{-1}. Most isolates of *Clostridium perfringens* are inhibited by < 4 gl^{-1}, but many isolates of *Clostridium difficile* are resistant. In addition, cefotaxime is also active against *Veillonella spp.,* propionibacteria, peptococci, peptostreptococci, and *Bifidobacterium spp.*.

Broadly speaking the pattern of simplification of anaerobic flora which we observed **(results Fig. 5 and 9)** could be partly explained in terms of this pattern of activity. However, it appears possible that there were additional effects that can not be readily be explained by the observed pattern of *in vitro* activity. This is a complex issue that deserves further detailed investigation.

The addition of systemic cefotaxime to PTA antibiotics was associated with striking additional effects on the intestinal flora of mice. As with PTA alone or CTX alone *E. coli* was eliminated after one dose of treatment and had not reappeared two weeks after cessation of treatment. However, this effect was accompanied by a marked increase by 2.6 $\pm$ 0.4 log (cfu) in the total count of enterococci during treatment. The increase was reversed to the normal level 7 days after stopping treatment.

Striking additional effects on the anaerobic faecal flora were also seen. Unlike either PTA or CTX used alone, the anaerobic TVC was reduced to non detectable levels in 50 % of mice. In the 50 % of mice where the anaerobic flora was retained, the TVC was reduced by 1 log and only 1 - 2 colony types were seen during treatment. These bacteriological findings were supported by the results of ß-aspgly. The concentration of ß-aspgly had increased during treatment with PTA plus CTX **(result Fig. 25)**, and the level of concentration of ß-aspgly in mouse faecal pellets was much higher than in mice treated with PTA alone or CTX alone.

Viewing this pattern of results, it is highly unlikely that the striking effect seen with PTA plus CTX could have been predicted from the very modest effect seen with PTA or CTX alone. Therefore there appears to have been a synergistic interaction which produced wholly unexpected results. It is extremely difficult to hypothesize about the nature of such interaction in the highly complex environment of the intestine.

This marked reduction in the TVC and the simplification of the anaerobic faecal flora during treatment with PTA combined with CTX were very surprising results it terms of the clinical application. The clinical application of PTA has almost invariably involved CTX and the suggestion of this work is that this combination may be associated with flora changes that may result in a major impairment of CR.

4.5 The Effect Of SDD Regimen On CR:

Different groups of 8 mice were treated with PTA (polymyxin, tobramycin, and amphotericin B) SDD drugs, and challenged with different doses of *Proteus mirabilis* s-R9. As already discussed, it was necessary to use different challenge strain from that used in TDD experiments.

It proved impossible to colonize control mice with an oral challenge of 10 log (cfu) of *Proteus mirabilis* s-R9. In contrast, after 5 days treatment with PTA alone the CR fell to 8 and this value was retained throughout a maximum of 14 days treatment. Overgrowth of *Proteus mirabilis* s-R9 in the gut did not occur - the TVC never exceeded 6.8 $\pm$ 0.3 log (cfu) / gram of faeces after challenge. This level of TVC was below the challenge level and it dropped to 4 $\pm$ 0.2 log (cfu) / gram of faeces 5 days after stopping treatment. SDD thus resembled TDD in producing a measurable reduction in CR but differed in that the effect was very much smaller and that the gut environment prevented extensive multiplication of the challenge organism.

This small reduction in the CR during treatment with PTA may have been due to the discussed simplification of the anaerobic flora, the elimination of *E.coli* from the gut, or the activity of SDD drugs on the challenge organism. Although the *Proteus mirabilis* s-R9 was resistant to high levels of the SDD drugs (material and methods. 2.3), there might have been sufficient activity to impair its growth in the faecal environment. The highest activity of SDD drugs were seen at 8 hours after treatment which might had small residual effect on *Proteus mirabilis* s-R9. Unfortunately faecal pellets studies were not done.

To summarize CR may have been reduced by the apparent elimination of *E.coli*, the simplification of anaerobic flora and a possible residual effect of the SDD drugs on the apparently resistant challenge strain. Conversely, this effect may have been minimized by the maintained levels of enterococci and the maintenance of the over all levels of anaerobic TVC. The problem of interpretation here well illustrates the complexities of this experimental system. Over simplified conclusions should not be drawn and experiments to discriminate between these various interactive effects in *in vivo* systems are difficult to design.

