A study of *Clostridium difficile* in biofilm mode of growth using the Sorbarod Filter System

By

Nuala H. O'Connell

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Medicine in Biological Sciences

University of Warwick, Department of Biological Sciences

April 2003

List of contents

List of Figures and Tables

Fig. 69 Effect of *L. paracasei* on growth of *C. difficile* in broth culture (*L. paracasei* titres)

(◆) - *C. difficile*

(■) - *L. paracasei*

(▲) -1mL *C. difficile*: 10mL *L. paracasei*: 10mL BHI,

(✖) - 0.5mL *C. difficile*: 10mL *L. paracasei*: 10mL BHI

(✱) - 0.1mL *C. difficile*: 10mL *L. paracasei*: 10mL BHI

(●) -0.01mL *C. difficile*: 10mL *L. paracasei*: 10mL BHI

Fig. 70 Effect of *L. paracasei* on growth of *C. difficile* in broth culture.

(◆)- *C. difficile* alone in broth culture

(■)- *L. paracasei* in broth alone

(▲)- 1mL *C. difficile*: 9mL *L. paracasei* (*C. difficile* titres)

(×)- 10mL *C. difficile*: 10mL *L. paracasei* (*C. difficile* titres)

(✱)- 9.9mL *C. difficile* : 100µL *L. paracasei* (*C. difficile* titres)

Fig. 71 Effect of *L. paracasei* on growth of *C. difficile* in broth culture.

(◆)- *C. difficile* alone in broth culture

(■)- *L. paracasei* in broth alone

(▲)- 1mLs *C. difficile*: 10mL *L. paracasei*: BHI (*C. difficile* titres)

(×)- 0.5mL *C. difficile*: 10mL *L. paracasei*: 10mL BHI (*C. difficile* titres)

(✱)- 1mL *C. difficile* : 10mL *L. paracasei* (*C. difficile* titres)

(●) 0.5mL *C. difficile*: 10mL *L. paracasei* (*C. difficile* titres)

Fig. 72 Effect of *L. paracasei* on growth of *C. difficile* in biofilm.

Control *C. difficile* biofilm (■)

Control *C. difficile* effluent (◆)

C. difficile biofilm with *L. paracasei* (▲) (*C. difficile* titres)

C. difficile effluent with *L. paracasei* (×) (*C. difficile* titres)

Fig. 73 Effect of *L. paracasei* on growth of *C. difficile* in biofilm.

Control *C. difficile* biofilm (■)

Control *C. difficile* effluent (◆)

C. difficile biofilm with *L. paracasei* (▲) (*C. difficile* titres)

C. difficile effluent with *L. paracasei* (×)(*C. difficile* titres)

Fig. 74 Effect of *L. paracasei* on growth of *C. difficile* in biofilm

Control*C. difficile* biofilm control (■)

Control *C. difficile* effluent (◆)

C. difficile biofilm (10mL *L. paracasei* added) (■)

C. difficile effluent (10mL *L. paracasei* added) (△)

C. difficile biofilm (1mL *L. paracasei* added) (✱)

C. difficile effluent titres (1mL *L. paracasei* added) (●)

C. difficile biofilm titres (0.1mL *L. paracasei* added) (▲)

C. difficile effluent titres (0.1mL *L. paracasei* added) (×)

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification at any other university or institute of learning.

Copy Right And The Ownership Of The Intellectual Property Rights

Dedication

This work is dedicated to my mother Joan, Marley, and all the wonderful staff at what was the Coventry Public Health Laboratory.

Acknowledgement

I would firstly like to express my sincerest gratitude to my chief supervisor Dr. J Keith Struthers, who was always patient, supportive and enthusiastic. Also, I extend my thanks to Professor Chris Dowson, my other supervisor, for his ideas and help.

I would also like to thank the following people; all the technical and media preparation staff of the Coventry PHLS for their technical support and for supplying the clinical isolates, as well as, staff at Beaumont Hospital Ireland, Brian Gee for his photographic expertise, Mrs Pat Tarpey of Manchester Royal Infirmary for her assistance in electron microscopy and photomicrography and my fellow work colleagues who were also doing research projects and who helped me whenever I needed it.

Finally, I would like to thank Dr Steve Rousseau, my PHLS Postgraduate Dean for help in securing funding towards this project.

Abbreviations

AAD	Antibiotic-Associated Diarrhoea
AIDS	Acquired Immunity Deficiency Syndrome
BEC	Biofilm Eradicating Concentration
BHI	Brain Heart Infusion
BSAC	British Society Antimicrobial Chemotherapy
CCFA	Cycloserine Cefoxitin Fructose Agar
CDAD	*Clostridium Difficile* Associated Diarrhoea
CDSC	Communicable Disease Surveillance Centre
CFU	Colony Forming Units
CPE	Cytopathogenic Effect
CPHL	Central Public Health Laboratory
EfMBC	Effluent Minimum Bacteriocidal Concentration
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
EPS	Extracellular Polymeric Substances
GISA	Glycopeptide Intermediate *Staphylococcus aureus*
GLC	Gas Liquid Chromatography
HCW	Health Care Worker
LAB	Lactic Acid Bacteria
LPS	Lipo-Polysaccharide
MBC	Minimum Bacteriocidal Concentration
MIC	Minimum Inhibitory Concentration
NCCLS	National Committee Clinical Laboratory Standards
NCTC	National Clinical Type Collection
PBS	Peptone Buffered saline
PCR	Polymerase Chain Reaction
PHLS	Public Health Laboratory Services
PMC	Pseudomembranous Colitis
PYG	Peptone Yeast-extract Glucose
TEM	Transmission Electron Microscopy
VRE	Vancomycin Resistant Enterococci
VRSA	Vancomycin Resistant *Staphylococcus aureus*

Abstract

In all ecosystems, bacteria are likely to grow in biofilms, and the organisms making up the natural flora of the colon are considered to be no exception to this. One of the most important hospital-acquired infections is antibiotic associated diarrhoea, with the causative agent being the Gram-positive anaerobe, *Clostridium difficile*. This organism elaborates two exotoxins, A and B, which cause disease by interaction with the enterocytes of the colon. Treatment of the condition can be difficult, with treatment failures not uncommon. As the organism produces a heat stable spore, it can survive with ease in the environment.

There are no previous reports concerned with the study of anaerobes in an *in vitro* biofilm system. The work described here is a study of *C. difficile*, using the Sorbarod filter system. This was to determine its suitability in establishing continuous culture biofilms of the organism. It was shown that *C. difficile* readily established itself on the filters, maintained a titre in excess of 10^9 cfu/filter for at least 72h. The lack of sporulation in the system confirmed that vegetative growth was being maintained. In the filters, the organism exhibited elongated forms, sometimes in excess of 10 microns in length. Electron microscopy showed that this was probably due to lack of cell separation at cell division. Toxin production appeared to be higher when *C. difficile* was grown in biofilm in comparison with growth in broth and biofilm effluent.

The susceptibility of the organism to benzylpenicillin, vancomycin, metronidazole and rifampicin was determined in broth culture, biofilm and biofilm effluent. Broth minimum inhibitory and minimum bacteriocidal (MIC/MBC) experiments showed, that apart from rifampicin, the organism was tolerant to the antibiotics. This was essentially repeated in the filter system. Rifampicin had a significantly better bactericidal activity against the organism, and synergistic killing was achieved when rifampicin was combined with vancomycin.

The growth of *C. difficile* in biofilm with other common members of the bowel flora was investigated in combination experiments with *Bacteroides fragilis*, *Enterococcus faecalis* and *Escherichia coli*. No antagonistic effect was demonstrated and antimicrobial susceptibility experiments using *C. difficile* and *E. coli* in combination showed resistance of both organisms to broad-spectrum antibiotics. Screening for bacteriophages and other biological agents with activity against *C. difficile* identified an isolate of *Lactobacillus paracasei*, which had marked activity against the clostridium as shown by the "sloppy agar" method, but there was a very variable effect in broth and the Sorbarod biofilms. Interestingly, the bacteriocin-like agent had activity against not only a number of other clostridial species, but also against *Streptococcus pneumoniae*.

The work here is thus a novel investigation of an important infection control problem, and is the basis for further detailed work examining the growth and pathogenic properties of *C. difficile* in biofilm.

Chapter One

1 Introduction

1.1 Infective antibiotic-associated diarrhoea

Pseudomembranous colitis (PMC) was first recognized in 1893 by Finney when he described a patient as having a "diphtheritic colitis", which manifested as plaque like membranes in the stomach and lower small bowel. There followed similar infrequent reports in the literature, and a comparative study of the incidence of the disease before and after the introduction of antibiotics did not show a statistically significant increase (Pettet *et al.*, 1954). However, as antibiotics were increasingly used, there was a notable increase in the incidence of PMC. A study by Tedesco *et al.* (1974) showed a 21% incidence of antibiotic-associated diarrhoea following clindamycin use; half of these patients had sigmoidoscopic evidence of PMC, prompting a warning on the package insert for clindamycin. Numerous other antibiotics, including ampicillin, were subsequently implicated in PMC.

Various theories have been postulated for the etiology of antibiotic-associated diarrhoea (AAD) and PMC, ranging from a direct effect of the antibiotic/antibiotic metabolite on the intestinal flora, to changes in enteric flora, or viruses. In numerous early studies, *Staphylococcus aureus* was considered a significant cause of AAD (Dearing *et al.*, 1960; Hummel *et al.*, 1964). Although *S. aureus* could be isolated from the stools of patients with abdominal distress and fever, there were no intestinal lesions at autopsy. Despite the discovery of *Clostridium difficile* in 1935, it's role as the major causative pathogen in AAD and PMC was not recognized until the late

1970s (Larson *et al.*, 1978). Since the link between *C. difficile* and AAD was established, the interest in *S. aureus* appeared to wane. However, a recent study by Gravet *et al.* (1999) found a ratio of one case of *S. aureus* AAD for every five cases of *C. difficile* AAD. Methicillin resistance was found in 92% of the implicated *S. aureus* isolates and significantly more AAD *S. aureus* isolates produced the recently described leucotoxin LukE-LukD and staphylococcal enterotoxin A than randomly detected strains (Gravet *et al.*, 1998). There is also evidence suggesting that *C. perfringens* may be pathogenic in some cases (Hancock, 1997). The enterotoxin gene of *C. perfringens* isolated from cases with AAD has been found to be located extra-chromosomally, differing from the chromosomal position of food-poisoning isolates, suggesting that these strains of *C. perfringens* are a distinct entity (Collie and McClane, 1998). Further studies are needed to determine the relative roles of the aforementioned bacteria in AAD and PMC.

1.2 *Clostridium difficile*

Clostridia are ubiquitous and are found in soil, decaying vegetation, marine sediment, and the intestinal tract of humans, other vertebrates and insects. More than 90 species of clostridia are recognized, however fewer than 20 are pathogenic to man. Hall and O'Toole (1935) first isolated this organism during a study of intestinal flora of newborn infants. It was initially called *Bacillus difficilis* due to the difficulty encountered in its isolation. They described an obligate anaerobic, spore-producing, motile Gram-positive rod, which was toxigenic and non-haemolytic. *B. difficilis* was renamed as *Clostridium difficile* three years later. Cells stain uniformly Gram-

positive in young cultures, but may become Gram-negative after 24-48h. Some *C. difficile* strains produce thin capsules, and fimbria-like structures (Borriello *et al.*, 1990). The production of oval, subterminal spores by *C. difficile* is not associated with toxigenic action. Cyloserine, cefoxitin, fructose agar (CCFA) is both selective and differential for *C. difficile*, which characteristically produces a "horse-dung" odor (Brazier, 1998). *C. difficile* liquefies gelatin and ferments fructose, glucose, mannose and usually xylose (Hathaway and Johnson, 1998). It produces acetic, isobutyric, butyric, isovaleric, valeric, isocaproic, formic and lactic acids in PYG (Peptone Yeast-extract Glucose) and converts tyrosine to ρ-cresol (Holdeman *et al.*, 1977). Other important biochemical reactions include production of H_2S (Theilman, 2000).

1.2.1 Epidemiology

The local importance of *C. difficile* is dependent on multiple factors including antimicrobial prescribing practices (spectra of activity and pharmacokinetic properties), endemic strains, patient susceptibility to *C. difficile* and criteria used to define AAD. Data on the epidemiology of *C. difficile* disease in England and Wales from the Communicable Disease and Surveillance Unit, Colindale, indicates a dramatic rise in the incidence of this predominantly hospital-acquired infection. Laboratory reports in 1997 from England and Wales showed that the total number of *C. difficile* toxin-positive cases was approximately 30% higher than in 1996. Although *C. difficile* AAD (CDAD) is considered primarily nosocomial, it is a problem that is not confined to hospitals. A study by Kyne *et al.* (1998) reported an outbreak of CDAD involving 139 patients in which 11% of new admissions presented

with *C. difficile* cytotoxin B positive diarrhoea; in some cases this was the sole reason for hospitalization. National data from Sweden indicates 42% of cases of *C. difficile* infection arise in the community (Karlstrom *et al.,* 1998). Community-acquired disease may be related to the increasing incidence of nosocomial CDAD, or the more widespread use of broad-spectrum antibiotics in General Practice. Sporadic, non-antibiotic-related CDAD occurs in the community but is rare.

A systematic review of the literature to identify risk factors associated with *C. difficile* infection was conducted (Bignardi, 1998). Risk factors for which there was evidence suggestive or consistent with an association with *C. difficile* diarrhoea were increasing age, severity of other underlying disease, non-surgical gastrointestinal procedures, presence of a nasogastric tube, anti-ulcer medications, stay on ITU, duration of hospital stay, duration of an antibiotic course, and administration of multiple antibiotics (Brown *et al.,* 1990; McFarland *et al.,* 1990). Some data also suggest an association between *C. difficile* and the use of antineoplastic chemotherapy although the effects of antineoplastic agents on the faecal flora remain unknown (Anand and Glatt, 1993). The frequent concomitant use of antibiotics and chemotherapeutic agents may well mask chemotherapy-induced infection by *C. difficile*, so that its incidence may well be underestimated. The occurrence of severe AAD in patients with the acquired immune deficiency syndrome (AIDS) is common but appears to be favored relative to both heavy antimicrobial use and general immunosuppressive treatment (Cozart *et al.,* 1993). The incidence appears to be the same as those in a non-AIDS control group.

Studies on the rate of intestinal carriage have been undertaken on all age groups. Asymptomatic colonization occurs in approximately 50% of neonates despite stool cytotoxin levels that may be similar to those in adults with severe colitis (Cooperstock *et al.*, 1983). A possible explanation is the absence of the enterocyte membrane toxin receptors in the first year of life. Reported carriage rates in healthy adults have varied from 0.3% in Europe to 15% in Japan (Mulligan, 1988). Serum antibodies to *C. difficile* toxins are found in 60% of children and adults in the United States and studies suggest that an inadequate immune response may predispose a patient to a relapse (Kelly, 1996).

The hands of personnel, as well as a variety of environmental sites within institutions, have been found to be contaminated with *C. difficile*, which can persist as spores for indefinitely (Samore, 1999). Contaminated commodes, bathing tubs, and electronic thermometers have been implicated as sources of *C. difficile* (Savage and Alford, 1983; Kaatz *et al.*, 1988; Brooks *et al.*, 1992). Symptomatic, and asymptomatic, infected patients are the major sources and reservoirs for environmental contamination and typing systems are available to aid epidemiological investigations (O'Neill *et al.*, 1996).

1.2.2 Clinical Manifestations

Infection with toxigenic *C. difficile* causes a spectrum of disease ranging from asymptomatic carriage, mild AAD to a fulminant, relapsing, and occasionally fatal colitis (Mylonakis *et al.*, 2001). Symptoms usually develop 4-7 days after starting, or shortly after stopping, antibiotic therapy, but can develop several weeks after therapy ends. It is always important to ask patients with diarrhoea about previous use of antibiotics. Most symptomatic patients have explosive, watery, foul-smelling diarrhoea with abdominal pain but little overall fluid loss (Tabaqchali and Jumaa, 1995). Recovery typically ensues within 3 to 10 days with or without specific treatment. In PMC, the presenting features may include profuse, persistent diarrhoea, fever, cramping abdominal pain and marked leucocytosis (Bulusu *et al.*, 2000). General malaise, occult colonic bleeding, dehydration and hypoalbuminemia may also be present. Sigmoidoscopy or colonoscopy reveals characteristic adherent yellow plaques involving the rectum and sigmoid colon in approximately 90% of cases. Proximal colon involvement will be missed if sigmoidoscopy alone is performed (Tedesco *et al.*, 1982). Toxic megacolon occurs infrequently but has a high mortality rate. Other intra-abdominal complications include: colonic perforation, transverse volvulus, protein-losing enteropathy, and recurrent, relapsing AAD (Fekety, 1997).

Extraintestinal manifestations occur more rarely and include bacteraemia, splenic abscess, osteomyelitis, wound infections, peritonitis and urogenital infections (Theilman, 2000). Like all other enteric pathogens, *C. difficile* has been associated

with reactive arthritis; in many cases patients are HLA-B27 antigen-positive (Mermel and Osborn, 1989).

1.3 Pathogenesis

A sequence of events occurs in the pathogenesis of AAD: disruption of the normal enteric flora by antibiotics or antineoplastic agents which have antibacterial activity, colonization with *C. difficile*, elaboration of toxin A and toxin B by the organism, and subsequent mucosal injury and damage.

1.3.1 The Role of Antimicrobials

The administration of antibiotics is considered the most significant and frequently reported predisposing factor for CDAD. The colon is home to more than 500 species of bacteria and maintenance of a normal bowel ecosystem is one of the main defences against *C. difficile* colonization (Borriello, 1998). Even when the organism is present in the bowel, suppression by other enteric flora, "colonization resistance," usually prevents disease (Borriello and Barclay, 1986). Almost any antibiotic may cause *C. difficile* disease, but broad-spectrum antibiotics active against enteric organisms allows *C. difficile* to become established in the bowel (Gorbach, 1999).

Clindamycin was one of the earliest antibiotics associated with PMC, prompting the condition to be called "clindamycin colitis" (Kabins and Spira, 1975). In current practice, however, many retrospective studies have shown broad-spectrum penicillins and cephalosporins are the commonest culprits, reflecting their widespread use.

Parenteral aminoglycosides have never been associated with *C. difficile* infection and other low risk antibiotics include rifampicin, quinolones, trimethoprim and the ureido-penicillins (Spencer, 1998). The reason for this may reflect the lack of effect of some of these antibiotics on endogenous anaerobic gut flora, but this is still uncertain. The use of multiple antibiotics has also been shown to increase the risk of infection. One study by Gerding *et al.* (1986) found that patients with *C. difficile* infection were more likely to have received multiple courses of therapeutic antibiotics when compared with matched controls. Interestingly, susceptibility to any particular antibiotic does not predict the likelihood of CDAD following exposure to that agent. Despite the fact that most strains of *C. difficile* are susceptible to aminopenicillins, even these antibiotics can precipitate *C. difficile* infection. *C. difficile* colonization and disease develop more commonly after prolonged antibiotic agents, but has also been reported with some antimicrobial agents used for short-term (≤ three doses) perioperative prophylaxis (Privitera *et al.,* 1991). A study by Kreisel *et al.* (1995) showed that not only is a prolonged prophylactic antibiotic course in elective surgical cases associated with increased risk of developing *C. difficile* toxin positivity, but the choice of antibiotic is also important. There are now sufficient reports in the literature to limit the widespread use of cephalosporins, especially in at risk populations such as the elderly, by the substitution, where possible, with antibiotics not associated with the promotion of AAD (Department of Health and Public Health Laboratory Service Joint Working Group, 1994). Widespread education about the rational prescribing of antibiotics in clinical practice remains of paramount importance.

1.3.2 Virulence factors of *C. difficile*

C. difficile produces a number of virulence factors including colonization factors and toxins, which contribute to infection. As with all pathogens, not all strains are equally virulent (Borriello *et al.*, 1987). One study in a hamster model demonstrated that some serotypes produced more toxins *in vivo* than others (Delmee and Avesani, 1990). Factors such as the production of spores, proteolytic and hydrolytic enzymes, expression of fimbriae and flagella, chemotaxis and adhesion to gut receptors, and production of capsules, may all play a part in pathogenesis by facilitating colonization or by directly contributing to tissue damage, or both (Borriello, 1998).

When established in the colon, pathogenic strains of *C. difficile* produce 2 major toxins that cause diarrhoea and colitis, as well as 3 minor toxins C, D, and E (Borriello *et al.*, 1990). These 2 major exotoxins have been well characterized: toxin A, a 308-kd enterotoxin, and toxin B, a 279-kd cytotoxin. Both toxins have contiguous repeating units at the COOH terminus and overall, the two toxins exhibit 49% homology at the amino acid level (Kyne *et al.*, 2001). Toxin A causes fluid secretion, mucosal damage, and intestinal inflammation and probably causes most of the clinical symptoms (Pothoukalis, 1996). Toxin A is also a chemoattractant for human neutrophils *in vitro*. Toxin B is approximately 1000 times more potent than toxin A as a cytotoxin in tissue culture but is not enterotoxic in animals (Pothoukalis *et al.*, 1986). The molecular organization and control of both toxins is now starting to be understood with the sequencing of the genes encoding them, which are arranged on a 19.6 kb toxicon in a chromosomal pathogenicity locus with the minor toxins

(Cohen *et al.*, 2000). Once the toxins gain access to the cytoplasm of the enterocyte, both inactivate *Rho* proteins, guanosine triphosphate-binding proteins, that regulate actin cytoskeleton and various signal transduction processes (Pothoukalis, 1996). Toxin-induced dysregulation of *Rho* leads to cytoskeleton disruption, cell rounding and retraction, and apoptosis in cell lines. Differential expression between strains of various combinations of these colonization and virulence factors may explain the apparent variability in virulence of *C. difficile* strains. Toxigenic strains of *C. difficile* had been reported to produce both toxins A and B nearly always, and non-toxigenic strains are reported to produce neither of these toxins. Recent studies indicate that this is not always true. Strains which produce toxin B but not toxin A (A-B+) have been described in a case report of PMC caused by such a strain, which suggests that toxin A may not be the main pathogenic factor of *C. difficile* infection, as previously thought. The prevalence of such strains still appears to be low, but is variable, with a described incidence of 31% in one Canadian hospital (Embil *et al.*, 1999). No strains have yet been described that express A but not B.

1.4 Diagnosis of *C. difficile*-related infection

The diagnosis of CDAD should be based on both clinical, as well, as laboratory findings. A review of the criteria for optimal diagnosis of infections due to *C. difficile* was performed by Gerding and Brazier (1993). Key elements included the patient's clinical history and symptoms, endoscopic evidence of a colonic pseudomembrane, laboratory evidence of *C. difficile* in stool, and laboratory evidence for toxin(s) in stool.

1.4.1 Clinical diagnosis

There are numerous risk factors and host factors that should prompt the attending clinician to consider *C. difficile* infection, in either the hospital or community setting. Advancing age (>65y) is one the cardinal factors associated with symptomatic infection. A carefully taken drug history detailing recent antibiotic use (strong associative risk but not a complete prerequisite), or other medications including cytotoxic drugs, antacids, and laxatives might alert to a diagnosis of *C. difficile* infection.

Diarrhoea is the most common clinical symptom, but this can be difficult to define as patients' bowel habits differ considerably and their "normal bowel habit" must first be elucidated. Diarrhoea is defined by a variety of criteria such as six watery stools over 36 hours, three unformed stools in 24 hours, or eight unformed stools over 48 hours (Gerding *et al.*, 1995). Other causes for diarrhoea should also be sought when suspecting AAD. Ward staff may also suspect AAD as it has a distinctive scent (horse manure) and this may also play an early role in diagnosis and reduction of horizontal transmission. A response to empirical treatment for AAD is also suggestive of the diagnosis but should be confirmed by the laboratory. Rarely, <1%, symptomatic patients do not have diarrhoea due to *ileus* (Kelly *et al.*, 1994). It is therefore important to communicate with the laboratory to test the non-diarrhoeal stool for *C. difficile* or it's toxins when suspected.

1.4.2 Endoscopy

Characteristic adherent yellow plaques, involving the rectum and sigmoid colon in 90% of cases, are the pathognomonic lesions found on endoscopy in the patient with PMC. The intervening mucosa may appear normal or erythematous and in severe cases, the plaques may coalesce to cover large areas of mucosa (Fekety, 1997). Biopsy may reveal changes associated with PMC in those cases where endoscopic findings are non-specific (Fekety *et al.*, 1993).

1.4.3 Laboratory diagnosis

The correct specimen for the diagnosis of AAD is a loose or watery stool, which should be submitted as soon as possible to the laboratory for testing. Numerous studies have shown that storage at ambient temperatures may result in possible denaturation of toxin so where it is not possible to test expediently, specimens should be refrigerated or frozen. In most cases of infection, toxin testing or *C. difficile* culture readily establishes the diagnosis; however repeat testing or endoscopy or both may be necessary. Studies by Bouza *et al.* (2001) showed that combination of direct-cell culture assay, culture for toxigenic *C. difficile* and "second-look" cell culture assay also enhances the potential for diagnosis of CDAD. Table 1 summarizes the characteristics of the different tests used to diagnose CDAD. Methods such as stool gram stain, analysis for faecal leucocytes, counterimmunoelectrophoresis of stools for toxin production, and direct chromatography of stool have only been marginally successful (Brazier, 1998). A combination of *C. difficile* culture and one nonculture

based test for toxin has been recommended as the optimal laboratory approach but stool toxin testing alone has also been recommended (Gerding and Brazier, 1993; Walker *et al.*, 1986).

Test	Sensitivity (%)	Specificity (%)	Clinical Utility
Endoscopy	51	~100	Diagnosis of PMC
Culture	89-100	84-99	Highly sensitive; confirmation of toxicity optional
Cell culture cytotoxin test	67-100	85-100	With clinical data, diagnostic of AAD
EIA toxin test	63-99	75-100	With clinical data, diagnostic of AAD
Latex test for *C. difficile* antigen	58-92	80-96	Less sensitive and specific than others tests, rapid results
PCR toxin gene detection	Undetermined	Undetermined	Research test

Modified from Gerding *et al.* (1995)

Table 1 Sensitivity and Specificity of Tests for Diagnosis of *C. difficile* –associated Diarrhoea

1.4.3.1 Culture

Traditionally, culture is the method for diagnosing bacterial infections.

Hafiz and Oakley attempted to produce the first selective media in 1976 using p-cresol. Others, however, subsequently found that p-cresol is in fact inhibitory to the growth of *C. difficile* leading to other selective media being devised (Brazier, 1998). The description of an egg yolk agar base medium containing cycloserine, cefoxitin and fructose (CCFA) by George *et al.* (1979) has led to a culture method which has a good success rate in recovery of *C. difficile* from stool specimens. Various modifications of antibiotic concentrations have been tested but, despite increasing the enhancement of growth of *C. difficile*, have made the agar less selective by allowing other microbes to grow. Mundy *et al.* (1994) suggested using CCFA plates that had been reduced anaerobically for 4 hours before use. Strains of *C. difficile* typically produce flat, yellow, ground glass appearing colonies with a surrounding yellow halo in the middle (Gerding *et al.*, 1995). Visual inspection of colonial morphology on this agar and confirmation by Gram stain is sufficient for a confirmation of *C. difficile* (Peterson *et al.*, 1986).

Further identification can be performed either biochemically or by Gas-Liquid Chromatography (GLC). As *C. difficile* is a spore-bearing bacillus, procedures such as alcohol-shock treatment of stool specimens have been reported to increase the culture yield by selecting for sporulating organisms (Bartley and Dowell, 1991). Culture may not be considered either cost-effective or timely by many laboratories

that prefer using more rapid tests such as ELISA-based assays for toxin detection, but it remains essential for epidemiological studies of outbreaks (Riley *et al.*, 1995). Laboratories that adopt culture as the sole means of testing for *C. difficile* diarrhoea can expect a high false positive rate as determination of toxin production is necessary to distinguish pathogenic toxigenic strains from non-pathogenic non-toxigenic strains (Shanholtzer *et al.*, 1992).

Latex testing for the *C. difficile* protein, glutamate dehydrogenase, is also available for direct use on stool specimens but various studies show sensitivity rates of 58% to 68% and a specificity of 94% to 96% (Gerding *et al.*, 1995). Disadvantages of this test are lack of an isolate for epidemiological investigations and no information on the toxigenicity of the isolate.

1.4.3.2 Toxin determination

Detection of toxin activity in stools from patients with antibiotic-associated colitis was the initial observation leading to the discovery of *C. difficile* as the causative agent of this infection (Larson *et al.*, 1977). Toxin B manifests itself as a cytopathic effect (CPE) on cell lines causing a rounding-up of cells and this forms the basis for the cytotoxic assay for toxin B in stools (Brazier, 1998). Toxin A is also cytotoxigenic and can be detected in this assay. Assay for the cytotoxins of *C. difficile* can be performed in almost any cell line but some are more sensitive than others (Thelestam and Bronnegard, 1980). Vero, Hep2, monkey kidney and HeLa cells are generally used. The detection of cytotoxin in tissue culture is considered the "gold

standard" with which other diagnostic tests are compared in evaluations of sensitivity and specificity (De Gromlami *et al.*, 1992). A faecal suspension is made in phosphate-buffered saline (PBS), centrifugued and the supernatant is then filtered through a 0.2μm membrane filter. The filtrate is then inoculated onto a cell monolayer, which is examined at 24h and 48h for a CPE. To confirm it's specificity, any CPE should be compared with a negative PBS control and be neutralizable with either *C. difficile* or C. *sordellii* antitoxin. Disadvantages of this methodology include its slowness (however, there have been some reports of detectable CPE in 70% of specimens in 6h) and having access to tissue culture cell lines (Brazier, 1998).

Several commercial enzyme immunoassays are now widely used for the detection of *C. difficile* toxins and are considered to be reasonable alternatives because of their good specificity, rapid turnaround time, convenience and cost. Most of the kits are designed to detect toxin A but a few detect both toxins (Lyerly *et al.*, 1998). There have been recent reports of A-B+ toxigenic strains and it is now recommended to adopt the use of a kit that tests for both toxins. An automated immunodiagnostic assay system for toxin detection has been made available from Vitek (Hazelwood, MO, USA) but sensitivity and specificity rates are poor by comparison with the gold standard of the cytotoxicity assay (Riederer *et al.*, 1995).

1.4.3.3 Molecular methods

The advent of methods based on the polymerase chain reaction (PCR) for detecting nucleic acid specific for a given gene or organism in a clinical specimen has led to

research in its applicability to the diagnosis of *C. difficile* infection (Wren *et al.*, 1990). Research has been mainly targeted at the direct amplification of the genes for toxin production. Early results look promising and it is thought that one major benefit of this direct method could be epidemiological typing of environmental and patient strains. Application of molecular methods will require that they have good sensitivity, specificity, and speed compared to culture and cytotoxin assay, and that they can be performed at a competitive cost (Gerding *et al.*, 1995).

1.5 Treatment of *C. difficile*

The treatment options for *C. difficile* infection remain limited, although promising agents are currently being assessed. Initial therapy of patients with PMC should include discontinuation of the offending antibiotic regimen, if possible, and replacement of fluid and electrolyte losses. Studies have indicated that approximately 15-25% of patients respond to this approach alone but most patients require specific antibacterial therapy (Olson *et al.*, 1994). If the patient has a concomitant infection that does require treatment, an antibiotic that has a reduced risk of exacerbating the *C. difficile* infection should be substituted along with the addition of an antibiotic to treat *C. difficile*. Antiperistaltic agents such as loperamide should be avoided as several case series have suggested they may predispose to an increased mortality with the development of toxic megacolon (Fekety, 1997). These agents promote colonic stasis so may theoretically contribute to further damage and inflammation due to prolonged mucosal exposure to *C. difficile* toxins.

1.5.1 Initial Treatment

Either oral metronidazole (400mg 8h) or oral vancomycin (125mg 6h) are the recommended first-line agents and are equally effective in treating *C. difficile* infection (Wilcox and Spencer, 1992; Zimmermann *et al.*, 1997). Symptomatic improvement generally occurs within 72h, and diarrhoea and colitis resolves in 95% of patients after 10 days of treatment. Although vancomycin has preferable pharmacokinetics (it is not appreciably absorbed from the gastrointestinal tract (GIT)) and good killing activity against *C. difficile* (Kucers and Bennett, 1987), restriction to second-line therapy reduces the emergence of glycopeptide resistance. Metronidazole is readily absorbed from the GIT and systemic side effects may occur, however, it is more cost-effective and does not pose a risk factor for colonization and infection with vancomycin resistant enterococci (VRE) (Delmee *et al.*, 1995). Metronidazole-resistant strains of *C. difficile* had been reported sporadically until a recent Spanish report worryingly described widespread metronidazole resistance in a HIV positive population (Pelaez *et al.*, 1997). There also have been reports of metronidazole inducing diarrhoea (Saginur *et al.*, 1980).

Patients who cannot tolerate oral medication because of paralytic *ileus*, nasogastric suctioning, intestinal obstruction or recent abdominal surgery can be effectively treated with intravenous metronidazole as excretion into the inflamed colon from the bile results in bactericidal levels in the faeces (Bolton and Culshaw, 1986). However, parenteral failures with metronidazole have also been reported (Guzman *et al.*, 1988). Reports of success with intravenous vancomycin are limited due to low intracolonic

levels. One case had undetectable faecal levels of vancomycin despite 5 days of parenteral therapy (Tedesco *et al.*, 1978). Vancomycin has also reportedly been administered as an enema and via a long catheter placed during colonoscopy but the safety and efficacy of this practice is questionable (Pasic *et al.*, 1993). It has also been suggested that intravenous empirical therapy should consist of both antibiotics (Wilcox, 1998).

1.5.2 Relapses

Many patients suffer a relapse of diarrhoea from *C. difficile* infection when initial therapy is discontinued. In a recent UK study, repeat courses of antibiotic treatment were given to 37% of those patients requiring antimicrobial therapy for *C. difficile* infection (Wilcox *et al.*, 1996). It was not know whether the high clinical failure was due to relapses or reinfections. Both may be related to the formation of antibiotic resistant spores (Tedesco *et al.*, 1985). Relapse is defined clinically as a re-occurrence of diarrhoea following a symptom free period (Wilcox, 1998). One molecular fingertyping study showed that 56% of relapses were in fact due to reinfection with a different strain (Wilcox *et al.*, 1998). The diagnosis of recurrent diarrhoea from *C. difficile* infection should be confirmed by a stool toxin study but it is not necessary to use this test as a confirmation of cure or eradication in asymptomatic patients. Many who continue to have positive toxin results do not develop a relapse. Treatment may not be necessary for mild symptomatic relapses, since they resolve spontaneously. It is common practice to switch from metronidazole to vancomycin (or vice versa) in patients with symptomatic recurrences. In recalcitrant relapse cases various

approaches have been suggested including a slow tapering of vancomycin therapy for 4-6 weeks (the rationale being primary killing of vegetative *C. difficile* and secondary killing of germinated resistant spores), the use of rifampicin in combination treatment or other antimicrobials including fusidic acid, bacitracin and teicoplanin (Kyne *et al.*, 2001).

New treatment options are still awaited. Pelaez *et al.* (2002) examined the *in vitro* activity of linezolid against *C. difficile*, including those with reduced susceptibility to metronidazole or vancoymcin and MICs were always ≤ 4mg/L, and thus, all isolates were susceptible. These data are promising but further studies on the efficacy of linezolid in cases of CDAD are needed. McVay *et al.* (2000) described the activity against *C. difficile* of a new nitrothiazole benzamide agent, nitazoxanide. *In vitro* activity was similar to that of vancomycin or metronidazole. Unfortunately, studies of the *in vitro* activity of new quinolones against *C. difficile* were not as promising (Alonso *et al.*, 2001). Alternatives to using antibacterials include biotherapy and immunological approaches (Roffe, 1996; Salcedo *et al.*, 1997).

1.5.3 Alternative therapies

Biotherapy aims to restore the commensal gut flora and hence colonisation resistance against *C. difficile*. Probiotics have special properties that make them useful in fighting infections of mucosal surfaces such as the gut and vagina. They are becoming increasingly available as capsules and dairy-based food supplements sold in health care shops and supermarkets. Several small series of patients have been

treated for, or received prophylaxis against, AAD with biotherapy, including *Lactobacillus acidophilus*, *Lactobacillus* GG, *Enterococcus faecium* SF 68, non-toxigenic *C.difficile*, yoghurt, brewer's yeast or *Saccharomyces boulardii* (Wilcox, 1998). Rectal infusions of normal faeces and bacterial enemas incorporating various facultatively aerobic and anaerobic bacteria are measures that have been tried in those that have failed to respond to traditional antibiotics (Tvede and Rask-Maden, 1989). A randomized placebo-controlled trial of *S. boulardii* in combination with standard antibiotics for *C. difficile* infection reported efficacy in terms of symptomatic recurrence rates but no data was provided to differentiate relapses from reinfections (McFarland *et al.*, 1994). It is thought that *S. boulardii* may effect the proteolytic digestion of toxins A and B by a secreted protease (Castagliuolo *et al.*, 1999). Two case reports of fungaemia have highlighted the inherent dangers associated with yeast formulations used in treating *C. difficile* infection (Niault *et al.*, 1999). In another study, two patients with relapsing *C. difficile* diarrhoea following standard treatments of both metronidazole and vancomycin were colonised with a non-toxigenic avirulent strain of *C. difficile* with symptomatic improvement (Seal *et al.*, 1987). A meta-analysis suggests that probiotics can be used to prevent AAD and that *S. boulardii* and lactobacilli have the potential to be used in this situation (D'Souza *et al.*, 2002). Further work to look at the costs of and the need for routine use of these agents needs to be undertaken.

The nature of the immune response to *C. difficile* infection is being investigated and may pave the way for vaccine development and other types of immunotherapy.

Serum IgG against toxin A is thought to play an important protective role against *C. difficile* in patients who remain asymptomatically colonised and likely explains an observation that patients who are already colonised with *C. difficile* on admission to hospital are significantly less likely to develop symptomatic infection (Kyne *et al.*, 2000). This study also reports significantly higher levels of both serum IgM and IgG antibodies against toxin A in patients who experienced single compared with multiple episodes of CDD. The role IgA plays in *C. difficile* infection is uncertain.

Nonetheless, passive or active immunotherapy could potentially be used to prevent or treat *C. difficile* infection. In a study of 6 children with relapsing *C. difficile* colitis, Leung *et al.* (1991) found that these children had low serum levels of IgG antibody against toxin A. Treatment with normal pooled intravenous gamma globulin, which contained IgG anti-toxin A, was associated with a marked increase in serum antitoxin antibody levels and resolution of recurrent *C. difficile* infection. A bovine immunoglobulin concentrate has been reported to inhibit the cytotoxicity and enterotoxicity of *C. difficile* toxin (Kelly, 1996). Immunotherapy looks a promising approach for treating *C. difficile* infection but further work is needed.

1.5.4 Surgery

Approximately 1 to 3% of patients with *C. difficile* colitis develop signs and symptoms of disease requiring admission to an intensive care unit (Synott *et al.*, 1998). Some of these severely ill patients require lifesaving emergency colectomy because of impending perforation, severe *ileus* with megacolon, or refractory

septicaemia. Indications for surgery include peritoneal signs, bacteremia unresponsiveness to antibiotics, progressive fever, rigors, an elevated white cell count, or CT scan evidence of significant pericolonic inflammation with increasing bowel wall odema (Synott *et al.*, 1998). Various surgical procedures have been described, including ileostomy, cecostomy, or decompressive colostomy but the recommended procedure is subtotal colectomy with ileostomy (Agnifili *et al.*, 1994). This can be converted to an ileorectal anastomosis after colonic inflammation has subsided. The overall mortality rate in cases requiring surgery is around 30 to 35% (Morris *et al.*, 1990).

1.6 Antimicrobial Susceptibility Testing and Resistance Patterns of *C. difficile*.

The mainstay of treatment for *C. difficile* colitis is either the imidazoles (metronidazole) or glycopeptides (vancomycin). Other antimicrobials such as rifampicin have been used in combination therapy (Buggy, 1993). *In vitro* determination of *C. difficile* susceptibility to these antibiotics is not routinely performed in most laboratories. The reference method for susceptibility testing of anaerobic bacteria is the agar dilution method as recommended by the British Society for Antimicrobial Chemotherapy (BSAC) and the National Committee for Clinical Laboratory Standards (NCCLS, 1997). This method is appropriate for reference laboratories only as it is time-consuming and poorly adapted to the analysis of a few isolates. Poilane *et al.* (1999) compared the Etest with the reference dilution method to determine the susceptibility of *C. difficile* to antibiotics used in therapy, in order to have a rapid, reliable and easy-to-use method. Ninety eight percent of the Etest

Minimum Inhibitory Concentrations (MICs) were within one dilution of the agar dilution MICs, however, the authors advised ensuring that the test conditions are well controlled for metronidazole testing.

Resistance patterns of *C. difficile* remain imprecise due to lack of laboratory surveillance data (Brazier, 1998). Studies have shown that this bacterium is highly susceptible to metronidazole (MIC 0.06 –2mg/L), vancomycin (MIC 0.125-4mg/L), and teicoplanin (MIC 0.03-2mg/L) (Dzink, 1980). Niyogi *et al.* (1992) found all *C. difficile* strains to be uniformly susceptible to metronidazole and penicillin G but highly resistant to gentamicin, trimethoprim, sulphamethoxazole, nalidixic acid, cycloserine and cefotaxime. The susceptibility of 50 random *C. difficile* isolates submitted for typing in 1996 (representing 18 PCR ribotypes and about 14% of the total number of isolates) was assessed by the Anaerobe Reference Laboratory in Cardiff, Wales (PHLS, 2002). This study showed no resistance to metronidazole, vancomycin or co-amoxiclav but resistance was reported to pipercillin/tazobactam (4%), chloramphenicol (6%), tetracycline (8%), penicillin (14%), clindamycin (20%), erythromycin (46%), imipenem (96%) and cefoxitin (100%).

After a report by a Spanish group who described an emerging problem of *C. difficile* strains with reduced metronidazole susceptibility (Pelaez *et al.,* 1998), reassessment of *C. difficile* susceptibility to metronidazole and vancomycin over an 8-year period (1993-2000) was undertaken by this group (Pelaez et al., 2002). The overall rate of resistance to metronidazole at the critical breakpoint (16mg/L) was 6.3% and

although full resistance to vancomycin was not observed, the overall rate of resistance was 3.1%. A French retrospective study looked at a collection of *C. difficile* strains from 1991 and 1997 (Barbut *et al.*, 1999). Strains with decreased susceptibility to metronidazole (MIC ≥ 8 mg/L, NCCLS breakpoint) were isolated from 6 patients (n=4 in 1991 and n=2 in 1997). Strains isolated in 1997 were surprisingly more susceptible than those isolated in 1991, and this trend correlated to a major change in serogroup distribution. Further reports of metronidazole resistance from China are documented in the literature (Wong *et al.*, 1999). Metronidazole resistance has also been reported in an environmental isolate from an elderly care ward in Leeds, which was typed as PCR ribotype 10, a non-toxigenic ribotype (Brazier *et al.*, 2001). Metronidazole remains an inexpensive, highly effective treatment for CDAD and it will be important to ascertain the clinical impact of metronidazole-resistant strains. Hopefully, a scenario similar to that experienced by 4 US hospitals, who had outbreaks of CDAD by a strain that was highly resistant to clindamycin, will not occur (Johnson *et al.*, 1999). In the current era of evolution of resistance, especially to glycopeptides, with the appearance of VRE, glycopeptide-intermediate *S. aureus* (GISA) and vancomycin-resistant *S. aureus* (VRSA), it is apparent that ongoing antimicrobial resistance surveillance is warranted.

1.7 Infection Control and Prevention

C. difficile can be isolated from the stools of asymptomatic patients as well as from symptomatic patients. Patients with diarrhoea excrete larger numbers of organisms in faeces and bacterial spores have been found in abundance in the environment of

patients with disease (Mulligan *et al.*, 1979). Contaminated environmental surfaces and healthcare workers (HCWs) handcarriage are considered important vectors for transmission in hospitals (McFarland *et al.*, 1989). The frequency of *C. difficile* handcarriage amongst HCWs significantly correlates with the intensity of environmental contamination (Strimburg *et al.*, 1989).

Infection due to *C. difficile* is almost entirely confined to persons who have been made susceptible by antibiotic therapy that removes the protective flora of the gastrointestinal tract. The key to prevention is good control of antibiotic use, use of infection control universal precautions and good environmental cleaning. Thus, an effective infection control programme should target the interruption of horizontal spread of the organism (to keep the patient from acquiring the organism), and minimalise the risk of organism exposure resulting in clinical infection (Worseley, 1998).

1.7.1 Control of Antibiotic Usage

Antimicrobial use is very common in all hospitalised patients with AAD; Clabots *et al.* (1992) found that 72% of patients with ward acquisitions had had antibiotics within the previous fourteen days. Specifically, clindamycin, ampicillin or cephalosporins pose as major risk factors for *C. difficile* infection as well as various dosing parameters (number of doses, number of antibiotic days and use of combination therapy) (Bignardi, 1998). Where appropriate, narrow spectrum antibiotics should be used whenever possible, preferably those antibiotics with a

lower potential for inducing *C. difficile* infection (Spencer, 1998). Empirical or "blind" therapy should usually be prescribed for serious infections. Ideally, where delay does not endanger the patient, microbiological results should be awaited to both confirm an infective disease and aid in choosing a more "bowel friendly" antibiotic. Review of drug charts regularly should be encouraged so that antibiotics can be discontinued when infections have been treated. Good control of antibiotics should be encouraged at all times, however, in the wake of an outbreak, it is essential that rational prescribing is strictly policed as spores of *C. difficile* can persist in the environment for months (Worseley, 1998). Changing of antibiotic policies to reduce the use of cephalosporins and clindamycin is also a useful measure in controlling outbreaks of *C. difficile*. A change of antibiotic policy for the treatment of community-acquired pneumonia was implemented at an UK hospital with a resultant decline in nosocomial acquisition of *C. difficile* and the number of bed days occupied by infected patients (Jones *et al.*, 1998). There may also be merit in the use of prophylactic agents that could reduce the risk of CDAD in patients who are receiving antibiotics, e.g. *S. boulardii* (Surawicz *et al.*, 1989).

1.7.2 Routine Infection Control Procedures

Primary prevention of *C. difficile* also includes good routine infection control practices. The use of isolation techniques (enteric precautions, private rooms, and cohorting of infected patients) has been employed for outbreak control (Worseley, 1998). The practice of cohorting patients when there are no available side-rooms may lead to reinfection from others members of the cohort or from a heavily contaminated

ward. Handwashing remains an essential infection control measure as poor handwashing plays a key role in the transmission of infection in the hospital setting (Johnson *et al.,* 1990). The literature shows evidence for poor compliance with this relatively simple task. There has been much debate about the merits of differing handwashing agents and the use of gloves. A study by Bettin *et al.* (1994) compared the effectiveness of liquid soap with that of Hibiscrub (4% chlorohexidine gluconate in 4% alcohol, Zeneca Pharma, UK) for decontaminating bare hands or gloved hands inoculated with an epidemic strain of *C. difficile*. The results showed no significant difference in residual counts on bare hands but on gloved hands the residual counts on bare hands were higher following a chlorhexidine wash than a soap wash. Enteric precautions should be adopted for all patients with diarrhoea, disposable gloves and aprons should be worn when handling body fluids. The patient also should be educated about basic infection control measures they can take in reducing nosocomial transmission.

1.7.3. Environmental Cleaning and Disinfection

C. difficile infection may arise from endogenous carriage or exogenous acquisition into the gastrointestinal tract. There is much evidence documenting the contamination of the hospital environment with *C. difficile*, so, primary prevention measures should be targeted towards it (Savage and Alford., 1983). Environmental contamination has been linked to spread of *C. difficile* via a contaminated commode chair; nursery baby baths and contaminated rectal thermometers (Larson *et al.,* 1982; Savage and Alford, 1983; Brooks *et al.,* 1992). The risk of transmission via contaminated endoscopes

appears to be low if scopes are properly cleaned and disinfected using 2% alkaline glutaraldehyde immersion for as little as 5 minutes (Hughes *et al.*, 1986). Studies have shown that the rate of environmental contamination rises in proportion to the status of the patients in the area, being lowest for rooms of culture negative patients (<8%), intermediate for rooms of asymptomatic patients (8-30%), and highest (9-50%) for rooms with patients with AAD (Gerding *et al.*, 1995). Ideally, ward rounds should begin with patients in the "clean" area, i.e. non-infected patients, and finish with infected patients.

There is a paucity of data regarding the efficacy of different cleaning agents and disinfectants for *C. difficile*. The American College of Gastroenterology recommends that instruments and contaminated surfaces in rooms of patients with *C. difficile* diarrhoea should be disinfected using alkaline gluteraldehyde, sodium hypochlorite, or ethylene oxide (Fekety, 1997). The UK guidelines recommend detergent-based hospital cleaning agents to remove environmental *C. difficile* (Department of Health and Public Health Laboratory Service, 1994), but findings from a study by Wilcox and Fawley (2000) imply that routine cleaning with detergent is often unsuccessful at removing *C. difficile* from the environment. Sporulation of the UK *C. difficile* epidemic strain, which produces significantly more spores than non-prevalent strains, was further enhanced when cultured in faecal emulsions exposed to non-chlorine-based hospital cleaning agents compared with two chlorine-releasing agents (Wilcox and Fawley, 2000). These findings question the UK guidelines as the use of detergent

cleaning agents may actually increase the environmental persistence of some *C. difficile* strains.

1.8 Bacteriocins

Amongst the variety of toxins produced by bacteria, some act against other bacteria. Those that do go under the umbrella term of bacteriocins and consist of a diverse collection of molecules ranging from short peptides to large 80-kDA proteins (James *et al.*, 1992). Bacteriocins are one of the most abundant and varied classes of antimicrobial molecules, having been detected in all major lineages of Eubacteria and in all Archaebacteria (James *et al.*, 1991). They are potent, often highly specific toxins that are usually produced during stressful conditions and result in the rapid elimination of neighbouring cells that are not immune or resistant to their effect. It has been proposed, given their narrow spectrum of activity, that their primary role is to mediate intraspecific, or population-level, interactions while causing little or no harm to the host bacterium due to posttranscriptional modification and/or specific immunity mechanisms (Riley and Gordon, 1999).

The state of bacteriocin classification requires constant review as the knowledge concerning various aspects of bacteriocin research rapidly accumulates and it appears that the term bacteriocin has been used to cover a wide range of chemically diverse substances that do not necessarily have much in common. One class of bacteriocins, the colicins, produced by *Escherichia coli*, has been the focus of numerous studies that suggest that colicins play a key role in mediating *E. coli* population dynamics. (Riley and Gordon, 1999). A variety of antimicrobial peptides are produced by lactic-

acid bacteria (LAB) and include Class 1 (lantibiotics) and Class 11 (small heat-stable non-lanthionine-containing peptides). The prototype LAB bacteriocin, nisin, was first discovered by Rogers (1928) when he observed metabolites of *Streptococcus lactis (*now reclassified as *Lactococcus lactis*) that were inhibitory to other LAB. The successful commercial application of nisin in the preservation of a number of processed foods and the growing concerns of the approaching "Post-Antibiotic Era" has generated considerable interest in developing potential novel applications for bacteriocins.

1.8.1 Nature and distribution

Hundreds of bacteriocins have been described in the past-century. These peptides can be differentiated into those, which are non-ribosomally synthesized (such as the gramicidins and bacitracins) and ribosomally synthesized (natural) peptides. Bacteriocins have a wide variety of sequences and structures but certain features are common (Hancock and Lehrer; 1998). These peptides are generally 12-50 aminoacids in length, tend to have a net positive charge due to an excess of basic over acidic residues and contain around 50% hydrophobic aminoacids. They fold into three-dimensional amphiphilic structures due to the presence of disulphide bridges. Thus, their hydrophilic domains are well separated from the hydrophobic domains. Bacteriocins fit into four structural classes which include β-sheet molecules stabilized by two or more disulfide bonds, amphipathic α-helices extended molecules, and loops due to a single disulphide bond (Hancock, 2001). The β-sheet and α-helical molecules are by far the most common in nature.

1.8.2 Mechanism of Action

The mechanisms by which bacteriocins act has become a complex issue. It can be difficult to predict either the activity of a bacteriocin or the secondary structure it will form by the amino acid sequence alone. Numerous model membrane studies all observe that the interaction of these peptides with the bacterial cytoplasmic membrane is an essential step in the peptides' bactericidal activity (Piers *et al* 1993, Oren and Shai, 1998) and a variety of hypotheses have been postulated to explain bacterial killing. Bacteriocins act by either permeabilizing the outer cell membrane of Gram-negative bacteria and disrupting its integrity, or having crossed the cell membrane (or the thick cell wall in Gram-positive bacteria) it has been proposed that they act on the nucleic acids of bacteria or trigger autolysis (Wu *et* al., 1999). A number of peptides have been shown to bind to lipopolysaccharide (LPS) and to permeabilize the outer membrane to self-promote uptake into Gram-negative bacteria. The affected outer membrane is thought to develop transient "cracks" which permit the passage of a variety of molecules, including the uptake of the peptide itself. After these initial interactions, the mode of bacterial killing is not clear. Many peptide molecules will then insert into the cytoplasmic membrane interface and are proposed to either aggregate into a micelle-like complex which spans the cytoplasmic membrane or flip-flop across this membrane under the influence of the large transmembrane electric potential gradient (Falla *et al.*, 1996). The micelle-like aggregates are proposed to have water associated with them, and this provides the channels for the movement of ions across the membrane and possibly leakage of large

water-soluble molecules. These aggregates will dissociate into monomers that may be disposed at either side of the membrane. The net effect is that some monomers will be translocated into the cytoplasm and can dissociate from the membrane and bind to cellular polyanions such as DNA and RNA (Hancock and Chapple, 1999). The bactericidal effect of bacteriocins tends to be extremely fast and it is difficult to monitor the stages of bacterial killing. It is important to understand how these peptides act to fully exploit their potential use as antimicrobial agents

1.9 Biofilms

C. difficile, being associated with AAD, is a major health problem, especially in the elderly, hospitalized patient. Contamination of the ward environment with spores of the organism, and the difficulty in their eradication, highlight the importance of *C. difficile* as an infection control issue. Most bacteria can grow as biofilms, and it is known that the growth characteristics and antibiotic susceptibility patterns of bacteria in biofilms can often be very different to those found in bacteria grown under standard laboratory conditions (Gander 1996; Donlan, 2001). It is recognized that, in the complex ecosystem of the colon, bacteria grow in biofilms and bacterial biofilms can cause environmental problems (MacFarlane *et al.*, 1997). A biofilm system seems appropriate to study the more natural state *C. difficile* in the outside environment and the colon.

1.9.1 Definition of Biofilms

It has long been appreciated that bacteria can adhere to solid surfaces and form a slimy coat, yet the fact that the biofilm mode of existence, with sessile bacteria, constitutes a major component of the bacterial biomass in many environments was only accepted in the 1970s. Biofilms have been described in many systems since Van Leeuwenhoek examined the "animalcules" in the plaque on his own teeth in the seventeenth century. The definition of biofilms has changed over the last 25 years with the increasing understanding of the genetic and molecular basis of this bacterial community. Marshall (1976) noted the involvement of "very fine extracellular polymer fibrils" that anchored bacteria to surfaces. Costerton *et al.* (1999) observed that communities of attached bacteria in aquatic systems were found to be encased in a "glycocalyx" matrix that was found to be polysachharide in nature, and this matrix material was shown to mediate adhesion. Characteristics of spatial and temporal heterogeneity and involvement of inorganic or abiotic substances held together in the biofilm matrix was defined by Characklis *et al.* (1990). Further studies showed that adhesion of bacteria to a surface triggered expression of genes controlling production of bacterial components necessary for adhesion and biofilm formation (Costerton *et al.*, 1995). The current definition of a biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced and through which channels for the circulation of nutrients are found, and exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan and Costerton, 2002).

1.9.2 Formation of Biofilms

Biofilms are universal and virtually any surface – animal, mineral, or vegetable (i.e. biotic or abiotic) – is suitable for bacterial colonization and biofilm formation. Substrata for biofilm formation vary greatly from very hydrophobic materials, various plastics, latex, and silicone, to highly charged hydrophilic materials such as glass and various metals. The characteristics of the substratum may have a significant effect on the rate and extent of attachment by microorganisms (Donlan and Costerton, 2002). Generally, rough hydrophobic surfaces develop biofilms more rapidly. The characteristics of the cell surface are equally important as the presence of flagella, pili, fimbriae, or glycoclayx may impact on the rate of microbial attachment (Mittleman *et al.*, 1992). Bacterial biofilms are formed from individual planktonic cells in a complex developmental process. The life cycle of a biofilm involves the transition from individual free-swimming cells to communities of bacteria adhered to a surface, followed by a subsequent return to a planktonic existence.

The process of biofilm formation can be divided into 3 steps: primary bacterial adhesion, secondary bacterial adhesion, and biofilm maturation (Costerton, 1999). The process of bacterial adhesion is dictated by a number of variables, including the species of bacteria, surface composition, environmental factors, and essential gene products. Primary adhesion involves the meeting of a planktonic microorganism and a conditioned surface. The organism is either propelled randomly or in an organized fashion (by chemotaxis and motility) towards the respective surface. Adhesion will ensue dependent on the net sum of the attractive or repulsive forces generated

between the organism and surface. These forces include electrostatic and hydrophobic interactions, steric hindrance, van der Waals forces, temperature, and hydrodynamic forces (Carpentier and Cerf, 1993).

The second stage of adhesion is the anchoring phase and employs molecularly mediated binding between specific adhesives and the surface (An *et al.*, 2000). Loosely bound organisms consolidate the adhesion process by producing exopolysaccharides that complex with surface materials and/or receptor-specific ligands located on pili and fimbriae, or both. Adhesion becomes irreversible in the absence of chemical or physical intervention, and the organisms become firmly attached to the surface (Dunne, 2002). Planktonic organisms can also stick to each other or different species of surface bound organisms, forming aggregates on the substratum.

Once bacteria have irreversibly attached to a surface, the process of biofilm maturation begins. The density of the biofilm increases as the surface-bound organisms begin to actively replicate (and die) and extracellular polymeric substances (EPSs) such as generated by attached bacteria interact with organic and inorganic molecules in the immediate environment to generate the glycocalyx. These EPSs consist primarily of polysaccharides and can be detected microscopically and by chemical analysis. They are highly hydrated (98% water) and bind tenaciously to the underlying surface. The structure of the biofilm is not merely a homogenous monolayer of slime but is heterogeneous with water channels throughout.

Microscopic images of biofilms reveal a structural complexity characterized by towers and mushroom-like shapes interspersed with open channels (de Beer *et al.*, 1994). Growth of the biofilm is dependent on many factors including the availability of nutrients to the biofilm, the removal of waste, internal pH, oxygen perfusion, carbon source, and osmolarity. The biofilm reaches a dynamic equilibrium whereby outermost organisms are shed to become planktonic cells, which are free to colonize other surfaces, and innermost organisms become quiescent or die. Stickler and Hughes (1997) have suggested that biofilm formation might be regulated at the level of population density-dependent gene expression, controlled by cell-to-cell signaling molecules such as acylated homoserine lactones.

1.9.3 Biofilms' Resistance to Antibiotics and Biocides

The development of a biofilm is a very effective survival strategy for bacteria. Biofilms growing in natural and industrial environments are resistant to bacteriophage, to amoebae, and to chemically diverse biocides used in industrial processes (Costerton *et al.*, 1987). Sessile bacteria pose many problems in medicine as they can withstand host immune responses, and are less susceptible to antibiotics than their nonattached individual planktonic counterparts (Nickel *et al.*, 1985). A β-lactamase-negative strain of *Klebsiella pneumoniae* had a minimum inhibitory concentration of 2μg/ml. with ampicillin in aqueous suspension. The same strain, when grown as a biofilm, was scarcely affected by 4 hour treatment with 5000μg/ml. ampicillin, a dose that eradicated free-flowing bacteria (Anderl *et al.*, 2000). The effect on susceptibility may be intrinsic (i.e., inherent in the biofilm mode of growth)

or acquired (i.e., caused by the acquisition of resistance plasmids). At least 3 reasons are suggested for the intrinsic antimicrobial resistance of biofilms. The first hypothesis is the failure of an antibiotic to penetrate the full depth of the biofilm. EPSs retard diffusion either by chemically reacting with the antimicrobial molecules or by limiting their rate of transport. Hoyle *et al.* (1992) showed that the EPSs of *Pseudomonas aeruginosa* was capable of binding tobramycin; dispersed cells were 15 times more susceptible to this agent than were cells in intact biofilms. Antibiotics have been shown to penetrate biofilms readily in some cases and poorly in others, depending on the particular agent and biofilm (Hoyle *et al.*, 1992). The second hypothesis depends on an altered chemical microenvironment within the biofilm. Studies using microelectrodes have shown that oxygen can be completely utilized in the outermost layers of a biofilm, such that deeper layers become anaerobic. Aminoglycoside antibiotics are less effective against the same microorganism in anaerobic than in aerobic conditions. Some bacteria in a biofilm experience nutrient limitation and exist in a slow-growing or quiescent state (Zhang and Bishop, 1996). As many antibiotics target rapidly dividing cells, these organisms manage to evade cell death.

A third speculative reason for reduced susceptibility is that a subpopulation of micro-organisms in a biofilm forms a unique, and highly protected phenotypic state (Stewart and Costerton, 2001). This phenotype is not a response to nutrient limitation; it is a biologically programmed response to growth on a surface. This hypothesis is lent support by findings from studies that show resistance in newly formed biofilms, even

though they are too thin to pose a barrier to the penetration of either an antimicrobial agent or metabolic substrates (Das *et al.*, 1998; Cochrane *et al.*, 2000).

A novel hypothesis has recently been postulated by Lewis (2001) for antimicrobial resistance in biofilm mode of growth and it relates to the potential of damaged bacterial cells to undergo apoptosis or programmed cell death. Lewis suggests that lysis of cells after bactericidial agents is secondary to a programmed apoptosis and not a direct result of the antibiotic. It is postulated that biofilm cells may be defective in programmed cell death and may persist, albeit damaged, during therapy (Broun *et al.*, 2000). After removal of the antibiotic, the damaged cells proliferate, engendering recalcitrance on the biofilm community.

With regard to acquired resistance, research has shown that plasmids can be exchanged in biofilms under many conditions. Ehlers and Bouwer (1999) demonstrated plasmid transfer by conjugation between different Gram-negative bacteria growing in biofilms. The transfer rates of plasmids horizontally were several orders of magnitude higher in biofilms than in liquid culture of the same organisms. This may be a result of the greater probability of contact between cells and the negligible effect of shear forces in either disrupting cell-to-cell contact or damaging the pili required for conjugation.

Standard susceptibility testing, which challenges planktonic cells with an antimicrobial agent, will not accurately predict the efficacy of an agent against

biofilm-associated organisms. Neither the NCCLS or BSAC have an approved method for evaluating the effectiveness of antimicrobial agents against biofilm-associated organisms.

In order for a biofilm system to have a use in routine antimicrobial susceptibility testing, it must be able to fulfill criteria including reproducibility, provision of a meaningful range of results, minimal risk of contamination to system, have a simple system for delivery of media to, and collection of effluent from all biofilms (Struthers, 2000). A variety of methods have been used to test several antimicrobial agents against biofilms of microorganisms (Table 2). A concern with any of these biofilm susceptibility testing methods is how closely they can approximate to an *in vivo* or an *in situ* biofilm.

Apparatus	Organism(s) Tested	Flow dynamics	Substratum	Method for removing and quantifying biofilm	Reference
Modified Robbins device	*P.pseudomallei*	Batch/mixing	Silastic disks	Not given.	Vorachit *et al.*, 1993
Calgary biofilm device	*P. aeruginosa, S.aureus, E.coli*	Batch/mixing	Plastic pegs	Viable count Sonicate peg, then viable count	Ceri *et al*, 1999
Disk reactor	Gram-negative bacteria	Batch/mixing	Teflon coupons	Sonicate, vortex, homogenize, then viable or direct count	Donlan *et al*, 1999
CDC reactor	Gram-negative Bacteria	Continuous/open	Needleless connectors	Sonicate, vortex, homogenize, then count	Murga *et al*, 2001
Sorbarod filter model	*P.aeruginosa S.pneumoniae*	Continuous/open	Cellulose-acetate filters	Vortex in ringers, then viable count	Struthers, 2000
Model bladder	Gram-negative bacteria	Continuous/open	Urinary catheters	Direct exam^n by Scanning or Transmission Electron Microscopy	Stickler and Hughes, 1999

Modified from Donlan and Costerton, 2002

Table 2. Apparatuses that have been used for growing and testing biofilms

1.9.4 Biofilms and Infection

It can be appreciated that the environments suitable for micro-organisms to colonize and establish biofilms are practically endless. To the benefit of human beings, they confer colonization resistance to infection, assist the absorption of nutrients from the bowel and are critical to biodegradation. Biofilms are, however, more often associated with problems. They not only cause oral disease such as dental caries and

peridontitis but also are strongly implicated in the pathogenesis of native valve endocarditis and cystic fibrosis (Donlan and Costerton, 2002). A biofilm link has also been suggested for otitis media and chronic prostatitis (Nickel *et al.*, 1994; Donlan and Costerton, 2002). Infections of implanted devices, industrial biofouling and public health issues related to food hygiene and provision of potable water are also associated with biofilms (Costerton *et al.*, 1987; Carpentier and Cerf, 1993). Biofilms of medical devices have been extensively studied over the last 20 years and infections of prosthetic heart valves, central venous catheters, urinary catheters, contact lenses, intrauterine devices, and dental unit water lines are well described (Donlan and Costerton, 2002). Research has elucidated the susceptibility of various materials, such as urinary catheters and contact lenses, to bacterial adhesion and biofilm formation.

Biofilm infections share clinical characterisitics. They develop preferentially on inert surfaces, or on dead tissue, and occur commonly on medical devices and fragments of dead tissue such as sequestra of dead bone (Lambe *et al.*, 1991); they can also form on living tissues, such as in the case of infective endocarditis. Biofilms grow and produce overt symptoms slowly. Antigens are released from biofilms and these stimulate antibody production (Cochrane *et al.*, 1988). This host immune response is ineffective in killing bacteria within the biofilm and may cause immune complex damage to surrounding tissues. Bacterial biofilms are a common cause of persistent infections, which show recurring symptoms, after cycles of antibiotics, until the sessile population is removed from the body. If an indwelling medical device is colonized by a biofilm, the problem will inevitably get worse, and the aging biofilm

will become increasingly difficult to treat. If organisms with acquired resistance are present in the biofilm, the probability of resistance-plasmid transfer might increase over time. Biofilms can act as "niduses" of acute infection if host defenses cannot eliminate the planktonic cells that are released at any one time during the infection (Costerton *et al.* 1999).

1.9.5 Biofilm Systems

Studying biofilms in the environment can be difficult because of their heterogeneous nature. Laboratory models can be used to provide more reproducible and defined conditions. In this way a number of different models have been developed to investigate biofilm growth (Buswell *et al.*, 2001). These include (i) flow cells; (ii) channel reactors; (iii) Robbins devices; (iv) rototorque; (v) packed bed reactors; (vi) airlift reactors; (vii) constant depth film fermentors; (viii) chemostat bioreactors and (xi) Sorbarod filter system. There is a vast range of experimental systems with which to develop biofilms, and the model used depends on the nature of the problem that one is investigating. Flow cells using glass are useful when the investigator wants to follow attachment using light microscopy combined with fluorescent dyes for species specificity or viability assays (Caldwell and Lawrence, 1988). Flow regimes in pipe work can be modeled using the Robbins device, which can simulate different scenarios such as urinary catheters (McCoy *et al.*, 1981).

1.9.5.1 Sorbarod Filter System

This is a simple model, which employs the use of Sorbarod filter plugs which are perfused with culture media (Struthers, 2000). Sorbarods are cylindrical cellulose fibre plugs. They are constructed with creped wood cellulose fibre running the length of the filter and wood cellulose wrap. There is a trace of polyvinyl acetate adhesive to seal the ends of the paper wrap. Initially they were used in the horticultural industry for the micropropagation of plant tissue (Conkie, 1988; Donkin and Price, 1989) and are now being adopted in microbiology for research. These cellulose Sorbarod filters provide a large surface area on which bacteria can adhere and grow and enable the rate of growth, expression of target proteins and effect of antibiotics on biofilm mode of growth to be studied (Hodgson *et al.*, 1995). The extensive inter-fibre spacing allows for the free flow of liquid media thus helping to prevent blockage of the system. The system can employ a 12 channel peristaltic pump, enabling 12 Sorbarod filters to be run in one experiment (Struthers, 2000). Hodgson *et al.* (1995) employed this biofilm technique for long-term culture and growth rate of *S. aureus* and *P. aeruginosa*. These organisms established steady states which were stable over several days at which the growth was reproducible, measurable and slower than in broth culture. Significant differences between the protein profiles of biofilms and the planktonic populations of *S. aureus* were demonstrated. The Sorbarod filter system has been used for antimicrobial susceptibility testing of biofilms. There are sufficient individual biofilms available in one experiment to cover a meaningful range of antibiotic concentrations, and it allows for 1 or 2 control biofilms to be run simultaneously. It has been used to investigate the susceptibility of S. *pneumoniae* to

various β-lactam antibiotics; with this organism it was shown that the biofilm mode of growth did not reduce it's susceptibility (Budhani and Struthers, 1997).

The Sorbarod system has also revealed unique structures associated with cell division in *Gardnerella vaginalis*, (Muli and Struthers, 1998) and that *S. pneumoniae* reversibly lost it's capsule when grown on these filters (Budhani *et al.*, 1998; Waite *et al.*, 2001). Unlike growth in batch culture, biofilms of type 3 pneumococci were found to generate small acapsular colonies at high frequency when plated on to blood agar. A significant proportion of these reverted to typical, large capsular colonies on subculture, although the frequency with which reversion occurred varied.

In this work, the Sorbarod filter system was used to study phenotypic characterisitics of *C. difficile* in the biofilm mode of growth.

Clostridium difficile ranks as one of the most important causes of hospital acquired infection. The Sorbarod biofilm system has been used in the investigation of a range of medically important bacteria. As anaerobes have not been investigated in biofilm, this system has the potential to provide useful information on *C. difficile*, which may be relevant to its survival in the environment and in the colon.

The aims of the study were:

1. To investigate the appropriateness of the Sorbarod filter system for biofilm growth of *C. difficile*.
2. To investigate the morphology of *C. difficile* grown in biofilm, broth and plate cultures to determine if there are any differences in the morphology.
3. To investigate the expression of pathogenic properties (toxins A and B) of *C. difficile* in Sorbarod biofilms and compare this to broth culture.
4. To determine the Minimum Inhibitory Concentrations (MIC), Minimum Bactericidal Concentrations (MBC), and Biofilm Eradicating Concentrations (BEC) of selected antibiotics used in treating *C. difficile* infection.
5. To investigate combination growth of various organisms with *C. difficile* and expose multiple organism biofilms to various antibiotics.
6. To screen stool specimens for bacteriophages and bacteriocins active against *C. difficile* and to characterize these entities where possible.

Chapter Two

2 Materials and Methods

2.1 Organisms and their maintenance

The major organisms used in this study were *Clostridium difficile* (a clinical strain from a patient at Walsgrave Hospital, Coventry, clinical strains from Beaumont Hospital, Dublin, and NCTC strain 11204). Other organisms also used were *Enterococcus faecalis* (clinical strain), *Escherichia coli* (clinical strain), *Bacteroides fragilis* (NCTC 9343), *Streptococcus pneumoniae* (clinical strain), *Listeria monocytogenes* (NCTC 11994), *Staphylococcus epidermidis* (NCTC 11047), *Bacillus cereus* (NCTC 7464), *Haemophilus influenzae* (NCTC 4560), *Neisseria lactima* (clinical strain), *Salmonella poona* (NCTC 4840), *Pseudomonas aeruginosa* (NCTC 10662), *Shigella sonnei* (NCTC 8574), *Klebsiella pneumoniae* (NCTC 9633), *Acetinobacteur baumanii* (clinical strain), *Clostridium perfringens* (NCTC 8237), *Clostridium bifermentans* (NCTC 506), *Clostridium novyi* (NCTC 538), *Clostridium tetani* (NCTC 279), *Clostridium sporogenes* (NCTC 532), *Clostridium butyricum* (NCTC 7432) and *Lactococcus paracasei (*isolated from horse manure).

For the storage of cultures for longterm preservation, a heavy suspension of organisms was inoculated onto beads (Microbank^{TM}, PRO-LAB Diagnostics, Ontario, Canada) which were then stored in a -70^{0}C freezer (Cryo King Ultralow). In order to obtain organisms for daily experimental work, one bead of the desired organism was removed from the –70^{0}C freezer, and subcultured onto 7% Columbia blood agar plates. Subsequent subcultures from beads were performed every 6 weeks.

2.2 Media, reagents and antibiotics

2.2.1 Media used

The commercially acquired agar, media and reagents for this study were Colombia Blood agar, Colombia Agar Base, Colombia Agar Technical (Agar No.3) Base, Colombia *C. difficile* Agar Base, Brain Heart Infusion media, C. *difficile* selective supplements (Oxoid, Basingstoke, Hampshire, UK), and defibrinated horse blood (Bioscience, Buckingham, UK). Media and agar were prepared as per the manufacturer's instructions using deionised water, dispensed into the required volumes and autoclaved at 121^0C for 15 minutes. Brain heart infusion (BHI) broth was used for the growth of *C. difficile* and feedbroth for biofilms. The stock formula of BHI is shown in Table 3. Sloppy agar, (0.5%) and (0.75%), and 1.5% agar base were prepared using Agar No.3 base with BHI media.

Ingredient	gram/Litre
Calf brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
pH 7.4	

Table 3 The stock formula of BHI

2.2.2 Selective Media

C.difficile selective media was prepared by the addition of 7% (v/v) defibrinated horse blood and *C. difficile* selective supplements (D-cycloserine 125mg and cefoxitin 4mg) after the selective agar base media (see Table 4 for stock formula) was autoclaved and cooled to 57^0C. For combination growth experiments, additional selective media were made with Agar Base No. 3 to which various antibiotics were added, depending on the selectivity required (Adatabs, Mast Laboratories Ltd., Merseyside, UK). Aztreonam (3.2mg), gentamicin (0.8mg) and trimethoprim (0.8mg) Adatabs were used.

Ingredients	gram/Litre
Proteose peptone	40.0
Disodium hydrogen phosphate	5.0
Potassium dihydrogen phosphate	1.0
Magnesium sulphate	0.1
Sodium chloride	2.0
Fructose	6.0
Agar	15.0

Table 4. The stock formula of *Clostridium difficile* agar base

2.2.3 Antibiotics

The following antibiotics were used in susceptibility testing, benzylpenicillin, (560:600 potency) (Britannia, Redhill UK), amoxicillin-clavulanic acid (5:1) (SmithKline Beecham, Worthing UK), meropenem (AstraZeneca, Luton, UK), metronidazole (Aventis Pharma, Kent UK), rifampicin (Aventis Pharma), and

vancomycin (Lilly, Basingstoke, UK). All antibiotics used were freshly prepared. The stock solutions were prepared in sterilized deionised water.

2.3 Growth conditions

Unless otherwise stated organisms were usually incubated in an anaerobic incubator (MACs, Don Whitley Scientific Ltd., Shipley, UK) at 37^0C.

2.4 Viable Counts

In order to determine the surface viable counts of bacteria, an adaptation of the technique of Miles *et al.* (1938) was employed. The Sorbarod filter was suspended in 5mL of Ringers' solution (Central Media Supplies, PHLS, Colindale, London, UK), disrupted by agitation using sterile loops and then vortexed. Twenty μL of either this filter suspension or effluent suspension were mixed with 180μL BHI broth in a microtitre well followed by titration in ten-fold serial dilutions in the same broth. Aliquots (10μL) of the appropriate dilutions were plated in triplicate onto Colombia blood agar plates, allowed to dry before inversion, and then plates were incubated anaerobically (or aerobically when selection of *C. difficile* was not desired) at 37^0C for 48h. The titre (colony forming units (cfu) per mL.) was then determined from the mean of the triplicate readings by employing the formula:

$$\frac{\text{Average number of colonies/dilution}}{\text{Volume of inoculum x bacterial dilution}}$$

Titres for biofilm effluent were expressed as cfu/mL, and the biofilm titre was expressed as total recoverable cfu/filter. This was obtained by multiplying the cfu/mL by a factor of 6.57 (accounts for the volume of the 20-mm length x 10-mm diameter filter plus the 5mL of Ringers solution added before vortex mixing).

2.5 Sorbarod Biofilm System

The Sorbarod filter system was assembled according to the method described by Struthers (2000), shown in diagrammatic and photographic representations of the system Fig 1, 2, and 3. Cellulose Sorbarods 10mm in diameter and 20mm in length (Ilacon, Tonbridge, Kent, UK) were placed in 10cm lengths of silicone tubing with an internal diameter of 10mm. These were then connected by tubing (0.8mm internal bore) to a feed bottle of BHI broth (Oxoid, Basingstoke, UK). The effluent end of the Sorbarod filter was connected via plastic adapters to replaceable 150mL sterile glass bottles which enabled collection of effluent fluid at any time interval during the investigation. The entire biofilm apparatus consisting of BHI feedbroth supply, connecting tubes, Sorbarod filters and effluent bottles were then autoclaved at 121^{0}C as a single unit. This minimised the chance of contamination. Each Sorbarod filter was pre-wetted with 3mL of BHI broth before inoculation with 3mL overnight broth culture of *C. difficile*, or other organisms such as *E. coli, E. faecalis, B. fragilis* or *L. paracasei* which were studied in combination experiments.

The BHI feedbroth was delivered by a 12 channel 205U peristaltic pump (Watson Marlow, Falmouth, UK) at a usual flow rate of 4.0mL/h. The whole apparatus with 12 biofilms was placed in an anaerobic incubator at 37^0C. At each assay time point, effluent was collected over 15 minutes so that the number of viable planktonic bacteria was determined. Biofilms were titrated as described previously.

Once the steady state of growth of organisms in biofilm was achieved (Chapter 3), each individual biofilm was exposed to a single antibiotic concentration for 18h for differing antibiotics. This change to antibiotic containing BHI broth was achieved by use of T connector tubing, upstream from the biofilm, enabling a switch from BHI feedbroth to a particular antibiotic concentration made up in 100mL of BHI broth. For each set of experiments, 12 individual Sorbarods were prepared to allow for one control antibiotic free biofilm and sufficient individual test biofilms which were exposed to different concentrations of antibiotics to cover a meaningful range. Each biofilm and effluent with and without antibiotic was titrated in triplicate.

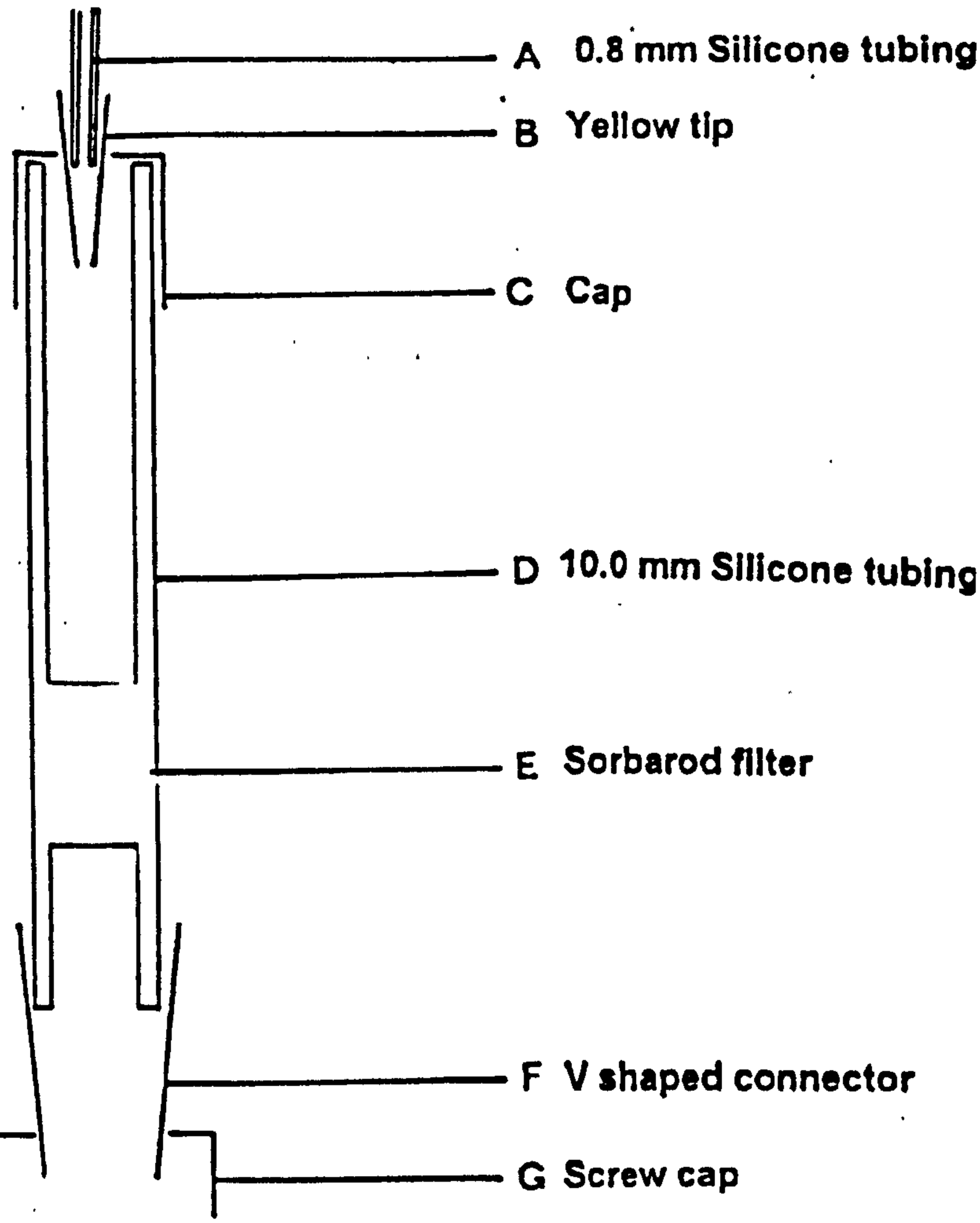

Fig. 1 A diagrammatic representation of the biofilm apparatus showing a 20 x 10mm biofilm in a silicon PVC tubing. Medium was supplied to each biofilm at a flow rate of 6mL/minute and effluent was collected in 150mL replacable bottles.

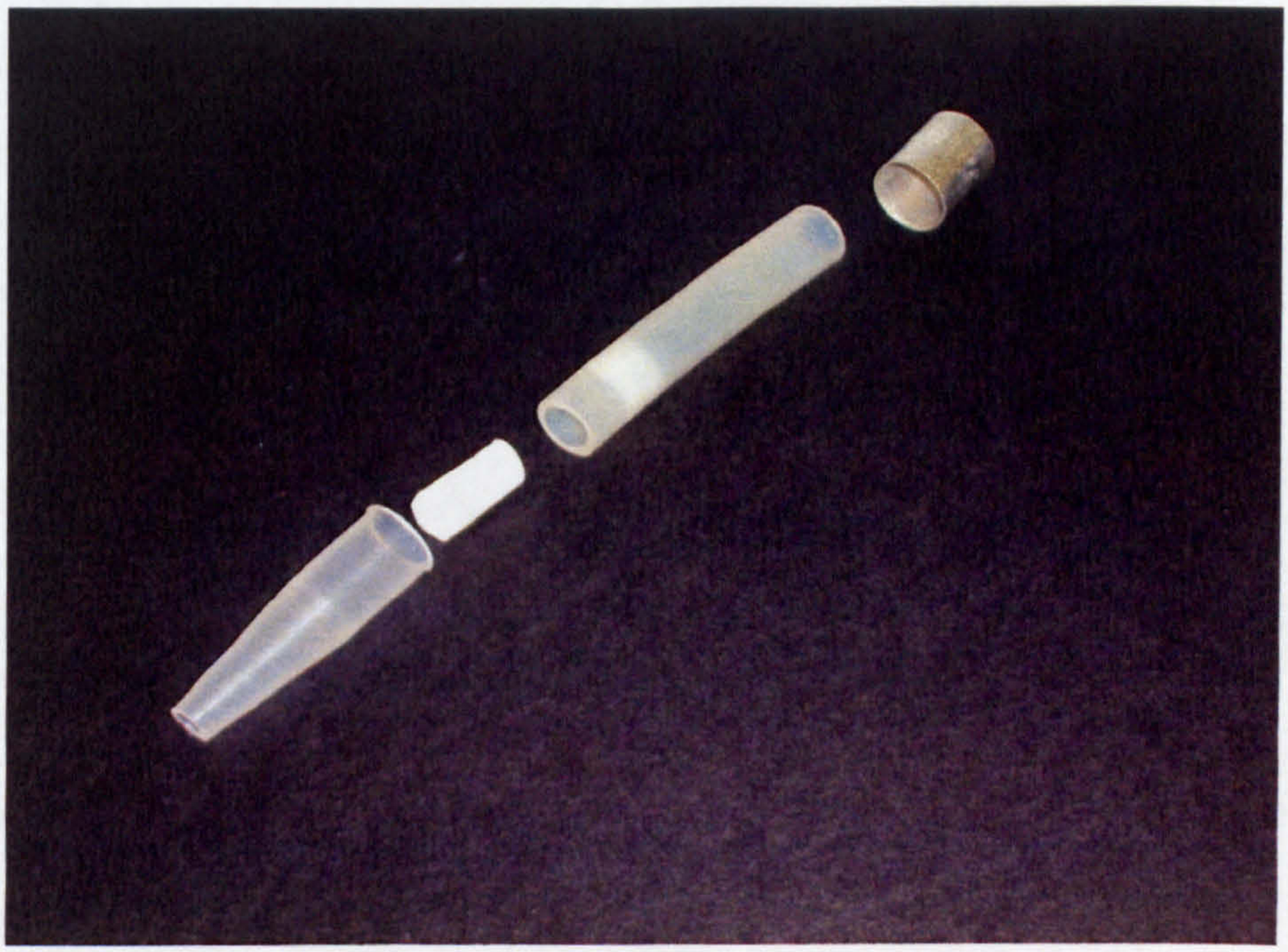

Fig 2. Photograph of Sorbarod filter (10mm x 20mm length) which consists of a paper sleeve encasing compacted concertina of parallel cellulose fibres running the length of the Sorbarod.

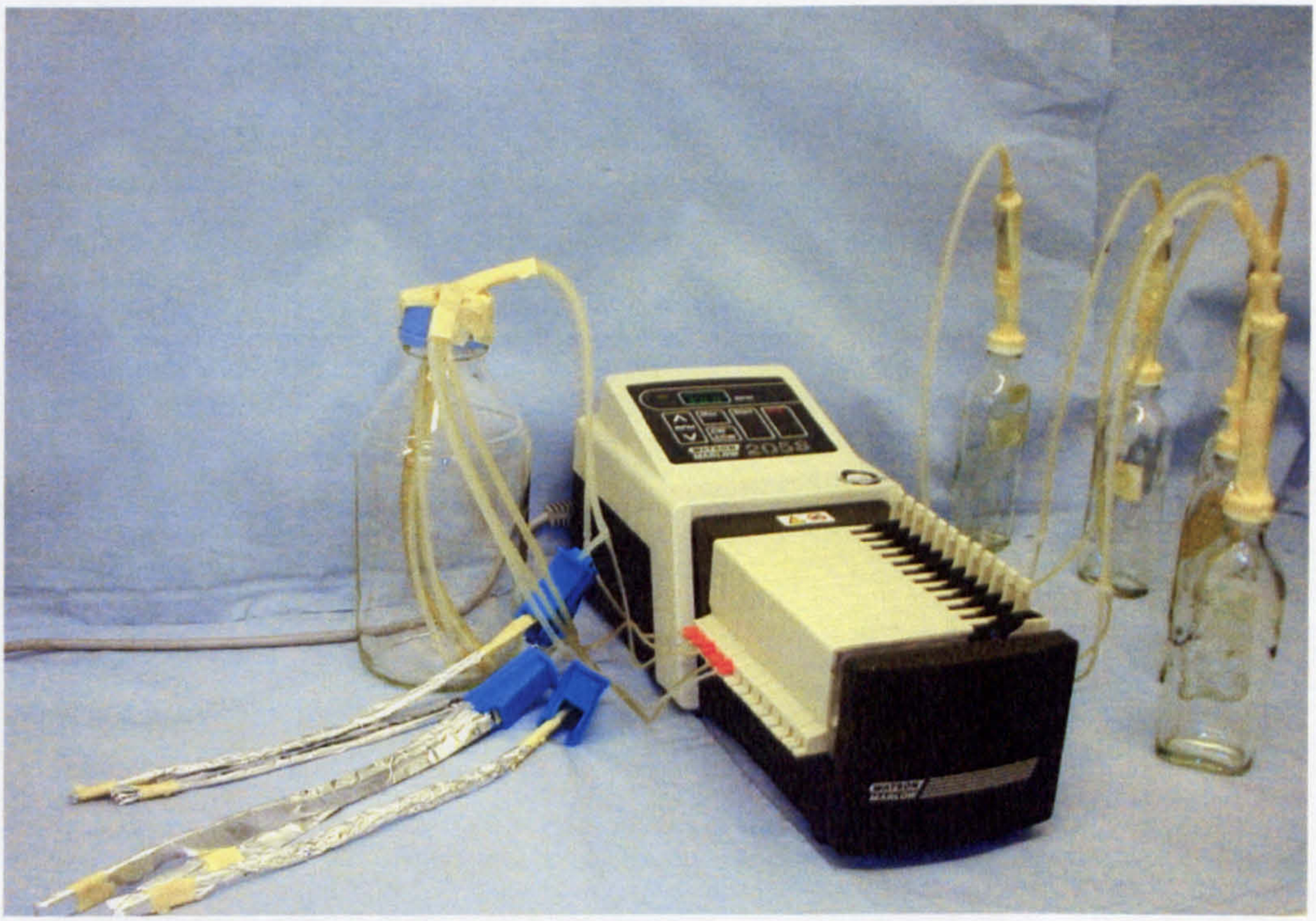

Fig 3. Photograph of the complete biofilm apparatus.

Medium was supplied to each biofilm at a flow rate of 4mL/minute and effluent was collected in 150mL bottles.

2.6 *C. difficile* Enzyme Immunassay for Toxins A/B

Toxin studies were performed on both *C. difficile* broth, biofilm and effluent specimens using an enzyme immunoassay kit (Ridascreen® *Clostridium difficile* Toxin A/B, R-Biopharm AG, Darmstadt, Germany; Table 5). Supernatant specimens of biofilms and effluents, which had been collected at different time intervals from the growth curve experiments, were stored at -70^0C. After thawing and mixing, test samples, positive controls and negative controls were pipetted in fifty µL aliquots into separate wells of the 96 microwell plate (Greiner BIO-ONE, Stonehouse, Gloucestershire, United Kingdom). A drop of biotin conjugate was added and mixed and the plate was incubated for 90 minutes at room temperature. All wells were decanted into a waste container with disinfectant. A total of 4 wash cycles, using 250µL washing buffer was performed. One hundred µL of streptavidin conjugate was then added to the wells and incubated at room temperature for 30 minutes. Washing was then performed 5 times. Finally a third incubation was done for 30 minutes at room temperature in the dark following the addition of 50µL substrate and 50µl of chromogen into each well. The reaction was stopped by adding 50µL of stop solution and the absorbency was measured at 450nm using a bioelisa plate reader (BIO-TEK Instruments, Winoosk, VT, USA).

Microwells coated with monoclonal antibodies against Toxin A and B
Universal stool diluent, containing buffered NaCl (negative control)
Washing buffer, pH 7.2, containing 0.1% thimerosal
Positive control, partially denatured toxin (0.1% sodium azide)
Biotin conjugate, biotinylated antibodies against Toxins A and B
Streptavidin Conjugate (HRP-conjugated)
Substrate, urea peroxide
Chromogen (tetramethylbenzidine)
Stop solution (1M sulfuric acid)

Table 5. Reagents in EIA test kit for *Clostridium difficile* Toxin A/B (Ridascreen®)

2.7 Susceptibility testing

2.7.1 Disk sensitivity testing

Disk sensitivity testing was performed on *C. difficile, E. coli, E. faecalis* and *B. fragilis.* The BSAC susceptibility methodology was used (www.bsac.org.uk). Ampicillin, aztreonam, colistin, gentamicin, rifampicin, trimethoprim and vancomycin were used.

2.7.2 Determination of Tube Minimum Inhibitory and Bactericidal Concentrations

Minimum inhibitory concentration (MIC) is the lowest concentration of antibiotic at which there is no visible growth of an organism (turbidity), while the minimum bactericidal concentration (MBC) is the lowest concentration that prevents growth after subculturing on antibiotic free medium (Lambert *et al.,* 1997). Stock solutions of the various antibiotics (benzylpenicillin (560/600), metronidazole, rifampicin and vancomycin) were prepared. Dilutions were carried out in order to achieve antibiotic concentrations to cover a meaningful range; 32μg/mL – 0.004μg/mL. The dilutions of antibiotic made in BHI broth (1mL) were inoculated in duplicate with diluted (10^{-2}) overnight broth cultures of *C. difficile* isolates (100μL). MICs were determined following overnight anaerobic incubation. Subculture of all non-turbid broths was done to determine the MBC.

2.7.3 Etest Susceptibility Testing

The Etest (Epsilometer) is a technique for quantitative antimicrobial susceptibility testing which allows for the direct determination of inhibitory concentration. In order to control MIC and MBC tests, the Etest (Cambridge Diagnostics Services, Cambridge, UK) was performed according to the manufacturers instructions. Etest strips contain a continuous gradient of antimicrobial agent (discontinuous twofold dilutions) on the underside of the strip, which diffuses out into the media, when the strip is placed on the surface of the agar plate. Inhibition of growth appears in the shape of an ellipse, and the MIC is read where the zone edge or ellipse intersects the Etest strip. From the fresh overnight culture of *C. difficile* on blood agar, a suspension was prepared in 0.85% sterile saline and adjusted to a 1 McFarland barium sulphate turbidity standard. Using a sterile swab, the inoculum was streaked onto a defibrinated horse blood agar plate and incubated anaerobically for 24-48 hours and the plates were then read.

2.7.4 Biofilm Eradicating Concentration (BEC) and Effluent Minimum Bactericidal Concentration (EfMBC)

Sorbarod filters were inoculated, according to the method of Budhani and Struthers (1997), with 3ml of overnight *C. difficile* broth to allow growth to become established for 24h. A single antibiotic concentration (range of 32mg/L – 0.004mg/L, prepared in 100ml of BHI broth) was run into each established Sorbarod biofilm at a flow rate of 4.0mL/h for eighteen hours. After this time effluent was collected for 15 min. in order

to determine the titre of planktonic bacteria. The individual Sorbarod filter was then harvested in 5mL of Ringers solution and vortexed to disintegrate the cellular matrix. Titrations were then performed on both effluents and the disintegrated filters in triplicate by an adaptation of the method of Miles *et al.* (1938) using sterile 96 well microtitre plates (Greiner BIO-ONE, Gloucestershire, UK). The biofilm eradicating concentration (BEC) and the biofilm effluent MBC were those concentrations of an antibiotic that eliminated the organisms from the biofilm filter and effluent respectively.

When titration of combination experiments of two organisms in biofilm were done, selective media was used. When *E. coli* and *C. difficile* were grown together, *E coli* was selected by aerobic growth and *C. difficile* was selected by growth anaerobically on BHI agar containing gentamicin. Enterococci were also selected by growth aerobically but were inhibited by growth on BHI agar containing trimethoprim. *B. fragilis* grew on agar containing vancomycin but not on BHI agar containing aztreonam, allowing for the selection of both *C. difficile* and *B. fragilis* when grown under anaerobic conditions. Selection of *Lactobacillus paracasei* was achieved by growth under aerobic conditions and *C. difficile* was selected by growth on *C. difficile* selective agar (Oxoid, Basingstoke, Hampshire, UK) when experiments were conducted using both organisms.

2.8 Microscopic techniques

2.8.1 Light microscopy

Cell suspensions from broth, biofilm and effluents were examined under the light microscope after they were stained by the Gram method which involves applying ammonium oxalate-crystal violet stain for 30 seconds, washing in water, applying Lugol's iodine solution for 30 seconds, then decolourising with a few drops of acetone before couterstaining with weak carbol fuchsin for 30 seconds and washing under tap water (Lillie, 1928).

These cell suspensions were also stained for spores by Schaeffer and Fulton's method (1933). Fixed slides were flooded with 5% aqueous malachite green and steamed for one minute. After washing under running water, the slides were counterstained with 0.5% aqueous safranin for 15 seconds. The slides were rinsed again with tap water and dried before viewing under the light microscope (Nikon Digitial Net camera DN100).

2.8.2 Transmission Electron Microscopy

Methods used were essentially those described in Kay (1967). In order to prepare samples for Transmission Electron Microscopy, two methods were employed that included the diffusion fixation of bacteria for preparation of bacterial sections and whole cell preparations for negative staining.

2.8.2.1 Preparation of bacteria for sectioning

Bacterial pellets were prepared from plates, broths and biofilms of *C. difficile* harvested at different time intervals. The pellets were resuspended and fixed for 2 hours in 5ml of 2.5% (v/v) glutaraldehyde in 0.1mL sodium cacodylate buffer pH 7.2, containing 0.15% (v/v) ruthenium red (Marrie and Costerton, 1984). Ruthenium red staining allows preservation of EPS matrix (Costerton *et al.*, 1981). After fixing, a pellet was collected after centrifugation and the supernatant discarded. The cells were washed five times in cacodylate buffer, post fixed in 2% osmium tetroxide in cacodylate buffer and dehydrated through a series of 50%, 70% and 90% (v/v) methanol for 10 minutes in each solution, and a further 30 minutes dehydration. All the solutions used in processing the specimens (from the wash after glutaraldehyde fixation to dehydration with 70% (v/v) methanol) contained 0.05% (v/v) ruthenium red. The samples were then infiltrated in equal parts of absolute alcohol and Spurrs resin for one hour. One part absolute alcohol and 3 parts Spurrs resin were infiltrated overnight.

Three fresh changes of 100% resin were made throughout the day and then the sample was embedded in fresh resin in correctly labelled capsules. These were left in the oven at 60^0C to polymerise overnight. 500nm sections of each block were cut on a Reichert Ultracut S microtome and these were mounted onto glass slides. These, so called semi-thin sections were stained with a 1% toluidine blue in 1% borax solution and examined on a light microscope to select appropriate areas for electron

microscope review. Sections of the selected blocks were cut at a thickness of approximately 100nm and mounted onto copper grids, stained with 10% (v/v) uranyl acetate for 20 minutes, washed in multiple changes of distilled water followed by staining in 0.1% (v/v) lead citrate (Reynolds, 1963) and further washes in distilled water. When the sections were completely dry, they were viewed under a JEOL 100CX Transmission Electron Microscope and electron micrographs were produced.

2.9 Screening for bacteriophages and bacteriocin producers

Stools specimens were initially screened for bacteriophages in the hope of identifying a phage that might have potential therapeutic effects in recalcitrant infections. Faecal specimens from multiple sources (farm sewage, goat, sheep, rabbit, cow, and horse) were each suspended in 10ml of Ringefs' solution and were centrifuged (Jouan Centrifuge, St Herblain, France). 0.5% (v/v)and 0.75 % (v/v) BHI sloppy agars were maintained in liquid form in a 50^0C water bath. To 3mL of a sloppy agar, 1mL of each faecal supernatant and 3 drops of an overnight broth of *C. difficile* were added and gently mixed before pouring over a BHI agar plate (1.5%). Plates were then incubated anaerobically for 24 hours and then examined for any lytic effects. No phage plaques were detected. However, clearings around suspected colonies with bacteriocin-like activity against *C. difficile* were observed. Suspect bacteriocin-producers were subcultured onto Colombia blood agar. Further identification was then carried out by Gram staining latex agglutination testing (Oxoid) and by an API 20 Strep (bioMérieux sa, Marcy-l'Etoile/France). Reference laboratory facilities (CPHL, Colindale, UK) were also used.

Chapter Three

3.1 Introduction

As noted in Chapter One, *Clostridium difficile*, being associated with antibiotic-associated diarrhoea, is a major public health concern, especially in hospitalised elderly patients (CDSC, 2000). The overuse of antibiotics that predispose to selection of the organism is well recognised (Gorbach, 1999). Contamination of the ward environment with spores of this clostridium, and the difficulty with eradication, highlight the importance of *C. difficile* as an infection control issue (Savage and Alford, 1983). It is recognised that most bacteria are likely to exist in nature within biofilms, a situation that will also include many organisms resident in the human body. MacFarlane *et al.* (1997) discuss growth of organisms in biofilm in the gastrointestinal tract. The human colonic ecosystem is an extremely complex environment comprised of several hundred different strains of bacteria. Their studies were undertaken to determine whether these colonic organisms formed metabolic or genotypically distinct assemblages in the gut microbiota in relation to polysaccharide fermentation. They showed communities of polymer-degrading bacteria and other groups of intestinal anaerobes growing on particulate matter that were essentially similar to those occurring elsewhere in the gut lumen.

As there is no previous report of an anaerobe being investigated in a biofilm mode of growth in the laboratory, the first aim of this work was to determine the growth characteristics of *C. difficile* using the Sorbarod filter system (Struthers, 2000).

3.2 The growth of *Clostridium difficile* in broth culture and biofilm

The growth patterns of the clinical strain of *C. difficile* in broth culture, and on Sorbarod filters, in the biofilm mode of growth, are shown in Figs. 4 (broth) and 5, 6, 7 (filters). In broth culture, maximum titres of about 10^8 cfu/mL were reached within 24 hours. There was a drop of about 1 log. after this period, but the titre then remained stable for at least a further 48 hours. As discussed later, this stable titre was probably due to this clinical isolate sporulating at high frequency. In the filters, the titres were maintained for at least 96 hours, with values of $>10^9$ cfu/mL being achieved. The titre in the filter effluent was in the order of 1 log. lower in most circumstances. There was little variation in the titre in the filters when different speeds were used to deliver the feed broth to the filters. The speed of the pump at 4 rpm equated to a flow rate of about 4 mL/hour, and this speed was used for subsequent experiments. The filters did begin to block from 72h – 96h.