In an attempt to further investigate the role of the aerobic faecal flora on the CR, we treated mice with clindamycin. As planned the anaerobic flora was severely affected; after one dose of treatment they were not detected in the faeces. The recovery of the anaerobic flora after stopping treatment was slow; by day 5 only 5 $\pm$ 1 colony types were seen compared to 12 $\pm$ 1 colony types for the mice before treatment. Similar results were reported by Nord et al., (1978) in humans. Unfortunately, the aerobic flora was also significantly disturbed. The TVC of *E.coli* greatly increased (by 3.5 $\pm$ 0.6 log cfu) and levels of enterococci were reduced to a non-detectable level during treatment.

The CR in mice treated with clindamycin was severely impaired. This may have been due to the elimination of the anaerobic flora by clindamycin, but the apparent elimination of enterococci can not be ignored. Certainly the increased levels of *E.coli* were inadequate to preserve CR. This result along with the findings from TDD and SDD experiments is compatible with the assumed important role of the anaerobic flora in maintaining the CR.

The CR in mice for *Proteus mirabilis* s-R9 immediately after stopping treatment and 7 days after treatment with SDD regimen varied depending on the duration of SDD treatment. The CR in mice treated for 5 days with SDD antibiotics was 8, which is similar to that during treatment, where as in mice treated for 10 days the CR was 7. These are results of a single experiments which showed that the longer the treatment period, the greater the residual effect on CR. However, flora studies did not show corresponding affects where there was almost a complete recovery of anaerobic flora at that time. As these were a result of a single experiment this area requires futher investigation.

The CR in mice during treatment with cefotaxime alone was not studied as the challenge organism *Proteus mirabilis* s-R9 was sensitive to cefotaxime. However, the CR in mice for *Proteus mirabilis* s-R9 one day after completion of

treatment with cefotaxime alone exceeded the maximum challenge 9.2 log (cfu). At this time, faecal pellets showed no antimicrobial activity.

This maintenance of CR was unsurprising in view of the small change seen in overall anaerobic TVC. However, there was a major simplification in the anaerobic flora in these mice, and the maintenance of CR in these circumstances suggests that such changes do not necessary result in a reduction in CR. This maintenance also may had been due to the very fast recovery of the anaerobic flora by day 3 of treatment and immediately after stopping treatment **(results section. 3.2.3.5)**, compared to the recovery of normal flora in mice treated with PTA regimen alone.

A striking effect on CR was observed when CTX was used in conjunction with PTA. Challenge one day after stopping cefotaxime showed marked decrease in the CR to < 4 (the lowest challenge dose tested was 4.4 log (cfu)). This marked impairment in CR compared to control mice (10) or to mice treated with SDD alone (8) parallels the marked effect of SDD and CTX on gut anaerobic flora. (The anaerobic flora in mice during treatment with PTA regimen combined with cefotaxime was almost identical to that of in mice treated with TDD antibiotic).

We found these results are very surprising. PTA antibiotics combined with systemic cefotaxime have been extensively used clinically for over 8 years **(Introduction Table. 3)**. During this time, there have been no reports of significant problems caused by colonization or overgrowth with organisms resistant to the drugs used. This implies maintenance of effective CR, and a corresponding maintenance of intestinal anaerobic flora has been assumed by the authors concerned. However, none of these studies included bacteriological data on faecal anaerobes, although enterococci were sometimes used as an indicator of anaerobe levels (a convenient but doubtful

assumption). The major discrepancy between these clinical studies and the CR data described here may reflect a number of factors:

1) A host difference (mice and men). A number of studies have suggested that the intestinal microflora of mice and humans appear to respond to antimicrobial agents in a similar fashion (van der Waaij, 1979; van der Waaij et al., 1982). However, the complexity of the systems and limitations of the technical procedures must limit the application of these conclusions.