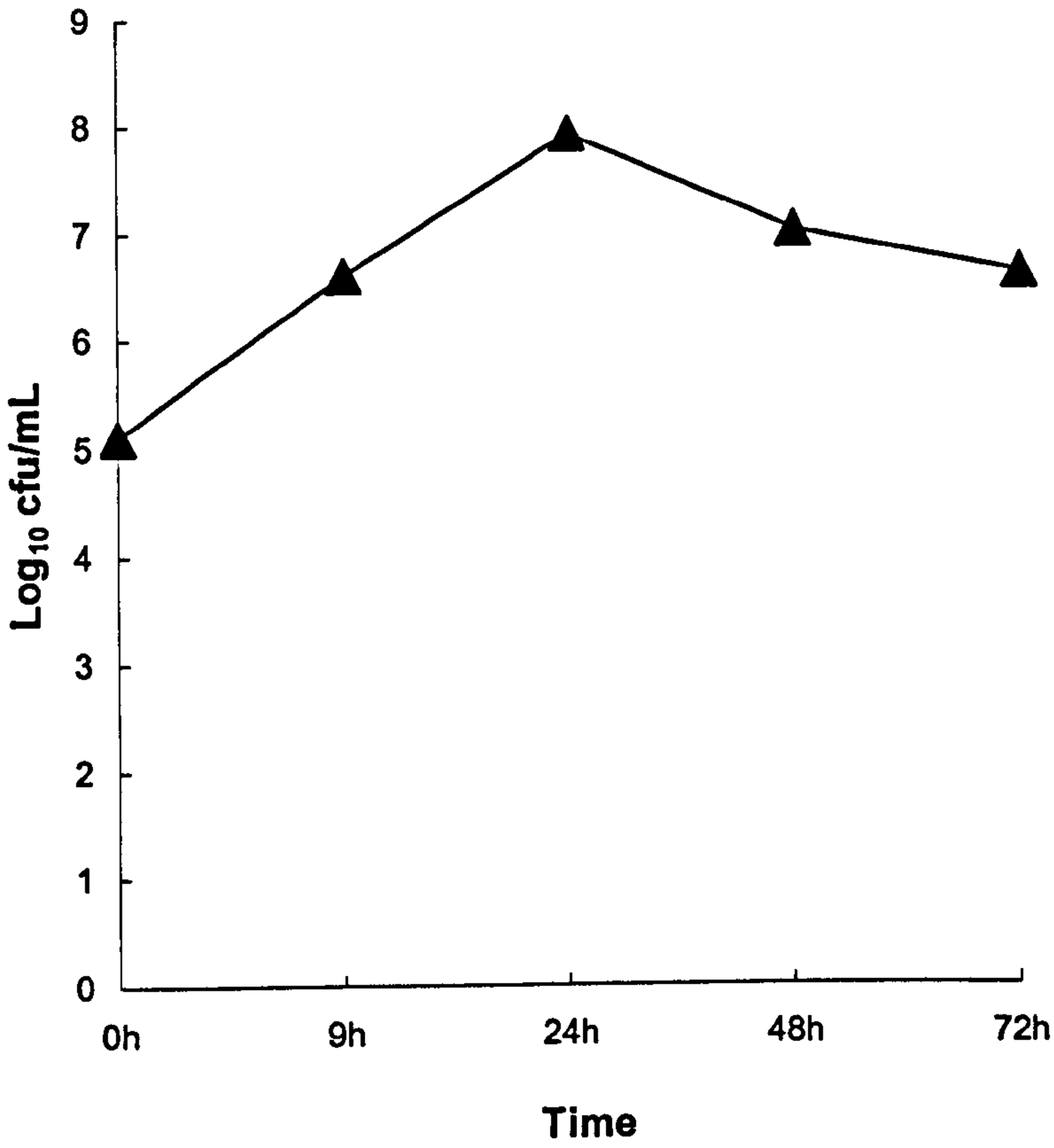

Fig. 4 Growth curve of *C. difficile* (▲) in broth culture

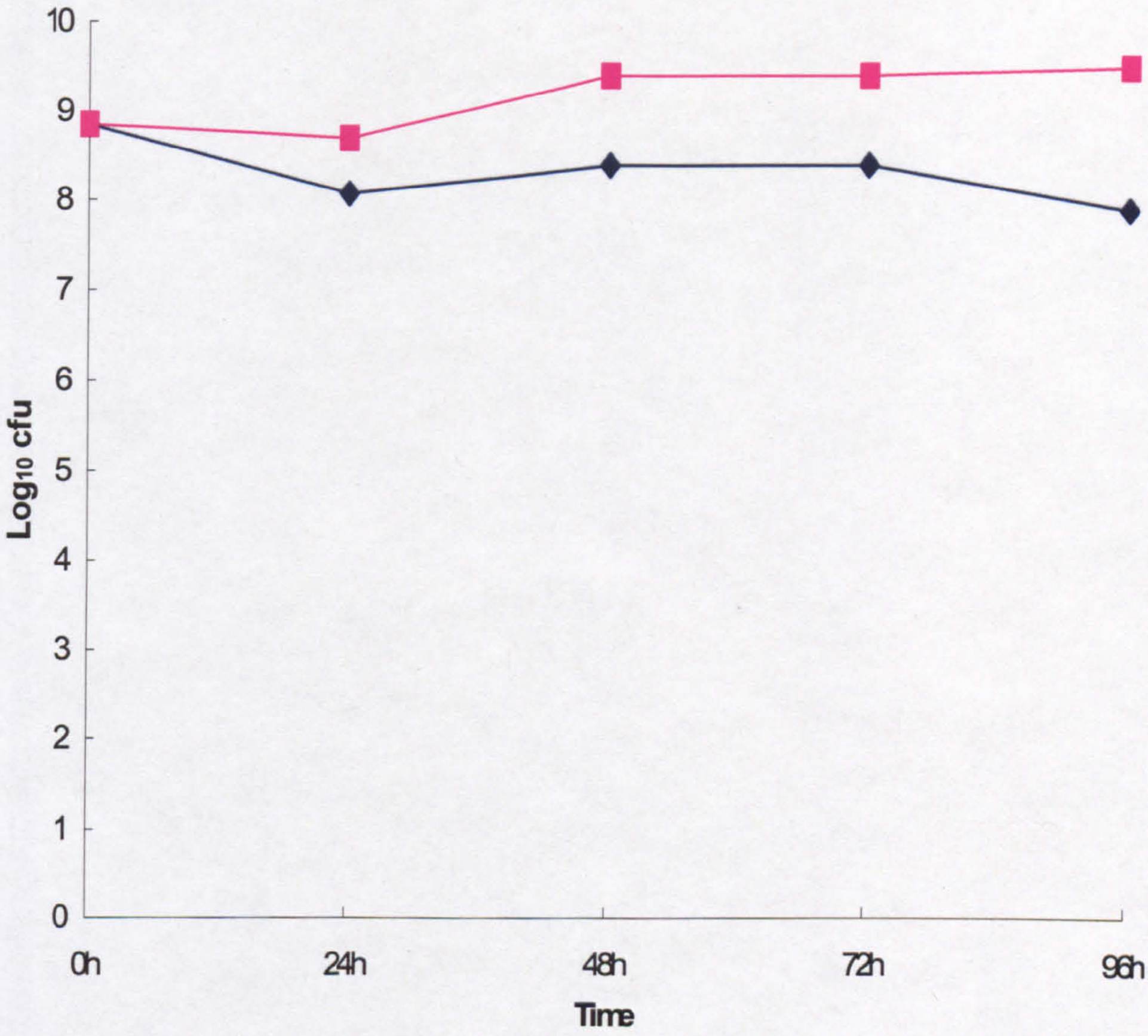

Fig. 5 Growth curve of *C. difficile*; feedbroth of BHI pumped at 2 rpm. Biofilm effluent titres cfu/mL (◆) and biofilm titres cfu/filter (■)

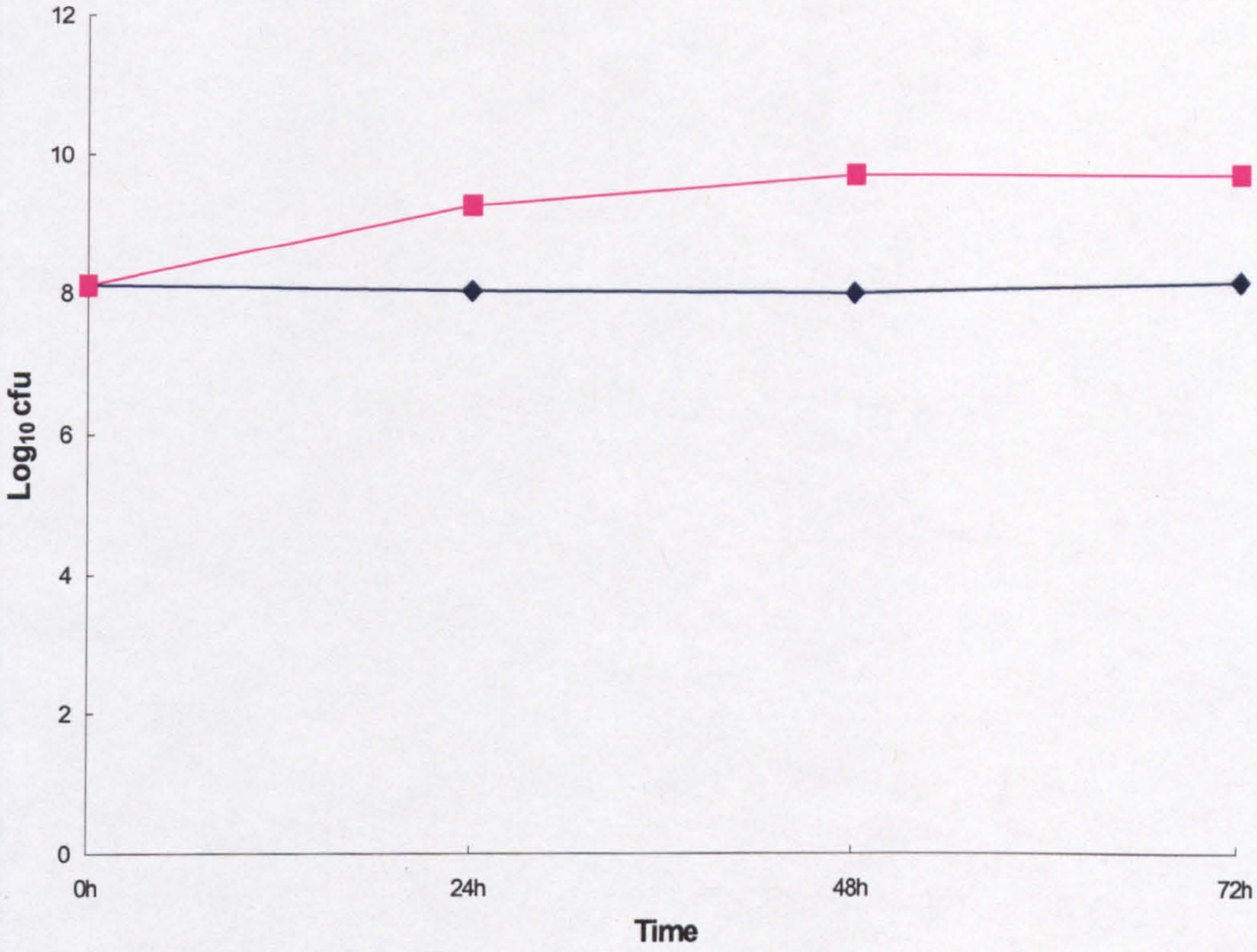

Fig. 6. Growth curve of *C. difficile*; feedbroth of BHI pumped at 4 rpm. Biofilm effluent titres cfu/mL (◆) and biofilm titres cfu/filter (■)

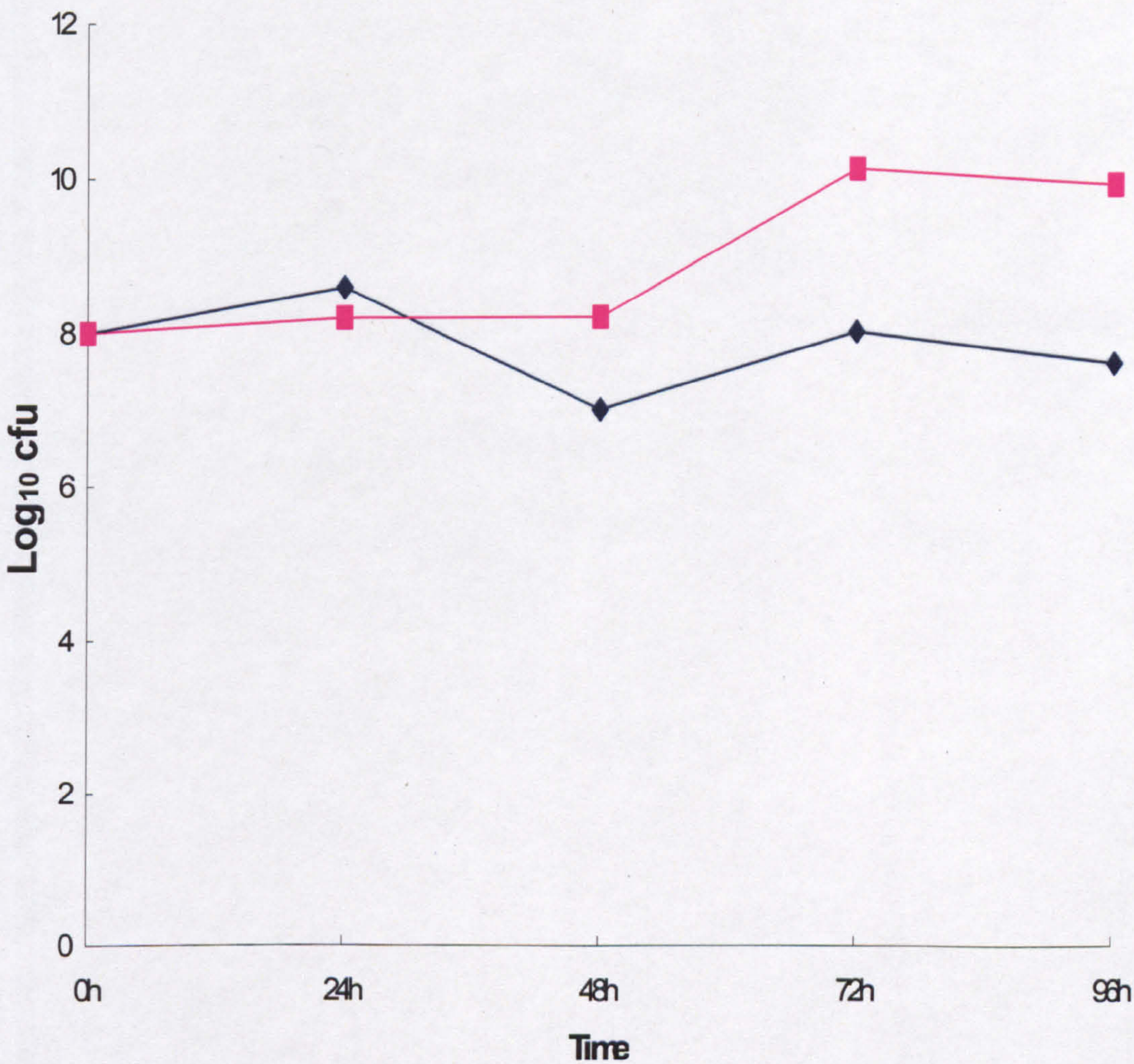

Fig. 7 Growth curve of *C. difficile*; feedbroth of BHI pumped at 6 rpm. Biofilm effluent titres cfu/mL (◆) and biofilm titres cfu/filter (■)

3.3 Light microscopic studies of *C. difficile*

Light microscopy studies revealed several features of the organism, depending on the growth medium used. In broth culture less than 24h in age, the typical rod-shaped structure of the bacterium was seen (Fig. 8). There was some degree of pleomorphism here, with variation in organism length between 1.8 and 4.5μ. By 48h, sporulating bacteria could be identified (Fig. 9). On blood agar plates *C. difficile* likewise exhibited the typical rod-shaped structure (Fig. 10). Here again there was some degree of pleomorphism, with the length of organisms varying from 1.2 to 4.5μ. By 72h, the majority of bacteria had sporulated (Figs. 11, 12).

In biofilm, the bacteria often exhibited a filamentous structure, which were occasionally curled (Fig. 13). The length of the organisms was often in excess of 10 microns. Sporulation was rarely observed.

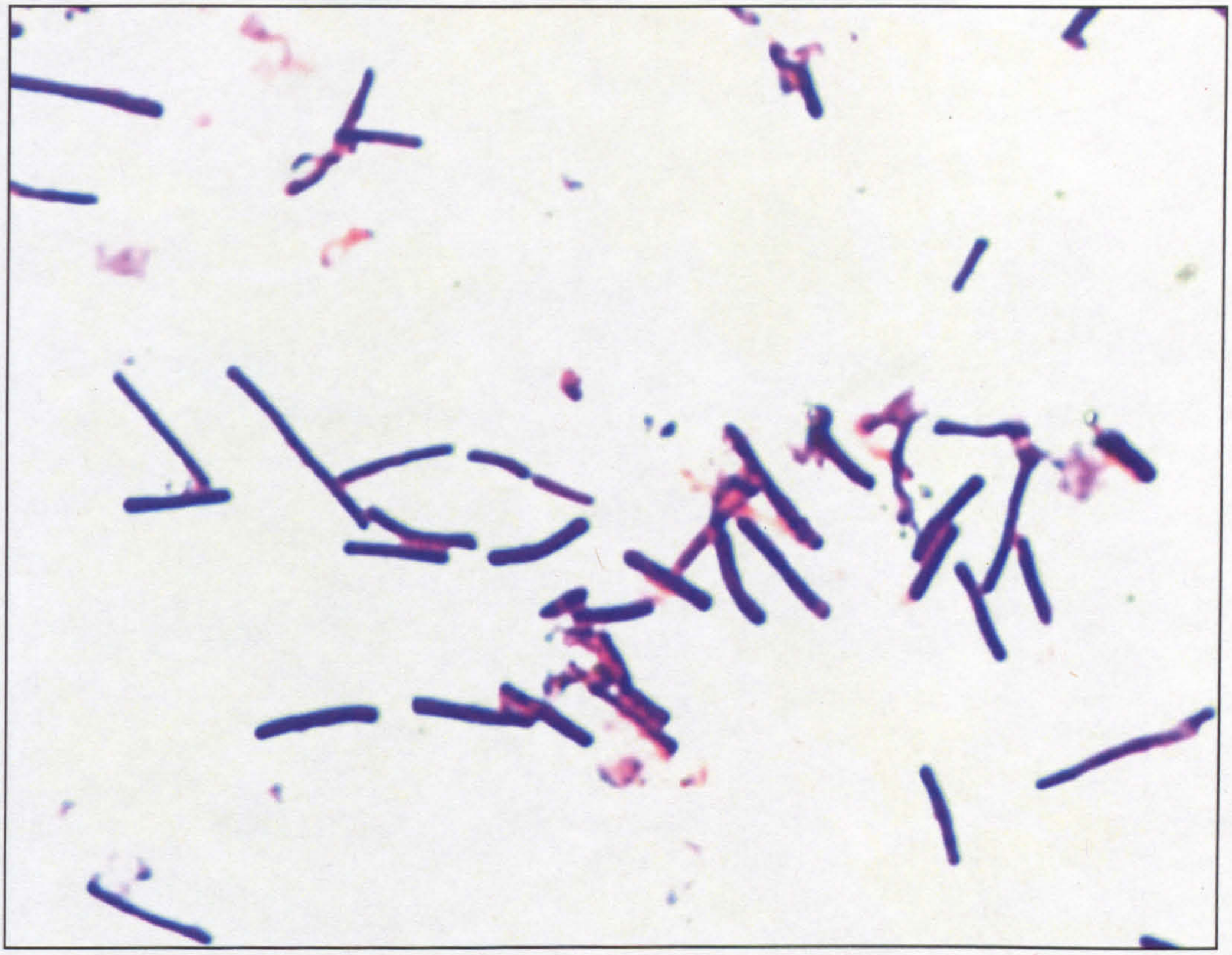

Fig. 8 Gram stain of broth culture at 24h (Scale: 1μm = 4mm)

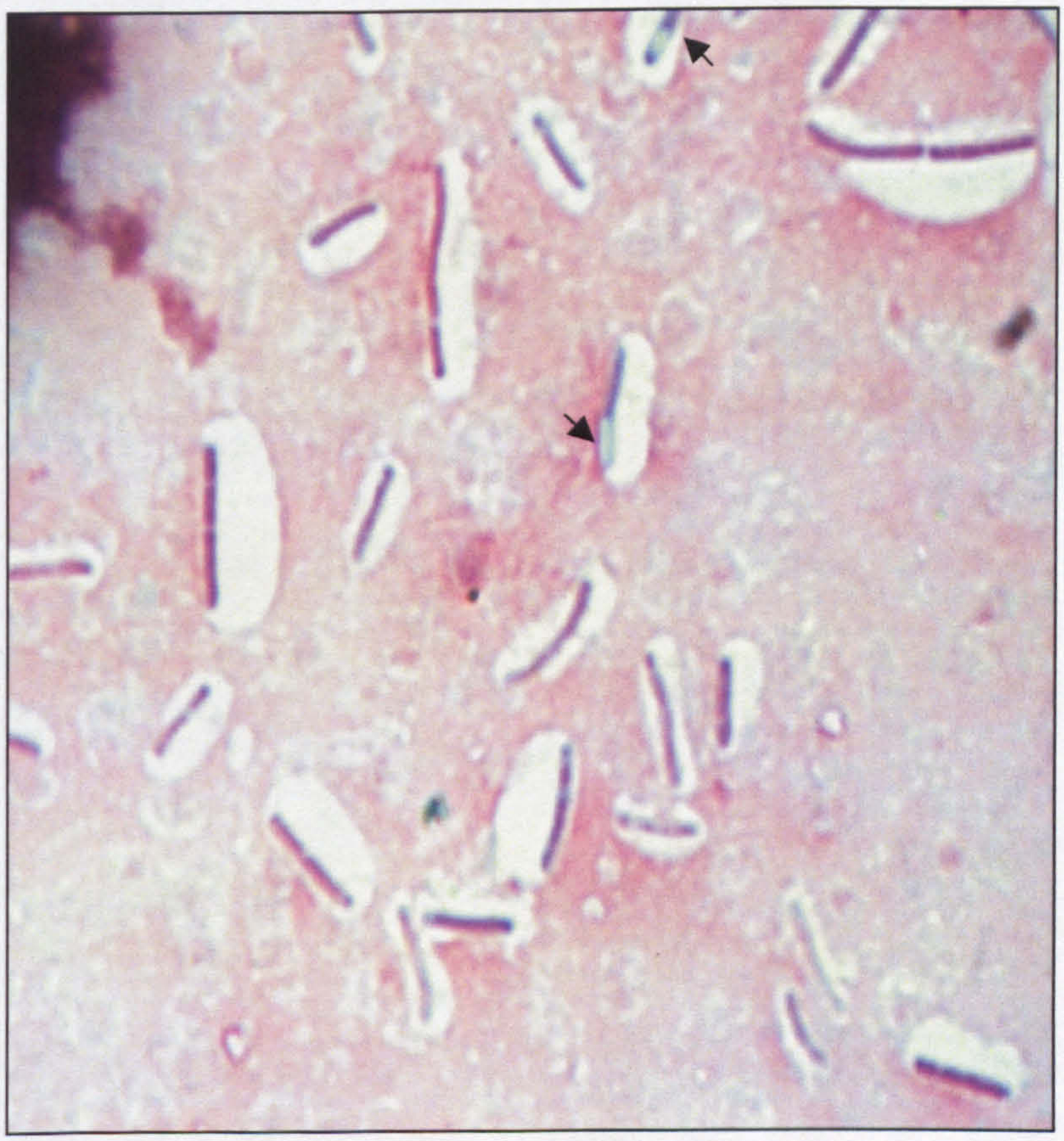

Fig.9 Spore stain of *C. difficile* broth culture at 48h showing spores (▲). (Scale: 1μm = 4mm)

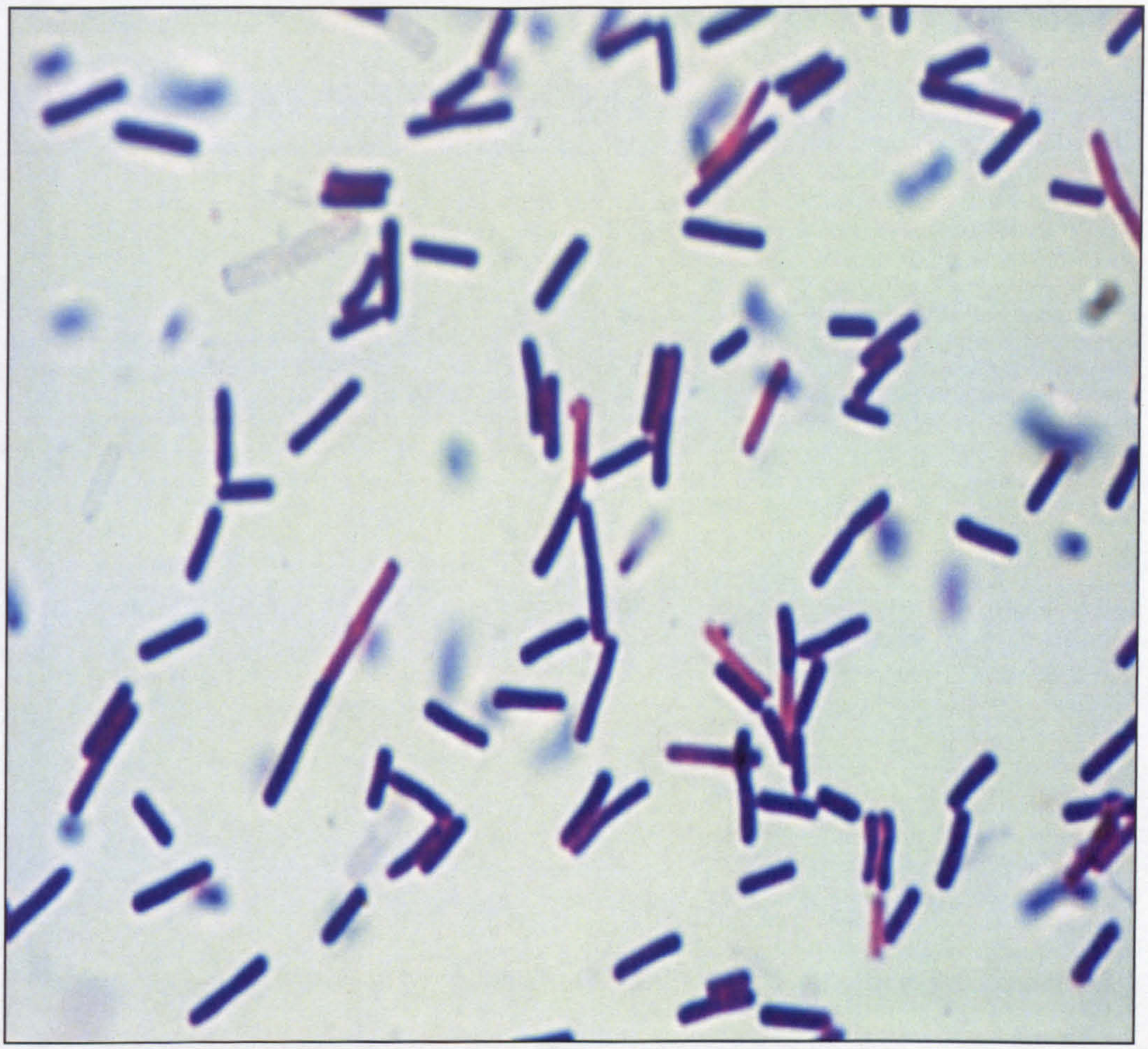

Fig.10 Gram stain of *C. difficile* from blood agar plate day 1 showing pleomorphic Gram-positive rods. (Scale: 1μm = 4mm)

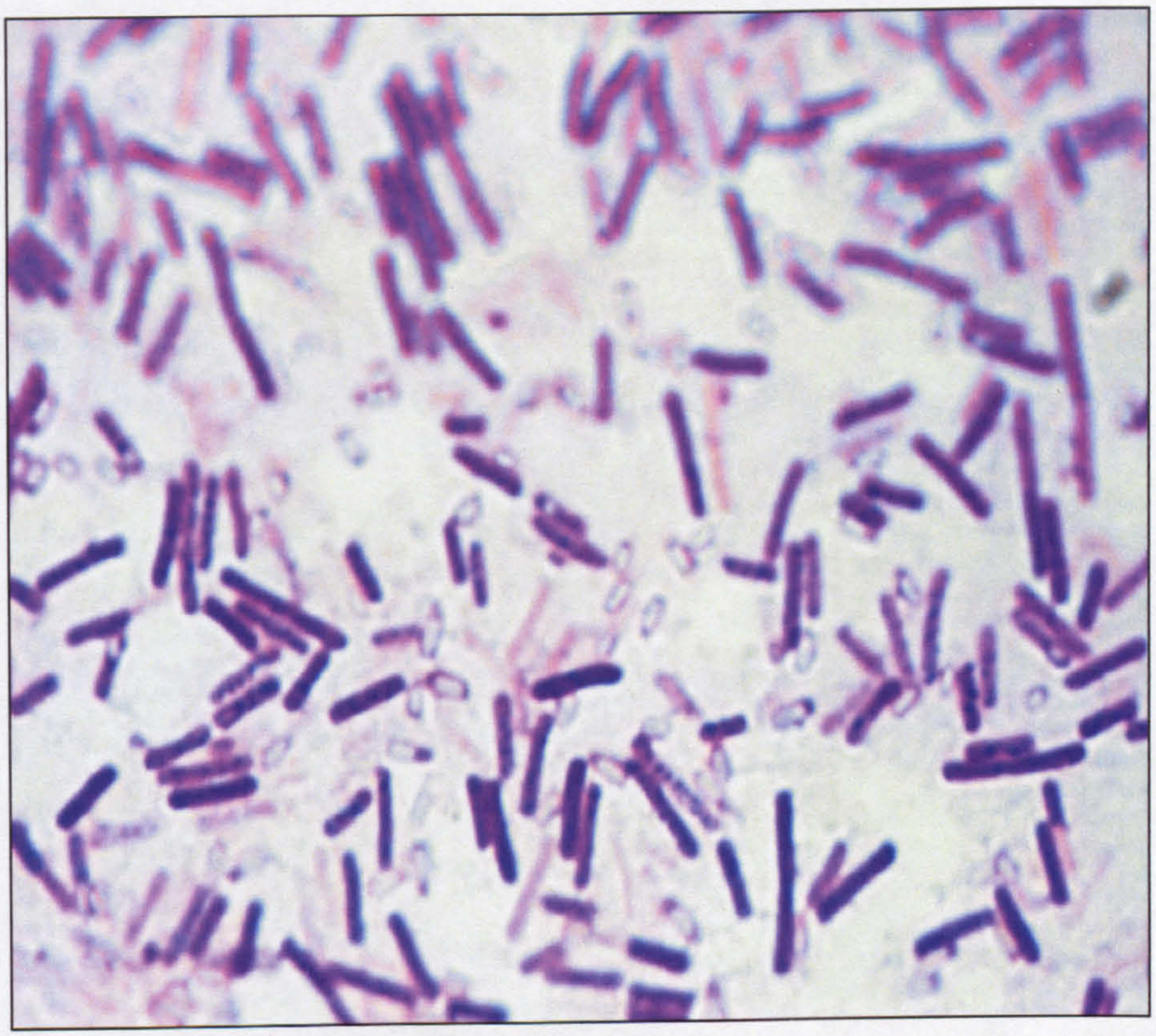

Fig. 11 Gram stain of *C. difficile* from blood agar plate day 3.

(Scale: 1μm = 4mm)

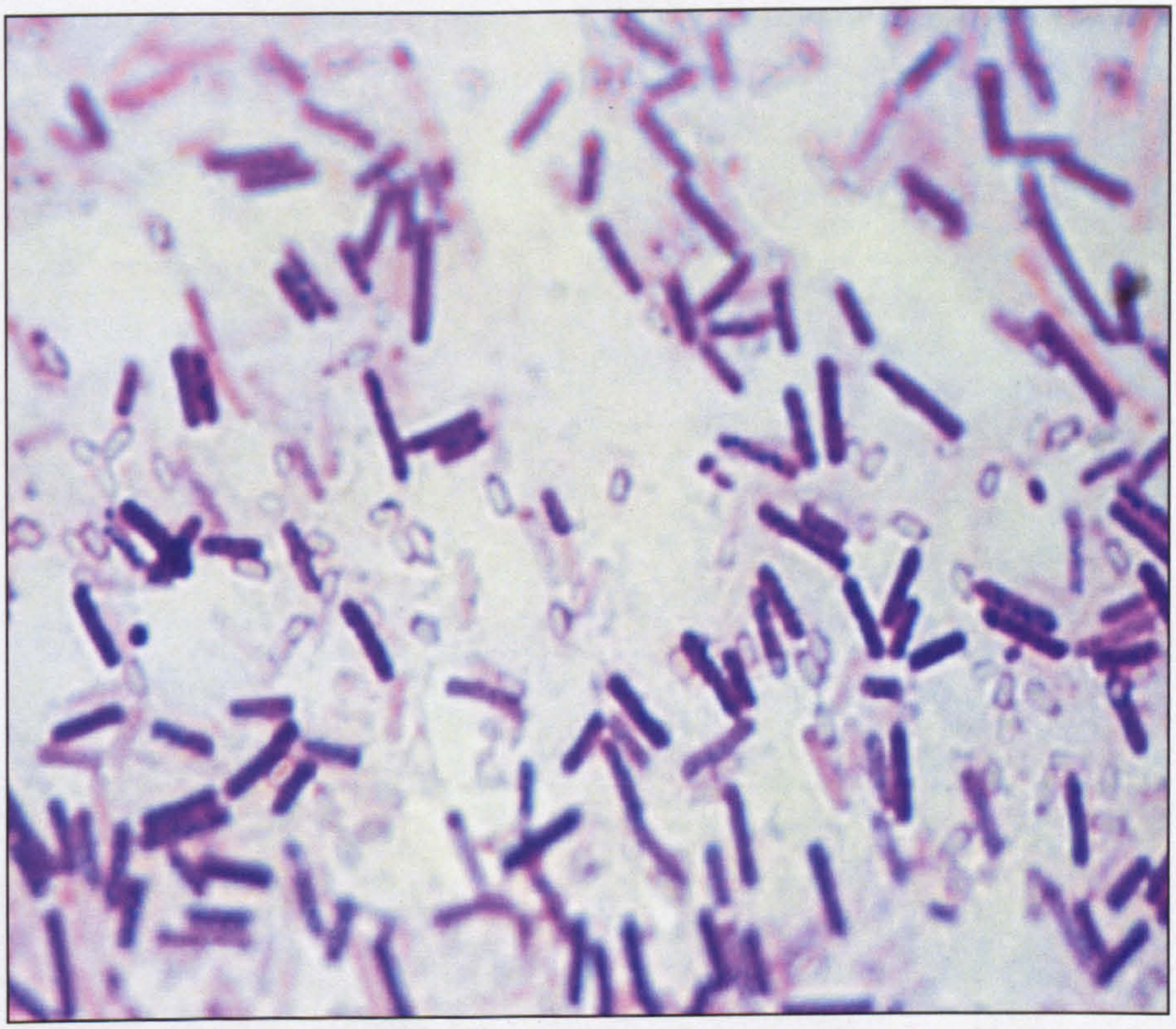

Fig. 12 Gram stain of *C. difficile* from blood agar plate day 8.

(Scale: 1μm= 4mm)

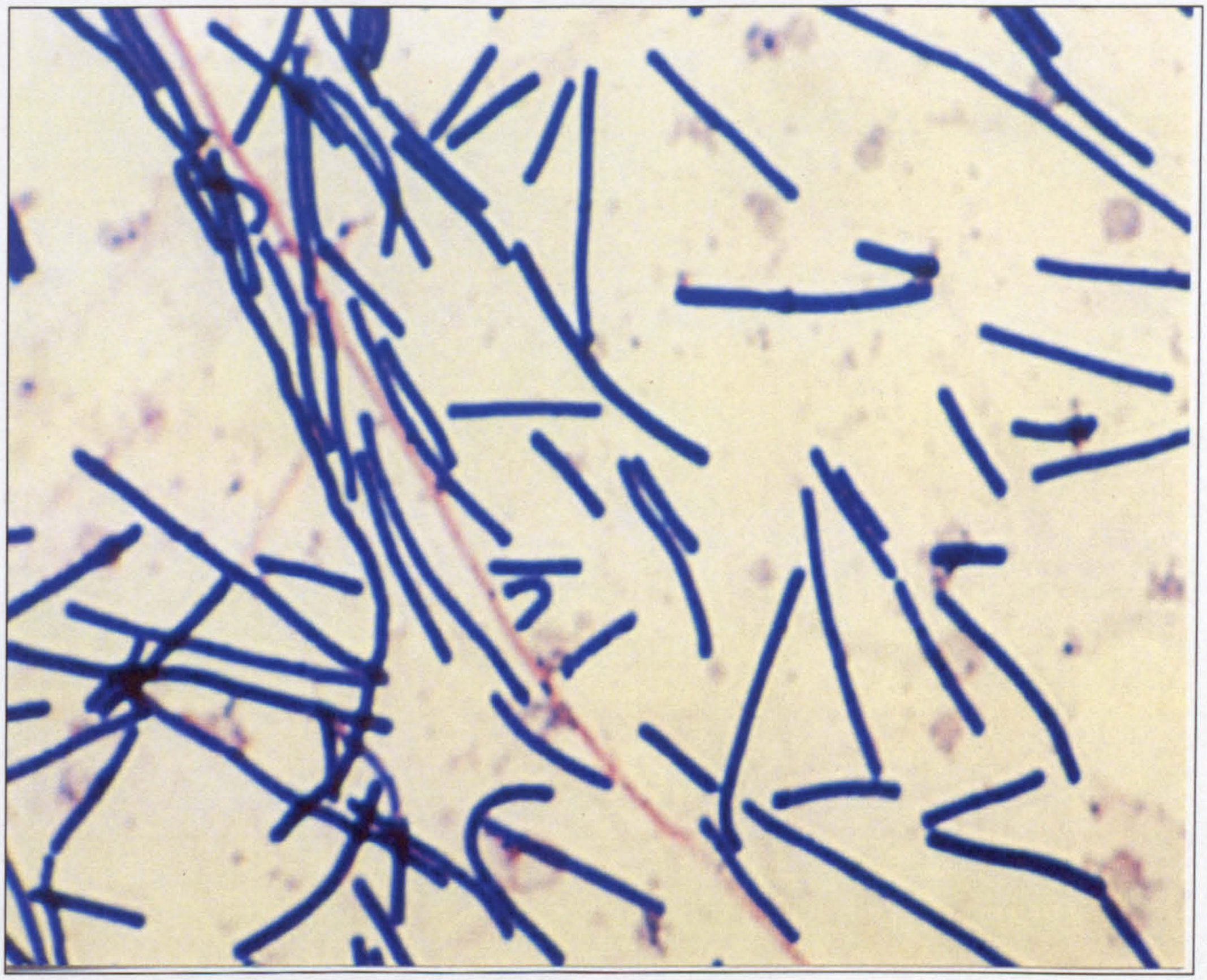

Fig. 13 Gram stain of *C. difficile* from 48h biofilm. (Scale: 1μm = 4mm)

3.4 Electron Microscopy

Transmission electron microscopy studies of the organism in broth, plate and biofilm culture are shown in Figs. 14- 25 . In broth culture at 24 hours, occasional spores were identified (Fig. 14), and by 48 hours this was a relatively common event (Fig. 15). On plates, at 24 hours, the typical rod-shaped structure of the organism was evident (Fig. 16), and some degree of sporulation was already evident (Fig. 17). Spores and spore formation showed the typical features of bacterial spores (Figs. 18,19, 20), and at higher magnification, the typical layers of the spore were readily identified (Fig. 21). These were identified as the inner membrane (A), inner dense cortical layer (B), outer less dense cortical layer (C), inner coat (D), outer coat (E) and exosporium (F) (Joklik *et. al.*, 1988). At 48 hours on the plates, the majority of organisms existed as spores (Fig. 22). In the filters, sporulation was not observed to any extent. (Fig. 23), and where elongated forms were identified by electron microscopy (Fig. 24), it was probable that these structures were due to non-separation of the bacteria at cell division (Fig. 25).

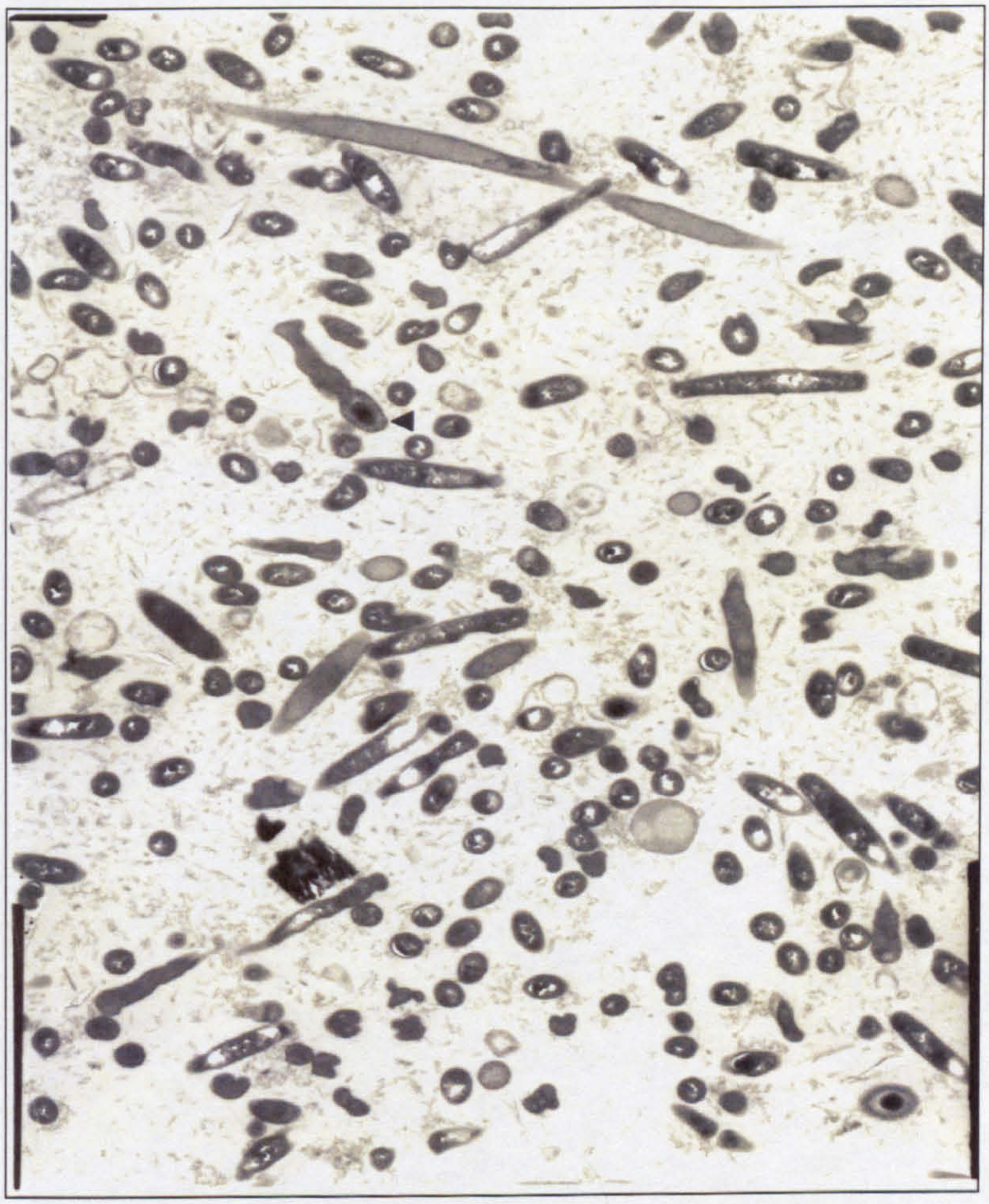

Fig. 14 Electron micrograph of broth culture at 24h showing occasional spores (see arrow ▼). (Scale: 1μm = 7mm)

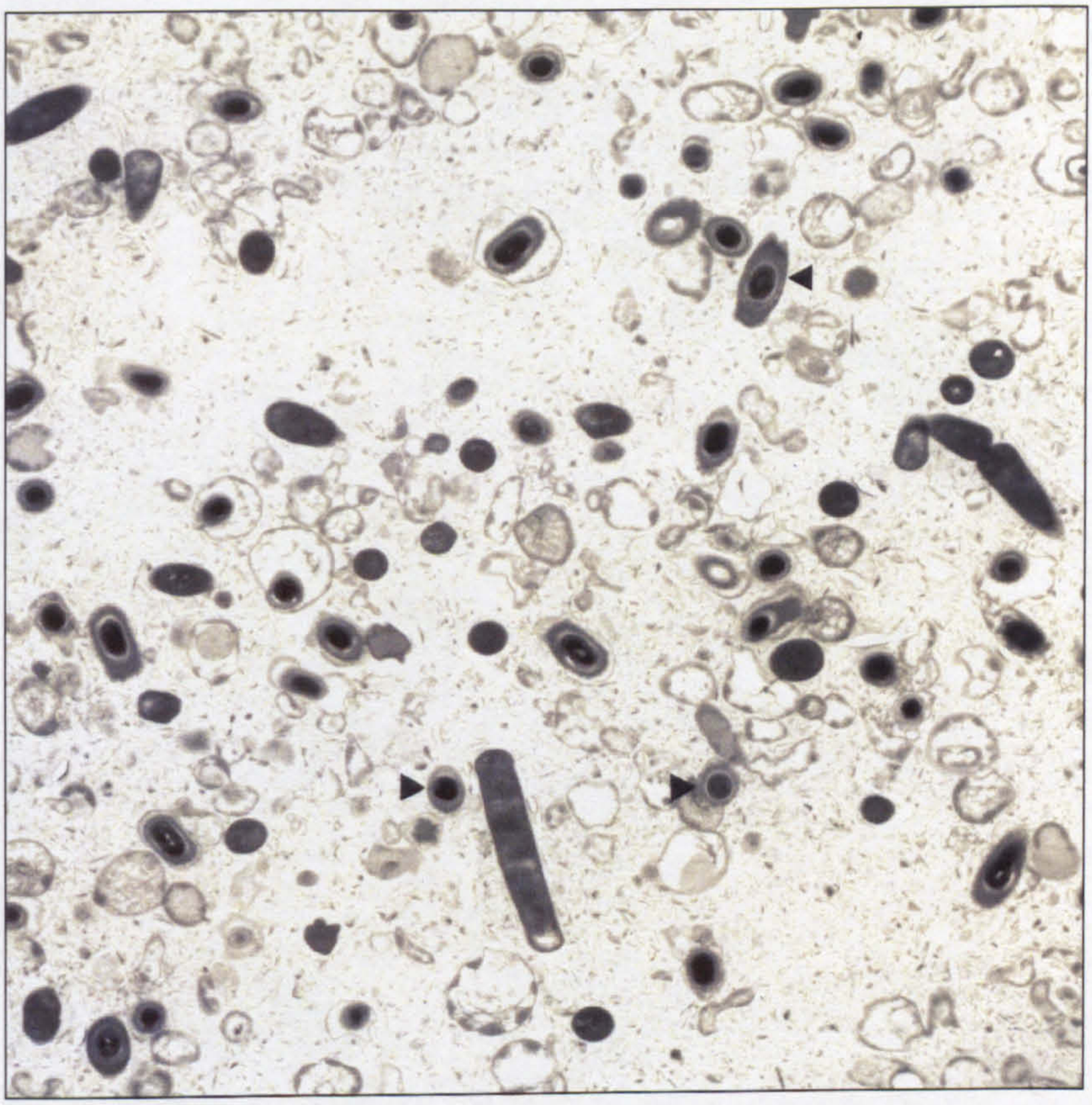

Fig. 15 48h broth culture of *C. difficile* showing numerous spores (see arrow ▸).

(Scale: 1μm = 7mm)

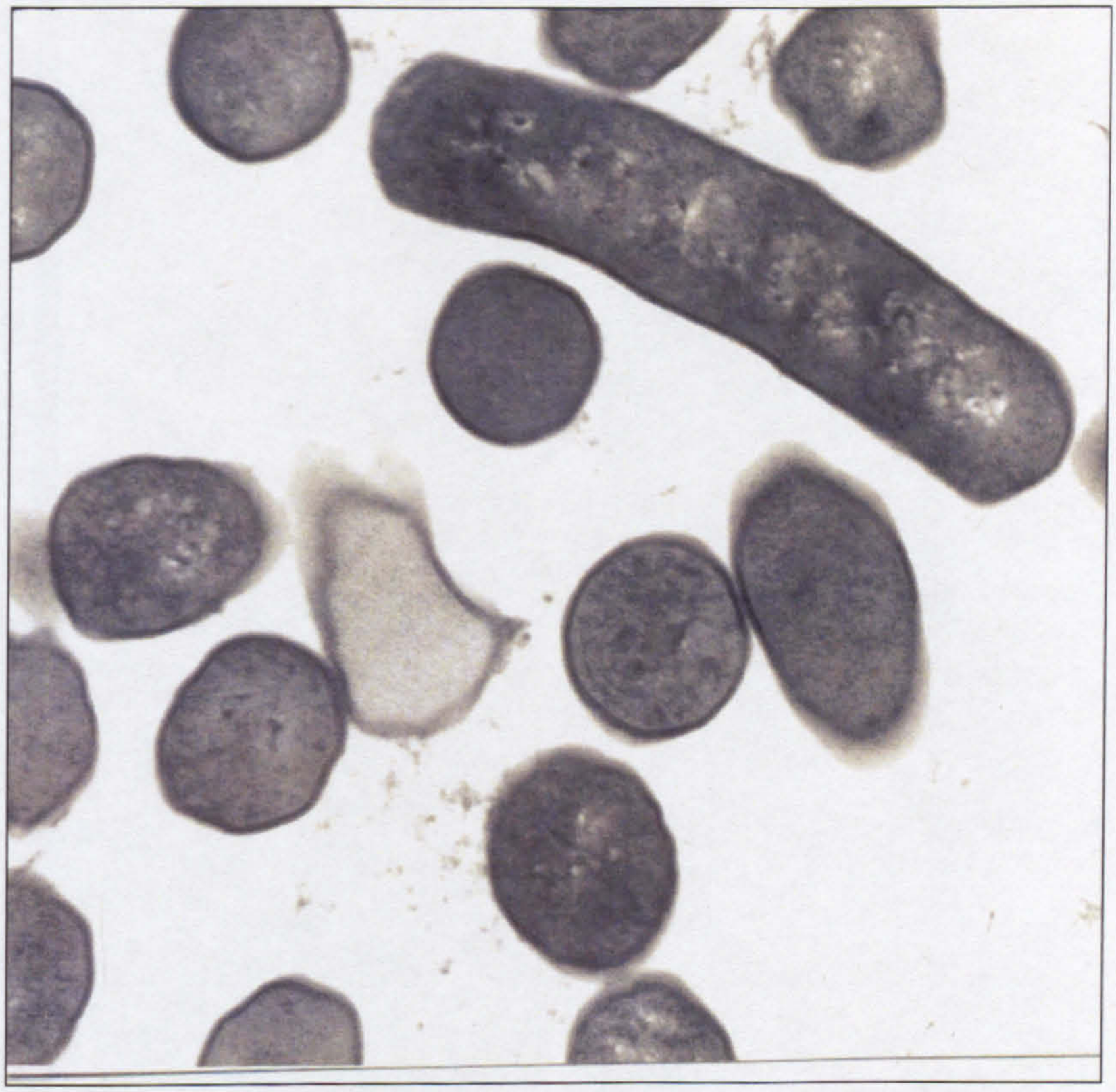

Fig. 16 The classic rod-shape structure of *C. difficile* from a 24h blood agar plate culture. (Scale: 1μm = 35mm)

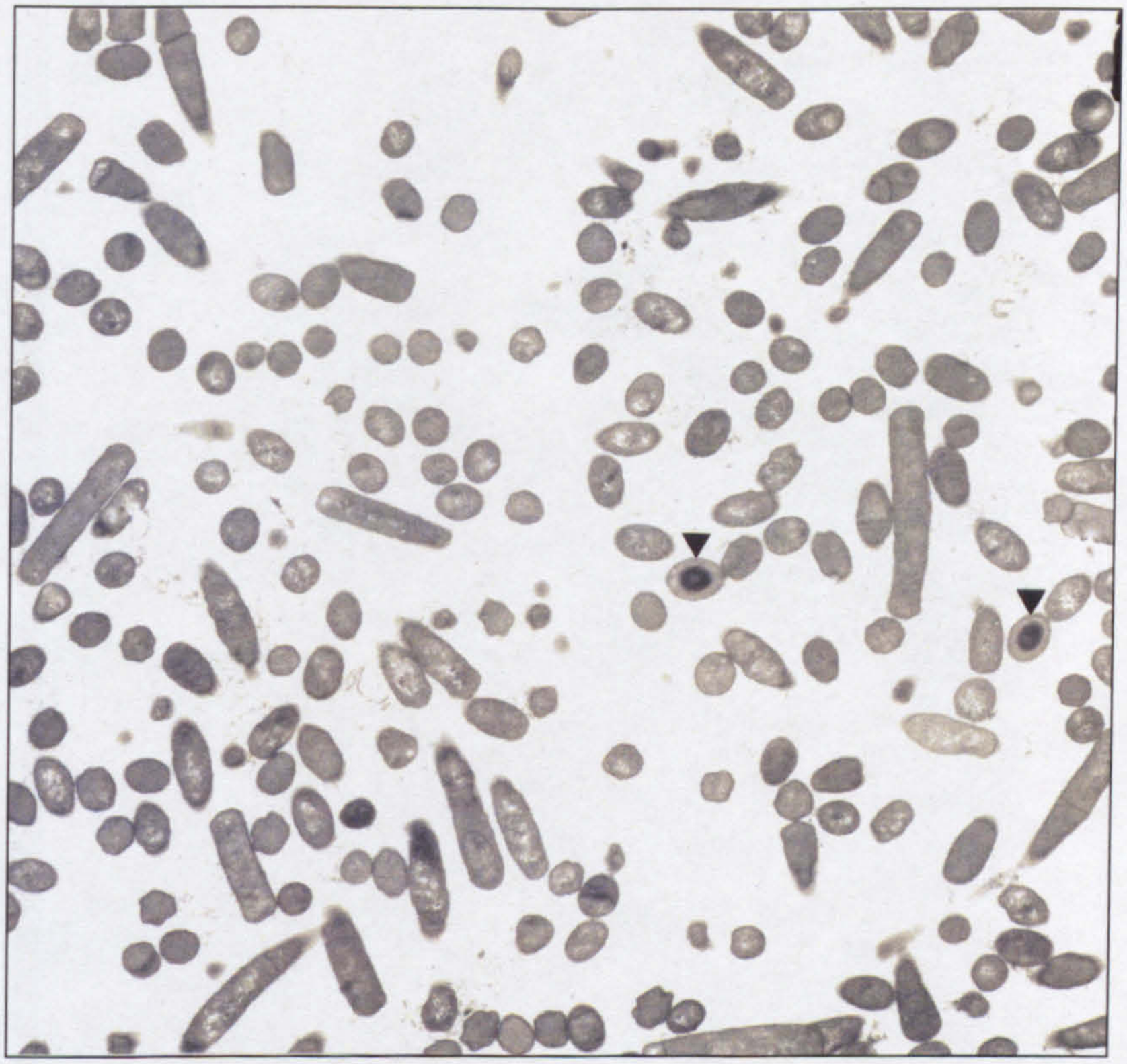

Fig. 17 Sporulation of *C. difficile* from 24h blood agar plates (see arrows ▼).

(Scale: 1μm = 7mm)

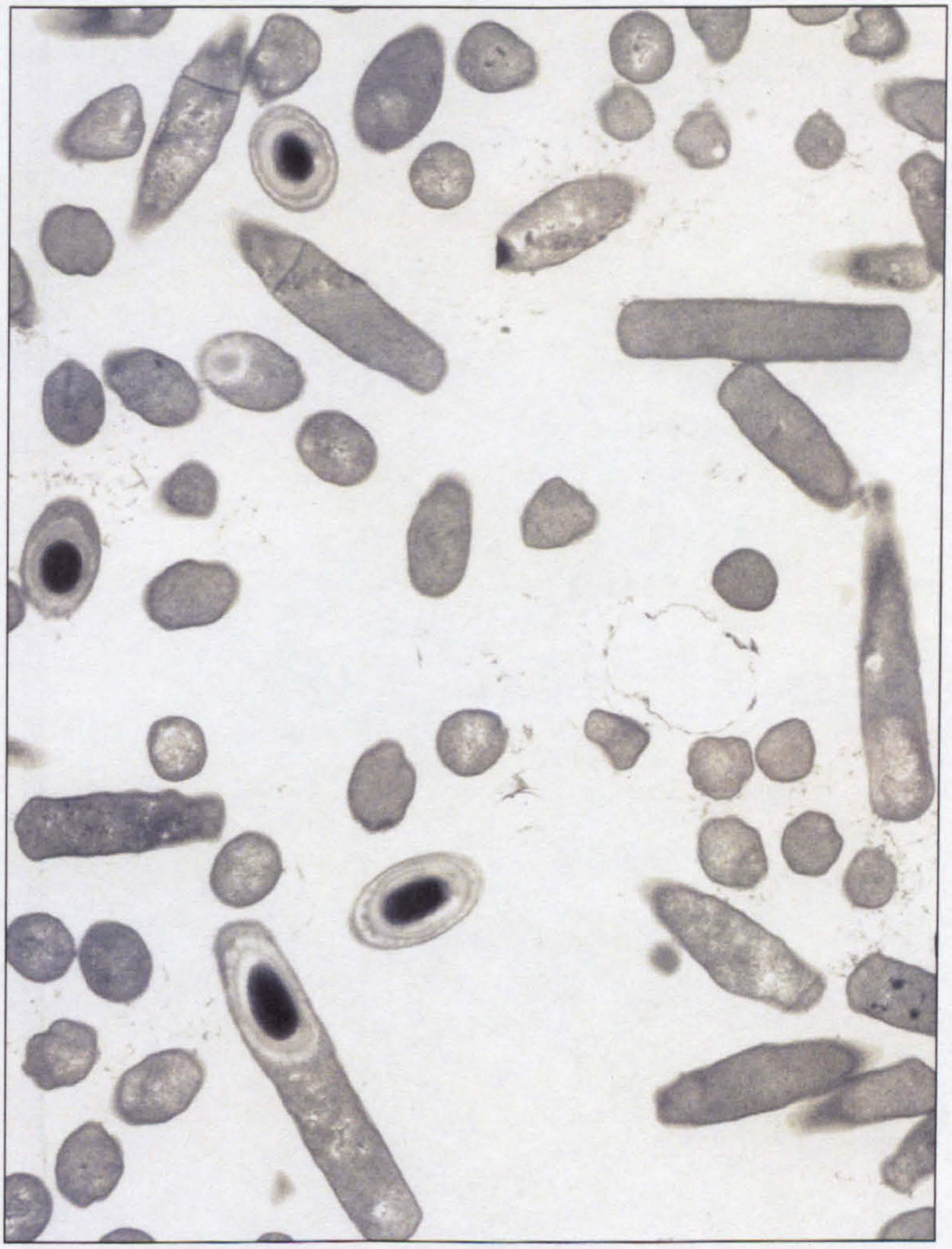

Fig. 18 Typical features of *C. difficile* spores at 24h from blood agar plates.

(Scale: 1μm = 14mm)

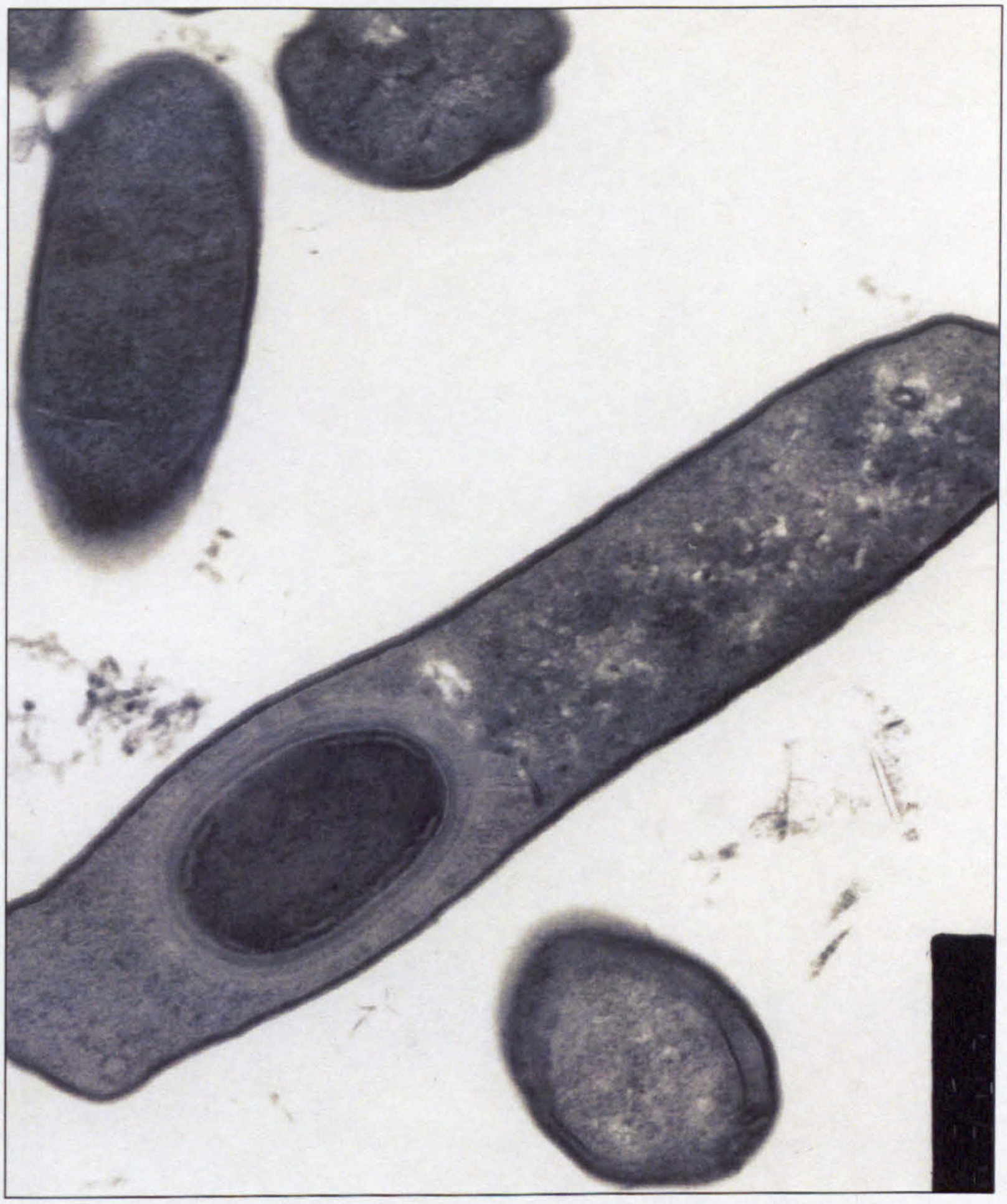

Fig. 19 Higher magnification of *C. difficile* spores from 24h blood agar plates.

(Scale: 1μm= 25mm)

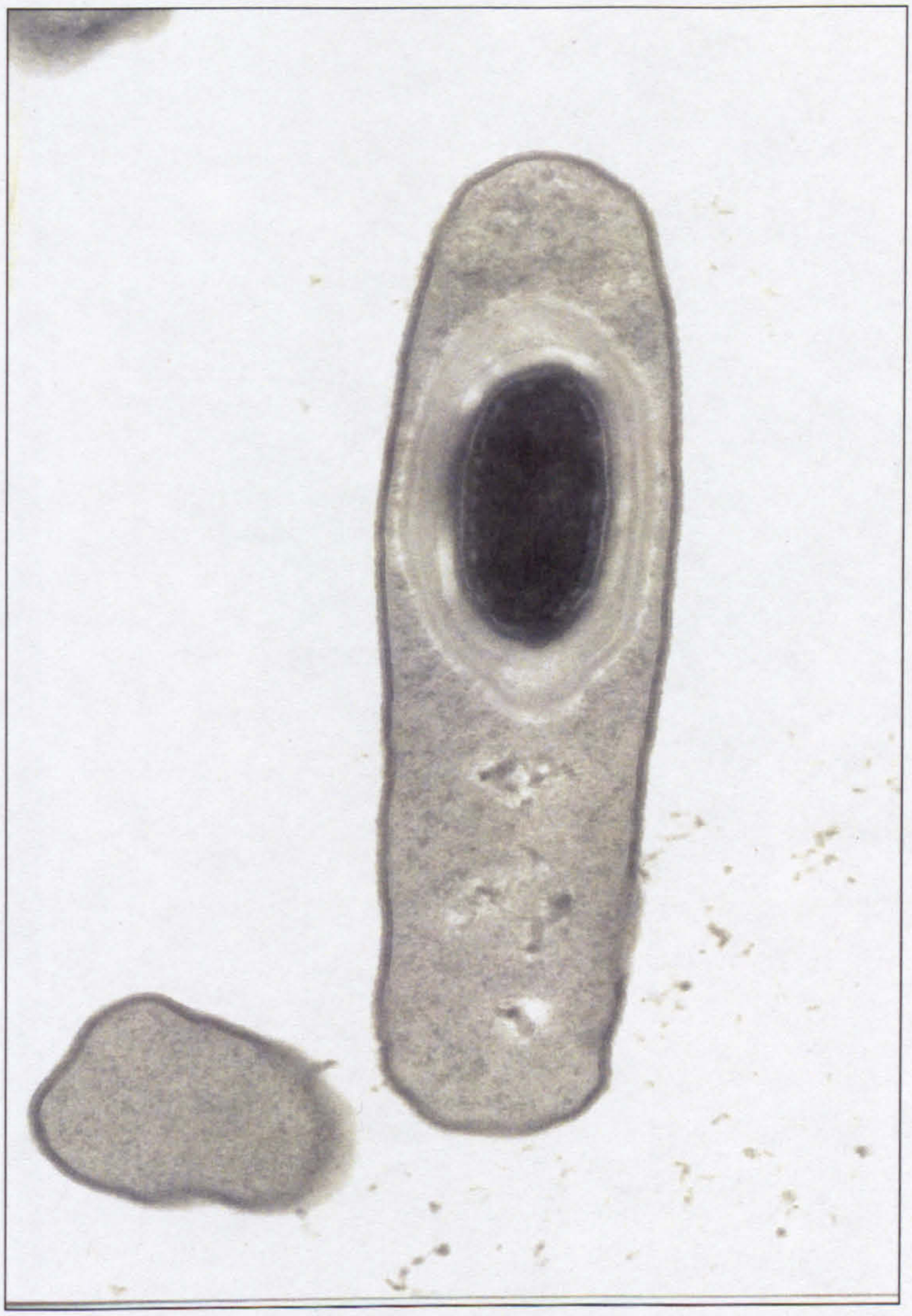

Fig. 20 Spore structure of *C. difficile* from 24h blood agar plate. (Scale: 1μm = 25mm)

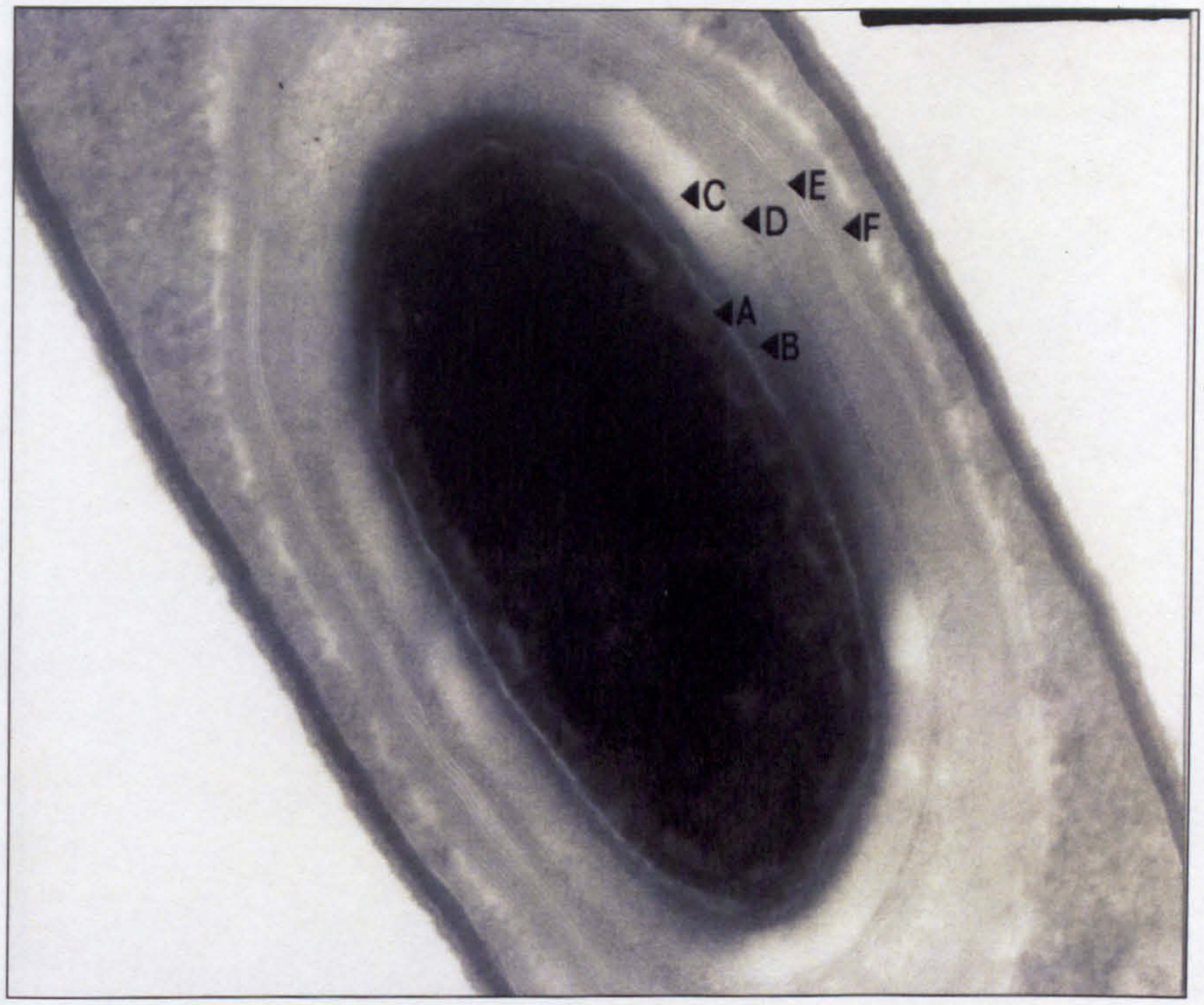

Fig. 21 The typical layers of a spore from a 24h blood agar plate; the inner membrane (A), inner dense cortical layer (B), outer dense cortical layer (C), inner coat (D), outer coat (E) and exosporium (F). (Scale 0.1micron= 10mm)

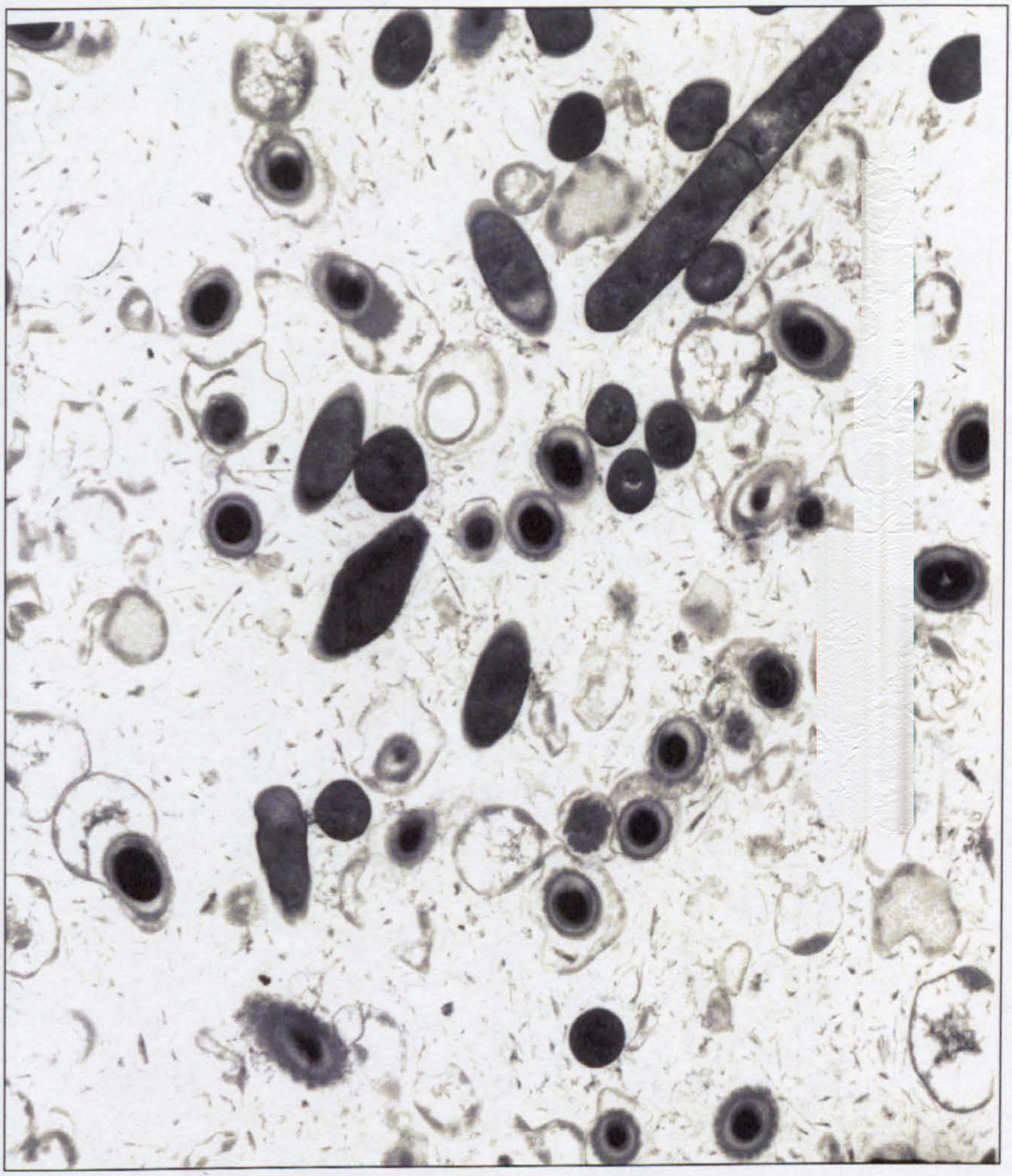

Fig. 22 Electron micrograph of 48h culture of *C. difficile* on blood agar plates showing numerous spores. (Scale 1μm = 14mm)

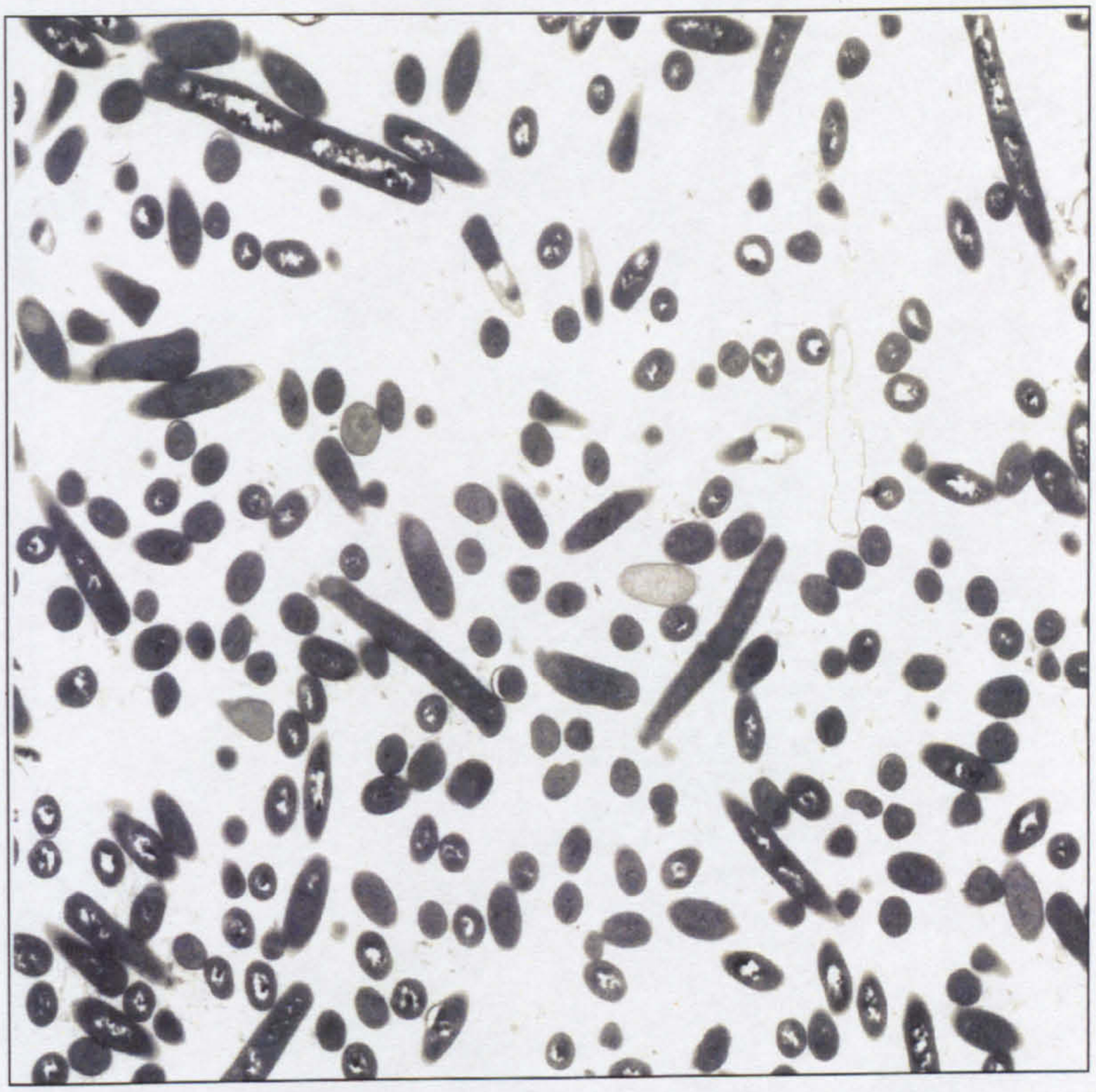

Fig. 23 Electron micrograph of *C. difficile* grown in biofilm for 48h. Sporulation was rarely observed. (Scale 1μm= 7mm)

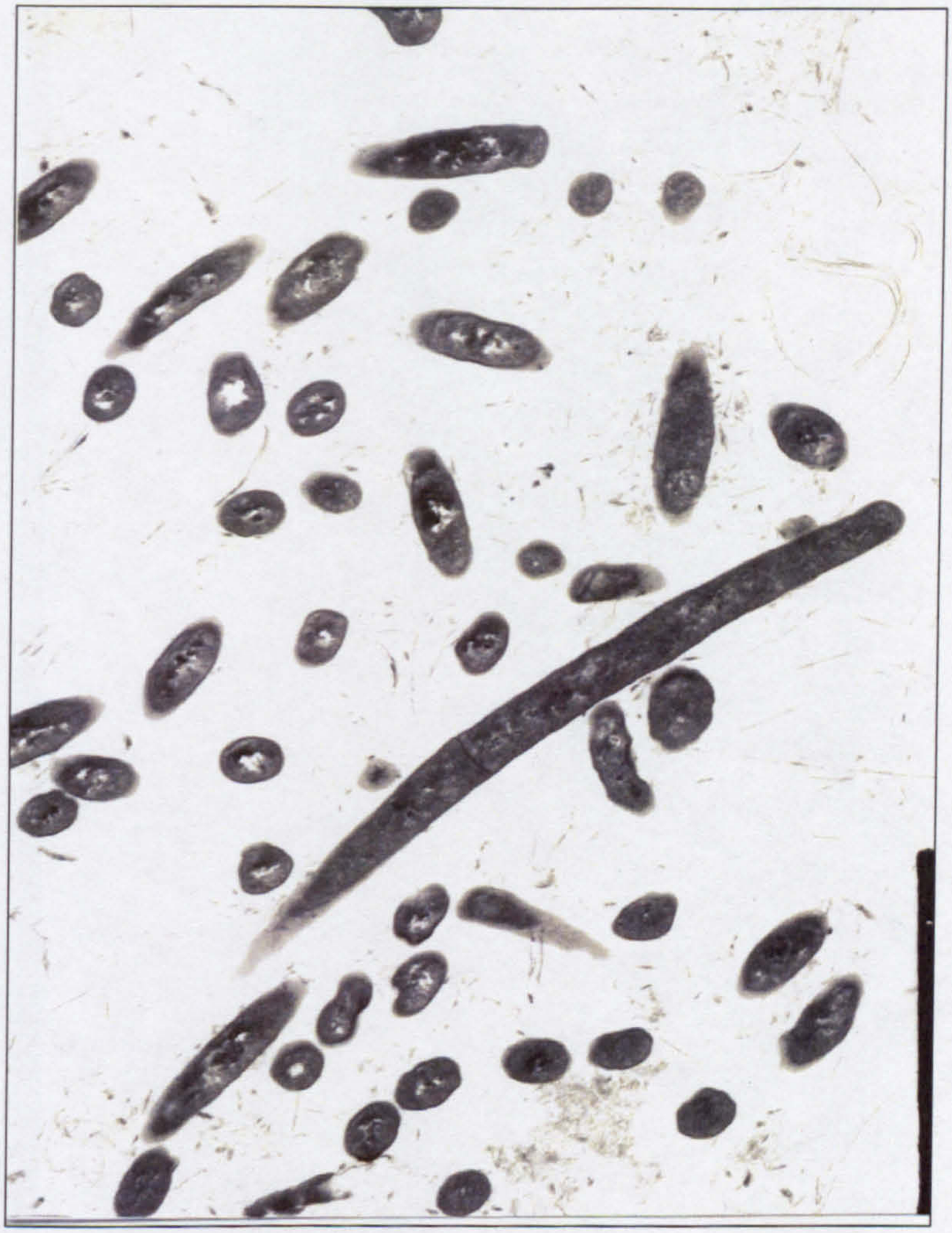

Fig. 24 Elongated filamentous structures of *C. difficile* when grown in biofilm (here at 48h). (Scale 1μm = 14mm)

Fig. 25 Higher magnification of the filamentous structures of *C. difficile* in biofilm at 48h. It is probable that there was non-separation of the bacteria at cell-division. (Scale 0.5μm = 36mm)

3.5 Characterisation of Toxins (A and B) production by *C. difficile* in broth and biofilm

C. difficile produces 2 major exotoxins that cause diarrhoea and colitis (Borriello *et al.*, 1990). Toxin A causes fluid secretion, mucosal damage and inflammation and toxin B acts as a cytotoxin (Pothoukalis, 1996). As previously mentioned, toxin determination in stool specimens was the initial observation that led to the discovery of *C. difficile* being responsible for AAD (Larson *et al.*, 1977). The detection of cytotoxin in faecal tissue by culture is considered "the gold standard" (Groschel *et al.*, 1996), however commercial enzyme immunoassays are more widely used because of their good specificity, rapid turnaround time, convenience and cost (Lyerly *et al.*, 1998). It is known that organisms in the biofilm mode of growth can exhibit an altered phenotype with respect to growth rate and transcription (Donlan and Costerton, 2002). The Sorbarod filter system was used to compare expression of toxins in biofilm with that found in broth culture of *C. difficile*. Toxin activity was determined using the Ridascreen® *Clostridium difficile* toxin A/B enzyme immunoassay as described in Chapter 2.

Toxin levels in the clarified supernatants obtained from growing *C. difficile* in broth, biofilm and biofilm effluent are shown in Fig. 26. Between 24 and 48 hours on the filter, the amount of toxin reached the maximum detectable by the Ridascreen method. The amount of toxin detected in broth and biofilm effluent was consistently of lower value.

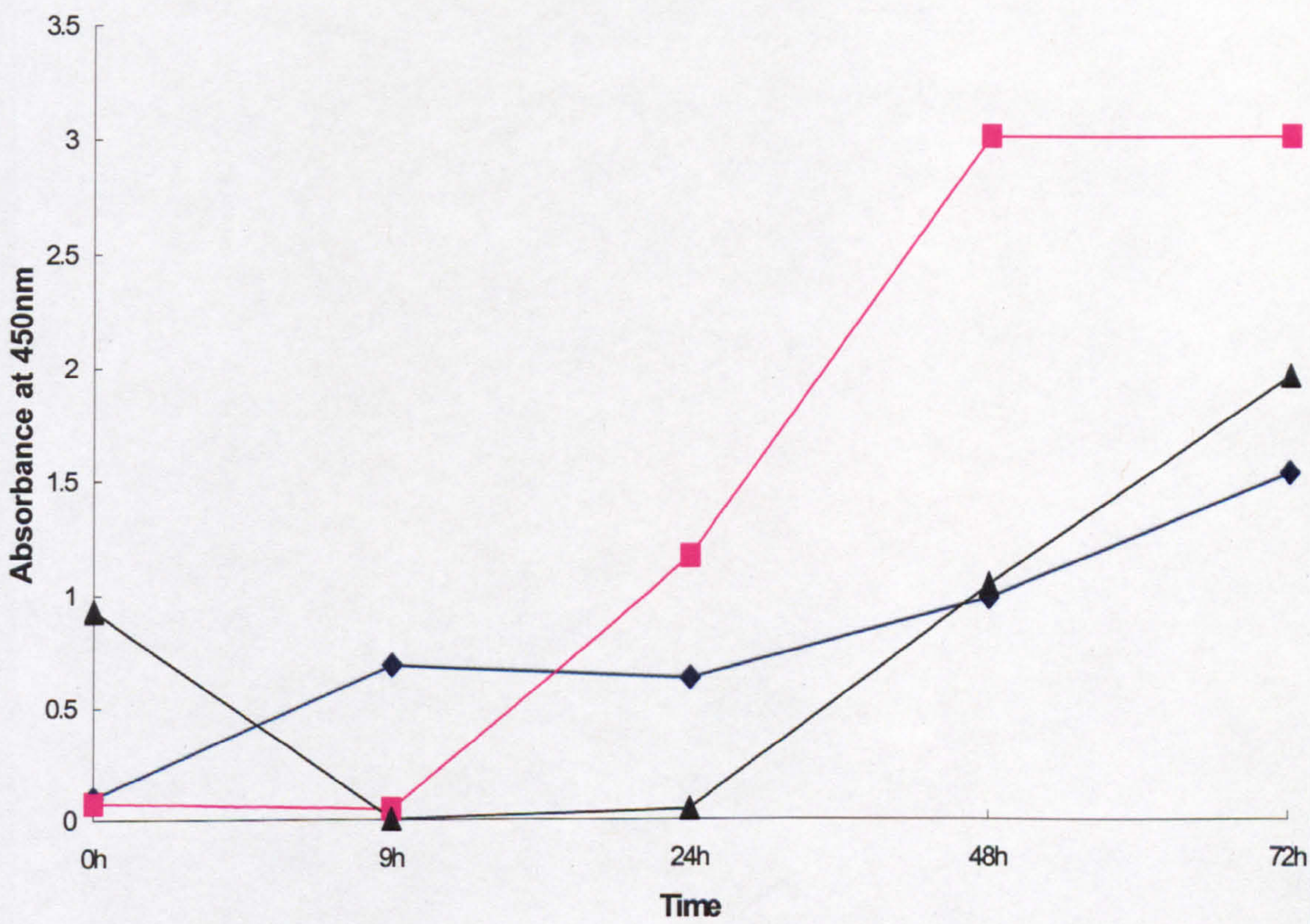

Fig. 26 Comparison of the toxin activity of *Clostridium difficile* in broth (▲), biofilm effluent (◆) and biofilm (■) over 72h.

3.6 Discussion

The aim of this chapter was to determine whether Sorbarod filters could be used to produce a model biofilm system for studying *C. difficile*. Using a clinical isolate, the typical features of the organism were shown in broth and plate culture, with the usual rod-shaped structure of vegetative bacteria being evident. While spores could be seen at 24 hours, by 48 hours they were a common feature with this isolate. High powered electron micrographs showed that these spores had the features typical of bacterial spores. In broth culture, the organism reached a maximum titre at about 24 hours. Titres were maintained at a similar level after this period; presumably this was due to the formation of spores, which germinated on titration. A degree of pleomorphism in the vegetative organisms was noted here with the length varying from 1.2 to 4.5 microns. Such pleomorphism is a recognised feature of clostridia (Hathaway and Johnson, 1998).

In biofilm, *C. difficile* maintained its titre in excess of 10^9 cfu/mL for at least 96 hours, in a steady state. The concentration of the organism in the biofilm effluent was of the order of 1 log. lower than that in the filter at the corresponding time. In the filters, the organism often showed elongated forms in excess of 10 microns in length, and on occasion these were seen as short spirals. While no readily identified difference in the cell wall structure of bacteria grown in the filters, broth or plate were seen, in the filter, *C. difficile* also exhibited a "Gram-negative" appearance on occasion. Studies of thin sections of filters fixed by standard histopathological methods, section and Gram-stained have shown irregular microcolonies of bacteria varying from 10-50 microns in width, which were adherent to the cellulose fibres of the filters (O'Connell *et. al*, 2003). It is therefore considered reasonable to conclude that on the Sorbarod filters that the organism can establish itself in continuous culture

for at least 96h, by which time the filters did begin to block. The lack of sporulation in this system confirmed that vegetative growth was being maintained. Simple experiments on the toxin titre showed that high concentrations of toxin were maintained up to at least 96h. It would appear that there are definite differences in toxin synthesis when the biofilm system is compared to broth and biofilm effluent.

This work thus established the experimental conditions for the antibiotic experiments in the next chapter, where the susceptibility of the organism in standard broth culture MIC and MBC experiments were compared with the antibiotic sensitivity profile in the biofilm mode of growth in the Sorbarod filters.

Chapter Four

4.1 Introduction

Enteral metronidazole and vancomycin are the mainstays of treatment of *C. difficile* infection and combination therapy with rifampicin has also shown good clinical response (Wilcox and Spencer, 1992; Buggy, 1993). Antimicrobial susceptibility testing of *C. difficile* is not routinely performed in most laboratories. The reference method for susceptibility testing is the agar dilution method (www. BSAC.org ; NCCLS, 1997), but it is considered both too time-consuming and labour intensive to be a routine test. Minimum inhibitory concentrations (MIC) of clostridial species are documented. For *C. perfringens* the penicillin MIC range is 0.06-0.25 mg/L, for *C. difficile,* the vancomycin MIC range is 1-8mg/L and that for metronidazole is 0.25-0.5 mg/L (Kucers and Bennett, 1987). *C. difficile* is also very sensitive to rifampicin. There are no minimum bactericidal concentrations (MBC) of these antibiotics to *C. difficile* documented in the literature. The aim of this work presented in this chapter was thus to compare the tube MIC/MBC results with that of the clinical strain grown in the Sorbarod filters.

4.2 The determination of the tube MIC/MBC, BEC and EfMBC

In initial experiments, the clinical isolate of *C. difficile* (459) and the NCTC strain 11204 (Type) were used. Benzylpenicillin, vancomycin, metronidazole and rifampicin were the antibiotics tested. The initial MIC/MBCresults are summarised in Tables 6 and 7. For the first three antibiotics tested, the MIC values were close to those published (Kucers and Bennet, 1987; Wilcox and Spencer, 1992; Buggy, 1993). With the exception of rifampicin, the other antibiotics showed that there was

significant degree of tolerance in the MBC experiments. The MBC for rifampicin was 0.25 mg/L, which is within the "sensitive" range. For the other antibiotics the MBC values were above 32 mg/L. In further experiments with the four antibiotics and the NCTC strain plus five clinical isolates, MIC/MBC experiments were conducted to 128 mg/L, and with the exception of rifampicin, tolerance was identified (Table 3). With metronidazole an endpoint between 8-128mg/Lwas reached for 4 clinical isolates in the range of concentrations tested.

The results of a BEC and EfMBC experiment controlled by a tube MIC are shown in Table 8. Here again with benzylpenicillin, vancomycin and metronidazole tolerance was noted on both the filter and in the effluent. With rifampicin a BEC of 8mg/L and EfMBC of 1.0mg/L was determined. This mirrored the effect in broth. In repeat experiments with rifampicin, some degree of variability in the susceptibility was noted in that tolerance was demonstrated in the filters, but in all cases the organism was eliminated from effluent at concentrations of 1-4mg/L.

	TubeMIC	Etest	MBC
Benzylpenicillin	4.0	1.0	>32
Vancomycin	2.0	1.0	>32
Metronidazole	0.5	0.25	>32
Rifampicin	0.004	<0.002	0.25

Table 6 Results of tube MIC/MBC of clinical strain 459 with benzylpenicillin, vancomycin, metronidazole and rifampicin. (Values in mg/L)

	MIC	MBC
Benzylpenicillin	1	>128
Vancomycin	2	>128
Metronidazole	0.5	64
Rifampicin	<0.004	0.25

Table 7 Results of tube MIC/MBC of NCTC *C. difficile* strain with benzylpenicillin, vancomycin, metronidazole and rifampicin. (Values in mg/L)

	MIC	MBC
Vancomycin		
2302	1	>128
1342	1	>128
459	2	>128
2236	0.5	>128
TYPE	2	>128
1790	1	>128
Metronidazole		
2302	0.128	32
1342	0.25	8
459	0.5	64
2236	0.25	>128
TYPE	0.5	64
1790	1	128
Penicillin		
2302	2	>128
1342	1	>128
459	4	>128
2236	2	>128
TYPE	1	>128
1790	2	>128
Rifampicin		
2302	<0.004	1
1342	<0.004	1
459	0.004	0.25
2236	<0.004	1
TYPE	<0.004	0.25
1790	0.016	1

Table 8 Tube MIC and MBC values of clinical strains *of C. difficile* and the type NCTC strain.

	BEC	EfMBC
Benzylpenicillin	>32	>32
Metronidazole	>32	>32
Vancomycin	>32	>32
Rifampicin	8.0	1.0

Table 9 Biofilm eradicating concentration (BEC) and biofilm effluent (EfMBC) of wild strain (clinical isolate 459) using benzylpenicillin, vancomycin, metronidazole and rifampicin. (Values in mg/L). A control tube MIC was done here for each antibiotic.

4.3 The features of antibiotic susceptibility on the Sorbarod filters

With the four antibiotics used, each filter and effluent which had been exposed to one concentration of the agent for 18 hours, was titred, along with the antibiotic free control. A tube MIC experiment was run in conjunction with each filter experiment. The results of duplicate experiments are shown in Figs. 27-34. For benzylpenicillin (Figs. 27, 28), vancomycin (Figs. 29,30) and metronidazole (Figs. 31, 32), while there was some fluctuation in the titre of the organism in the filters, the bacteria were present in high titres up to a concentration of 32 mg/L. In the case of the biofilm effluent, there was wider fluctuation, and a significantly lower titre, however the organism was still present in effluent at 32 mg/L. Representative experiments with rifampicin, are shown in Figs. 33, 34. With this antibiotic a discernable end point was achieved in the effluent (1-4 mg/L), and an endpoint was also achieved with one BEC experiment (Fig. 33).

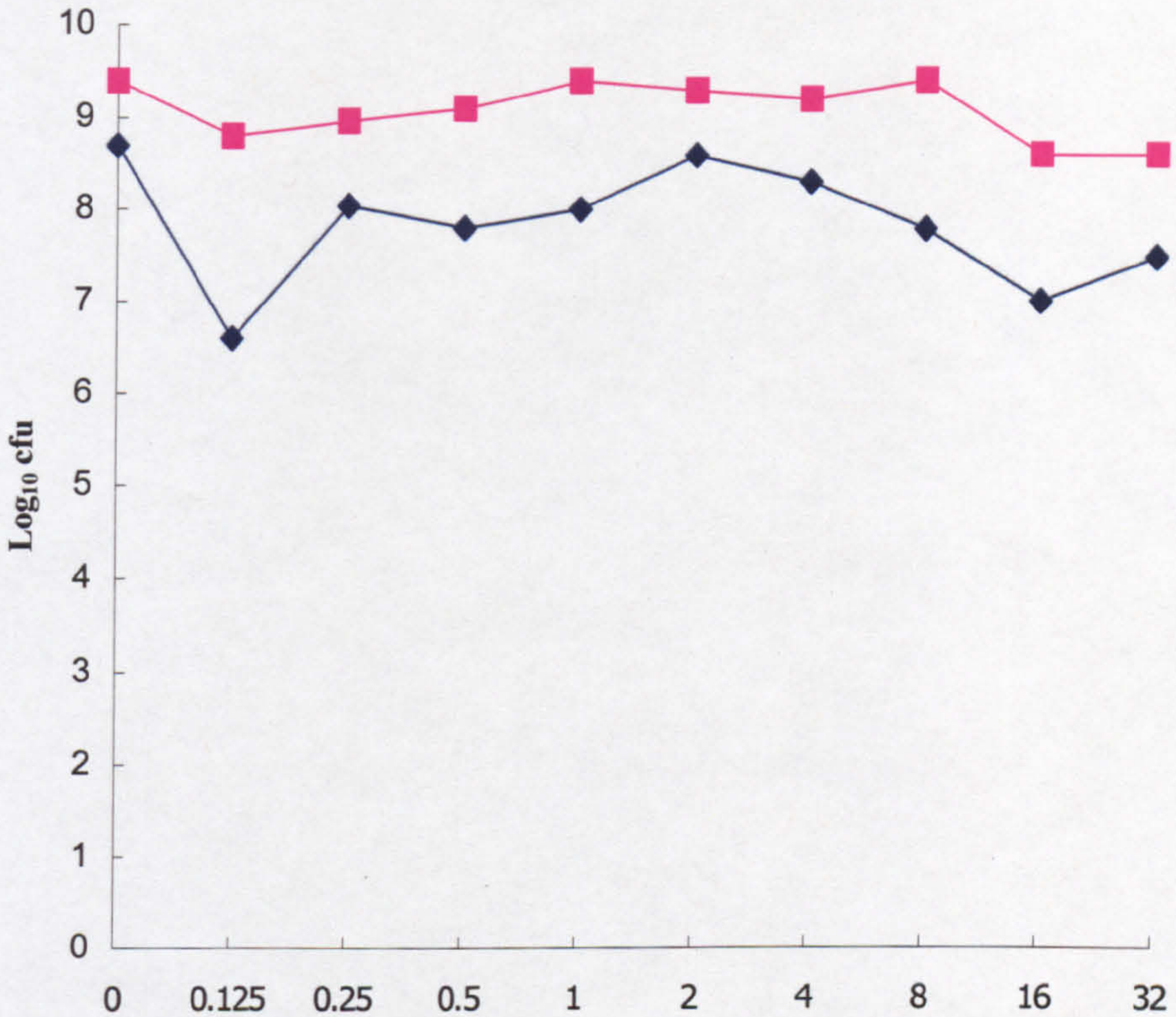

Fig. 27 Effect of penicillin on growth of *C. difficile* on Sorbarod biofilm. Individual biofilms were exposed to single concentration of antibiotic for 18h; after collection of effluent, biofilm was harvested. Titres were done as described in the text to determine BEC and effluent MBC. Effluent titres; cfu/mL (◆) and biofilm titres; cfu/filter (■).

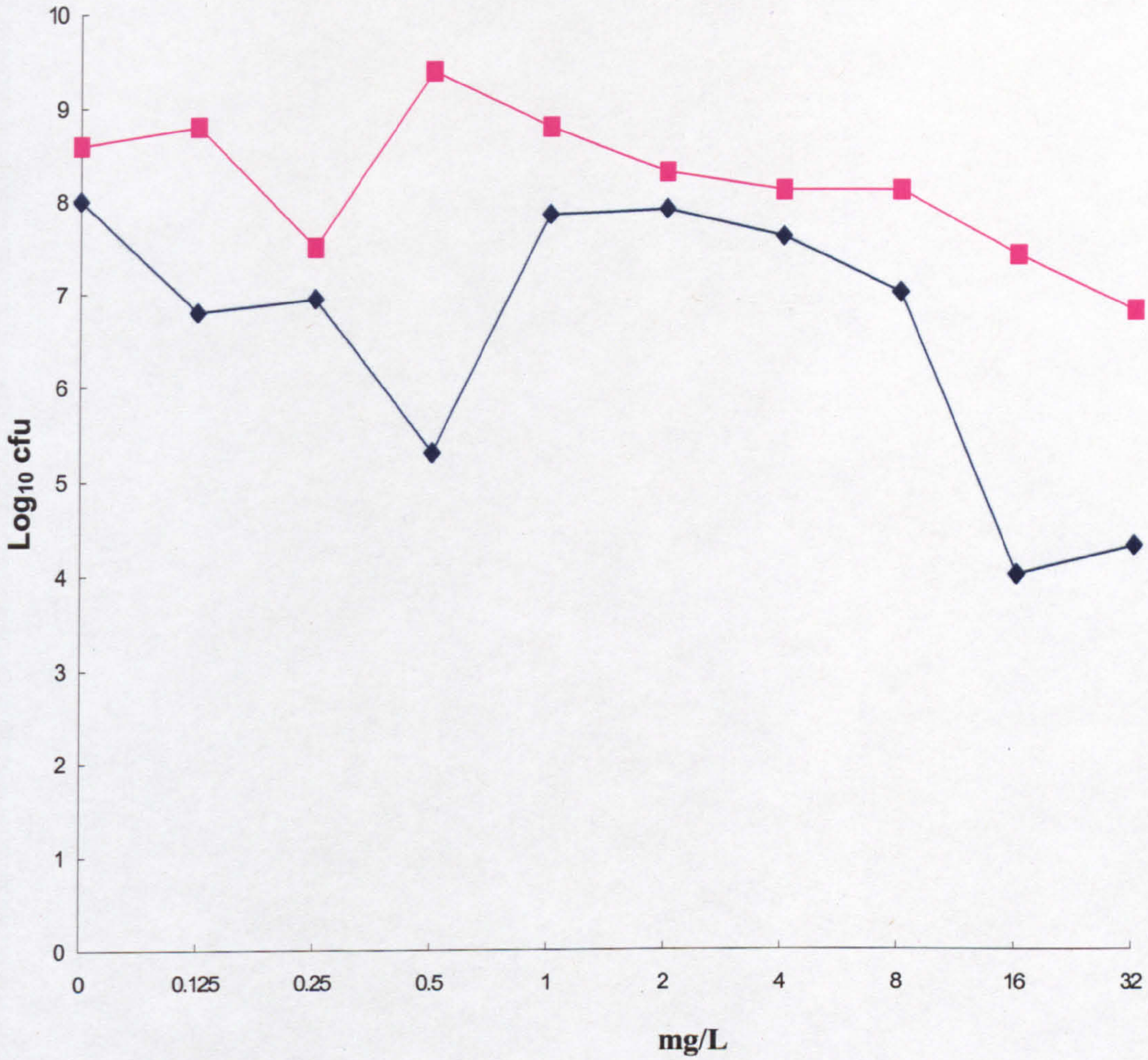

Fig. 28 Repeat experiment – effect of penicillin on growth of *C. difficile* in biofilm

Effluent titres; cfu/mL (◆) and biofilm titres; cfu/filter (■).

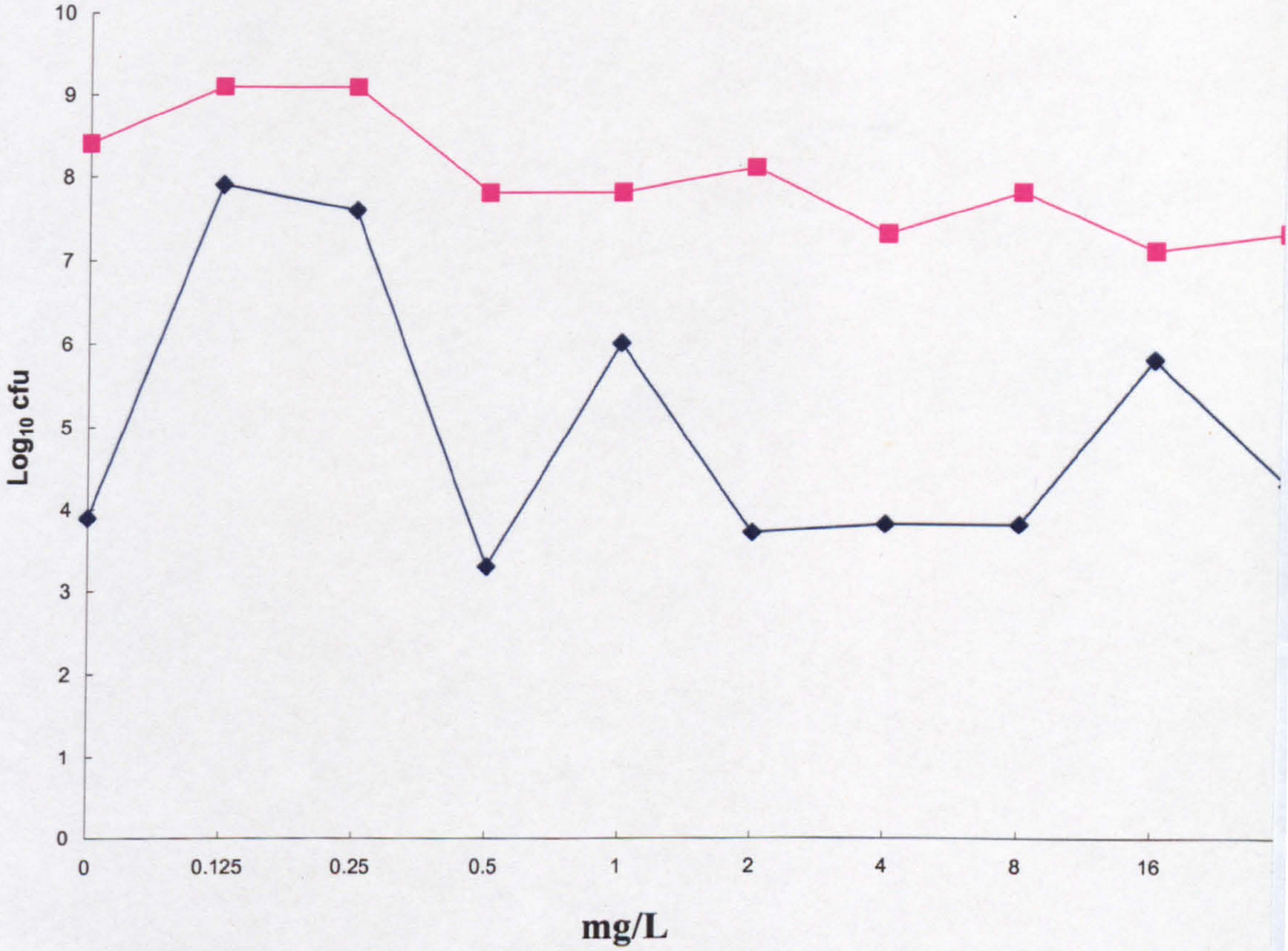

Fig. 29 Effect of vancomycin on growth of *C. difficile* on Sorbarod biofilm.

Individual biofilms were exposed to single concentration of antibiotic for 18h; after collection of effluent, biofilm was harvested. Titres were done as described in the text to determine BEC and effluent MBC.

Effluent titres; cfu/mL (◆) and biofilm titres; cfu/filter (■).