2) A challenge strain effect. Only one strain of *Proteus mirabilis* was used in these experiments. Therefore this may be a result unique to this particular challenge strain in a particular strain of mice. In contrast, clinical studies refer to a much more complex situation. Again, it was unfortunate that we were unable to procure multiple challenge strains for the SDD experiments.

3) The use of drugs (tobramycin, polymyxin and cefotaxime) showing a very wide spectrum of activity, with overlap between their antimicrobial spectrum. The drugs were also bactericidal and used in very high local concentrations. In these circumstances it may be that the drugs were sufficient in themselves to prevent colonization with SDD resistant strains in patients, despite a significant reduction in CR. However, these considerations may be applied equally in the mouse model. Finally, even a resistant organism might not have the ability to establish itself and colonize the gut. In a study performed in mice, I was unable to colonize TDD mice with a high challenge dose of 10 log (cfu) of *Pseudomonas cepacia* (from human origin) entirely resistant to the TDD antibiotics.

4) The probable absence in patients of an explicit oral challenge administered as a bolus of highly resistant organisms as used in the mouse model.

Despite these factors, the contradictory pattern of results remains disturbing. On one hand we have experimental data which suggest that the

PTA and CTX regimen may be associated with a major reduction in CR, with a demonstrated colonization with drug resistant strains following oral challenge. On the other hand, we have 8 years of clinical usage of this SDD regimen which suggests that no such colonization takes place. Certainly our results indicate the need for considerable care in the clinical use of the PTA and CTX regimen and further reinforces the absolute requirement for detailed microbiological monitoring to allow early detection with drug resistant strains.

4.6 Conclusions:

In this study we first, repeated the total decontamination studies described by van der Waaij and colleagues between (1971-1974) in order to verify or modify their conclusions concerning the nature of CR. Secondly, we used the same mouse model to study the CR in mice treated with the SDD regimen that has been most commonly used in clinical ICU practice (PTA). Considerable problems were experienced in the preparation of challenge strains, particularly for the SDD experiments. Suitable challenge strains are a prerequisite for studies of this type. Despite considerable efforts we failed to solve many of the difficulties encountered in this area and further investigation is required.

Broadly speaking, our results support the concept of CR described by van der Waaij et al., (1971). We confirmed that the application of their technique allowed reproducible quantification of CR, that CR could be markedly reduced by TDD drugs, and that the recovery of CR after termination of TDD drugs was associated with recovery of faecal anaerobic flora. However, our results showed slow recovery of the anaerobic faecal flora after stopping treatment, with a corresponding delayed recovery in CR. This disagreed with van der

Waaij et al.'s observation that CR had returned to normal within 14 days of stopping treatment. In addition, unlike van der Waaij, we observed a marked variation in the value of CR associated with different challenge strains. Further, the precise detail of experimental technique exerted a major influence on the results obtained. It was thus apparent that the phenomenon of CR reflects more complex mechanisms than those described by van der Waaij.

Results of SDD studies provided the first detailed data of the effect on CR of the PTA SDD regimen with and without the addition of systemic cefotaxime. PTA alone showed a small but significant reduction in CR whilst cefotaxime alone produced no significant effect on CR. However, striking and surprising results were seen in mice treated with PTA and cefotaxime. A marked reduction or simplification of the anaerobic faecal flora and overgrowth of enterococci was observed and this was associated with a marked reduction in CR. These results of the combined regimen were only partly compatible with a summation of the effects observed when PTA and cefotaxime were used separately. It seems that some form of synergistic interaction may have occurred by an as yet unexplained mechanism.

These results are surprising in view of the absence of drug-resistance problems associated with this widely used clinical regimen. They indicate the need for considerable care in the clinical use of the PTA and cefotaxime regimen, and further reinforce the absolute requirement for detailed microbiological monitoring to allow early detection of colonization with drug resistant strains. More generally, they establish the need for further experimental studies to supplement the numerous clinical trials in this complex area.

Future, lines of research should further examine the effect of SDD and cefotaxime on aerobic and anaerobic faecal flora and on CR using mice and, if possible human models. In addition, extension of the present SDD data to include challenge with different bacterial species must be an early priority.

4.7 Addendum:

The origins of the concept of Colonization Resistance can be traced to the work of Nissle in 1916, whilst clinicians have attempted to modulate intestinal flora since the work of Garlock and Seley in 1939. Selective decontamination regimen, first used clinically by Guiot and van Furth in 1977, explicitly combine these two strands of work, aims to remove potentially pathogenic flora whilst retaining CR thus preventing overgrowth or colonization with drug resistant strains.

These results presented in this Thesis showed a reduction in CR whenever antibiotics were administered to mice (although the size of this effect varied markedly with the regimen used). Some reduction of CR during the clinical use of SDD may also be inferred from the described outgrowth or colonization with enterococci and coagulase-negative staphylococci (Preheim et al., 1987 and Vandenbroucke-Grauls et al., 1991).

These findings suggest that it may be impossible to modulate gut microbial flora with antibiotics without changing the gut CR. An ideal SDD regimen may be unattainable. Lowering of CR during SDD may be associated with a reduction of components of the gut flora that contribute to CR and are not intentionally targeted by the antibiotic regimen. Alternatively, the organisms targeted by SDD (primarily AGNB) may themselves materially contribute to CR.

In the present study, flora changes other than those intended by SDD were indicated by the observed simplification of the anaerobic gut flora during treatment. As the methods used to detect this simplification were crude, it is probable that undetected changes in the strains of dominant organisms and in the minor components of the flora also occurred.There was no direct evidence from my experiments that AGNB (the targets of SDD) contributed to CR. However, the experimental design did not directly address this issue.

If some reduction of CR is an inevitable result of SDD, then this must be matched with the observed lack of problems associated with drug resistance that characterized ten years clinical use of SDD regimens. It seems highly likely that factors other than CR alone are involved. These probably include the very high gut level of decontaminating antibiotics (many times higher than attainable serum concentrations), and the very low likelehood of thee emergence of simultaneous resistance to the combinations of the drugs used.

The final emphasis must centre on the great complexity of this field andthe challenge that it continues to present.

APPENDICES

Appendix 1

Media: Medium 10 (M 10; Caldwell and Bryant, 1966).

Composition of Medium 10 (M 10 Agar):

Trypticase (BBL)	2 g
Yeast extract (Difco)	0.5 g
Agar (Difco)	15 g
Glucose	0.5 g
Cellobiose	0.5 g
Starch	0.5 g
Mineral sol. 1[a]	37.5 ml
Mineral sol. 2[b]	37.5 ml
VFA sol.[c]	3.1 ml
10 % Antifoam sol.	5 ml
0.1 % Resazurin sol.	1 ml
0.1 % Hemin sol.	1 ml
Cystiene. $HCl.H_2O$	0.5 g
Ascorbic acid	0.5 g

a. Mineral sol. 1 (per litre): K_2HPO_4, 7.8 g.

b. Mineral sol. 2 (per litre): KH_2PO_4, 4.7 g; $(NH_4)_2SO_4$, 12 g; $CaCl_2$, 1.2 g; $MgSO_4.7H_2O$, 2.5 g.

c. Volatile fatty acid mixture: acetic, 17 ml (2.9×10^{-2} M); propionic, 6 ml (8.0×10^{-3} M); butyric, 4 ml (4.3×10^{-3} M); isobutyric, 1 ml (1.1×10^{-3} M); N-valeric, isovaleric, and DL-alpha-methylbutyric, 1 ml each (9×10^{-4} M).
( ) indicate the final concentration in 1000 ml medium.

Appendix 2

Participation in Congresses:

A 2-1 Selective Decontamination of the Gut: effect of Oral Antibiotics on Colonization by Gram-Negative aerobes in Mice. A. S. Ameen, A. B. J. Speekenbrink, S. R. Alcock. *Society for General Microbiology 116th Meeting,* Warwick, April 1990, Abstract No. 1415, p.53.