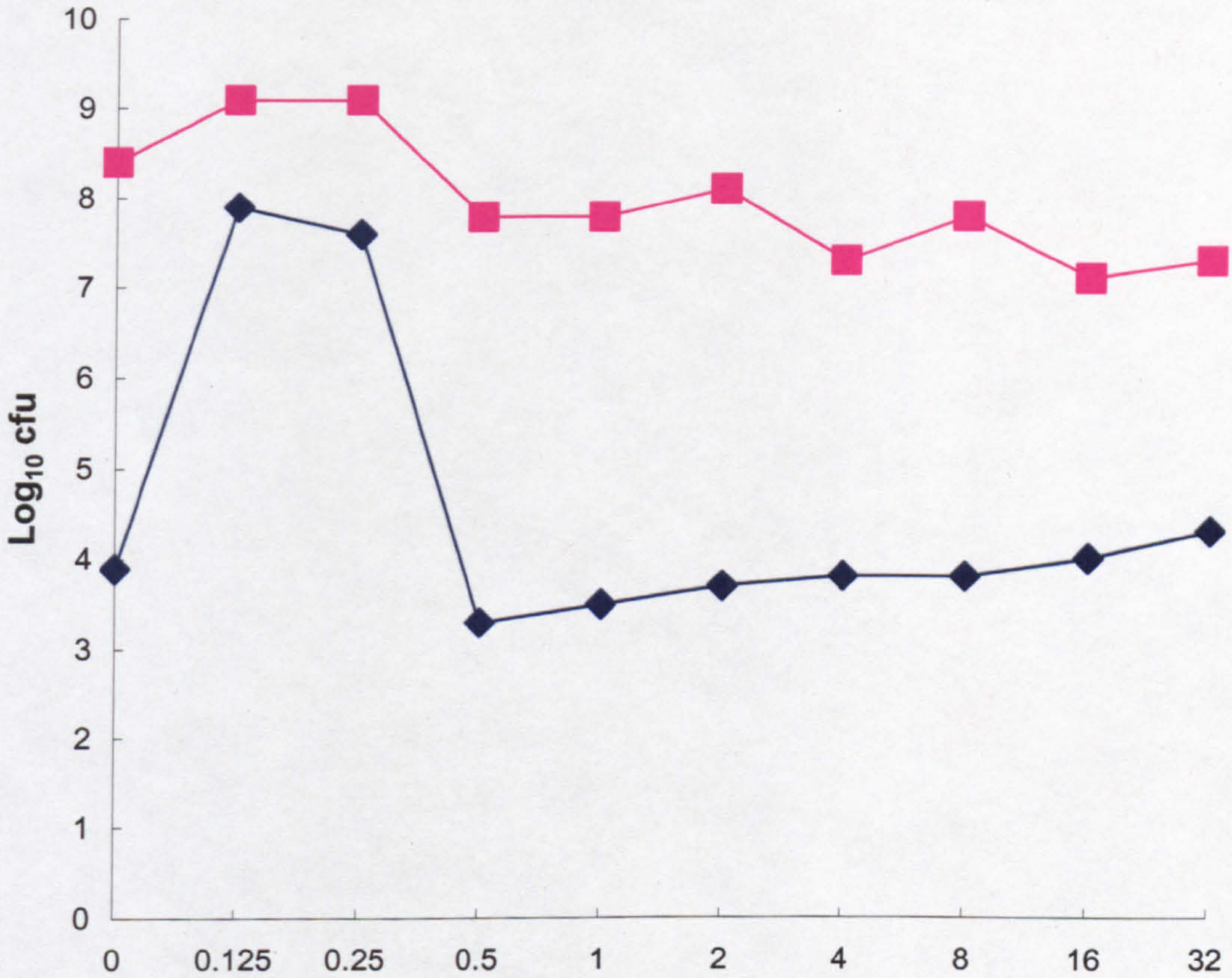

Fig. 30 Repeat experiment – effect of vancomycin in biofilm.

Effluent titres; cfu/mL (◆) and biofilm titres; cfu/filter (■).

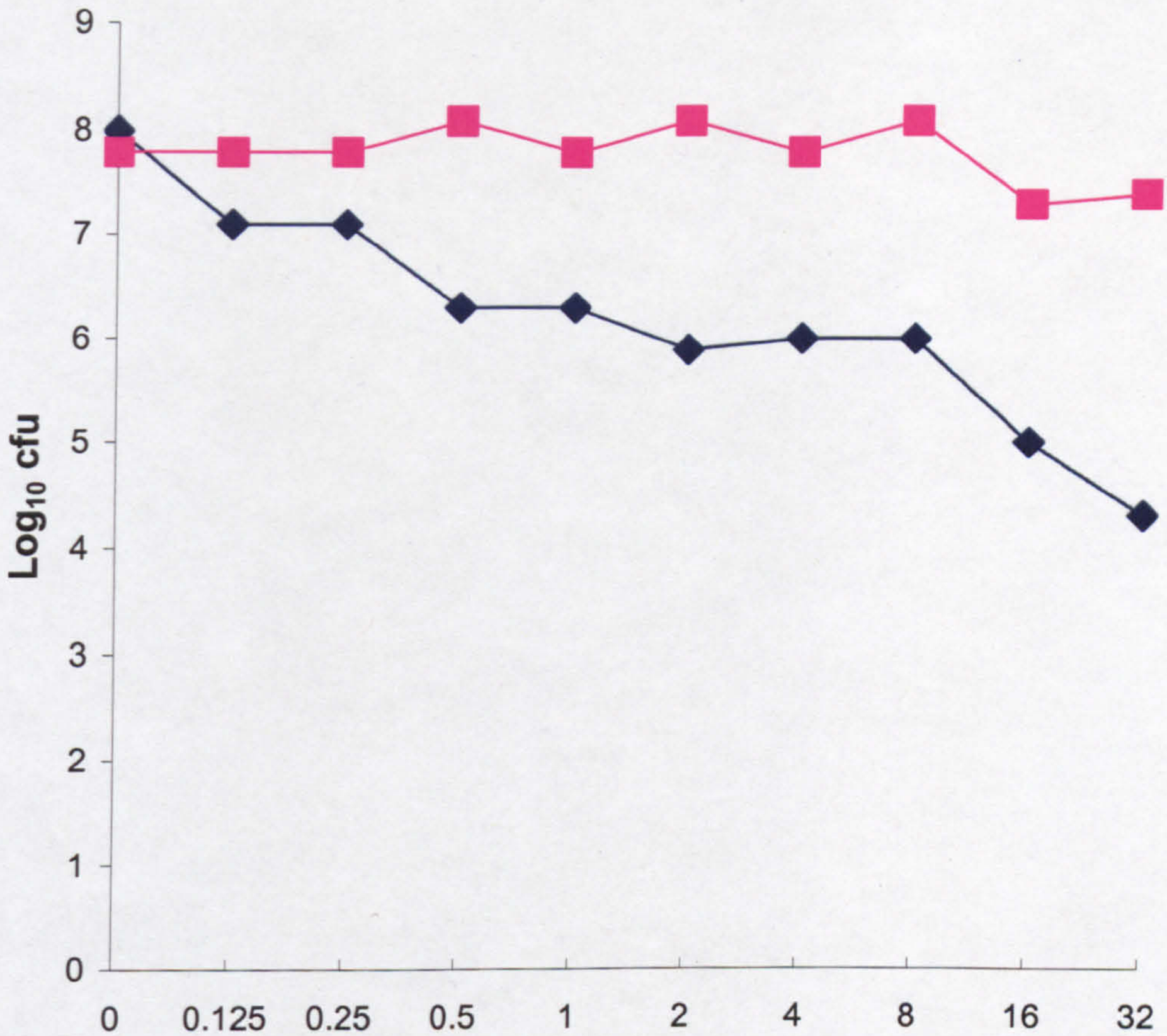

Fig. 31 Effect of metronidazole on growth of *C. difficile* on Sorbarod biofilm.

Individual biofilms were exposed to single concentration of antibiotic for 18h; after collection of effluent, biofilm was harvested. Titres were done as described in the text to determine BEC and effluent MBC. Effluent titres; cfu/mL (◆) and biofilm titres; cfu/filter (■).

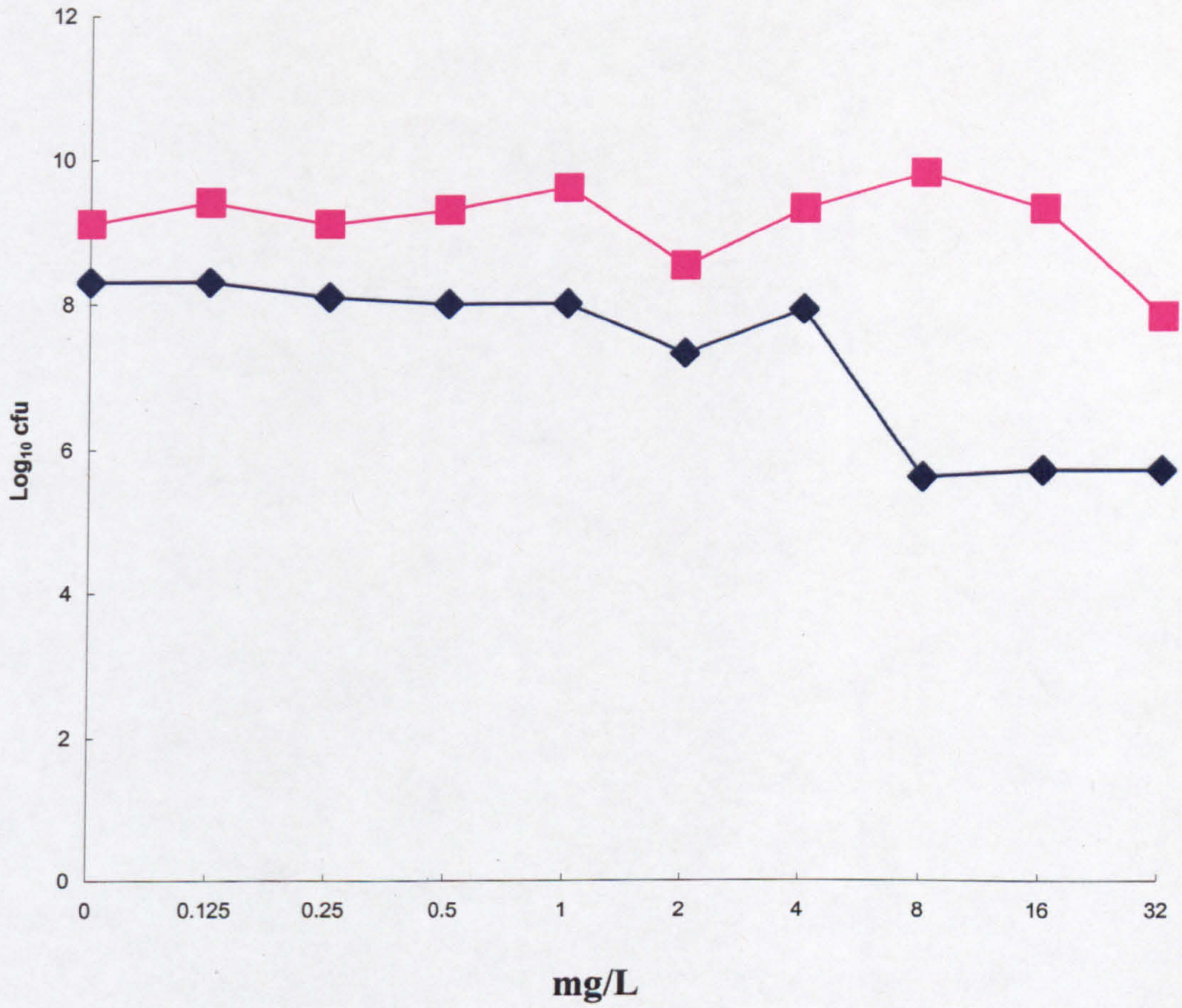

Fig. 32 Repeat experiment – effect of metronidazole on growth of *C. difficile* in biofilm. Effluent titres; cfu/mL (◆) and biofilm titres; cfu/filter (■).

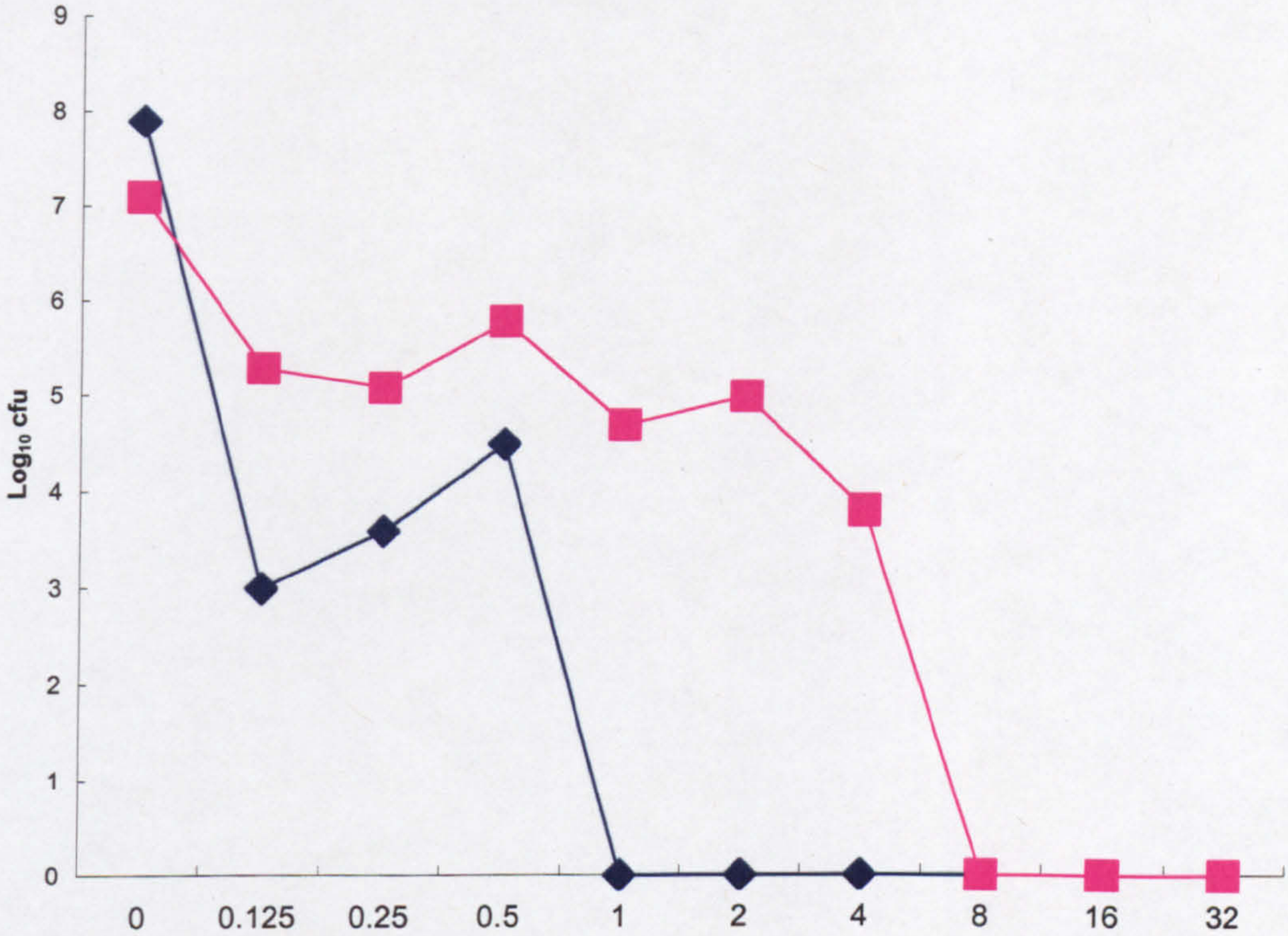

Fig. 33 Effect of rifampicin on growth of *C. difficile* on Sorbarod biofilm. Individual biofilms were exposed to single concentration of antibiotic for 18h; after collection of effluent, biofilm was harvested. Titres were done as described in the text to determine BEC and effluent MBC. Effluent titres; cfu/mL (♦) and biofilm titres; cfu/filter (■).

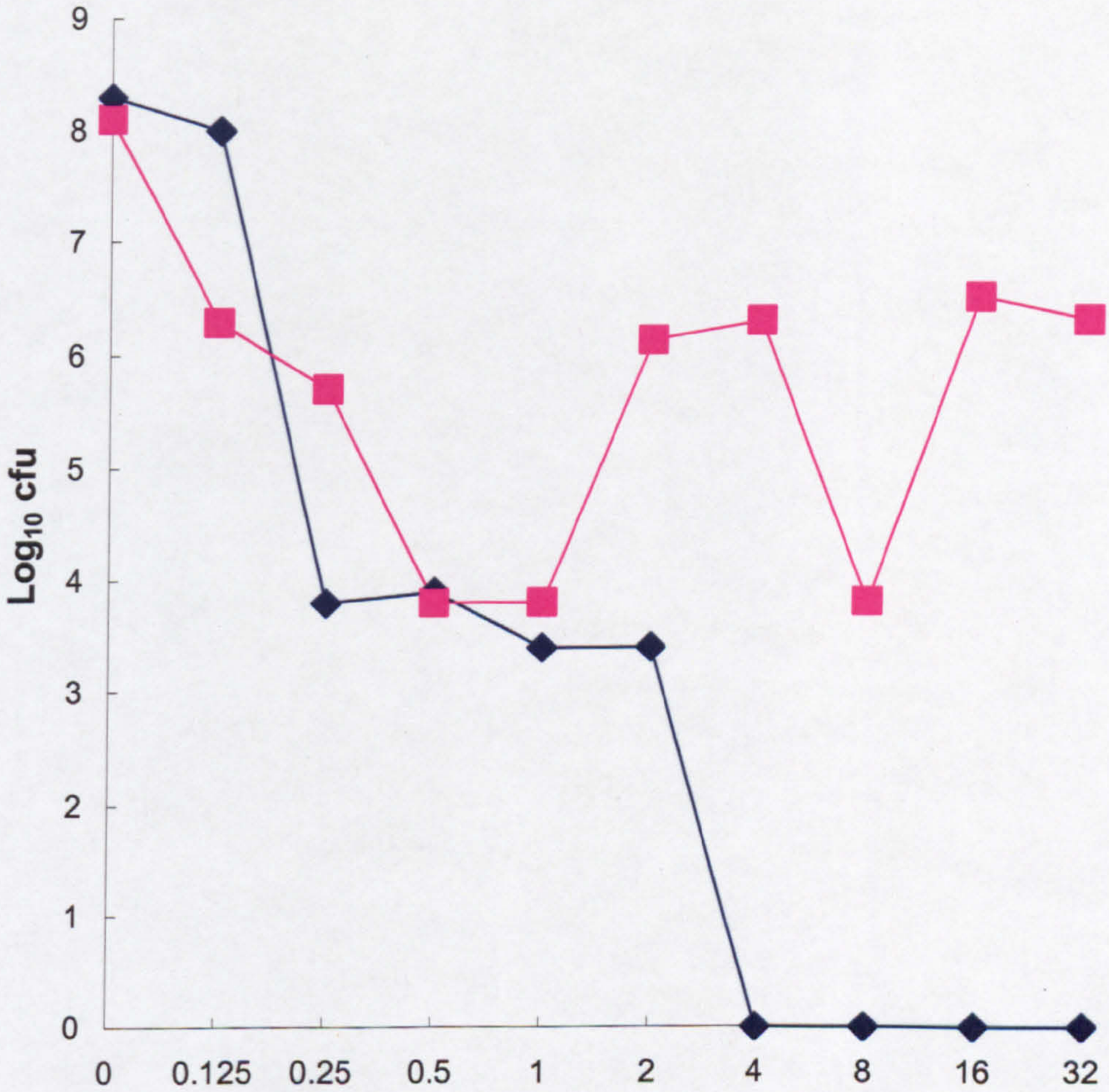

Fig. 34 Repeat experiment – effect of rifampicin on growth of *C. difficile* in biofilm. Effluent titres; cfu/filter (◆) and biofilm titres; cfu/filter (■).

4.4 The effect of high concentration of vancomycin alone or combined with fixed concentrations of rifampicin.

Filter and effluent experiments were conducted where the organism was exposed to vancomycin at concentrations up to 256 mg/L. These are shown in Figs. 35 and 36. Here again *C. difficile* was resistant to this antibiotic at these very high concentrations. The results of combining vancomycin over the same concentration range with rifampicin at 1 or 8 mg/L are shown in Figs. 37 and 38 respectively. This identifies a form of synergy between the two antibiotics, with a discerned killing effect with rifampicin at a fixed concentration. For 1mg/L of rifampicin, the organism was eliminated at 128mg/L of vancomycin in both the biofilm and effluent. For 8mg/L the organism was eliminated at 32mg/L of vancomycin.

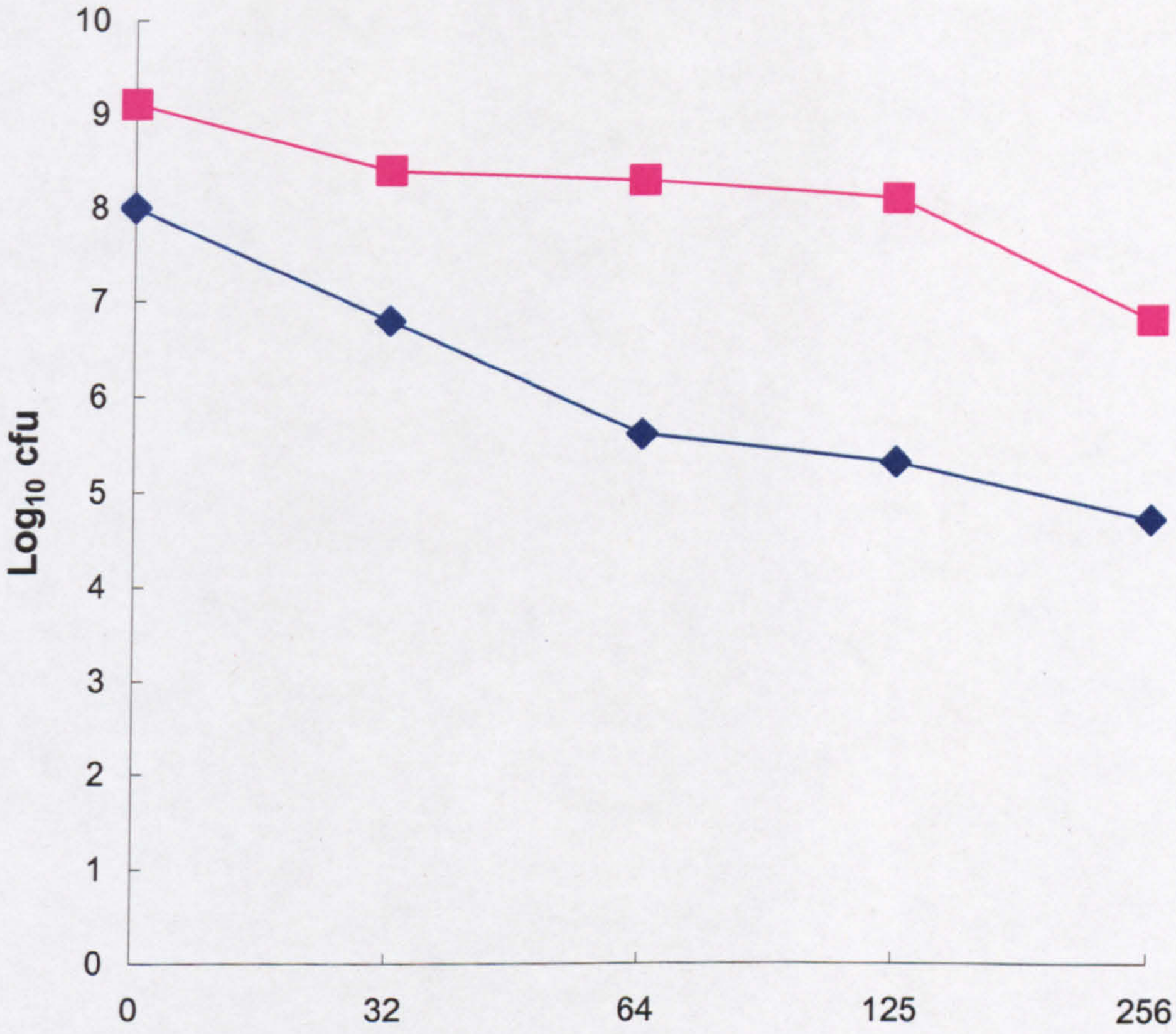

Fig. 35 Effect of high concentration of vancomycin on growth of *C. difficile* in biofilm. Effluent titre; cfu/filter (◆) and biofilm titre; cfu/filter (■).

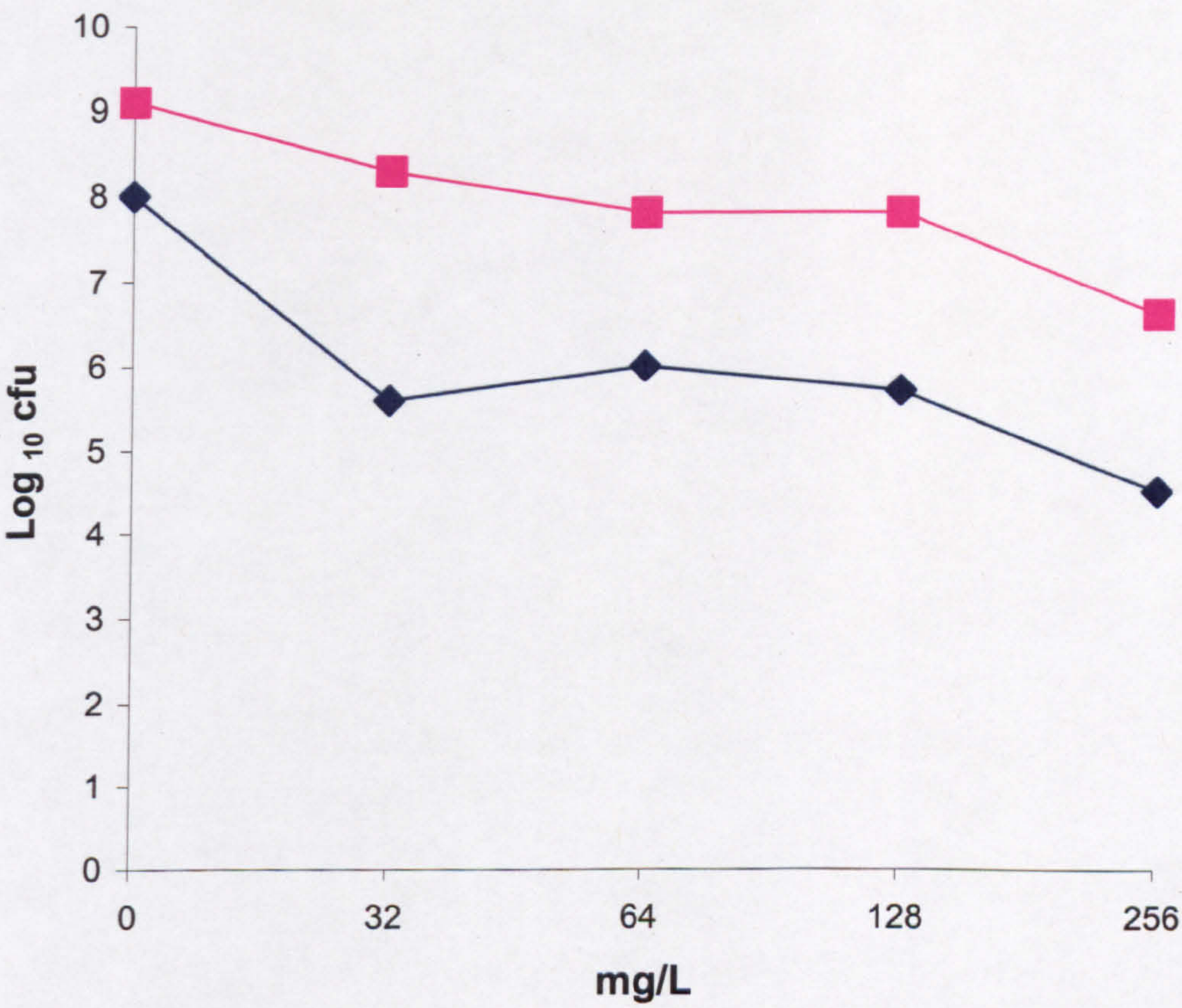

Fig. 36 Repeat experiment of effect of high concentration of vancomycin on *C. difficile* in biofilm. Effluent titre; cfu/filter (♦) and biofilm titre; cfu/filter (■).

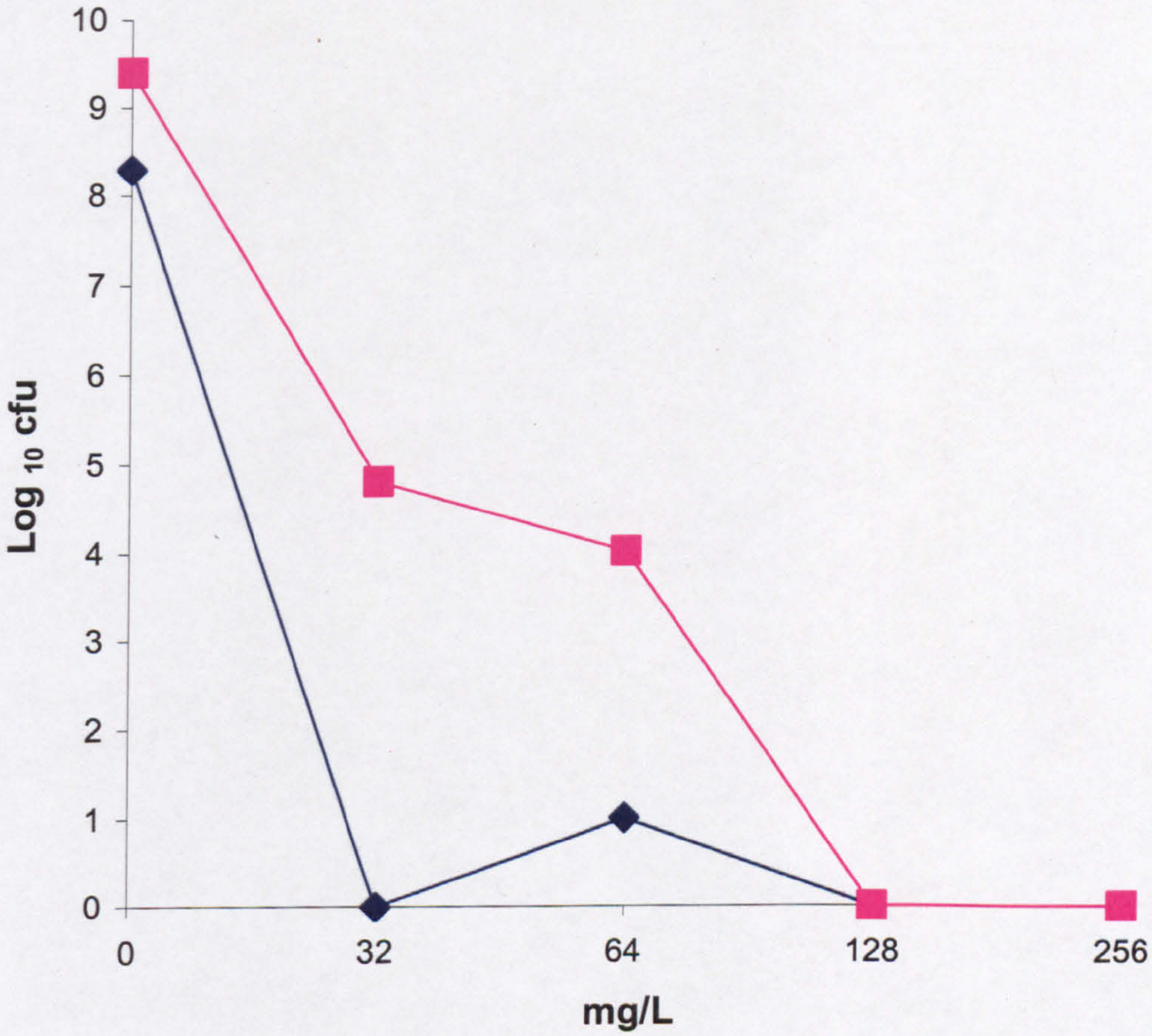

Fig. 37 Effect of rifampicin (1mg/L) + differing vancomycin concentrations on growth of *C. difficile* in biofilm. Effluent titre; cfu/filter (♦) and biofilm titre; cfu/filter (■).

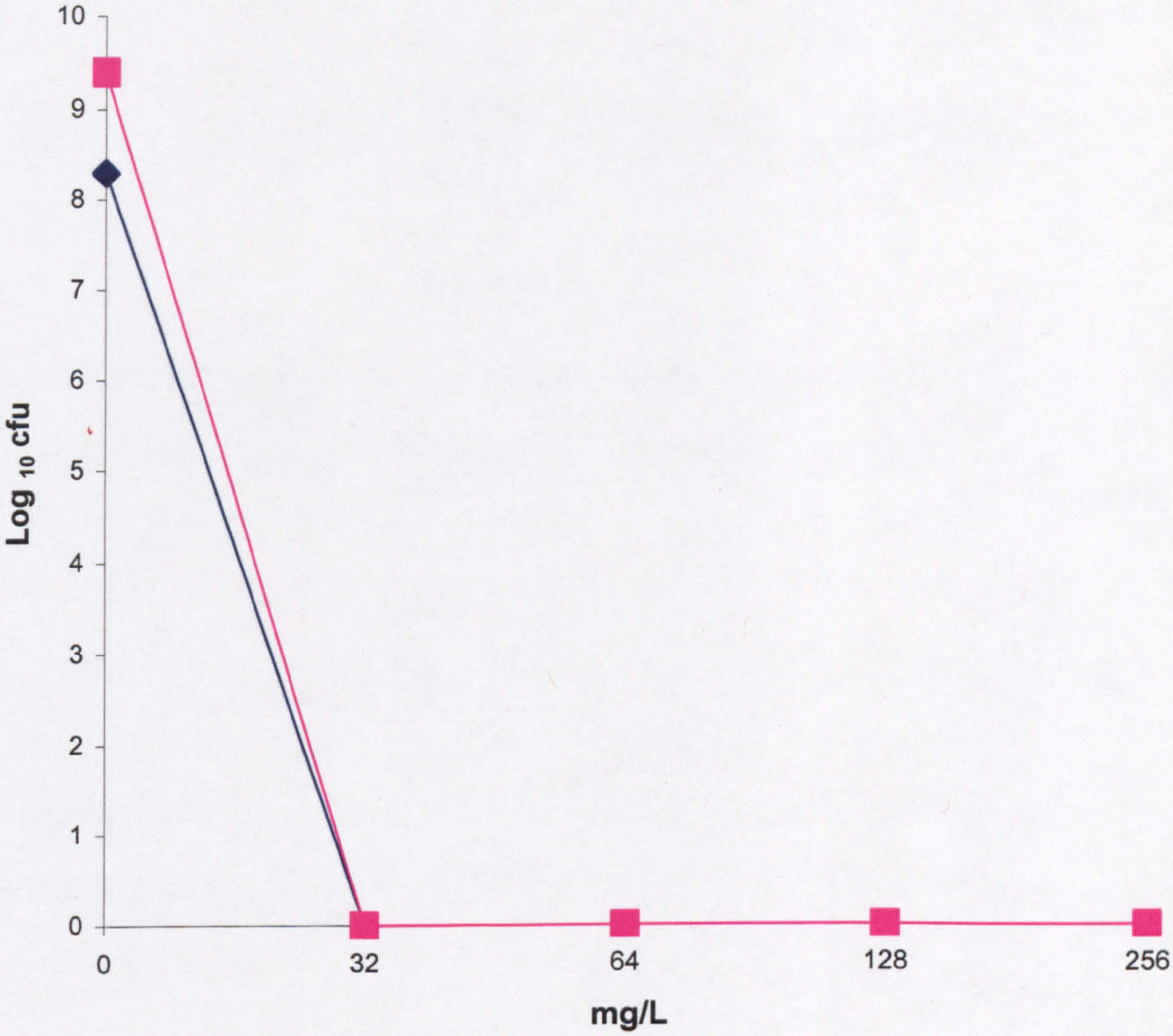

Fig. 38 Effect of combination of rifampicin (8mg/L) + differing vancomycin concentrations on growth of *C. difficile* in biofilm. Effluent titre; cfu/filter (◆) and biofilm titre; cfu/filter (■).

4.5 The effect of antibiotics on toxin production by *C. difficile* in biofilm

Following antimicrobial susceptibility testing of *C. difficile* in biofilm, effluents and vortexed filters were centrifuged for each antibiotic, at differing concentrations, in duplicate. Toxin activity was determined, as previously described in Chapter 2.

In general, toxin activity was always present in biofilm for all antibiotics. Absorbance values were maximum at the lowest concentration of antibiotic. This contrasts with toxin activity for biofilm effluents, although absorbance measured was lesser in value, activity appeared to increase when antibiotic concentrations increased.

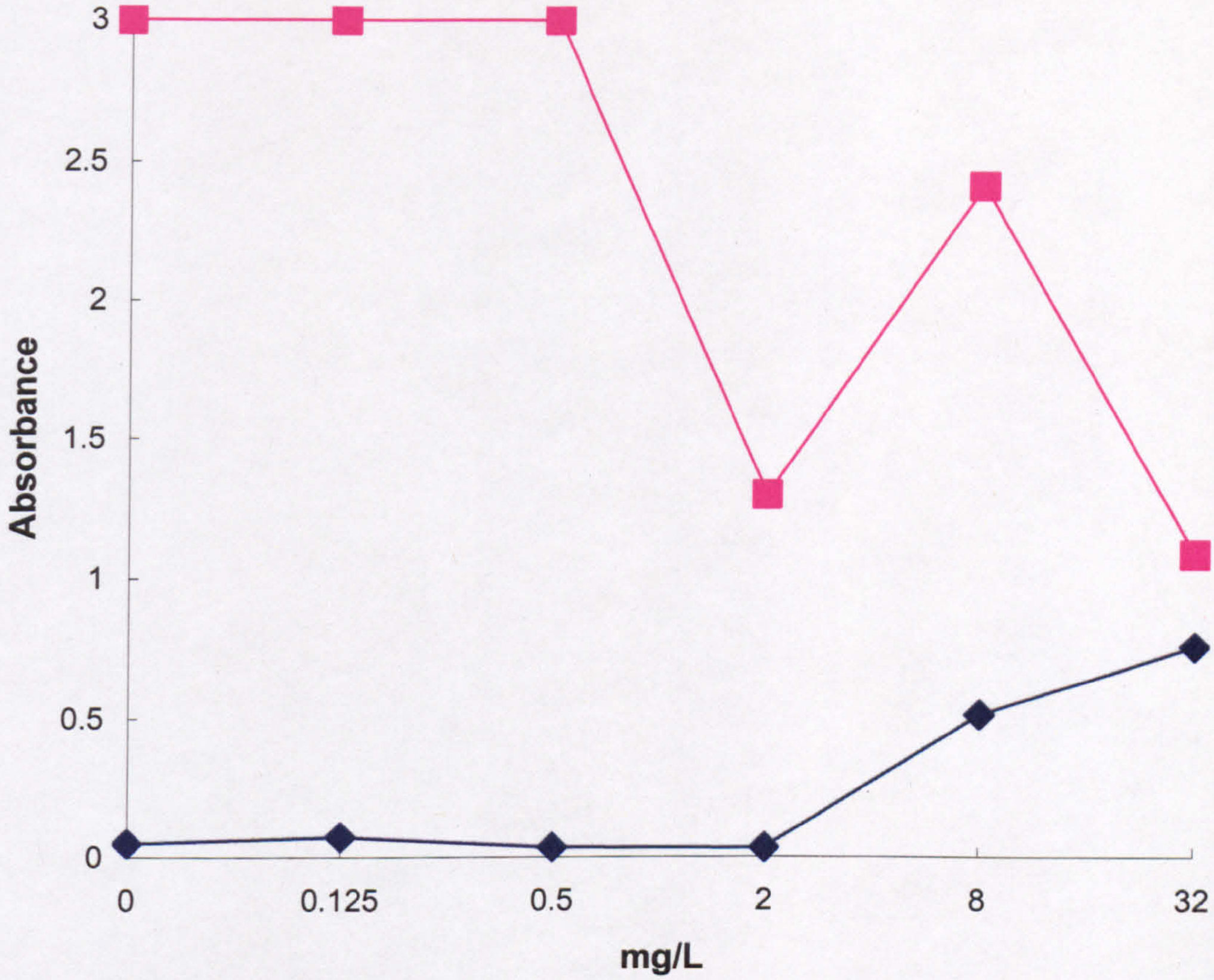

Fig. 39 Effect of vancomycin on toxin production of *C. difficile* in biofilm.

Absorbance values of biofilm effluent (◆) and biofilm (■).

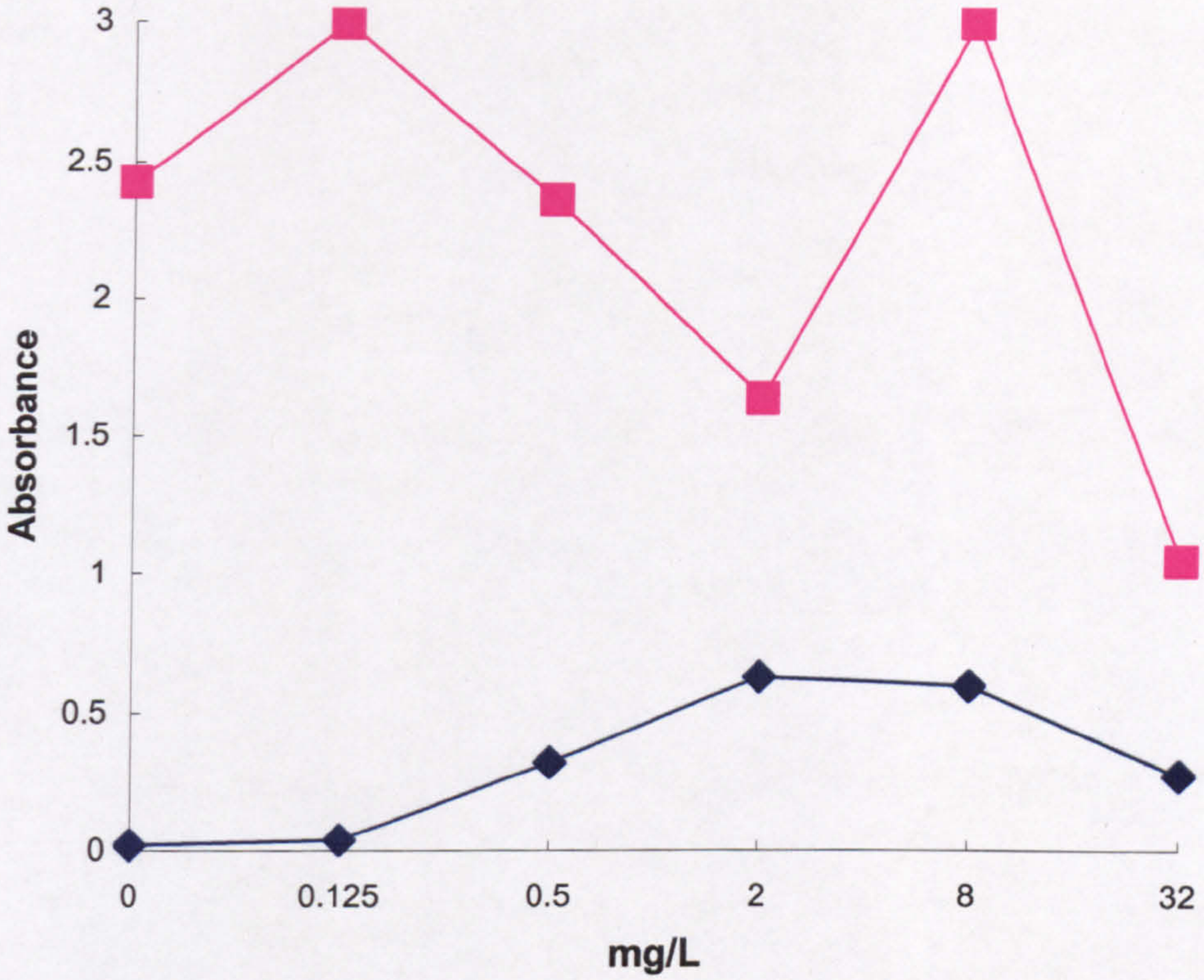

Fig. 40 Effect of vancomycin on toxin production of *C. difficile* in biofilm.

Absorbance values of biofilm effluent (◆) and biofilm (■).

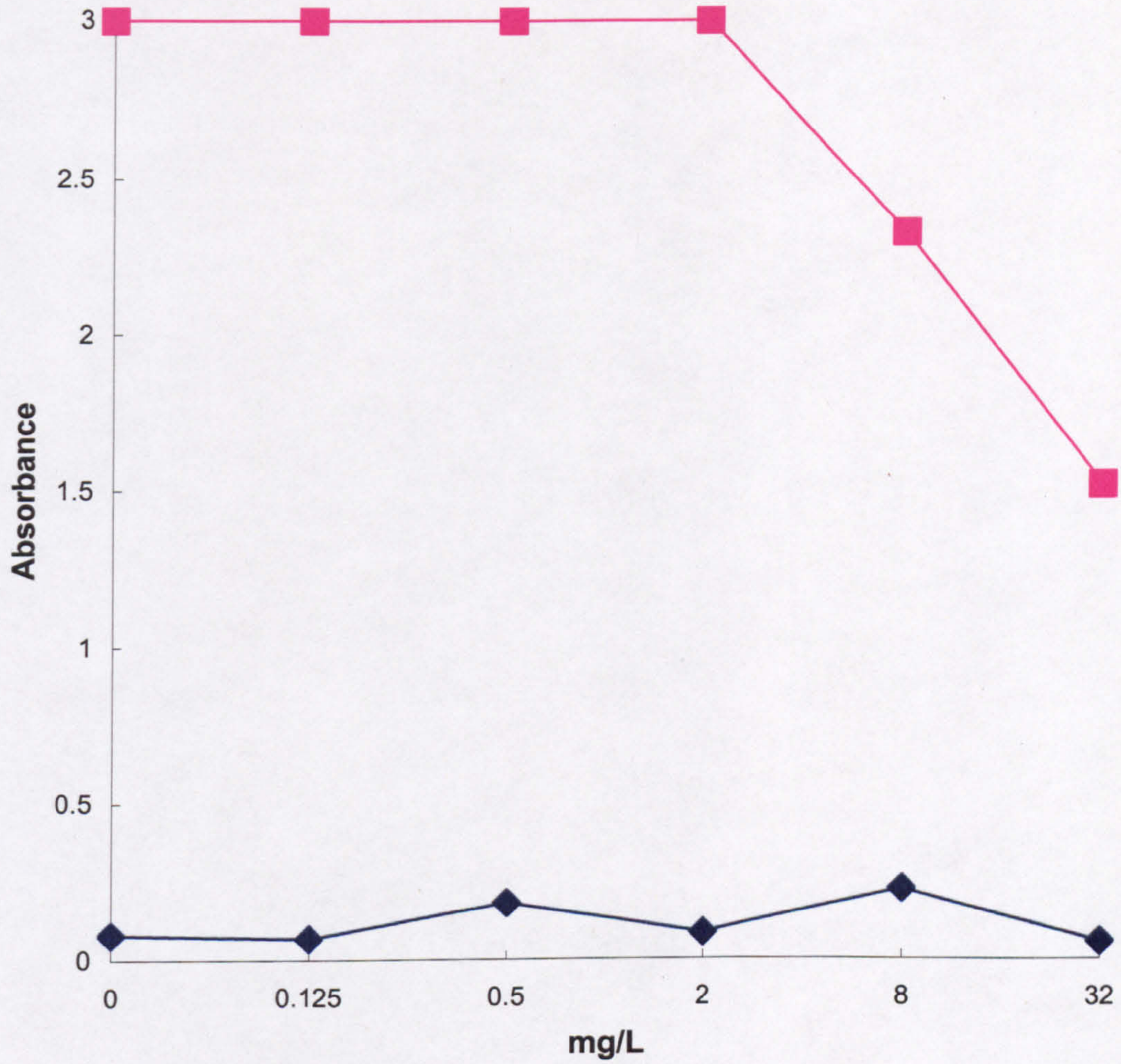

Fig. 41 Effect of metronidazole on toxin production of *C. difficile* in biofilm.

Absorbance values of biofilm effluent (◆) and biofilm (■).

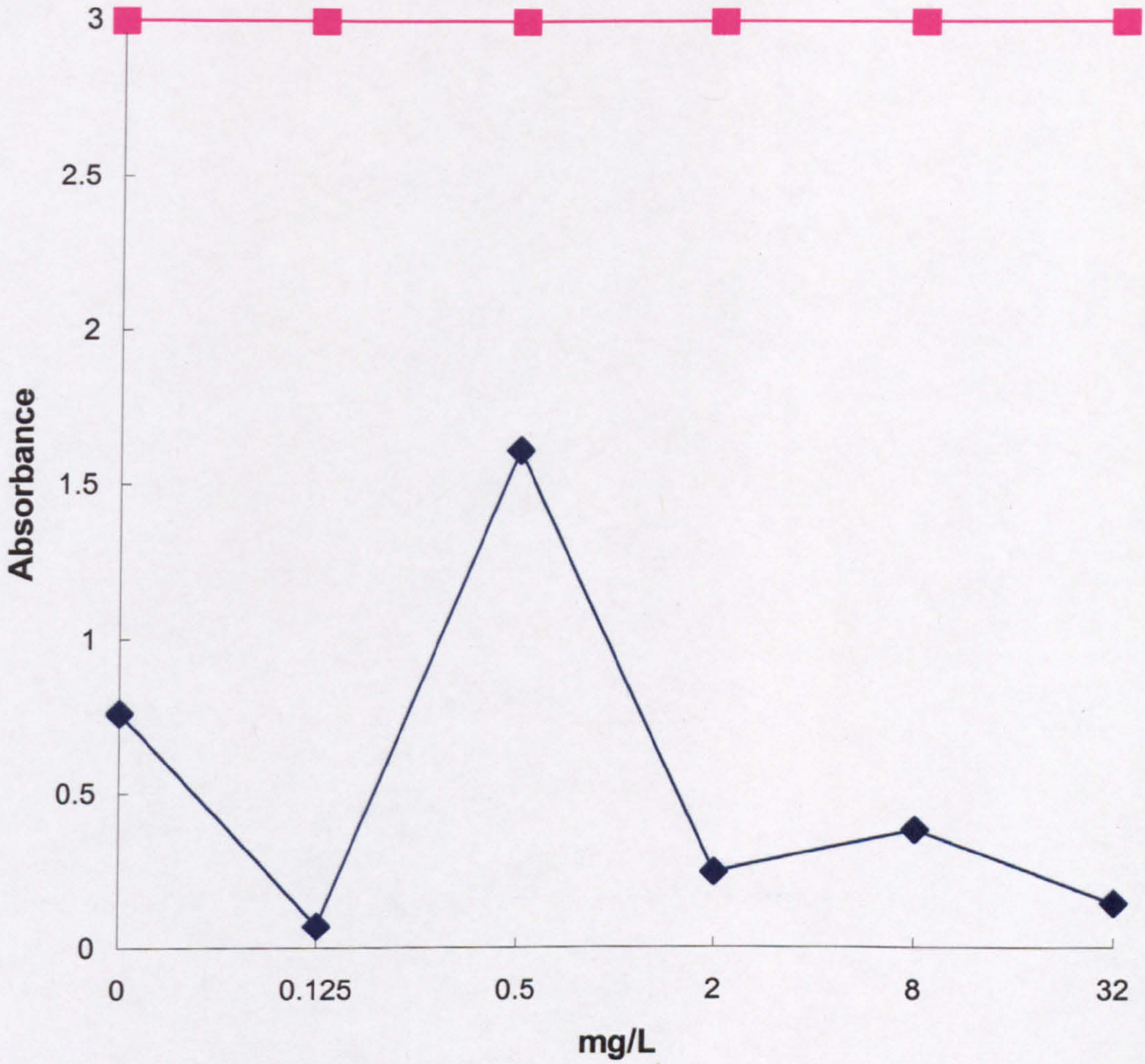

Fig. 42 Effect of metronidazole on toxin production of *C. difficile* in biofilm.

Absorbance values of biofilm effluent (◆) and biofilm (■).

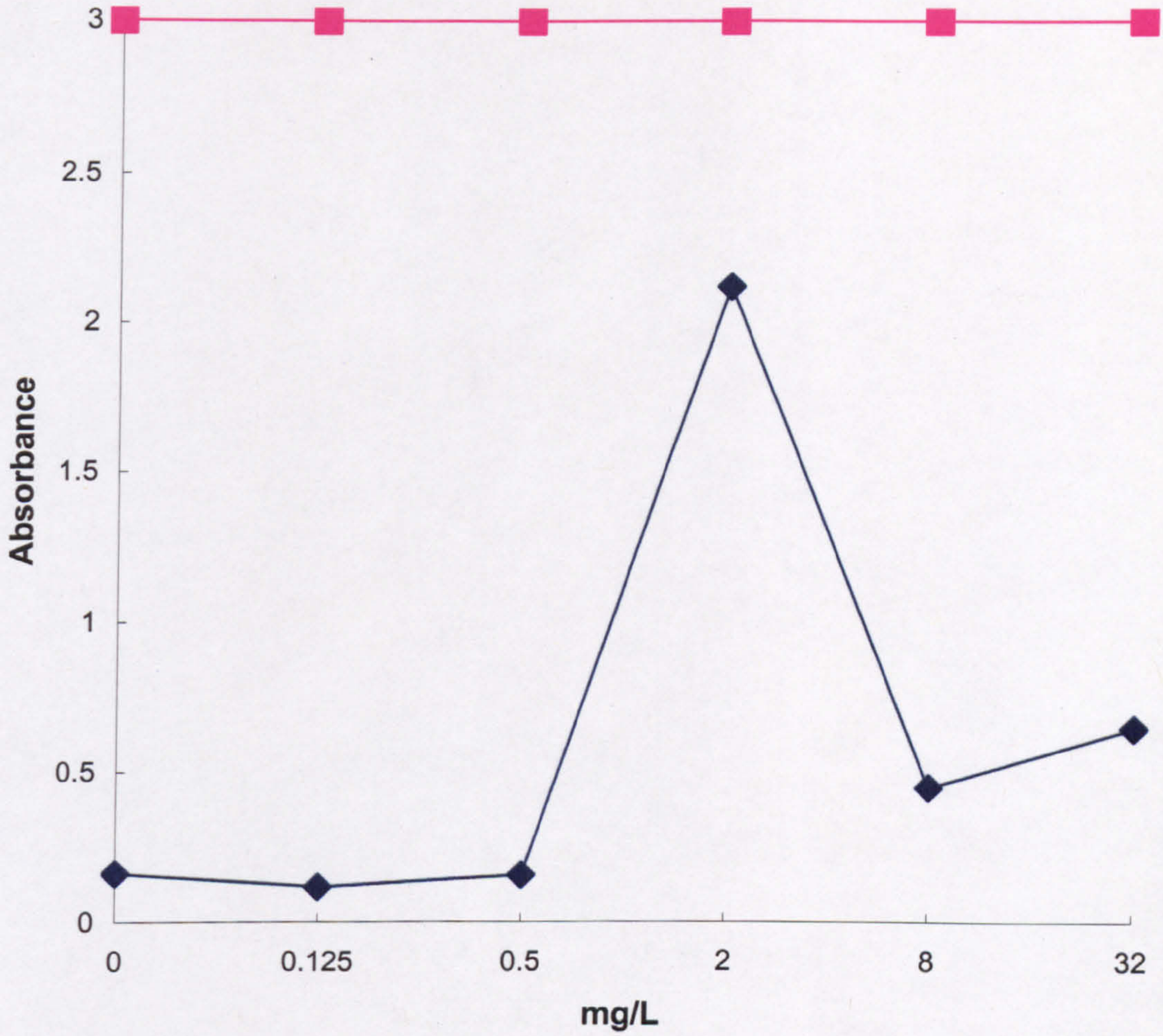

Fig. 43 Effect of penicillin on toxin production of *C. difficile* in biofilm. Absorbance values of biofilm effluent (◆) and biofilm (■).

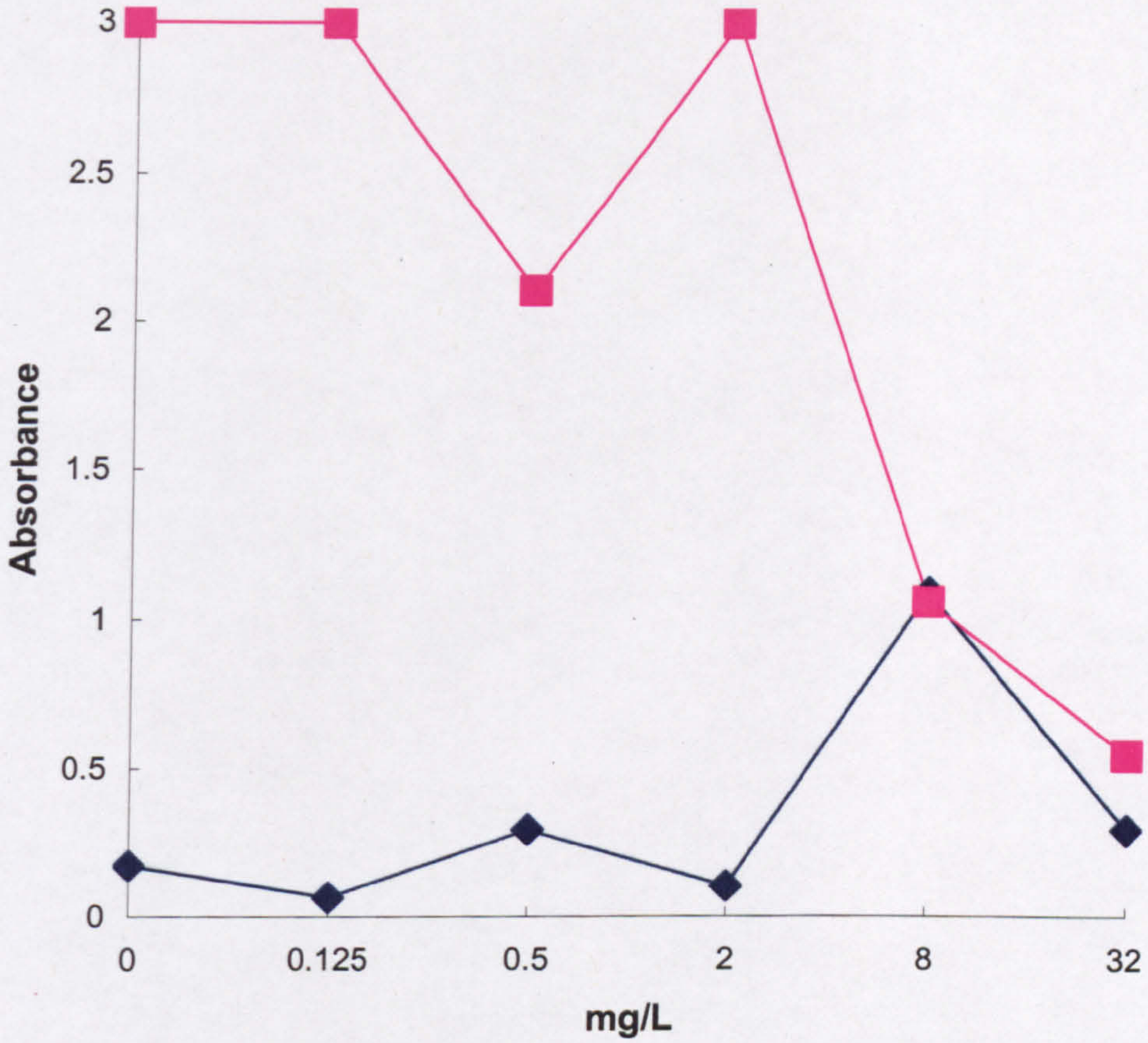

Fig. 44 Effect of penicillin on toxin production of *C. difficile* in biofilm. Absorbance values of biofilm effluent (◆) and biofilm (■).

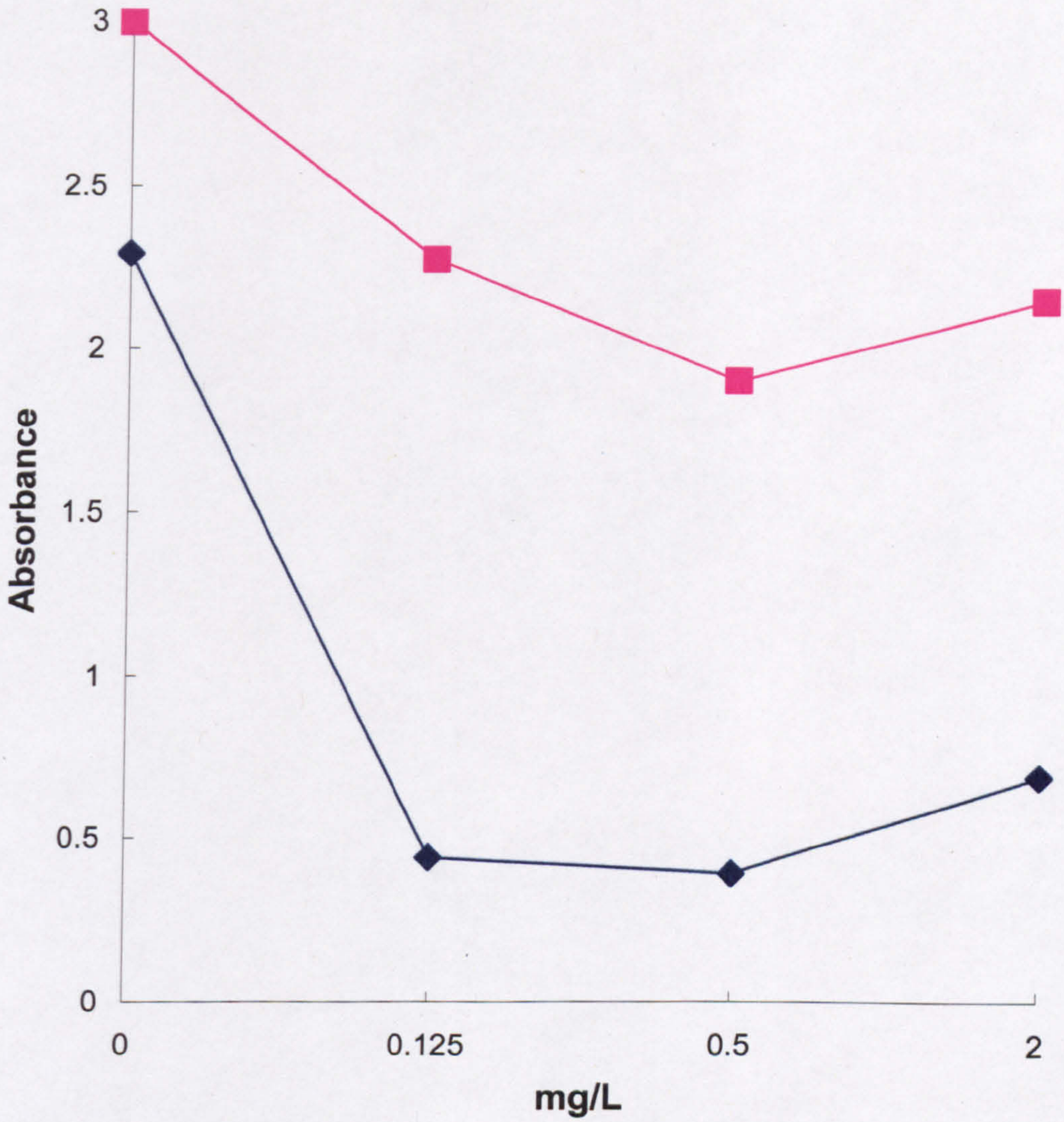

Fig. 45 Effect of rifampicin on toxin production of *C. difficile* in biofilm.

Absorbance values of biofilm effluent (◆) and biofilm (■).

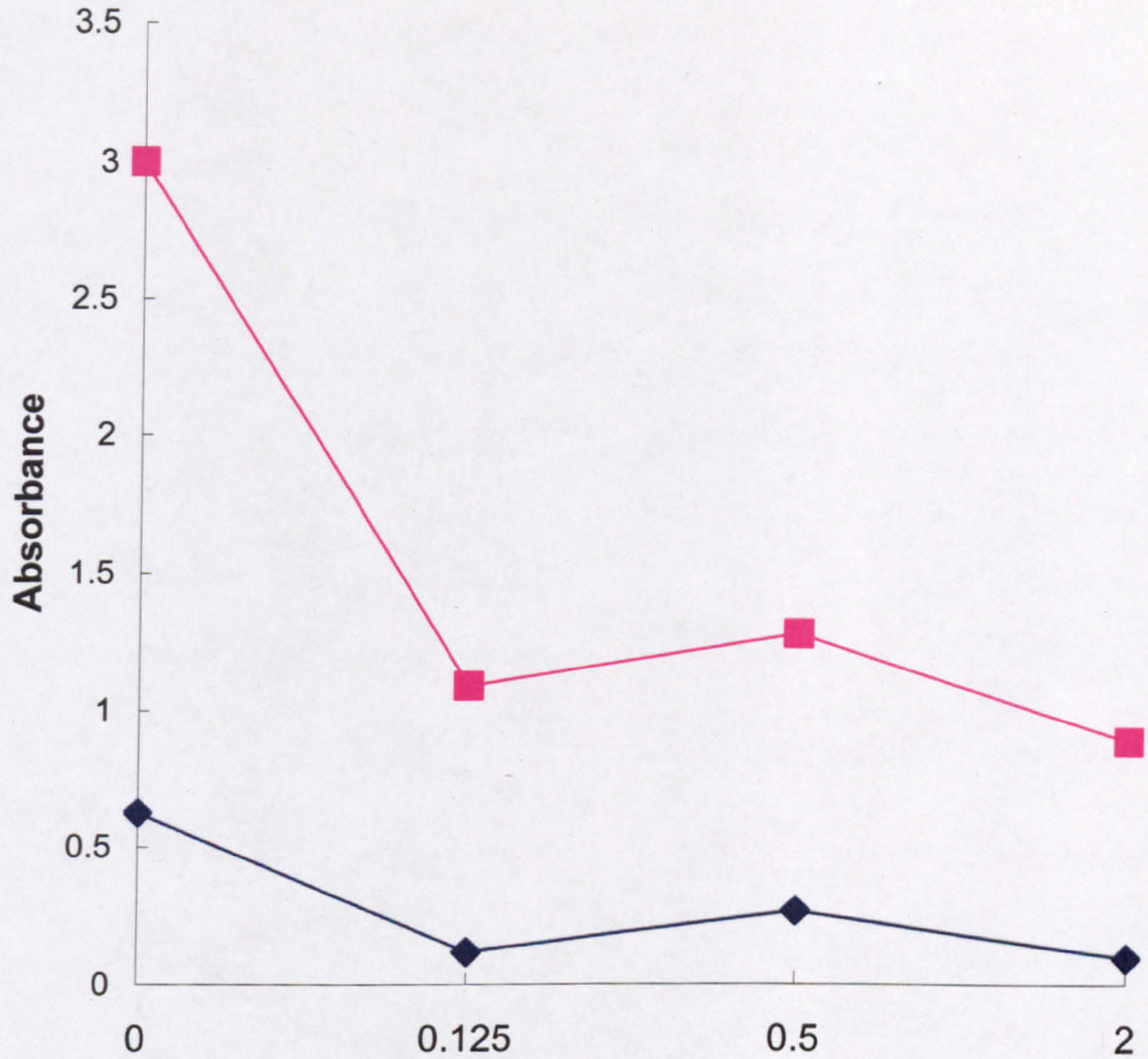

Fig. 46 Effect of rifampicin on toxin production of *C. difficile* in biofilm.

Absorbance values of biofilm effluent (◆) and biofilm (■).

4.6 Discussion

The MIC values of both the clinical and ATCC strains of *C. difficile* to benzylpenicillin, vancomycin and metronidazole correlated with the MIC values in the literature (Kucers and Bennett, 1987). However a high degree of tolerance, usually in excess of 128 mg/L, was identified in the tube MBC. This feature was mirrored in the Sorbarod filter experiments with these antibiotics, reflecting the accepted resistance to antibiotics in a biofilm system (Gander, 1996; Struthers, 2000). The tolerance in the biofilm effluent reflected that reported previously with other organisms (Muli and Struthers, 1998; Struthers, 2000). It should be noted that when bacteria from the filters and effluent were plated onto agar and examined directly by disc and Etest methods, that the bacteria had reverted to the sensitive phenotype.

With rifampicin, the MIC confirmed the susceptibility of *C. difficile* to this agent, and a MBC value of 0.25 to 1.0 mg/L was obtained. In the Sorbarod filters, the effluent MBC varied from 1-4 mg/L, and in at least one experiment, controlled by a tube MIC experiment, a BEC of 8 mg/L was obtained. This showed that rifampicin appeared to have significantly better bacteriocidal activity against the organism when it was in broth, as a biofilm in the filters, or in filter effluent, than the other three agents.

Further experiments showed that tolerance to vancomycin occurred in the filters and effluent up to 256 mg/L at least, but with the addition of rifampicin at appropriate pharmacokinetic levels, significant synergistic killing was achieved in the filter system.

With benzylpenicillin, metronidazole and rifampicin, conducting experiments with concentrations of up to 32 mg/L are appropriate, as the range up to this value relates to the pharmacokinetics of these agents in the human (Kucers and Bennett, 1987). With vancomycin, the situation is different, for via the oral route, it is poorly absorbed, and high concentrations, in the order of 200-300 mg/L are obtained in the colonic fluid (Kucers and Bennett, 1987). The tolerance of the *C. difficile* to three of the agents in the filters and effluent could be of relevance to the treatment of CDAD. It may be reasonable to assume that the addition of rifampicin to vancomycin, based on the work here, could have a clinical basis to it, considering that vancomycin achieves high concentrations in the colon, and noting the report that the combination of vancomycin and rifampicin is effective in treatment (Buggy, 1993).

As discussed in chapter 3, the testing of toxin by the EIA did have a degree of variability associated with it, and this is reflected in the toxin experiments done here. It is worthwhile to note that at the lower antibiotic concentrations, that the level of toxin was consistently lower in the effluent than that in the filter. This to an extent may reflect the difference in the titres of organisms in the two circumstances, but it may be that there is some control process in the biofilm form of growth. In two experiments (Figs. 42 and 43), the maximum toxin levels were maintained up to 32 mg/L in the filters.

This work laid the basis for the next chapter, where the growth of *C. difficile* in biofilm was investigated in combination with other common bowel bacteria, and the effect of several antibiotics was investigated.

Chapter Five

5.1 Introduction

The colon harbors approximately 10^{11} organisms per mL of fluid and bacteria account for 30% of faecal dry weight (Reese and Betts, 1996). More than 400 different species of bacteria can be found in a single faecal specimen. The bacterial flora of the gastrointestinal tract has been the subject of investigation for many years. This is a site of proliferation of a large number of species of micro-organisms, with domination of different strains being influenced by many factors, and competition and interference being widespread (Reid *et al.*, 1990). Some gastrointestinal tract studies have focused on bacterial interactions and their impact on anatomic, physiologic, and immunologic parameters of the colon (Rolfe, 1984). Antagonisitic and co-operative reactions, which lead to interference in the establishment of pathogenic organisms, are known to occur. This process, whereby the microflora resists incursion by harmful microorganisms, has been referred to as colonization resistance (Reese and Betts, 1996).

Maintenance of a normal bowel ecosystem is one of the main defences against *C. difficile* colonization (Borriello, 1998). Almost any antibiotic may cause *C. difficile* disease, but broad-spectrum antibiotics allow C. *difficile* to become established in the bowel (Gorbach, 1999). It has been shown previously that two organisms can be grown together on Sorbarod filters (Muli and Struthers, 1998; Budhani and Struthers, 1999). In the case of *S. pneumoniae* and *Moraxella catarrhalis*, both organisms maintained similar titres on the biofilms (Budhani and Struthers, 1999). The Sorbarod

filter system was used here to investigate the growth of *C. difficile* in combination with recognized members of the bowel flora and included *Bacteroides fragilis, Enterococcus faecalis* and *Escherichia coli.*

Human and animal stool specimens were screened for bacteriophage and bacteriocin activity against *C. difficile,* and if such activity were identified, some basic investigations were to be conducted. The aim of the chapter was to use the Sorbarod filter system as an investigative procedure for combinations of *C. difficile* with other bowel bacteria.

5.2 Growth of *Bacteroides fragilis, Enterococcus faecalis* and *Escherichia coli* in biofilm

Initially, before combination experiments could be considered, it was important to establish if *B. fragilis, E. faecalis* and *E. coli* could readily grow as single organisms in biofilm on the Sorbarod filters. Growth of each organism could be achieved in this biofilm model and was also maintained for up to 72h.

B. fragilis reached biofilm titres in excess of 10^{10} cfu/filter (Figs. 47, 48). The titre of the *B. fragilis* effluent was in the order of 1-3 log. lower. *E. faecalis* reached high biofilm titres of up to 10^{11} cfu/filter (Figs. 49, 50). Again, the effluent for this organism was of the order of 1-2 log. lower. Similarly, *E. coli* sustained growth for 72 h on the filters with titres in excess of 10^{9} cfu/filter (Figs. 51, 52).

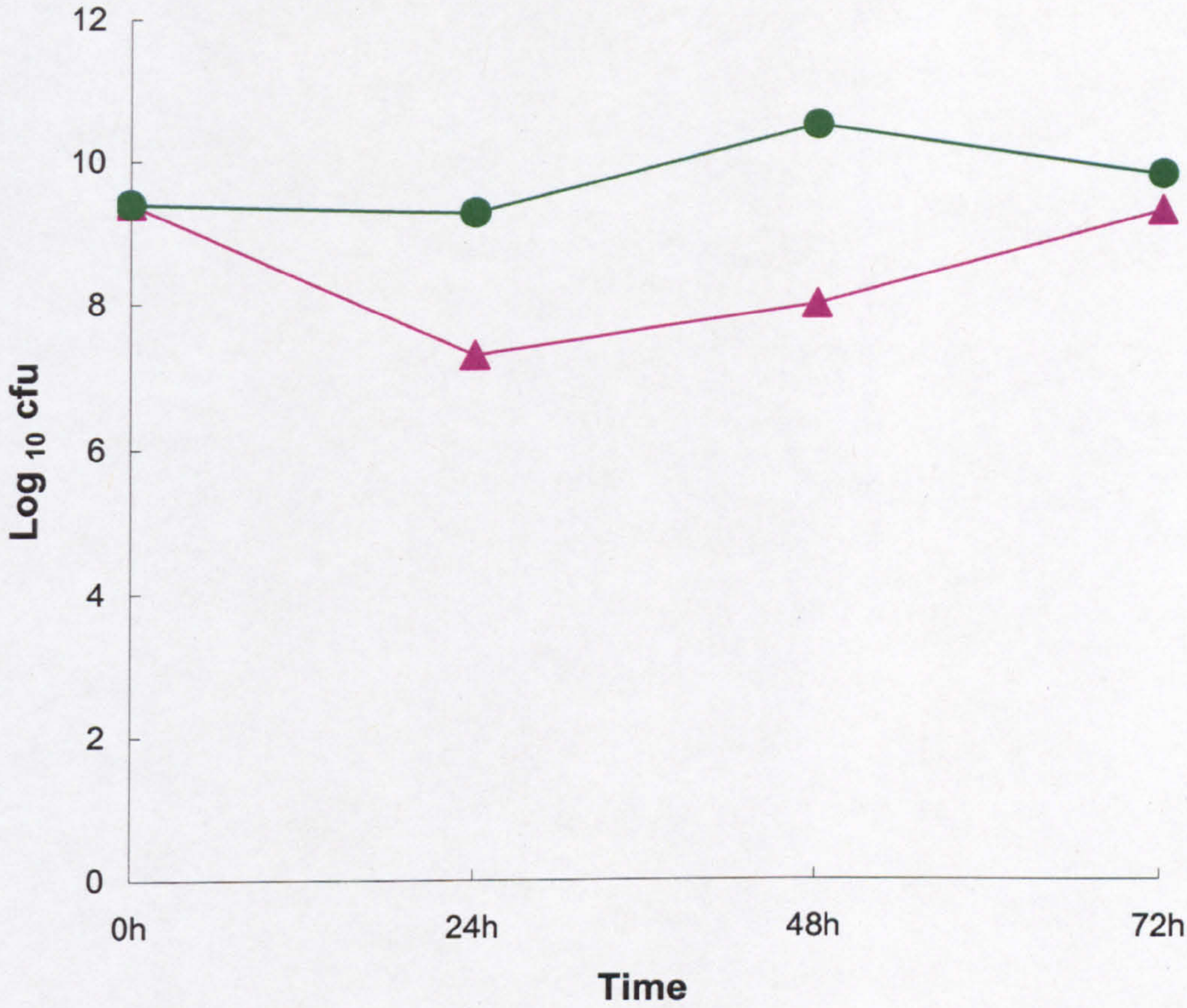

Fig. 47 Growth of *B. fragilis* in biofilm. Effluent titres (▲) and biofilm titres (●).

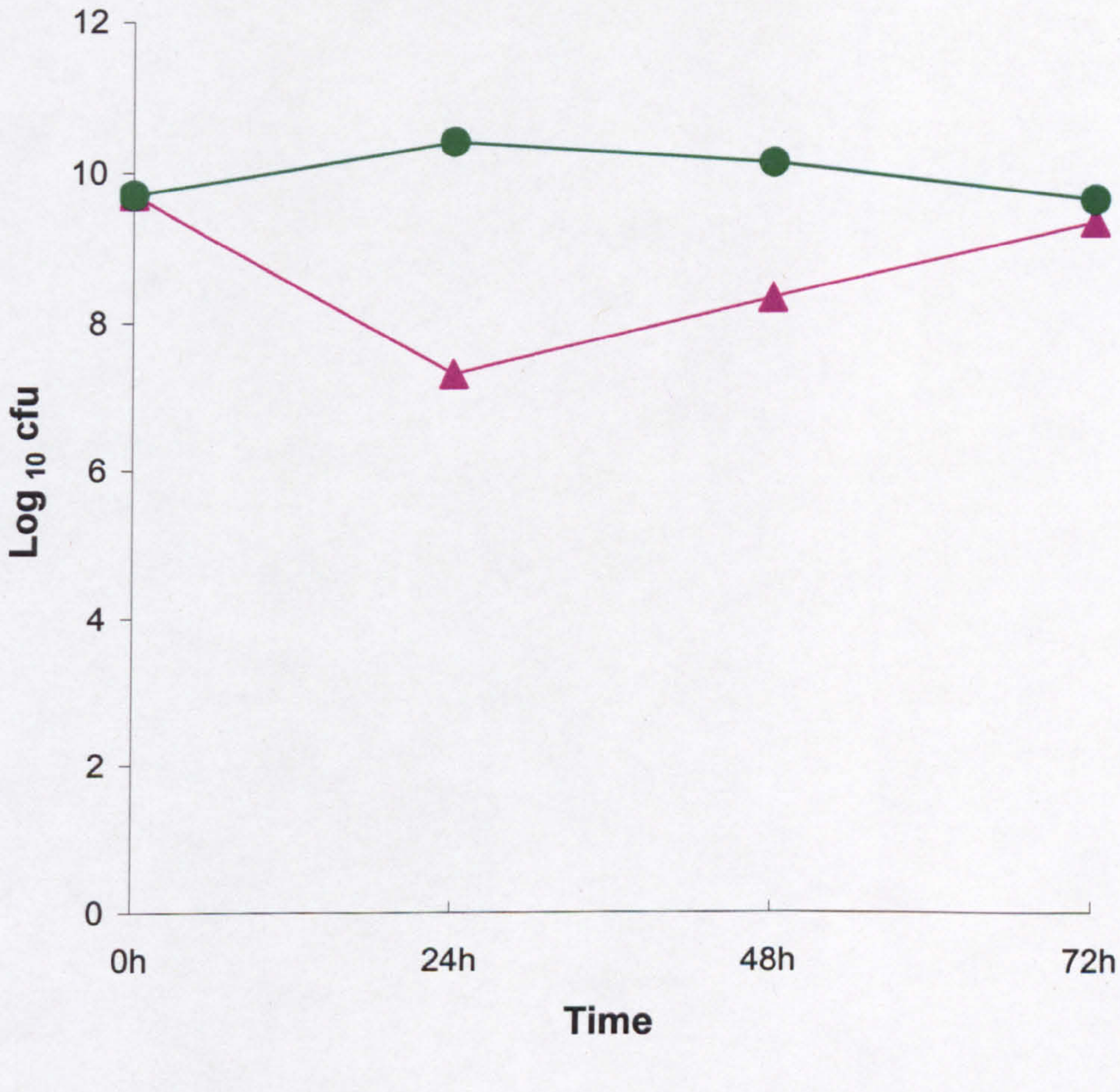

Fig. 48 Repeat experiment of growth of *B. fragilis* in biofilm. Effluent titres (▲) and biofilm titres (●).

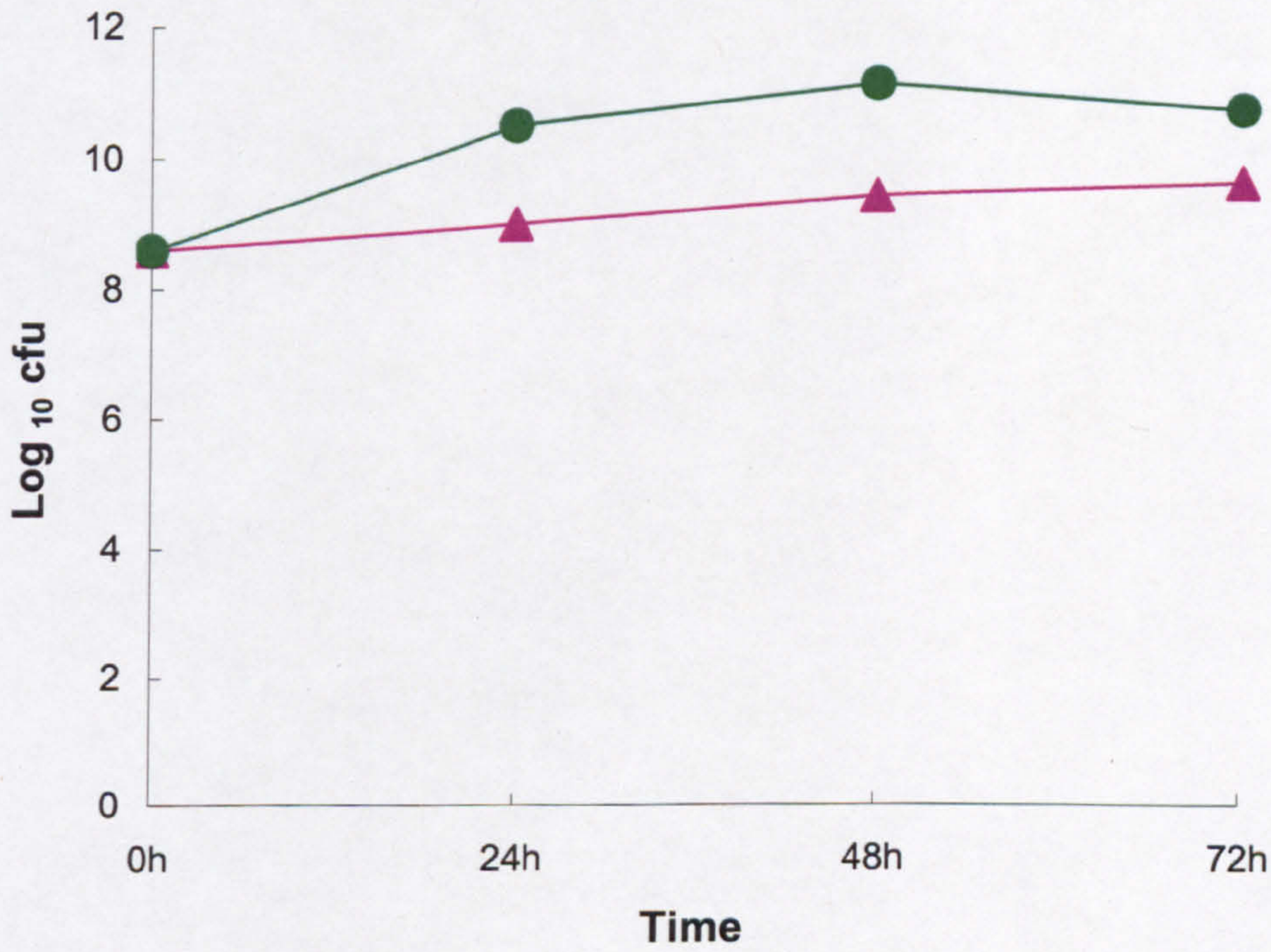

Fig. 49 Growth curve of *E. faecalis* in biofilm. Effluent titres (▲) and biofilm titres (●).

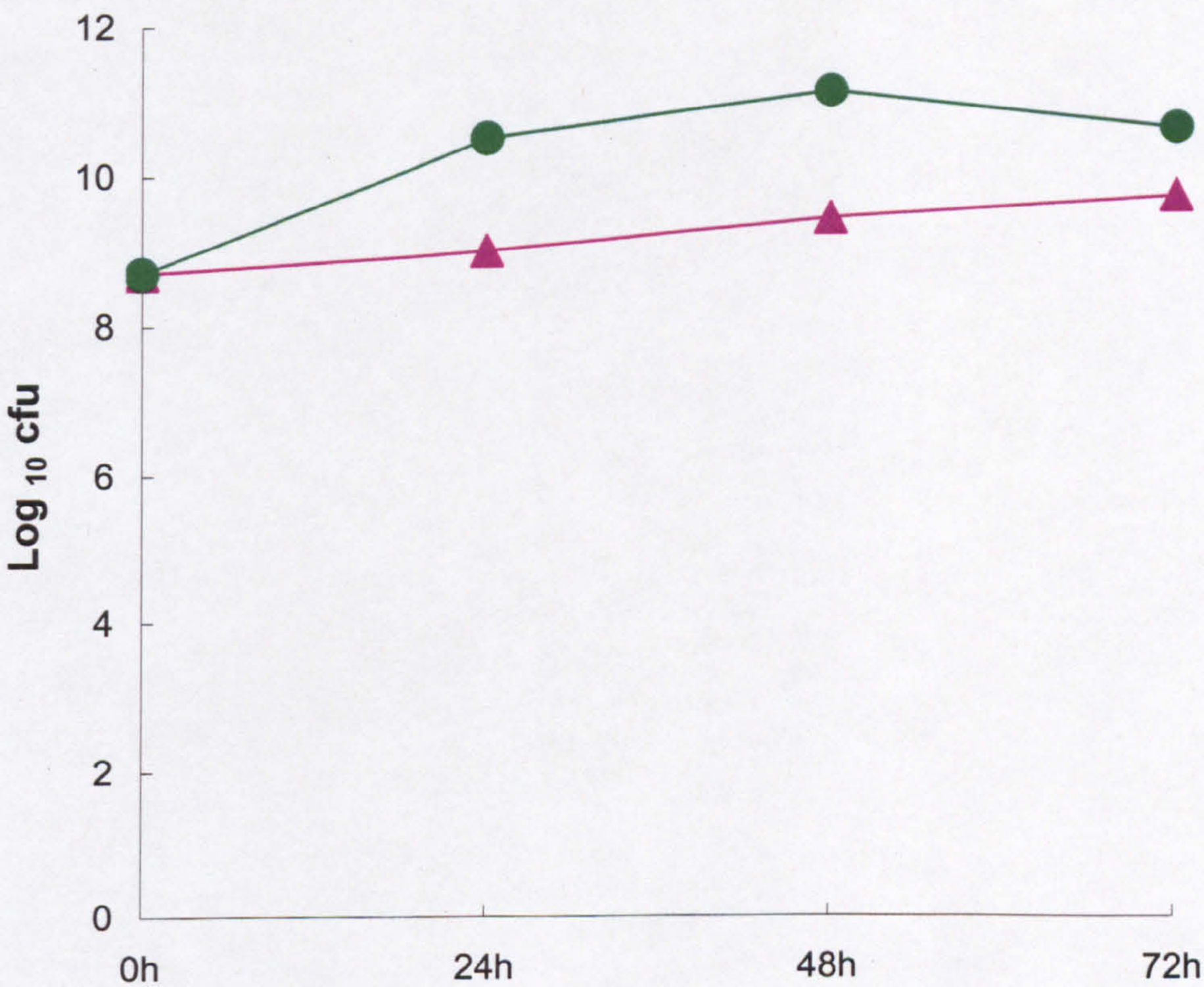

Fig. 50 Growth curve of *E. faecalis* in biofilm. Effluent titres (▲) and biofilm titres (●).

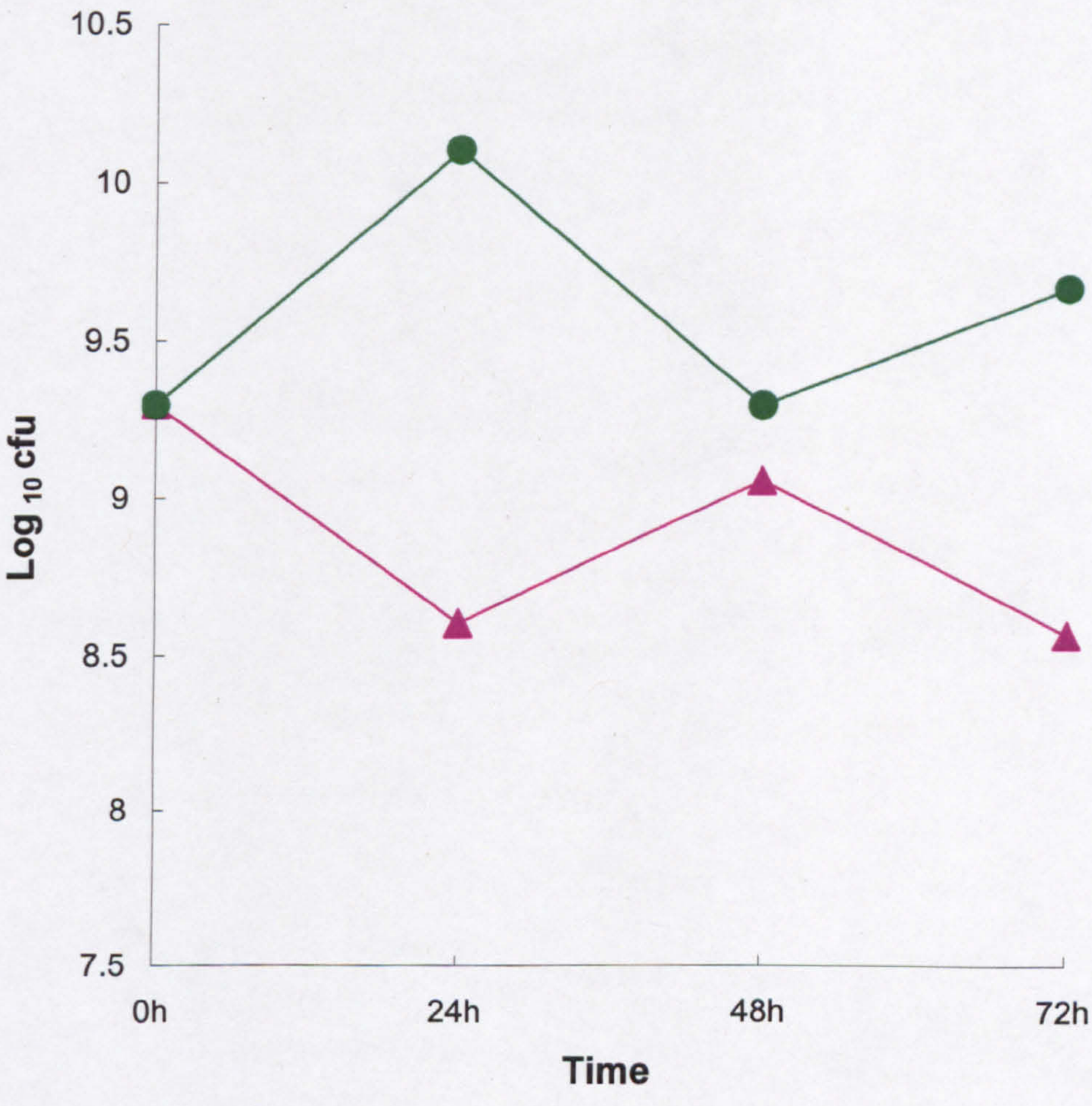

Fig. 51 Growth curve of *E. coli* in biofilm. Effluent titres (▲) and biofilm titres (●).

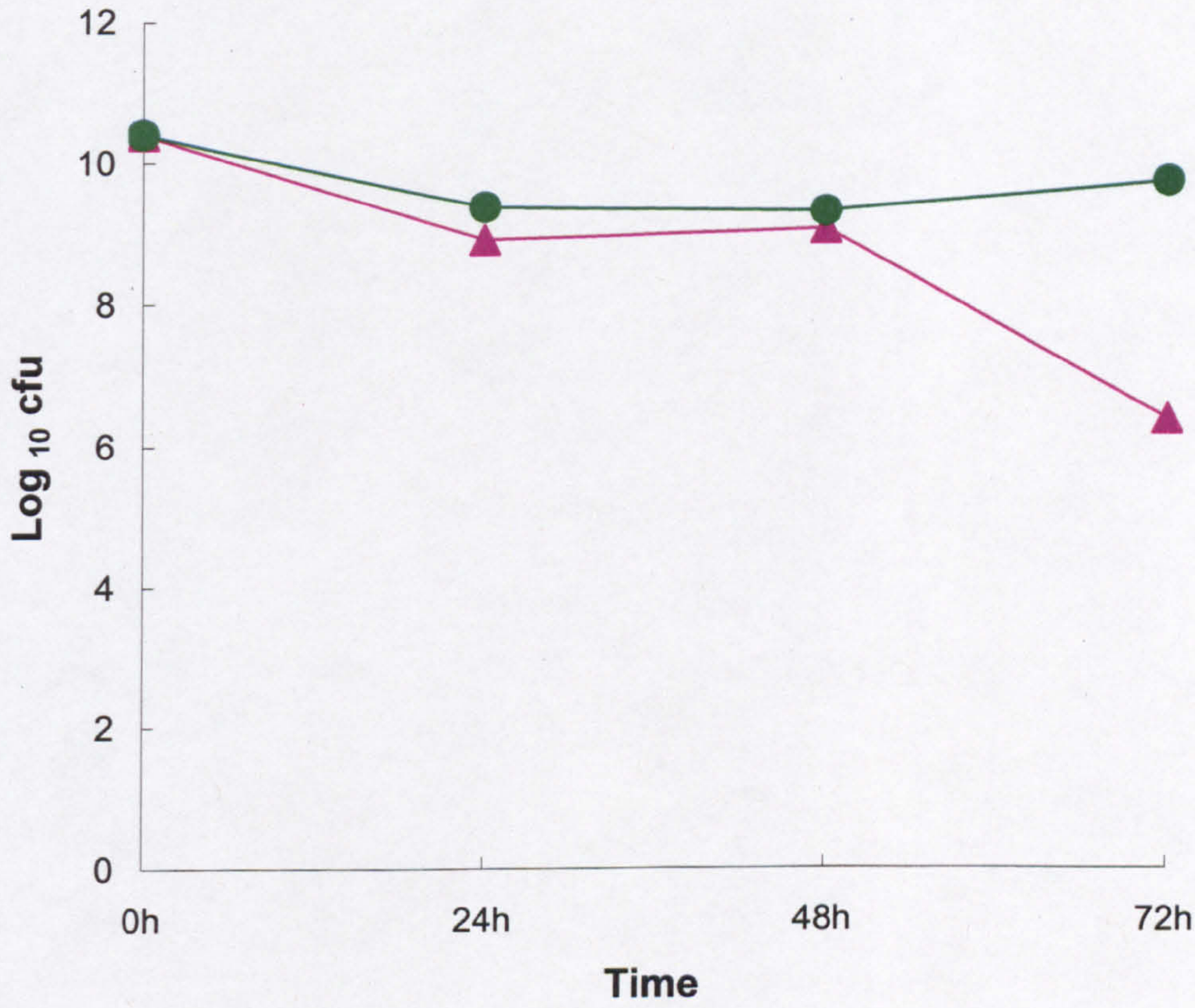

Fig. 52 Growth curve of *E. coli* in biofilm. Effluent titres (▲) and biofilm titres (●).

5.3 Selective media for combination experiments

In order that organisms could be selectively isolated from combination growth experiments, selective media needed to be developed. The combination experiments involved growth of *C. difficile* with either *B. fragilis, E. faecalis* or *E. coli*. Table 10 summarises both the atmospheric conditions and antibiotic susceptibilities of the organisms. Both *E. faecalis* and *E. coli* could be selected by growth on agar plates under aerobic conditions at 37^0C. Trimethoprim was added to BHI agar plates to select for *C. difficile* when grown with *E. faecalis*. Gentamicin was added to BHI agar plates to select for *C. difficile* when grown with *E. coli*. When *C. difficile* and *B. fragilis* were grown together, aztreonam and vancomycin were used to select out the respective organism.

	C. difficile	*E. faecalis*	*E. coli*	*B. fragilis*
Aerobic	✗	✔	✔	✗
Anaerobic	✔	✔	✔	✔
Vancomycin	✗	✗	✔	✔
Aztreonam	✔	✔	✗	✗
Trimethoprim	✔	✗	✔	✔
Gentamicin	✔	✔	✗	✔

Table 10 Conditions used for selection of each organism in dual organism biofilm experiments. (✔ = growth of organism, ✗ = inhibition of growth)

5.4 Combination experiments of *B. fragilis, E. faecalis* and *E. coli* in biofilm.

The results of combination experiments in the Sorbarod filters are shown in Figs. 53, 54 (*C. difficile* and *B. fragilis*), 55 (*C. difficile* and *E. faecalis*), 56, 57 (*C. difficile* and *E. coli*). To an extent, the titres in the filters mirrored those of the organisms grown individually.

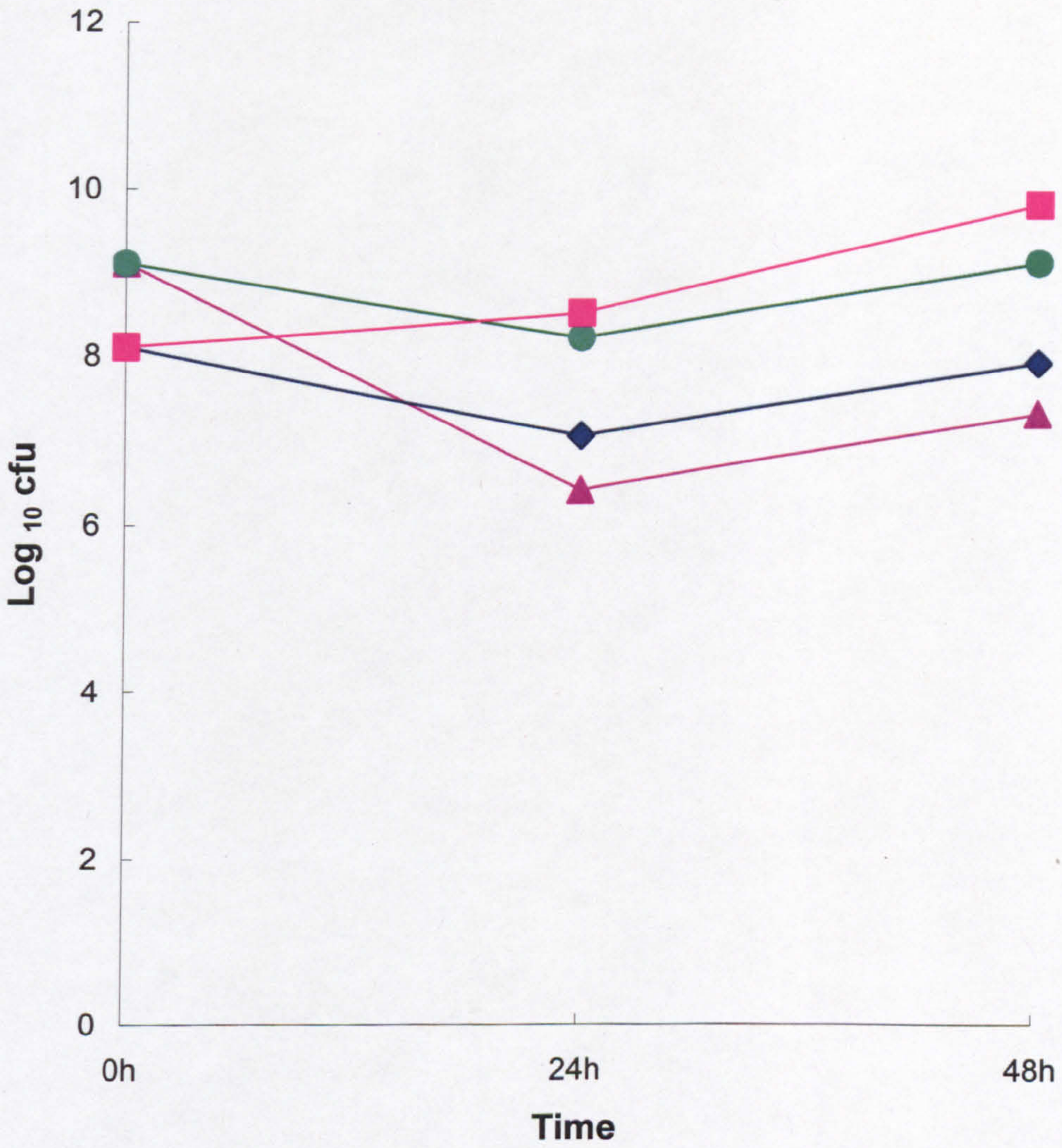

Fig. 53 Growth curve of *C. difficile* with *B. fragilis* in biofilm. *C. difficile* effluent titres (♦) and biofilm titres (■); *B. fragilis* effluent titres (▲) and biofilm titres (●).

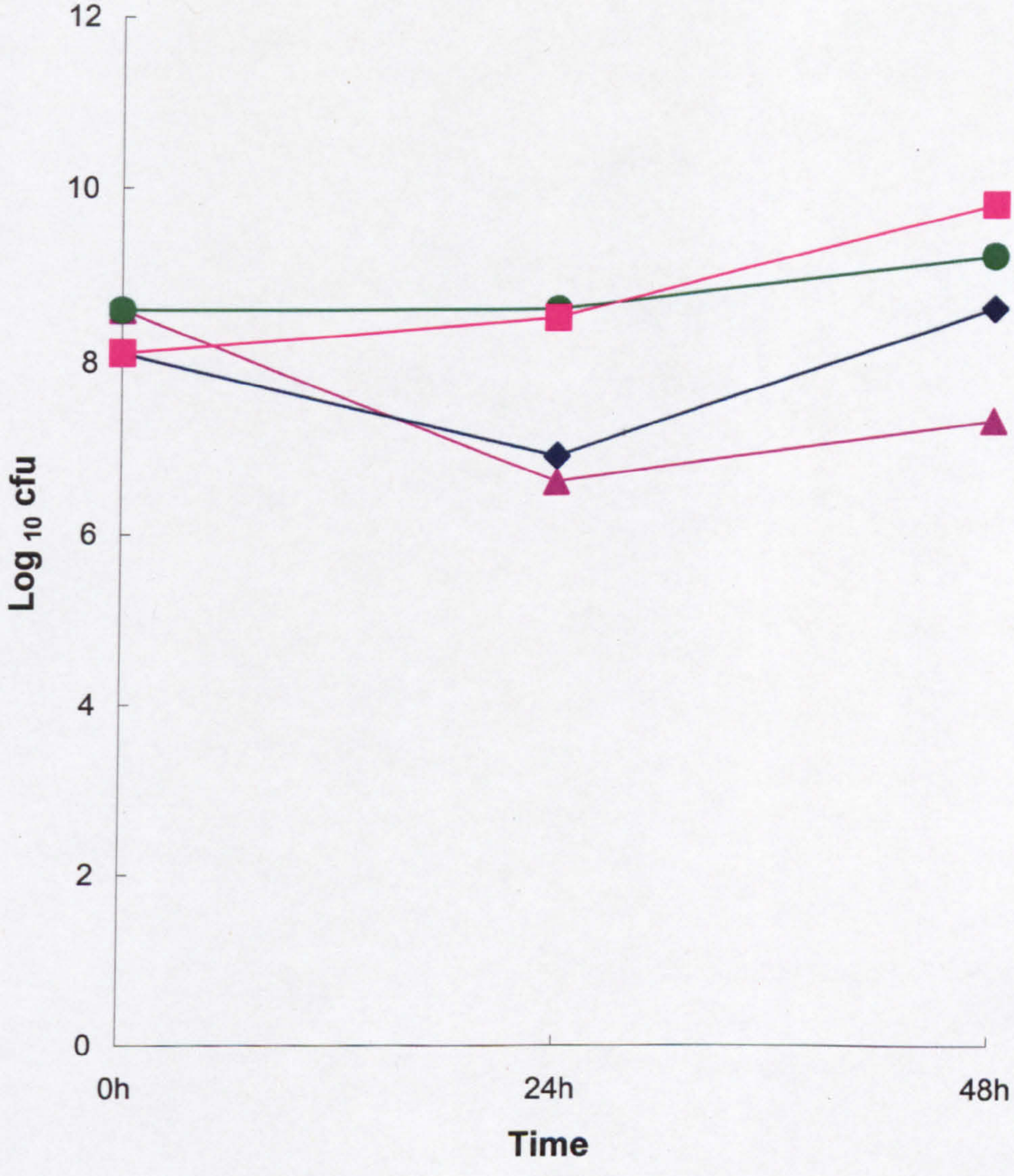

Fig. 54 Growth curve of *C. difficile* with *B. fragilis* in biofilm. *C. difficile* effluent titres (♦) and biofilm titres (■); *B. fragilis* effluent titres (▲) and biofilm titres (●).