Infection is a major problem in intensive care units (ICU) and in the management of neutropaenia. A high proportion of infections in these vulnerable patients groups are though to be endogenous with aerobic gram-negative bacilli (AGNB) colonizing the oropharynx, stomach and intestines are the principle source of infection. Selective decontamination of the digestive tract (SDD) has been employed for infection prophylaxis in neutropaenia since 1977 and has been increasingly applied ti ICU patients since 1984. SDD employs non-absorbable antibiotics to eliminate or markedly reduce the numbers of AGNB and of yeasts in the gastrointestinal tract, thus reducing the risk of endogenous infection, whilst retaining the normal anaerobic flora, thus preventing colonization or overgrowth with drug-resistant strains. This protective action of the anaerobic flora is of central importance to the clinical use of SDD regimen and it is one manifestation of a more general phenomenon termed colonization resistance (CR). This concept has attracted considerable controversy. The experimental basis for the concept of CR is dependent to a surprising degree on a series of studies performed in 1971-1972 by van der Waaig et al., (1971; 1972), and involving total rather than selective decontamination of the digestive tract. Problems arising during our experimental work suggested that we repeat these early studies. We challenged mice with *E.coli* before, during and after decontamination using streptomycin and neomycin, and we confirmed the results of the original studies. (We present our results and discuss their significance).

A 2-2 Reduction of Colonization Resistance in Balb / c mice. A. S. Ameen, A. B. J. Speekenbrink, S. R. Alcock. *91st General Meeting of the American Society for Microbiology,* Dallas, May 1991, abstract No. 251, p. 113.

Selective decontamination of the digestive tract (SDD) uses antibiotics to reduce aerobic gram-negative rods, thus preventing endogenous infection with these organisms. Retention of the predominant anaerobic flora is thought to prevent colonization with strains resistant to the SDD agents (colonization resistance (CR)). The mechanisms underlying the observed clinical success of these regimens remain contentious. A clinical regimen of tobramycin and polymyxin E was used for SDD in Balb / c mice. Other mice were given streptomycin and neomycin for the elimination of aerobic and anaerobic flora (total decontamination (TDD)). Oral challenge of 10^{8-10} *E.coli* was required to achieve intestinal colonization of untreated mice. This finding was species specific in that colonization of untreated mice with *Proteus sp., Pseudomonas aeruginosa* and *Klebsiella pneumoniae* was impossible at doses of 10^{9-10} cfu. A marked reduction in CR was seen following total decontamination: $< 10^2$ cfu of *E.coli* achieved colonization in 16 / 16 mice following oral challenge both during and one day after discontinuation of TDD. Contrary to previous reports a small but significant reduction in CR to *Proteus sp.* was also seen in 7 / 8 SDD mice which showed no evidence of reduction in intestinal anaerobic flora.

A 2-3 Selective Decontamination of the Digestive Tract (SDD) and Colonization Resistance (CR) in Mice. A. S. Ameen, A. B. J. Speekenbrink, S. R. Alcock. *92nd General Meeting of the American Society for Microbiology,* New Orleans, May 1992, Abstract No. 366, p. 87.

SDD uses antibiotics to reduce the number of aerobic gram-negative rods colonizing the digestive tract, thus preventing endogenous infection with these organisms. SDD also prevents colonization with drug-resistant strains by retaining the anaerobes of the tract; this is known as CR. Balb / c mice were given a clinical SDD regimen of oral streptomycin cefotaxime (CTX). Alternatively, a regimen of oral streptomycin and neomycin achieved total decontamination of the digestive tract (TDD). Intestinal colonization of untreated mice with *E.coli* required an oral challenge of 10^{8-10} cfu. In TDD mice there was major impairment of CR: a challenge dose of 10^{2} cfu of *E.coli*, administered during and 1 day after treatment, colonized 36 / 36 mice. In SDD mice, a small but significant reduction in CR was seen: over 50 % of mice challenged during treatment with 10^{8} cfu of *Proteus* strain were colonized, whereas it was impossible to colonize untreated mice with a challenge dose > 10^{10} cfu. Although CTX alone did not impair CR, a marked reduction in CR occurred during treatment with SDD and parenteral CTX: 24 / 24 mice were colonized when challenged with 10^{4} cfu of the proteus strain. Simplification of the anaerobic intestinal flora was also observed with the regimen of SDD and parenteral CTX.

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