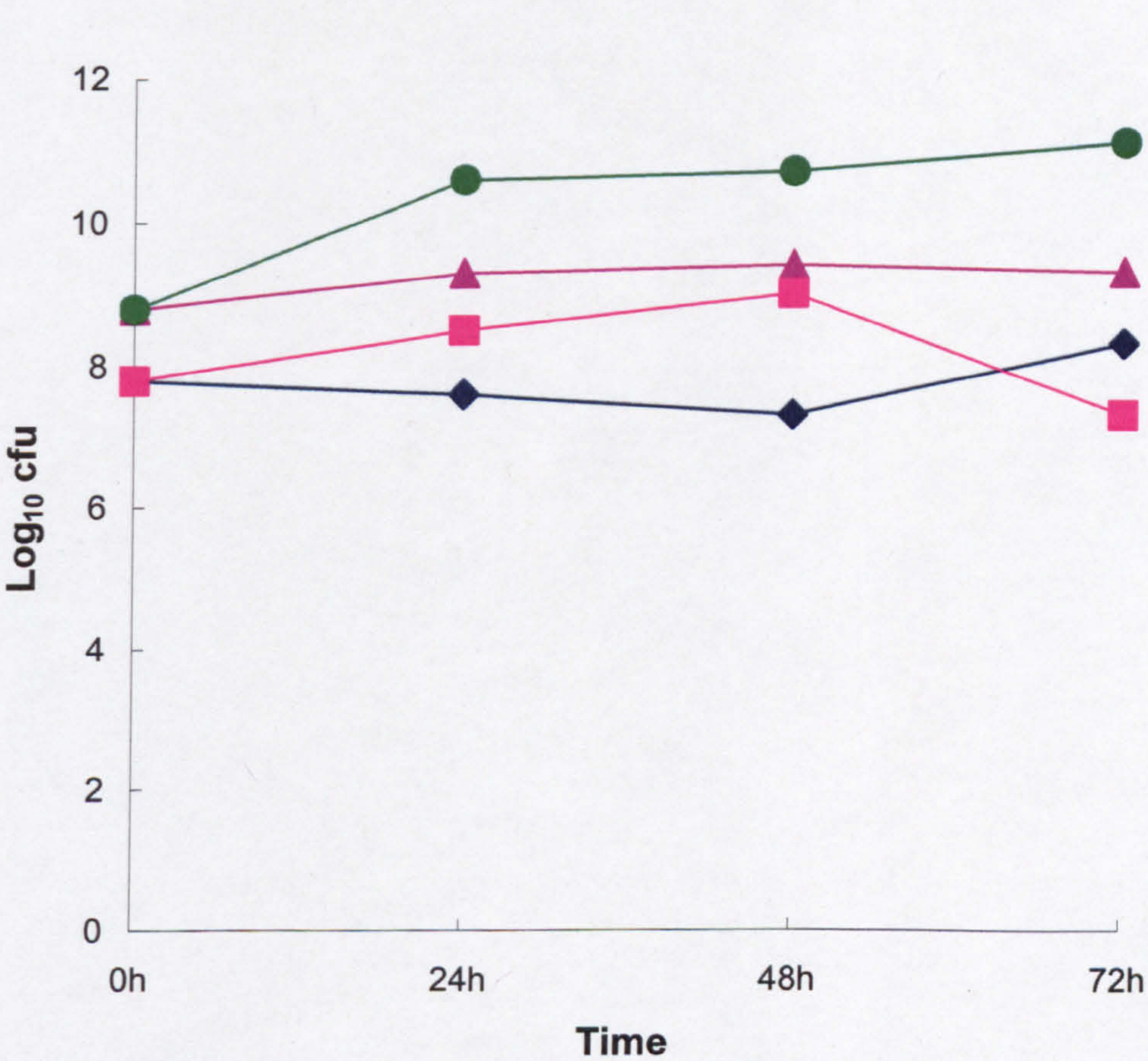

Fig. 55 Growth curve of *C. difficile* and *E. faecalis* in combination. *C. difficile* effluent titres (◆) and biofilm titres (■); *E. faecalis* effluent titres (▲) and biofilm titres (●).

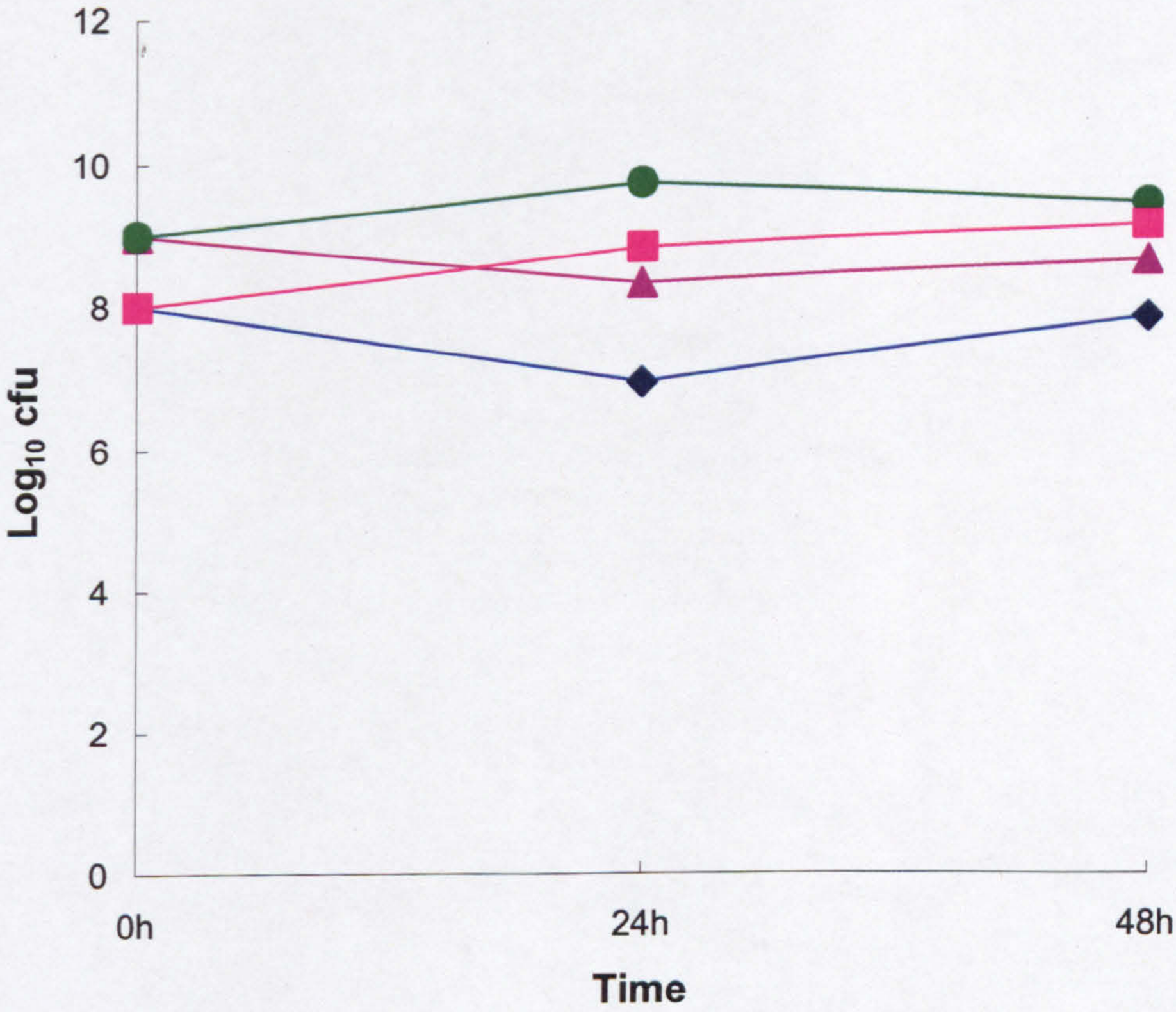

Fig. 56 Growth curve of *C. difficile* and *E. coli* in combination. *C. difficile* effluent titres (◆) and biofilm titres (■); *E. coli* effluent titres (▲) and biofilm titres (●).

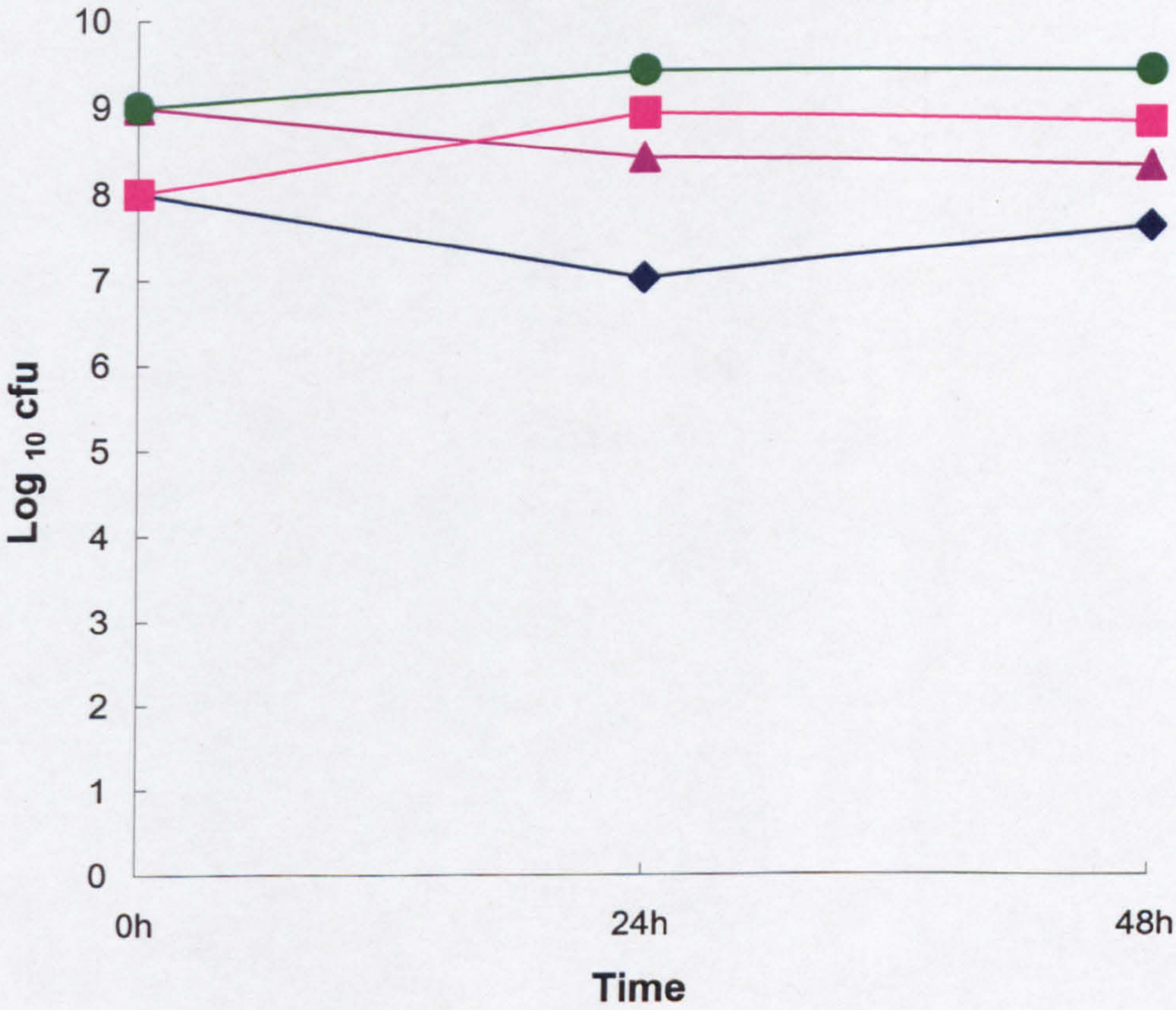

Fig. 57 Growth curve of *C. difficile* in combination with *E. coli*. *C. difficile* effluent titres (◆) and biofilm titres (■); *E. coli* effluent titres (▲) and biofilm titres (●).

5.5 Antimicrobial susceptibility testing of *C. difficile* and *E. coli* in biofilm

The administration of antibiotics is considered the most significant predisposing factor for *C. difficile* infection. Although any antibiotic can potentially cause *C. difficile* disease, broad-spectrum antibiotics against enteric organisms are considered the main predisposing agents. Two broad-spectrum antibiotics, co-amoxiclav and meropenem were used against a combination of *C. difficile* and *E. coli* grown in biofilm on Sorbarod filters. The MIC of both organisms for co-amoxiclav and meropenem was determined by the Etest (Table 11).

The results for experiments with co-amoxiclav and meropenem are shown in Figs. 58, 59 and 60, 61 respectively. While there was a degree of variation in the titres, both organisms maintained their titres up to at least 32mg/L. The exception was the repeat co-amoxiclav experiment, where *C. difficile*, at a lower starting titre of 10^6 cfu/mL, was eliminated from the effluent and biofilm at 32mg/L.

	Co-amoxiclav MIC mg/L	Meropenem MIC mg/L
C. difficile 459	0.125	0.38
E.coli	0.023	4

Table 11 Etest determination of MIC of *C. difficile* and *E. coli* to co-amoxiclav and meropenem

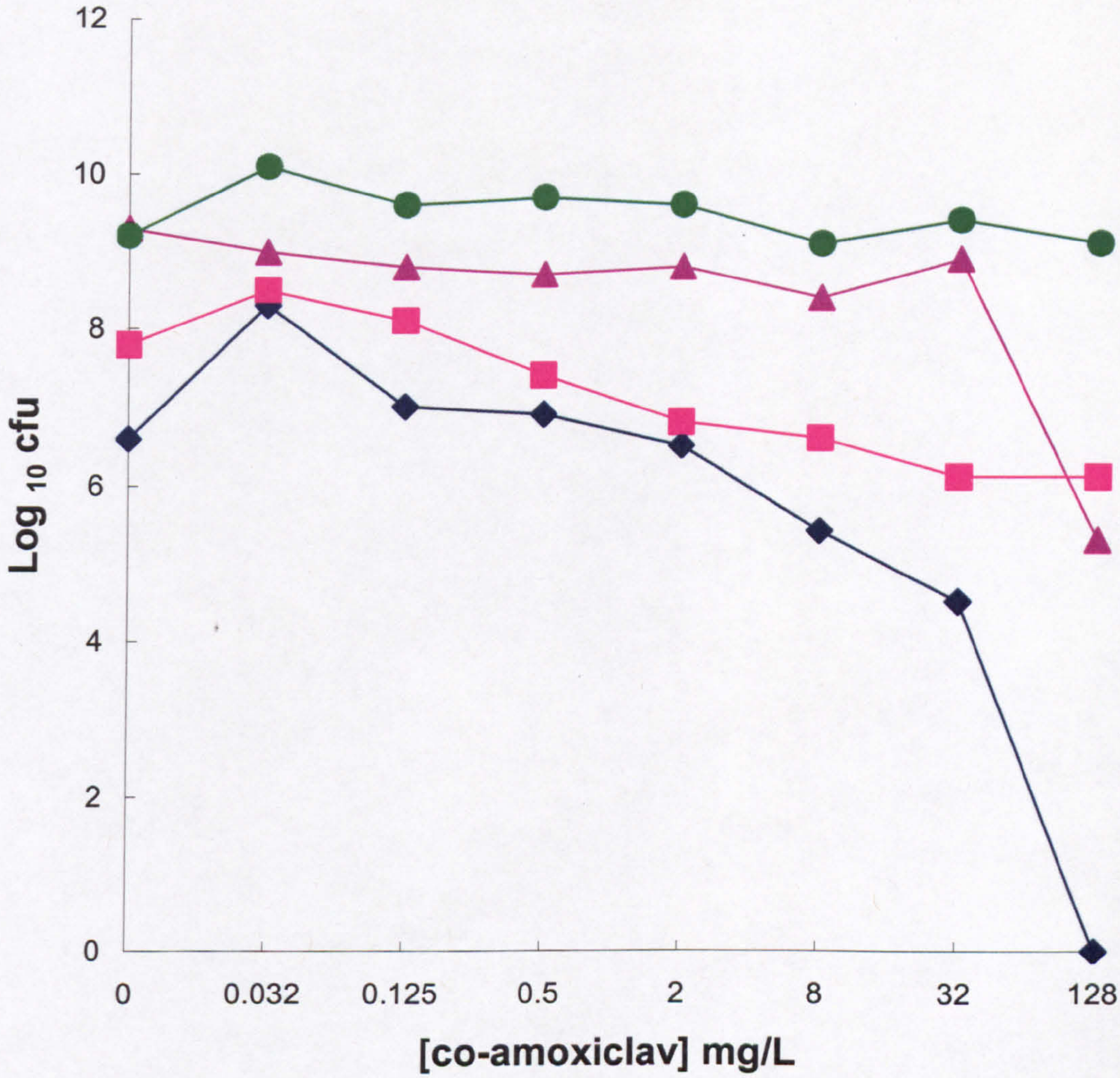

Fig. 58 Effect of co-amoxiclav on growth of both *C. difficile* and *E. coli* in biofilm. *C. difficile* effluent titres (♦) and biofilm titres (■); *E. coli* effluent titres (▲) and biofilm titres (●).

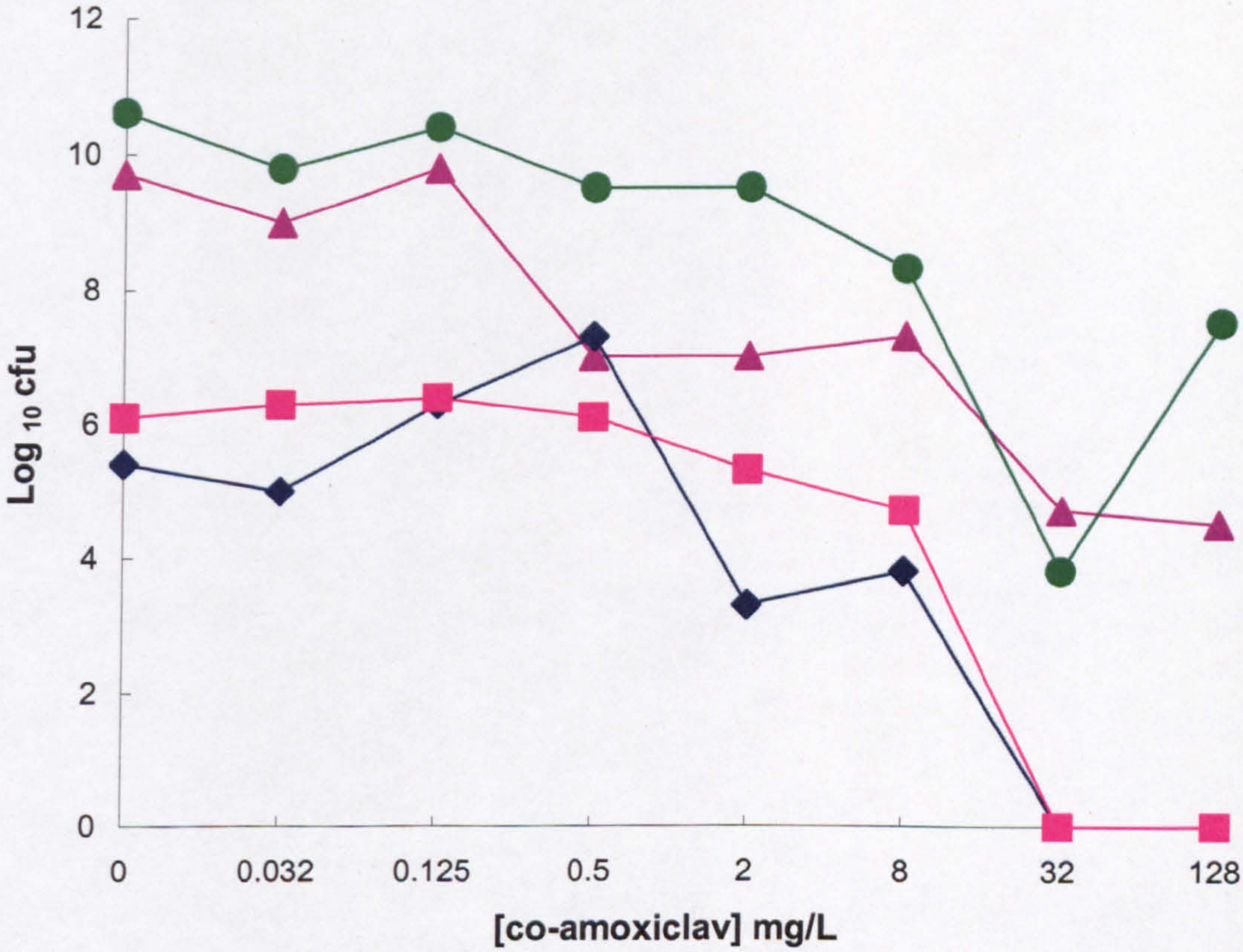

Fig.59 Effect of co-amoxiclav on growth of *C. difficile* and *E. coli* in biofilm. *C. difficile* effluent titres (◆) and biofilm titres (■); *E. coli* effluent titres (▲) and biofilm titres (●).

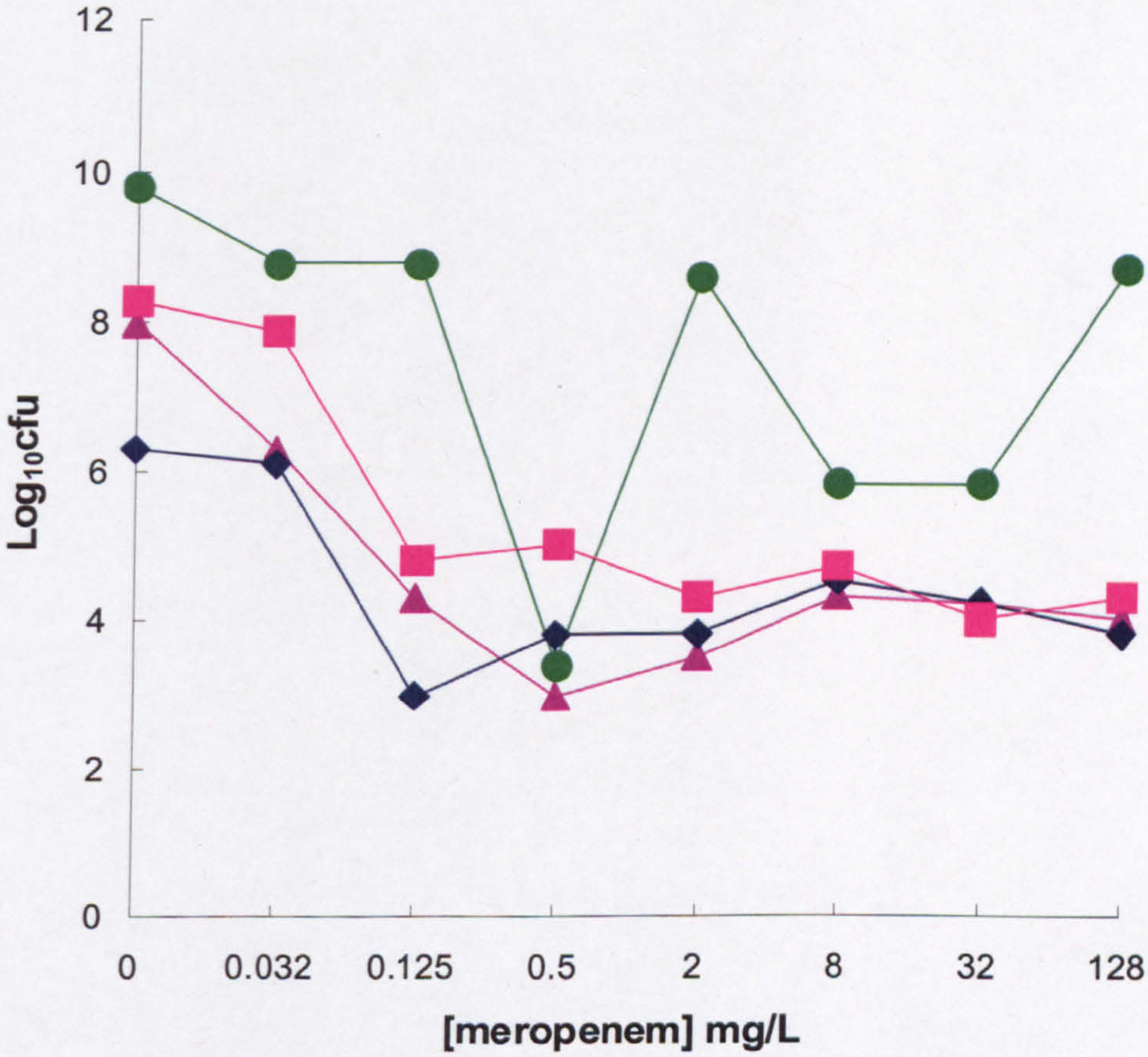

Fig. 60 Effect of meropenem on growth of *C. difficile* and *E. coli* in biofilm.
C. difficile effluent titres (◆) and biofilm titres(■); *E. coli* effluent titres (▲) and biofilm titres (●).

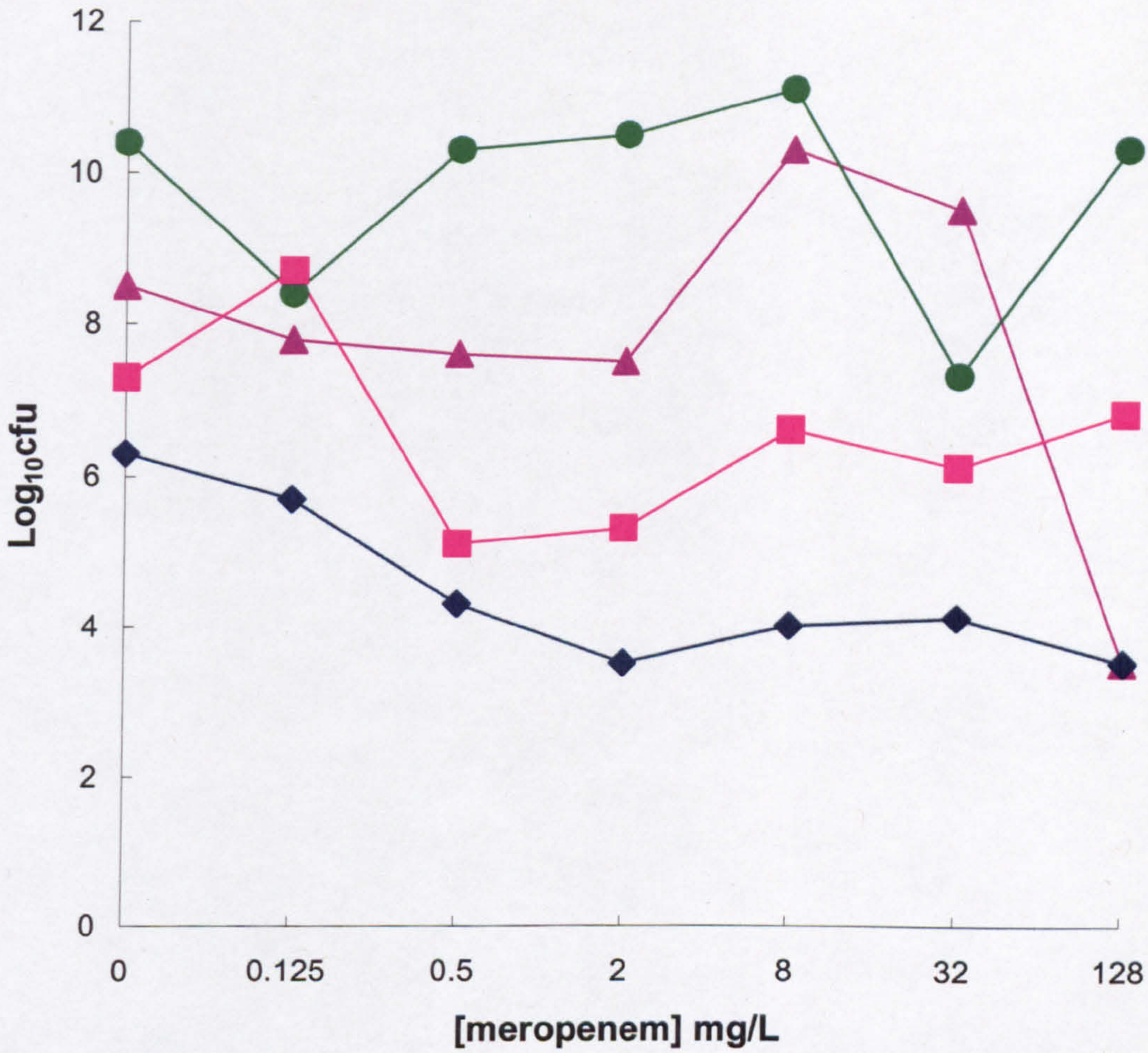

Fig. 61 Effect of meropenem on growth of *C. difficile* and *E. coli* in biofilm.
C. difficile effluent titres (♦) and biofilm titres (■); *E. coli* effluent titres (▲) and biofilm titres (●).

5.6 Screening for bacteriophage and bacteriocin-producing microorganisms

Stool specimens from human, goat, cow and horse sources as well as sewage effluent samples, obtained from the outflow at the Coventry Sewage Works, were screened for the presence of bacteriophage and bacteriocin-producing micro-organisms. Typical bacteriophage plaques were not noted, however "clearing zones" were observed in the *C. difficile* lawn when the horse faecal specimen was screened (Fig. 62).

5.7 Characterisation of a bacteriocin producing micro-organism

Small orange colonies were noted in the centre of the clearing zones or plaques (Fig. 62). The orange colonies were subcultured onto blood agar plates which were incubated aerobically and anaerobically at 37^0C. The organism grew in both atmospheric conditions and staining revealed a Gram-positive coccus in chains. API strep 20 profiling identified that this isolate was *Streptococcus uberis* (99.9% certainty). However, the isolate was referred to the Streptococcus Reference Laboratory at Colindale for confirmation. The isolate in fact proved to be *Lactobacillus paracasei.*

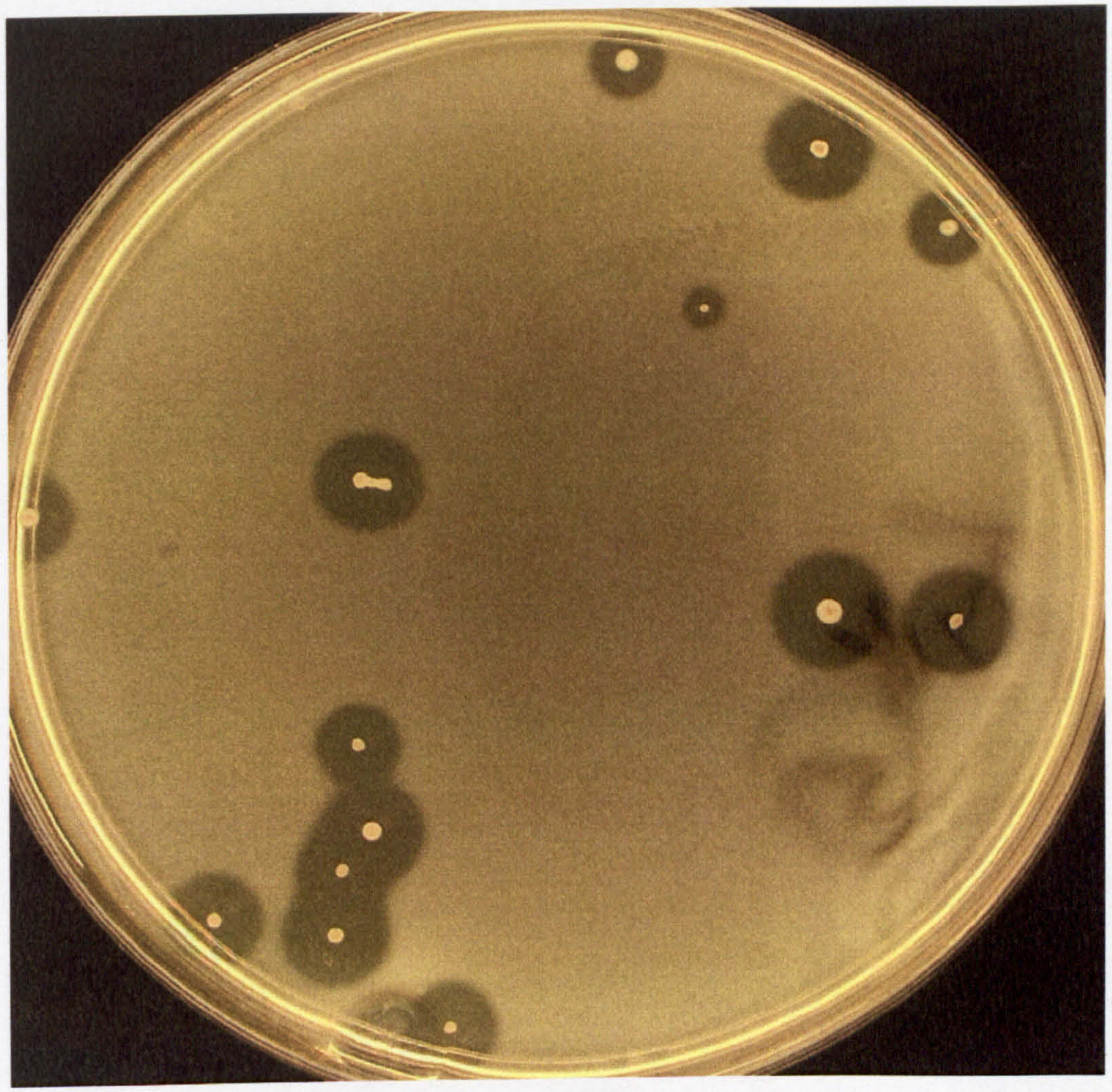

Fig. 62 Photograph of clearings zones on *C. difficile*- enriched BHI agar plate around small orange colonies

5.8 Bacteriocin from *Lactobacillus paracasei*: determination of spectrum of activity

There have been many studies of bacterial antagonism since this phenomenon was reported originally by Pasteur and Joubert (1877). Characterisation of the spectrum of activity of the bacteriocin from *L. paracasei* was undertaken. A modification of the method described by Gillies and Govan (1966) was initially used. Streaks of *L. paracasei* were made centrally onto blood agar plates. Test organisms were inoculated in perpendicular streaks to *L. paracasei*. Zones of inhibition for the test isolates were noted. Table 11 shows the spectrum of activity of the bacteriocin against various isolates. It appeared to have an inhibitory effect on the growth of *C. difficile, S. pneumoniae* and *B. cereus*. Fig. 63 is a photographic representation of the zones of inhibition of the bacteriocin to *C. difficile* and *S. pneumoniae*.

Further characterization of this bacteriocin's activity against other clostridial strains was undertaken using both a modification of Gilles and Govan (1966) (Table 12), and a modification of the method described in Chapter 2 for the screening for bacteriophage / bacteriocins. A suspension of *L. paracasei* was made in Ringer's solution (McFarland 0.5) and serial dilutions to 10^{-6} were done. One hundred μL of each dilution of *L. paracasei* was added to 3mL of sloppy agar plus 1mL of overnight broth of each clostridial strain. Activity was demonstrated by zones of inhibition to *C. difficile* strains, *C. tetani, C. bifermentans* and *C. novyi.* Titres of *L. paracasei* were determined in experiments against *S. pneumoniae*, NCTC *C. difficile* and the clinical strain 459. Titres were between 2 - 4x 10^8 cfu/mL.

	Bacteriocin effect
Listeria monocytogenes	✗
S. aureus	✗
Staphylococcus epidermidis	✗
E .faecalis	✗
Bacteroides fragilis	✗
E. coli	✗
C.perfringens	✗
C. difficile 459	✔
Type *C. difficile*	✔
C. difficile 2302	✔
C. difficile 1462	✔
C. difficile 2223	✔
Streptococcus pneumoniae	✔
Bacillus cereus	✔
Haemophilus influenzae	✗
Neisseria lactima	✗
Salmonella poona	✗
Pseudomonas aeruginosa	✗
Shigella sonnei	✗
Klebsiella pneumoniae	✗
Acetinobacteur baumanii	✗

Table 11 Spectrum of activity of bacteriocin (inhibition of growth ✔ and no inhibitory effect ✗)

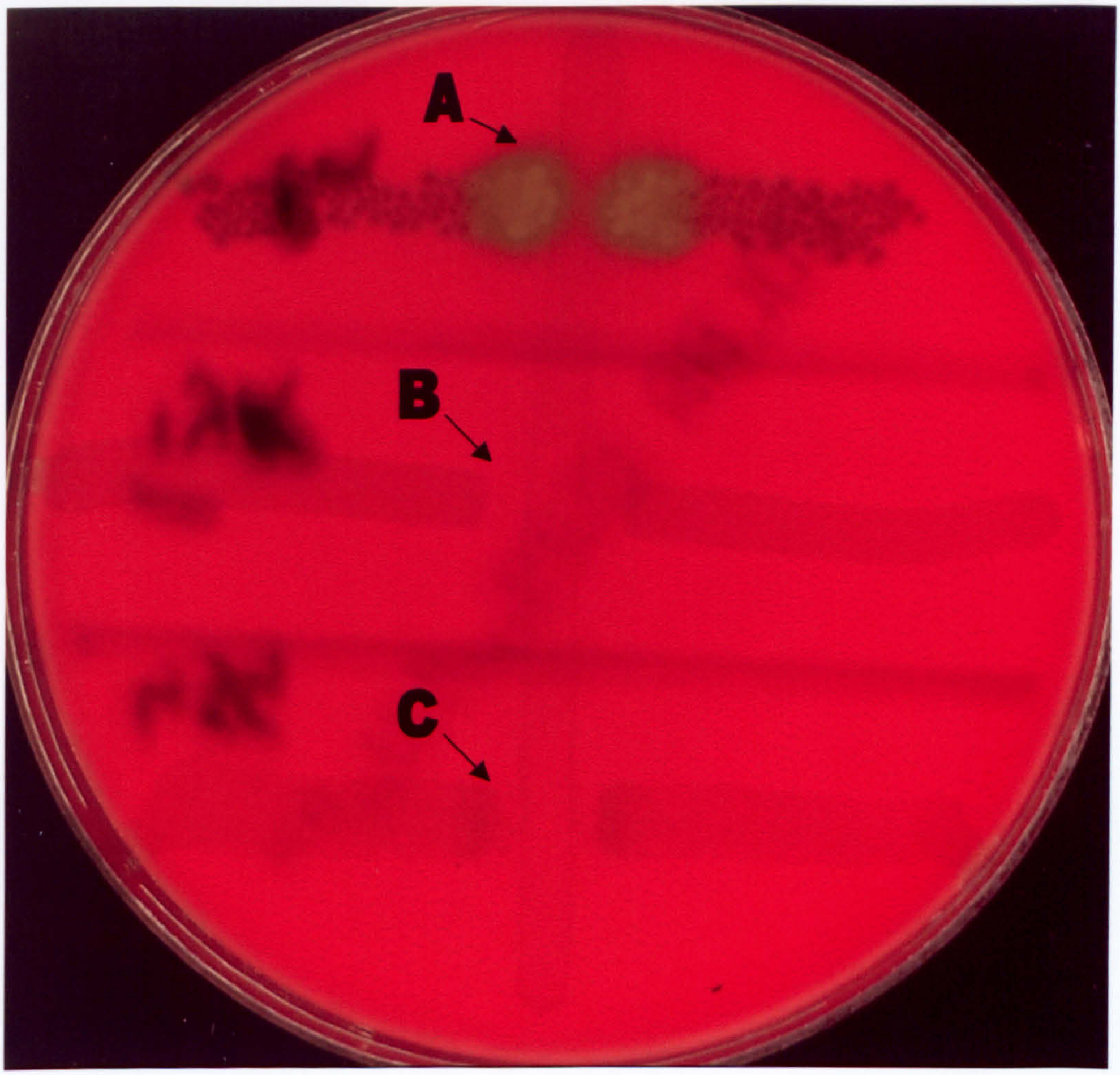

Fig. 17. Photograph of blood agar plate showing bacteriocin effects of *L. paracasei* (central vertical streak) on (A) *S. pneumoniae*. and 2 strains of *C. difficile* (B) 459 and (C) NCTC 11204. A distinct zone of α haemolysis was seen with the interaction of *S. pneumoniae* and the bacteriocin of *L. paracasei* (see arrow).

C. difficile 459	✔
Type *C. difficile*	✔
C. bifermentans	✔
C. sporogenes	✘
C. novyi	✔
C. butyricum	✘
C. tetani	✔
C. perfringens	✘

Table 12 Spectrum of activity of bacteriocin against clostridia species (inhibition of growth ✔; no effect ✘)

5.9 Growth of *Lactobacillus paracasei* in broth and in biofilm

L. paracasei exhibited the typical logarithmic growth curve in BHI broth culture, reaching titres of 10^7 cfu/mL at 48h (Figs. 64, 65). *L. paracasei* readily established growth on Sorbarod filters reaching titres in excess of > 10^{11} cfu/filter (Figs. 66, 67). Effluent titres of *L. paracasei* were of the order of 2 log. lower than biofilm titres.

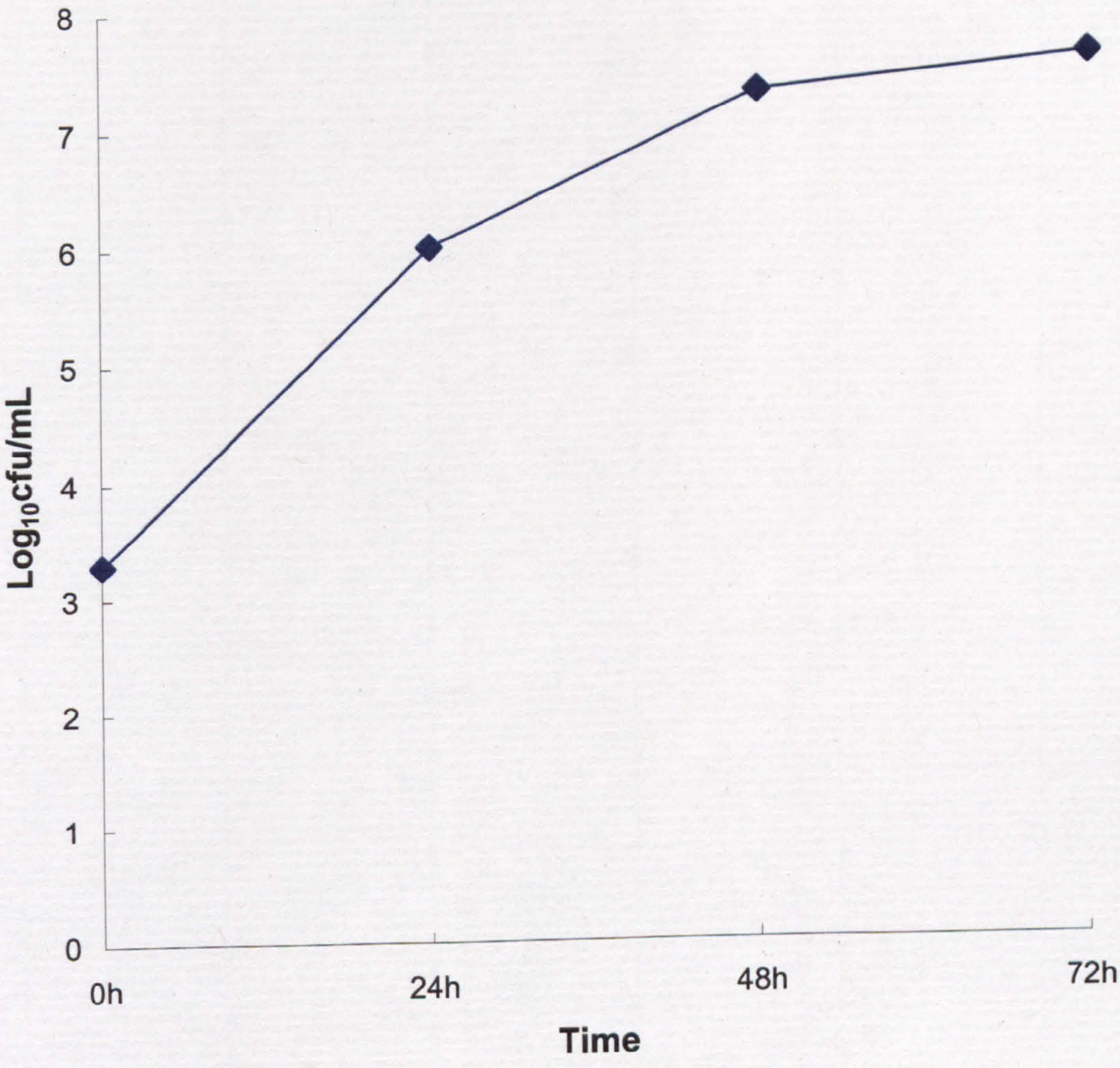

Fig. 64 Growth curve of *L. paracasei* (◆) *in* broth culture

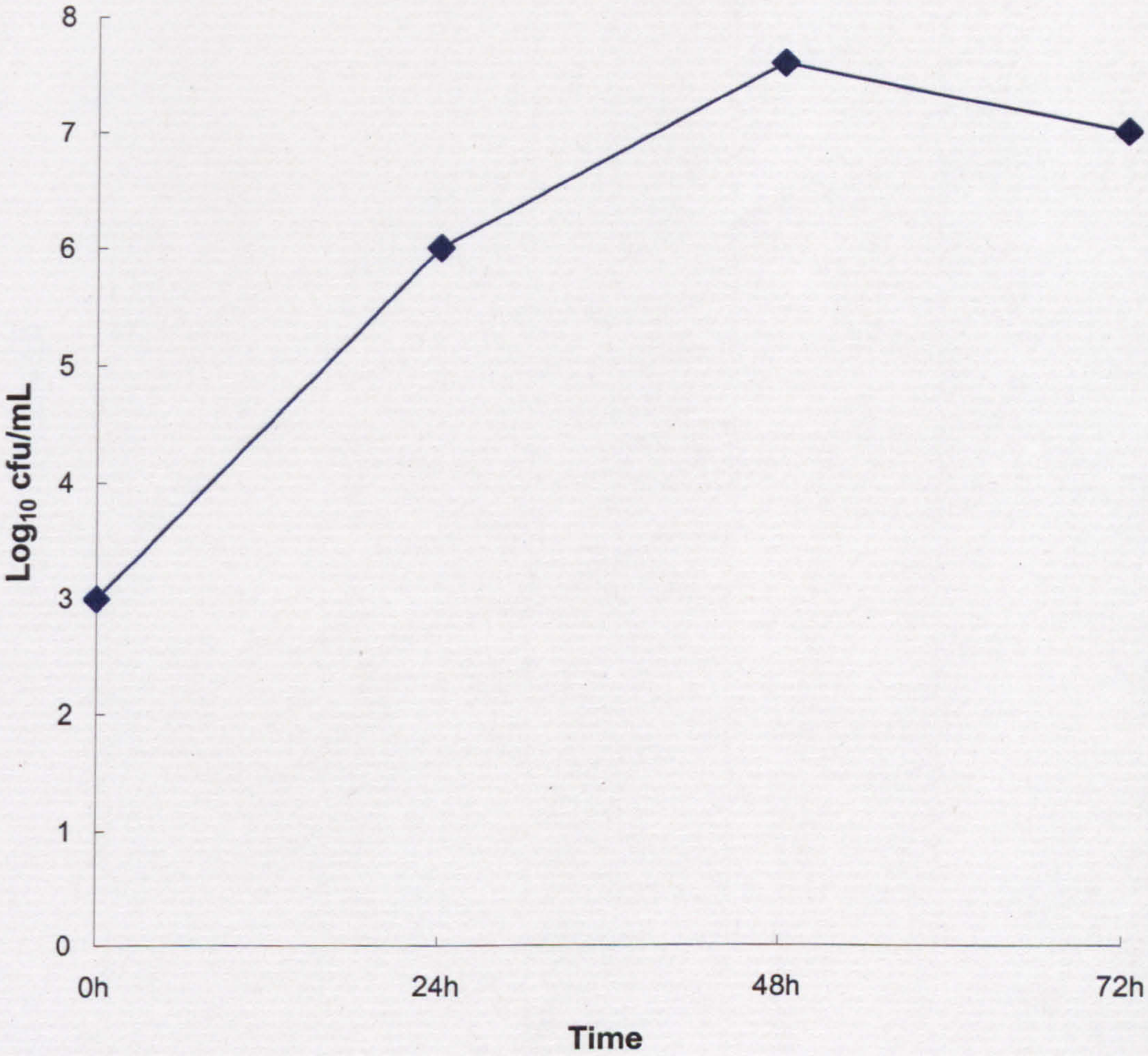

Fig. 65 Growth curve of *L. paracasei* (♦) in broth culture

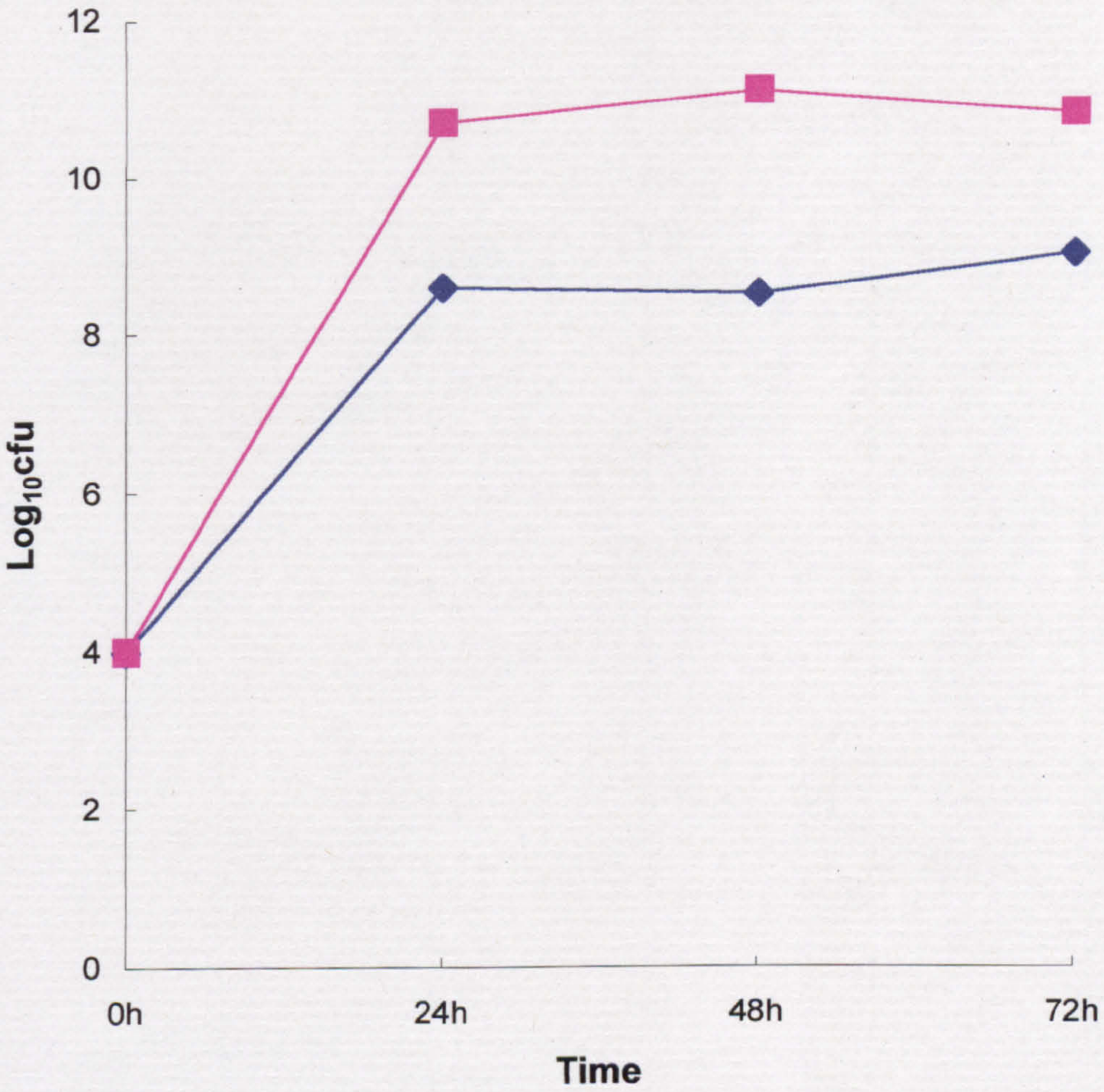

Fig. 66 Growth curve of *L. paracasei* in biofilm. Effluent titres (◆) and biofilm titres (■).

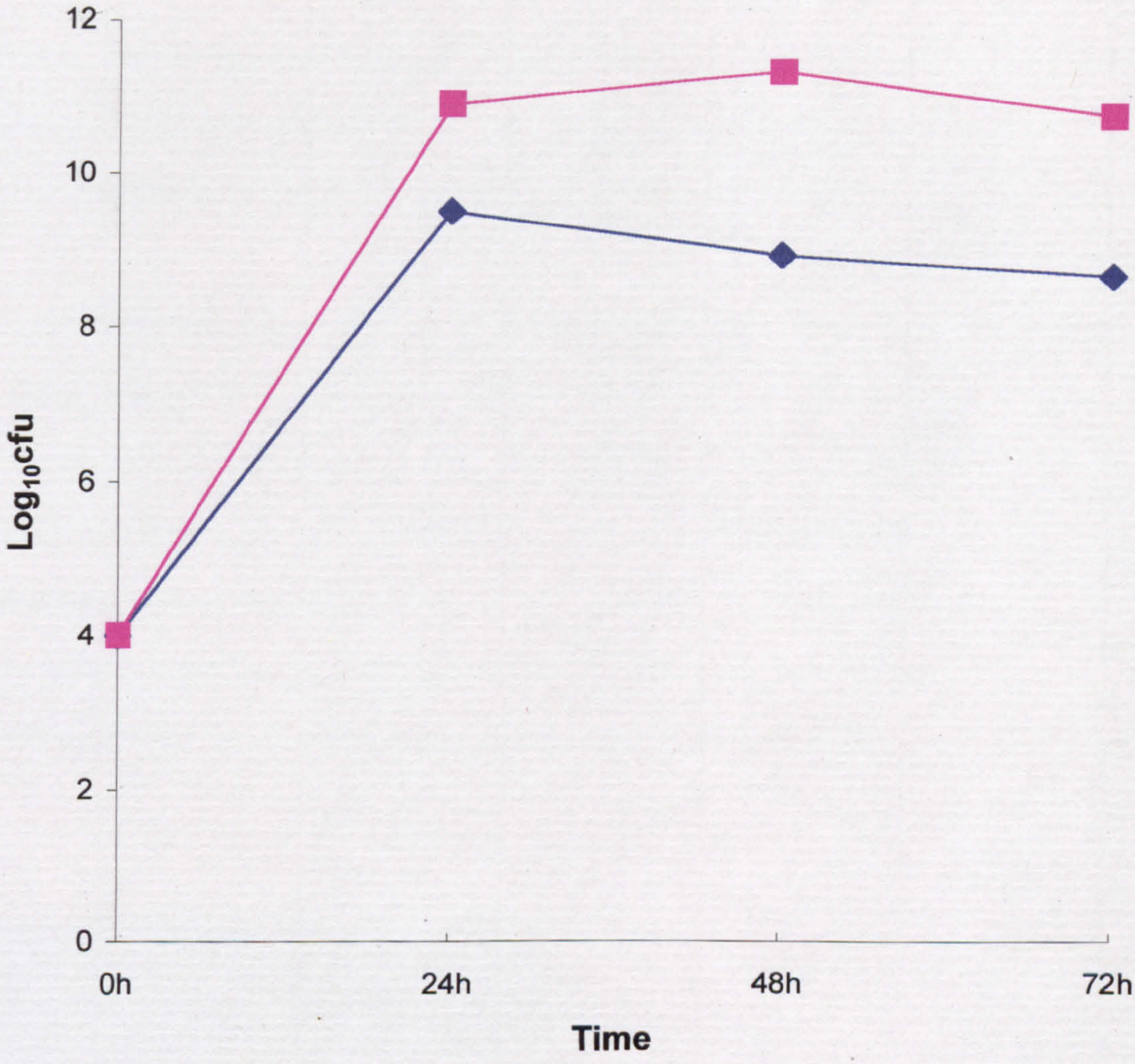

Fig. 67 Growth curve of *L. paracasei* in biofilm. Effluent titres (◆) and biofilm titres (■).

5.10 Effect of *L. paracasei* on growth of *C. difficile* in broth culture

Numerous studies were undertaken to determine the effect of *L. paracasei* on the growth of *C. difficile* in broth culture. *L. paracasei* was selected by growth under aerobic conditions and *C. difficile*, by growth on *C. difficile* selective media.

An initial study was to determine the time interval at which the bacteriocin was produced. Overnight broth cultures of *C. difficile* (40mL) and *L. paracasei* (10mL) were added to 50mL of BHI broth. This was incubated anaerobically and 5mL was removed and centrifuged at 3h, 6h, 12h and 24h intervals. The supernatant and filtered supernatant (0.6μ filter) were inoculated into wells of a *C. difficile* BHI agar plate. Table 13 shows that bacteriocin activity was present at 3h in the unfiltered supernatant but the filtered supernatant did not show any activity at any time.

	Supernatant	Filtered supernatant
3h	5mm	0
6h	10mm	0
12h	8mm	0
24h	8mm	0

Table 13. Time interval at which zone of inhibition of *C. difficile* developed, size of radius of zone around each well measured in mm.

Fig. 68 shows a graphical representation of the results of the effect of *L. paracasei* on growth of *C. difficile* in broth culture using differing ratios of *C. difficile* to *L. paracasei* to BHI. *C. difficile*, alone in broth, exhibited the typical log. growth curve. However, when combination experiments with *L. paracasei* were conducted, a degree of inhibition was shown at a ratio of *C. difficile*: *L. paracasei*: BHI of 1:10:10. At ratios of 5:100:100, and 1:100:100 and 1: 1000: 1000, various degrees of inhibition were noted, but titres recovered at 48h. The titres of *L. paracasei* showed no significant inhibition (Fig. 69).

Fig. 70 again demonstrates some inhibitory effect of the bacteriocin on *C. difficile*, here occurring only when the ratio of *L. paracasei* was 10 times greater than *C. difficile*. Again, this killing effect was not sustained from 24h.

Fig. 71 shows the variability with these experiments with an ongoing killing effect of the bacteriocin at 12-48h when the ratio of *L. paracasei* exceeded *C. difficile* by a factor of >10.Elimination of the clostridium from broth in three of the samples occurred here.

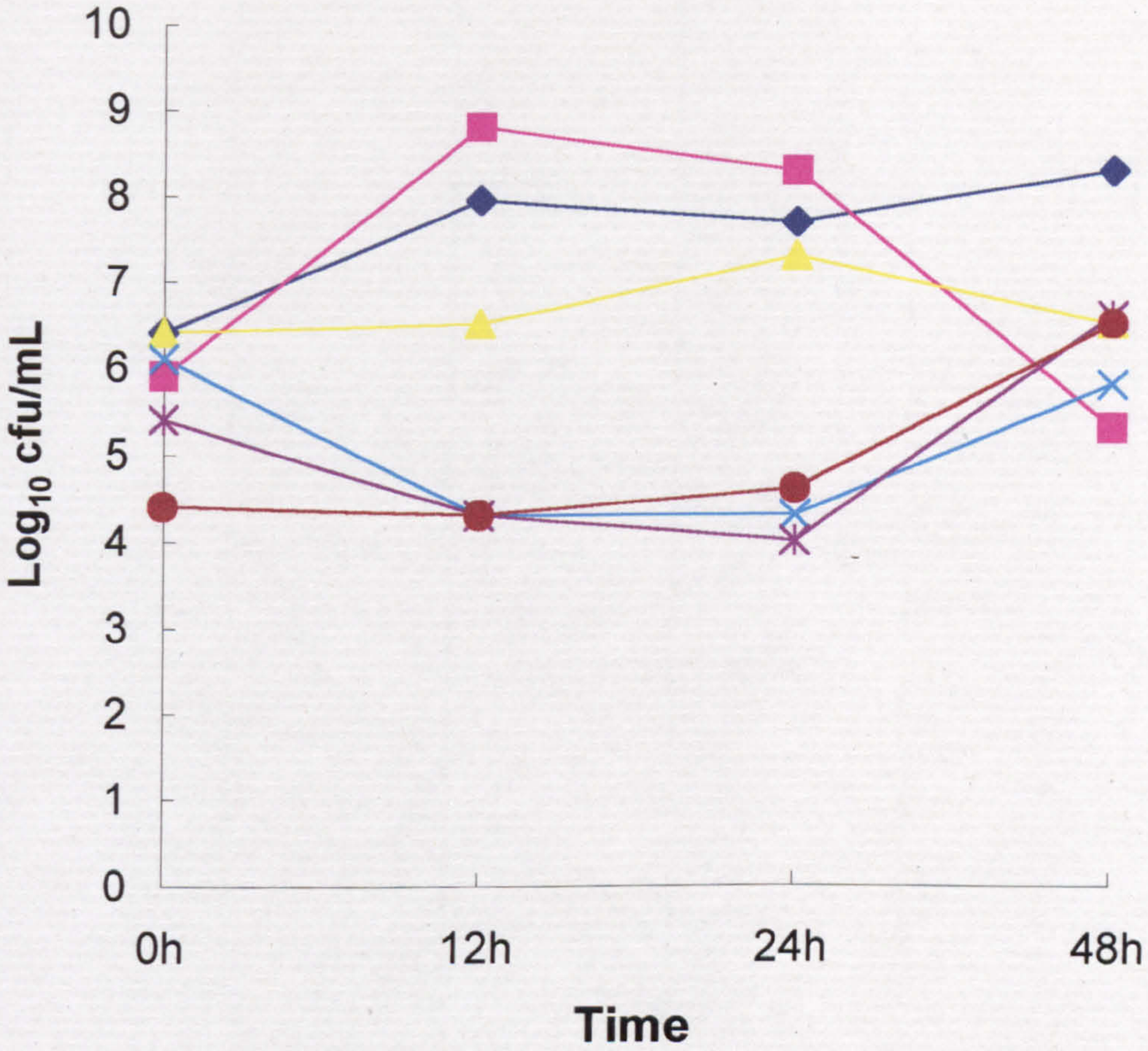

Fig. 68 Effect of *L. paracasei* on growth of *C. difficile* in broth culture (*C. difficile* titres)

(◆) - *C. difficile*

(■) - *L. paracasei*

(▲) -1mL *C. difficile* : 10mL *L. paracasei* : 10mL BHI (1:10:10)

(✖) - 0.5mL*C. difficile*: 10mL *L. paracasei*: 10mL BHI (5:100:100)

(✳) - 0.1mL *C. difficile*: 10mL *L. paracasei*: 10mL BHI (1:100:100)

(●) -0.01mL *C. difficile*: 10mL *L. paracasei*: 10mL BHI (1:1000:1000)

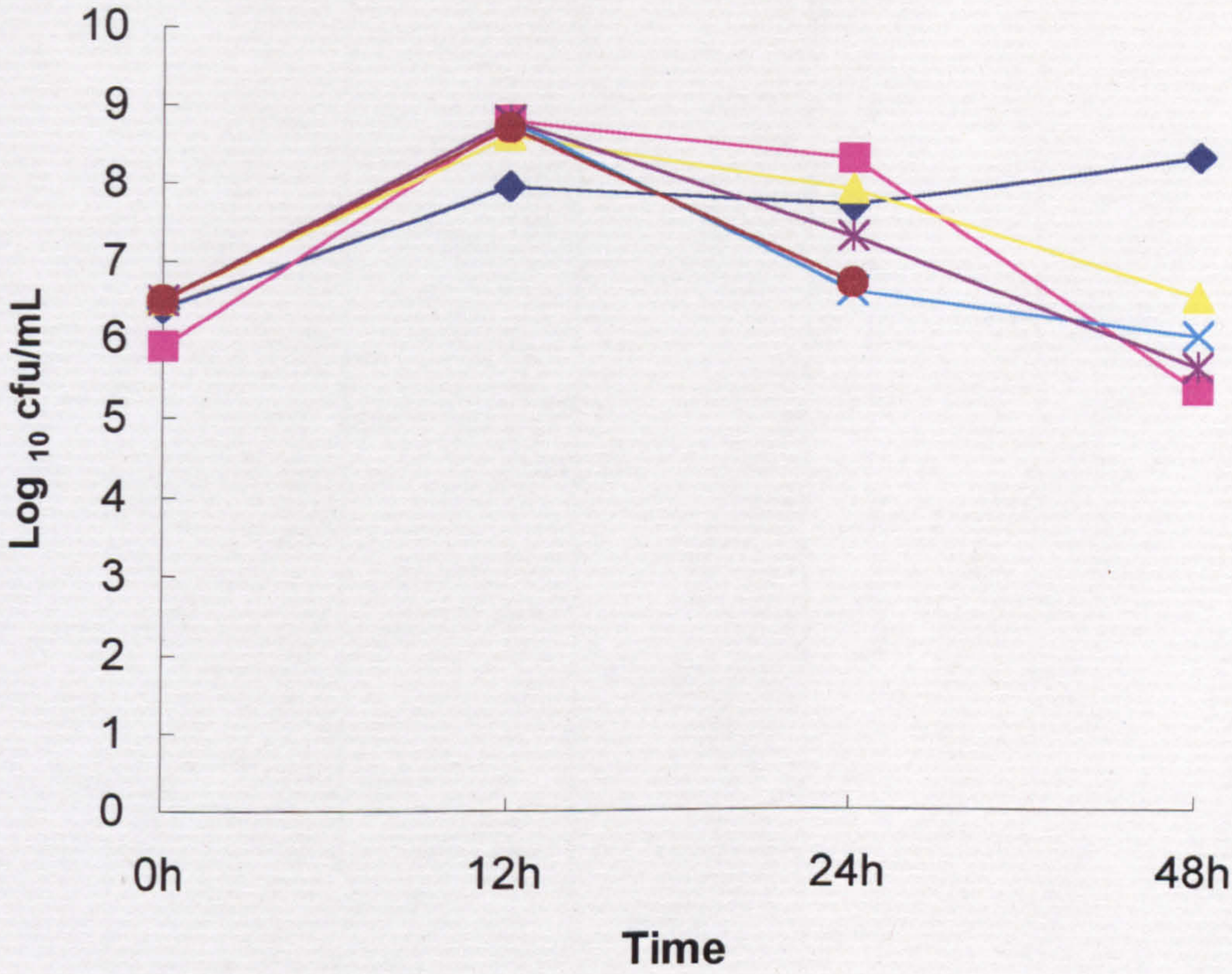

Fig. 69 Effect of *L. paracasei* on growth of *C. difficile* in broth culture (*L. paracasei* titres)

(◆) - *C. difficile*

(■) - *L. paracasei*

(▲) -1mL *C. difficile* : 10mL *L. paracasei* : 10mL BHI,

(✖) - 0.5mL*C. difficile*: 10mL *L. paracasei*: 10mL BHI

(✱) - 0.1mL *C. difficile*: 10mL *L. paracasei*: 10mL BHI

(●) -0.01mL *C. difficile*: 10mL *L. paracasei*: 10mL BHI

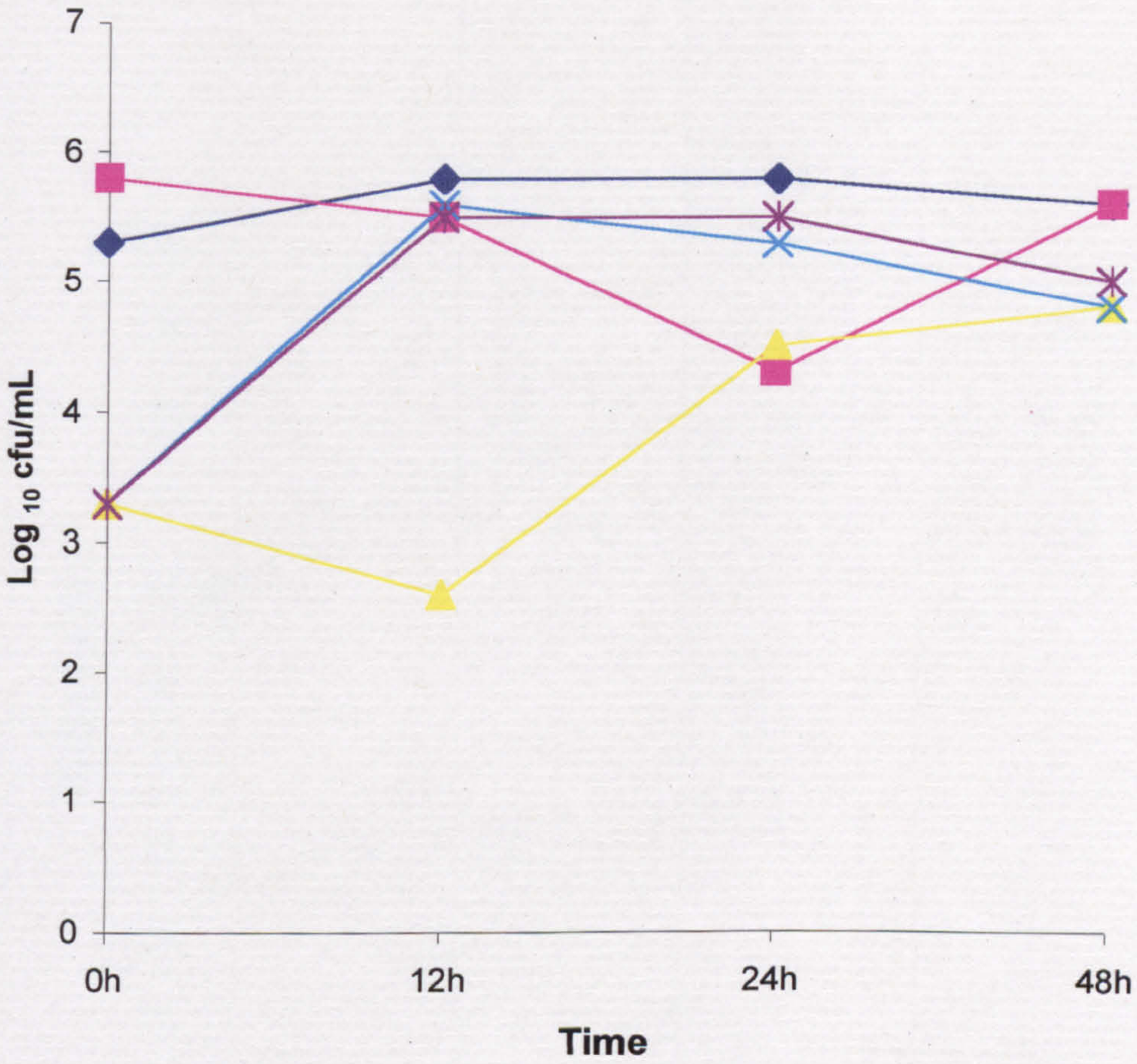

Fig. 70 Effect of *L. paracasei* on growth of *C. difficile* in broth culture.
(◆)- *C. difficile* alone in broth culture
(■)- *L. paracasei* in broth alone
(▲)- 1mL *C. difficile*: 9mL *L. paracasei* (*C. difficile* titres)
(×)- 10mL *C. difficile*: 10mL *L. paracasei* (*C. difficile* titres)
(✱)- 9.9mL *C. difficile* : 100µL *L. paracasei* (*C. difficile* titres)

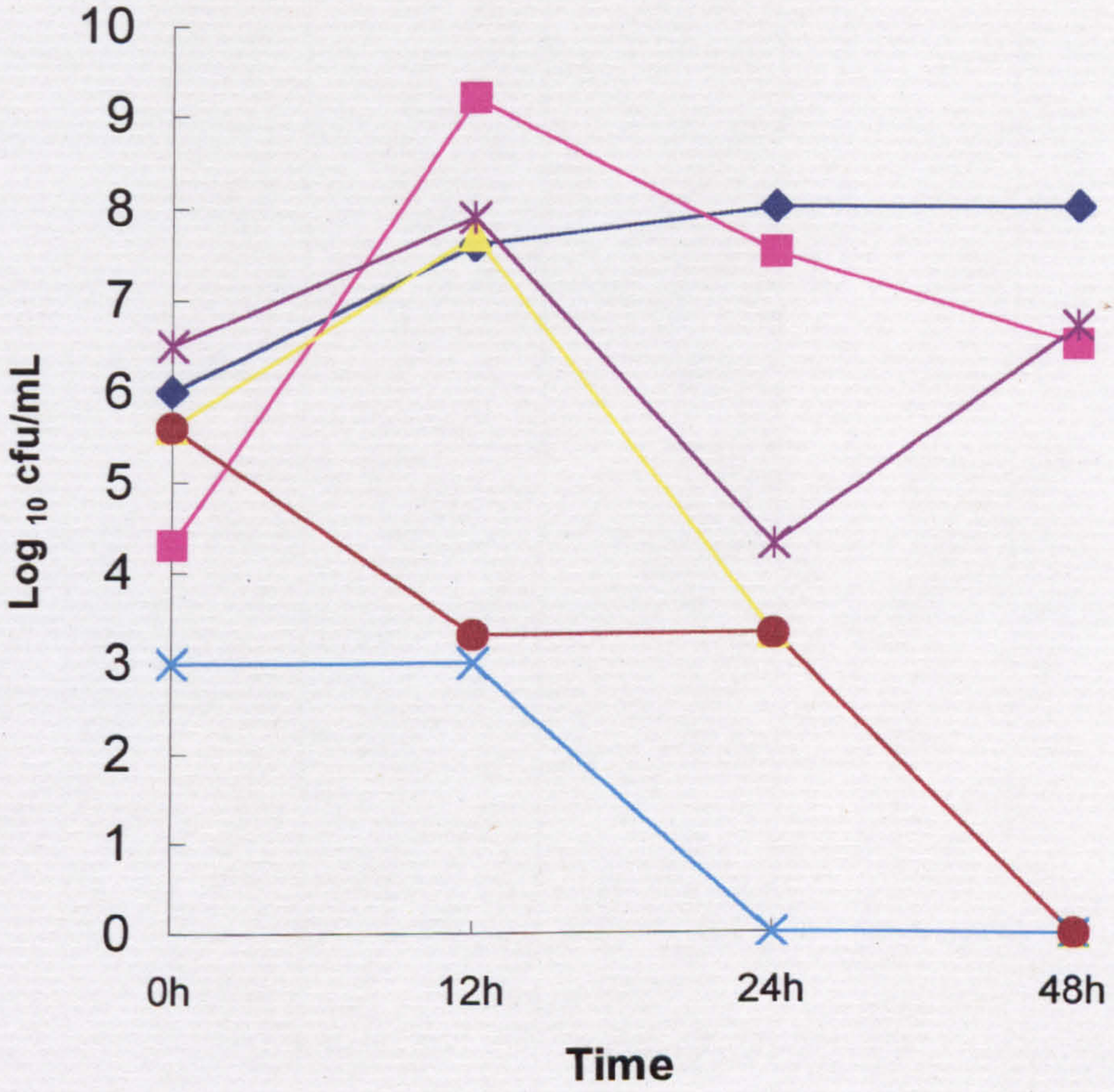

Fig. 71 Effect of *L. paracasei* on growth of *C. difficile* in broth culture.
(◆)- *C. difficile* alone in broth culture
(■)- *L. paracasei* in broth alone
(▲)- 1mLs *C. difficile*: 10mL *L. paracasei*: BHI (*C. difficile* titres)
(✕)- 0.5mL *C. difficile*: 10mL *L. paracasei*: 10mL BHI (*C. difficile* titres)
(✳)- 1mL *C. difficile* : 10mL *L. paracasei* (*C. difficile* titres)
(●) 0.5mL *C. difficile*: 10mL *L. paracasei* (*C. difficile* titres)

5.11 Effect of *L. paracasei* on growth of *C. difficile* in biofilm

C. difficile was inoculated onto Sorbarod filters and growth was established for 24 h. Two mL of an overnight culture of *L. paracasei* was then added directly to the filters for 24h. There appeared to be little killing effect in these experiments apart from an approximate log. reduction in both biofilm and effluent titres when *L. paracasei* was present for 24h. (Figs. 72, 73).

Fig. 74 illustrates an experiment using different volumes of *L. paracasei* added to established biofilms of *C. difficile*. Maximum bacteriocin activity occurred with the addition of 10mL of *L. paracasei*.

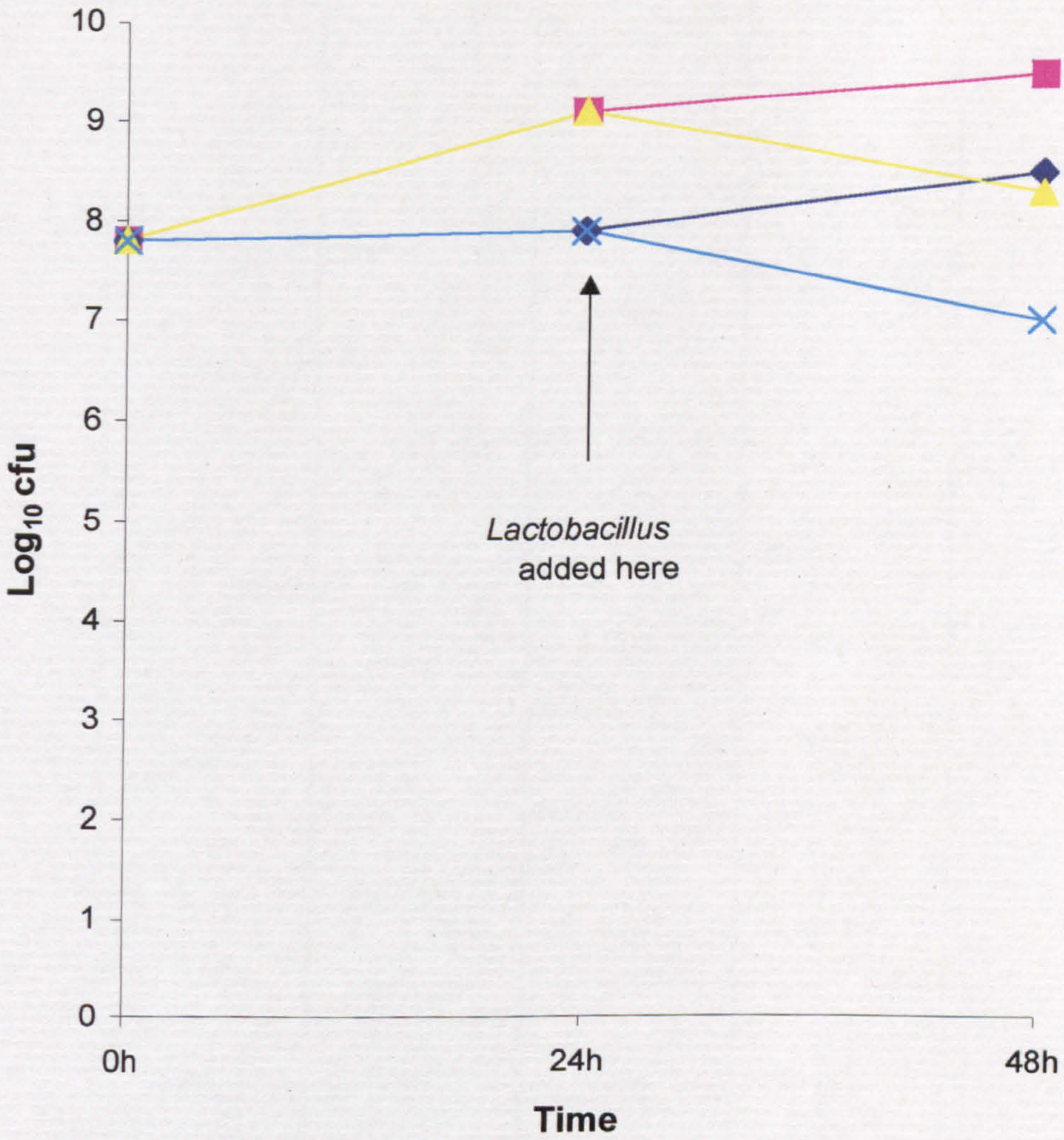

Fig. 72 Effect of *L. paracasei* on growth of *C. difficile* in biofilm.
Control *C. difficile* biofilm (■)
Control *C. difficile* effluent (◆)
C. difficile biofilm with *L. paracasei* (▲) (*C. difficile* titres)
C. difficile effluent with *L. paracasei* (×) (*C. difficile* titres)

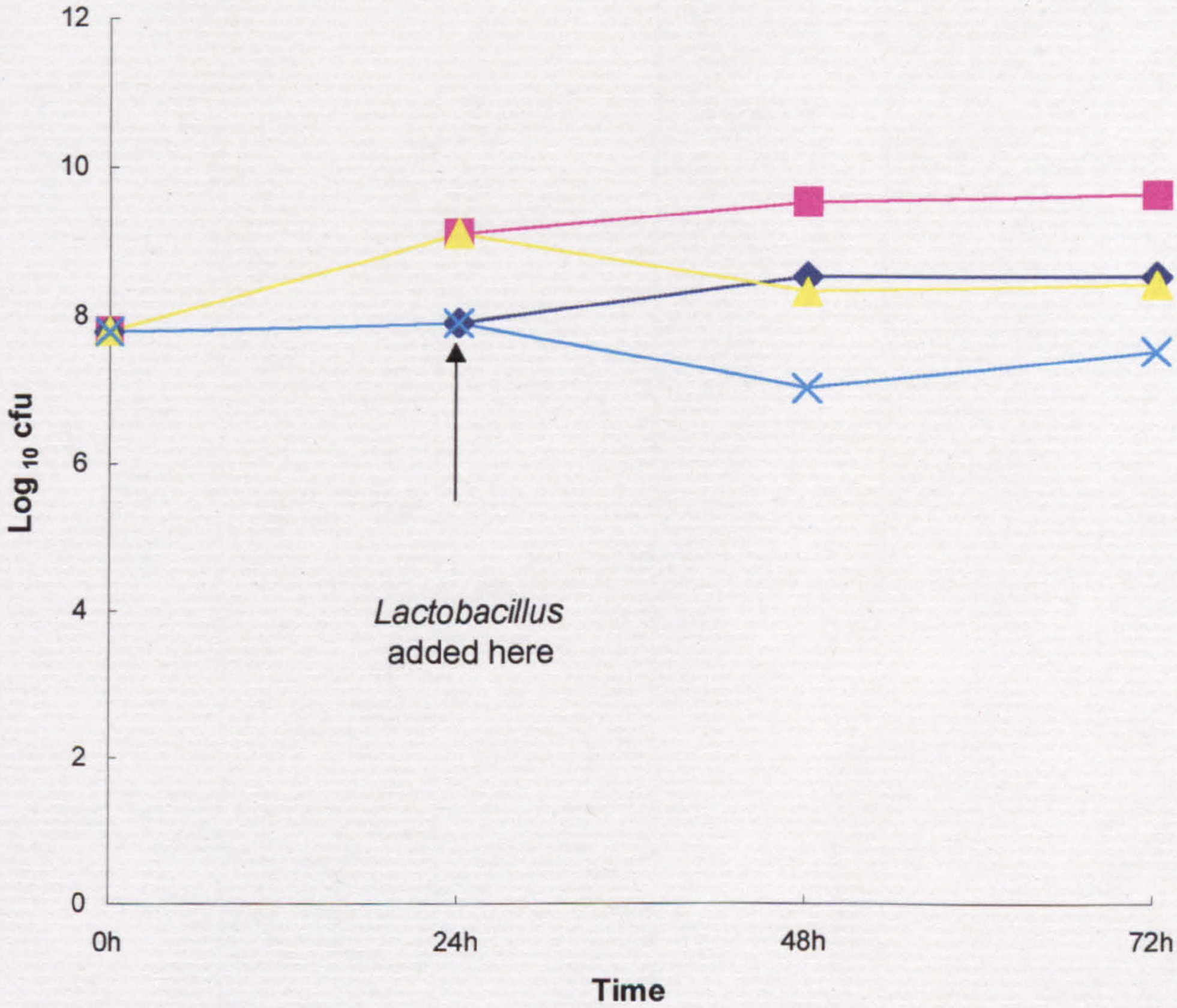

Fig. 73 Effect of *L. paracasei* on growth of *C. difficile* in biofilm.
Control *C. difficile* biofilm (■)
Control *C. difficile* effluent (◆)
C. difficile biofilm with *L. paracasei* (▲) (*C. difficile* titres)
C. difficile effluent with *L. paracasei* (×)(*C. difficile* titres)

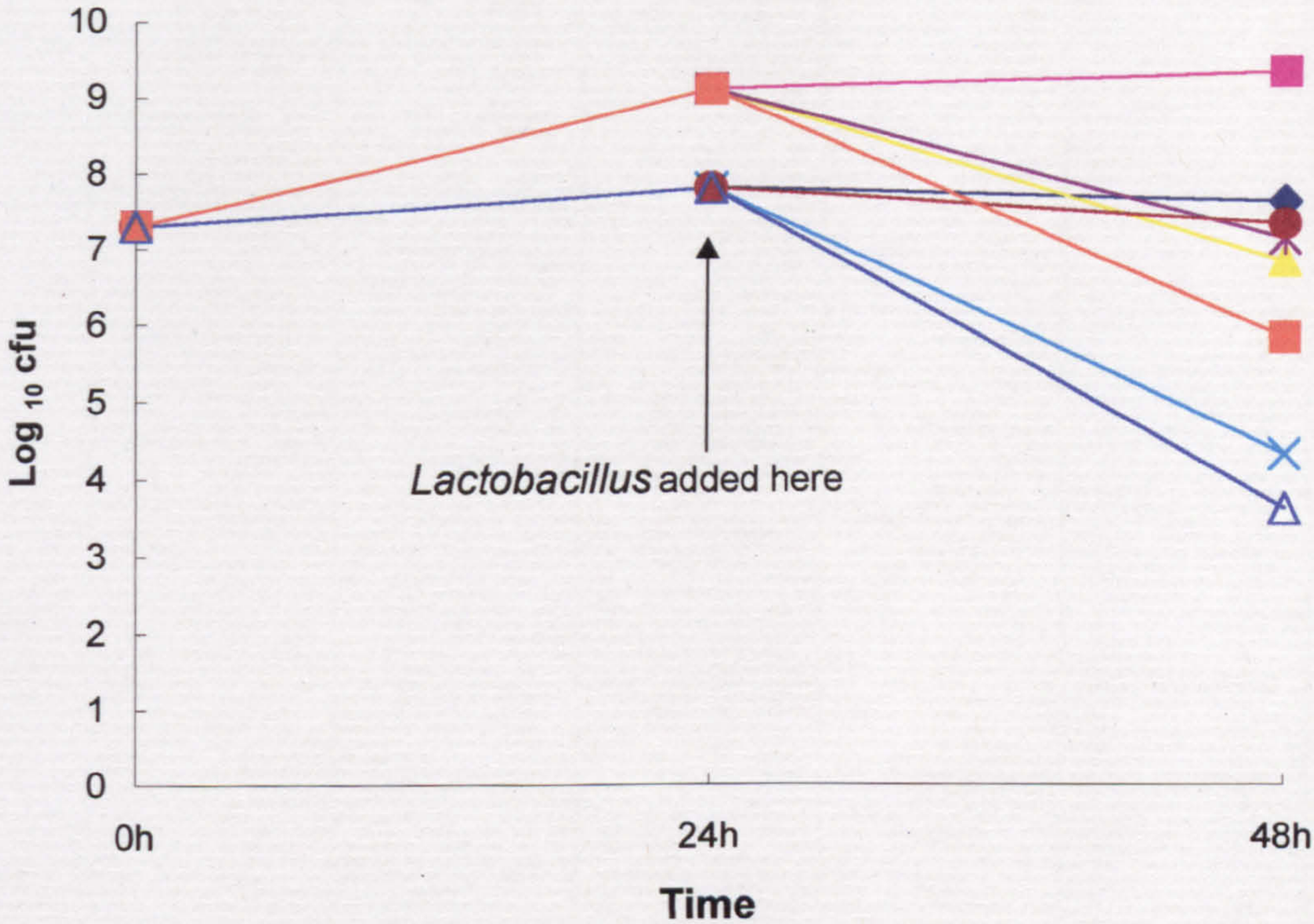

Fig. 74 Effect of *L. paracasei* on growth of *C. difficile* in biofilm
ControlC. *difficile* biofilm control (■)
Control *C. difficile* effluent (◆)
C. difficile biofilm (10mL *L. paracasei* added) (■)
C. difficile effluent (10mL *L. paracasei* added) (△)
C. difficile biofilm (1mL *L. paracasei* added) (✶)
C. difficile effluent titres (1mL *L. paracasei* added) (●)
C. difficile biofilm titres (0.1mL *L. paracasei* added) (▲)
C. difficile effluent titres (0.1mL *L. paracasei* added) (×)

5.12 Effect of *C. difficile* on growth of *L. paracasei* in biofilm

An experiment was also conducted to see if *C. difficile* had any antagonistic effect on the established growth of *L. paracasei* in biofilm (Fig. 75). There was no demonstrable effect, especially in the filters.

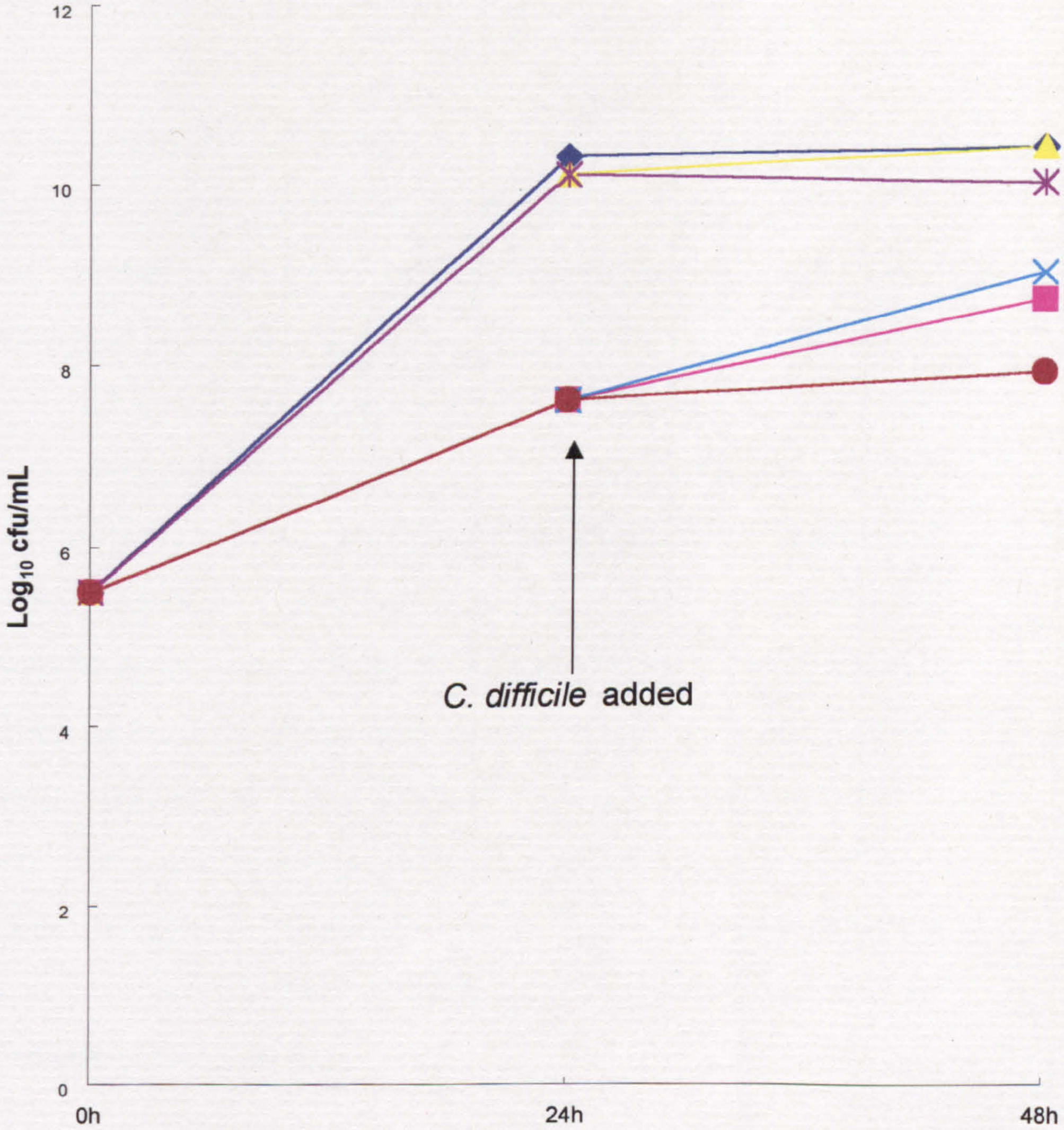

Fig. 75 Effect of *C. difficile* on growth of *L. paracasei* in biofilm

Control *L. paracasei* biofilm (◆)
Control *L. paracasei* effluent (■)
L. paracasei biofilm after addition of 1.0mL *C. difficile*(✳)
L. paracasei effluent after addition of 1.0mL *C. difficile* (●)
L. paracasei biofilm after addition of 0.1mL *C. difficile* (▲)
L. paracasei effluent after addition of 0.1mL *C. difficile* (×)

5.13 Discussion

The concept of colonisation resistance is very important when considering *C. difficile* infection. As raised previously in this chapter, the colon contains a complex ecosystem, with many individual bacterial species making up the "normal flora". If the interaction of a pathogen such as *C. difficile* with the members of the normal flora is to be studied, a model biofilm system needs to be developed. Three representative bacteria were chosen here, *E. coli, B. fragilis* and *E. faecalis.* All these bacteria grew readily on the Sorbarod filters, and in combination experiments with *C. difficile* there was little interference in the titres of the bacteria, at least within the variance of the titres in this system. Several experiments were conducted with *C. difficile* and *E. coli* with the broad spectrum antibiotics, co-amoxiclav and meropenem. Essentially here both organisms exhibited resistance to the antibiotics up to a concentration of 16 mg/L, but for *C. difficile* in the presence of co-amoxiclav, it was eliminated from the biofilm at 32 mg/L when the starting titre was significantly less, by a factor of 3 logs, than the *E. coli*. In both co-amoxiclav experiments, *C. difficile* was eliminated from the effluent at a concentration of 32 mg/L.

Screening various possible sources for an agent with antibacterial activity against *C. difficile* identified an isolate of *L. paracasei* which had distinct activity against the clostridium as shown by the "sloppy agar" method on a solid agar base (Fig. 62). This organism and its bacteriocin activity had an interesting host range; in addition to *C. difficile, C. tetani, C. bifermentans, C. novyi*, and from a surprisingly different

bacterial group, *S. pneumoniae,* were inhibited. When titred out, overnight broth cultures of the Lactobacillus gave the same titre against the different indicator strains, as identified by the zones of clearance around all the lactobacilli colonies.

Attempts to develop a model for this activity in broth and filter biofilms were frustrating. In broth some degree of activity against *C. difficile* could be demonstrated, but this effect was very variable. In the biofilm system, a significant killing effect was difficult to define. Clearly the role that any bacteriocin-like substance can play in the modulation of the growth of *C. difficile* in broth or this biofilm system is not as straightforward as identifying the activity on solid media.

Chapter Six

General Discussion

It is over twenty years since the association between *C. difficile*, AAD and pseudomembranous colitis was first noted. Since then, much has been learned about the epidemiology and treatment of this disease, which occurs almost exclusively in patients who have had prior exposure to antibiotics (Gorbach, 1999). The normal bowel flora is thought to prevent infection via colonization resistance (Reid *et al.*, 1990). When this is disrupted by antimicrobial therapy, the patient, usually over 70 years of age, becomes susceptible to infection with *C. difficile*. Most classes of antibiotics have been associated with *C. difficile* infection, particularly the cephalosporins and clindamycin (Gorbach, 1999). *C.difficile* is an anaerobic spore-producing organism. Patients with diarrhoea due to *C. difficile* liberate large numbers of spores into the environment, allowing the bacteria to survive in adverse conditions indefinitely, providing a means for cross-infection (Verity *et al.*, 2001). Other patients may ingest small numbers of these spores but provided their bowel flora has not been disrupted, they are unlikely to develop infection. Diarrhoea develops only with the release of toxins (A and B), that cause mucosal damage and inflammation. The diagnosis of *C. difficile* infection depends on the detection of *C. difficile* toxins in the stool using cell-culture or immunoassay (Brazier, 1998). Metronidazole or vancomycin enterally is the mainstay of treatment, while control mechanisms include

antibiotic stewardship, use of infection control universal precautions and good environmental cleaning.

Many problems remain to be resolved. The numbers of *C. difficile* infections continue to rise and *C. difficile* is now the commonest cause of diarrhoea in the elderly and is associated with substantial morbidity and mortality (Wilcox, 1998). A particular problem is recurrence of symptoms after successful treatment of initial *C. difficile* infections. A study by Wilcox and colleagues (1998) confirmed the findings of smaller studies that more than 56% of the recurrences of symptoms were due to reinfection rather than relapse. Reinfection obviously suggests inadequate infection control precautions and relapse was either thought to be due to inadequate treatment regimens or germination of spores in the colon post cessation of treatment.

An analysis of the detailed ecology of the colon must be important in understanding its diseases, and for bacteria, their growth in biofilm and interactions must be relevant. MacFarlane *et al.* (1997) discuss growth of organisms in biofilm in the gastrointestinal tract. They showed communities of polymer-degrading bacteria and other groups of intestinal anaerobes growing on particulate matter. It could be argued that growth of *C. difficile* in biofilm may account for treatment failures in those who have "true relapses" of infection, and other aspects of this infection.

To date there are no detailed studies of anaerobes in biofilm. It is known that *Porphyromonas gingivalis* is associated with dental plaque but the only work looking

at biofilm formation by this organism looked at the role of polyphosphate kinase (Chen, 2002). Other work involving anaerobes looked at butanol production by *Clostridium beijerinckii* BA101 in an immobilized cell biofilm reactor (Lienhardt *et al.*, 2002).

There is no previous report characterising an anaerobe in a biofilm mode of growth in the laboratory, and the first aim of this work was to determine the growth characterisitics of *C. difficile* using the Sorbarod filter system (Struthers, 2000). The work presented in Chapter 3 compared the growth of *C. difficile* in broth culture to biofilm growth. Differences were observed in titres in biofilm growth and the broth culture (Figs. 4-7). In broth culture, in excess of 10^8 cfu/mL were reached in 24h followed by a 1 log. drop, but the titre then remained stable as the isolate used sporulated at high frequency (Fig. 9). The titres in the filters were maintained for 72h with values of $>10^9$ cfu/filter being achieved, and these titres were often stable over the 72 h period (Fig. 6). It is thus reasonable to state that the organism was in a steady-state growth. Thin sections of filters growing *C. difficile* showed irregular microcolonies of bacteria about 10-50 μm in dimensions. (O'Connell *et al.*, 2003). These results are similar to other bacteria studied in this system, including *Gardnerella vaginalis*, *Lactobacillus acidophilus* (Muli and Struthers, 1998), and *Streptococcus pneumoniae* and *Moraxella catarrhalis* (Budhani and Struthers, 1997).

The Sorbarod system has revealed unique structures associated with cell division in *Gardnerella vaginalis* (Muli and Struthers, 1998), and that *S. pneumoniae* reversibly

lost its capsule when grown on these filters (Budhani and Struthers, 1998). In this latter situation, the Sorbarod biofilms revealed a previously unknown mechanism for regulating capsule expression in pneumococcus (Waite *et al.*, 2001). It was thus considered relevant to study *C. difficile* grown in biofilms by both light microscopy and TEM. In broth and plate culture, sporulation could be readily detected by 24h, and the vegetative bacteria showed the polymorphism recognized in the clostridia, with the length varying between 1.2 and 4.5 μm. In the biofilm system, longer elongated structures in excess of 10μm in length, and occasionally there were short spirals (Fig. 13). Electron microscopy studies showed that these were probably due to cell division, without cell separation actually occurring (Fig. 24). Apart from this feature, there was no other identifiable difference between the vegetative cell in broth, plate or biofilm. The major difference between the different sources of bacteria was the absence of spores in the filters. This clinical strain readily formed spores in broth and on plates, so it is reasonable to consider that the Sorbarod filter system allows the organism to maintain a continuous culture biofilm mode of growth for at least 72 hours. The relevance of this is discussed later.

Determination of toxin by the EIA showed higher concentrations of toxin were achieved when the organism was grown biofilm in comparison to broth and the effluent. While this may have reflected to some extent the differences in titres between the various growth modalities, there may be differences in the control of toxin expression. Although there were inconsistencies in repeat experiments, it seems reasonable that these differences are real. Clearly there are no reports on the kinetics

of toxin expression in biofilm or broth culture, and this would be a worthwhile area to investigate in future work.

With the first three aims of the thesis addressed, and the biofilm system for *C. difficile* in the filters being defined, the important area of antibiotic susceptibility was then investigated. Initial studies involved antimicrobial sensitivity testing using tube tests. The MIC values of *C. difficile* to benzylpenicillin, vancomycin and metronidazole correlated with those documented (Kucers and Bennett, 1987). However, MBC values showed a degree of tolerance, usually in excess of 128 mg/L. A number of clinical isolates were investigated here, and it is probably worthwhile to embark on a more detailed national study of MIC and MBC values, especially as MBC determination has not been considered before with this organism. This may be of considerable importance, considering that the site where CDAD is being treated, as the contents of the colon are to an extent unusual. High enough concentrations of antibiotics need to reach the colonic contents to effect killing. The roles that adherence of the organism to enterocytes, the presence of IgA antibodies and the presence of phagocytic cells such as neutrophils needs to be further defined, in this context.

This resistance pattern was not surprisingly found in the filters using benzylpenicillin, vancomycin and metronidazole, reflecting the accepted resistance to antibiotics in a biofilm system (Gander, 1995; Budhani and Struthers, 1997; Muli and Struthers, 1998; Struthers, 2000). It should be noted that when these resistant bacteria in the

filters and effluent were plated onto solid agar and examined directly by disc and Etest methods, that the bacteria reverted to the sensitive phenotype.

With rifampicin, the results were different in that an "acceptable" MBC value was obtained in broth culture. This result was consistently repeated in the filter effluent, with an EfMBC of between 1 and 4 mg/L being achieved in three experiments. On one occasion, in an experiment controlled by a tube MIC, a BEC of 8 mg/L was achieved (Fig. 33). Vancomycin BEC experiments up to 256 mg/L were also conducted, and these showed resistance up to this value (Figs. 35, 36). Addition of rifampicin at fixed concentrations of 1 and 8 mg/L showed a definite killing effect, probably due to some form of synergy.

The range of antimicrobial concentrations used in these experiments was based on the known pharmacokinetics of each antibiotic. With benzylpenicillin, metronidazole and rifampicin, conducting experiments with concentrations of up to 32 mg/L are appropriate, as the range up to this value relates to the pharmacokinetics of these agents in the human (Kucers and Bennett, 1987). It is appreciated that benzylpenicillin is not used in the treatment of CDAD, but has a usual half-life of only 30min with only some active penicillin excreted via the bile (5-10%) with the rest excreted in the urine or is inactivated in the liver. Rifampicin does not reach high levels in the lower GIT, and only 60% of a single dose is excreted in the faeces. Metronidazole is well absorbed from the GIT with 14% of an administered dose being excreted in the faeces. With vancomycin, the situation is different, for via the

oral route, it is poorly absorbed, and high concentrations, in the order of 200-300 mg/L are obtained in the colonic fluid (Kucers and Bennett, 1987). The tolerance of the *C. difficile* to vancomycin and metronidazole and the possible synergistic effect of rifampicin could be of relevance to the treatment of CDAD, particularly in light of the frequency of treatment failures, and noting the report that the combination of vancomycin and rifampicin is effective in treatment (Buggy, 1993).

As discussed above, the testing of toxin by the EIA did have a degree of variability associated with it, and this is reflected in the toxin experiments done here. It is worthwhile to note that at the lower antibiotic concentrations in particular, the level of toxin was significantly lower in the effluent than that in the filter. The reasons for this are unclear, but may indicate that significant toxin production is associated with biofilm formation. It is worthwhile to note that in their investigations of β-lactamase production by *M. catarrhalis,* Budhani and Struthers (1998) showed that levels of this enzyme in the Sorbarod biofilms were in the order of 200-fold lower in the filters than in broth or effluent based on relative organism numbers. In a comparison of protease production in planktonic and biofilm, Evans *et al.* (1994) reported that the production of protease by *S. epidermidis* was higher in biofilm populations but in a study by Tait and Sunderland (2002), they showed that single species planktonic and single species biofilm cultures of *Enterobacter gergoviae* produced similar quantities of protease and microcin.

Colonisation resistance is very important when considering *C. difficile* infection as the colon contains a complex ecosystem, with many individual bacterial species making up the "normal flora". Antagonistic and cooperative reactions, which lead to the establishment of pathogenic organisms, are known to occur (Reid *et al.*, 1990). A further aim of this work was to investigate combination growth of various organisms with *C. difficile* to determine if there were any obvious interactions. *E. coli, B. fragilis* and *E. faecalis* grew readily on the Sorbarod filters (Figs. 47-52). In combination experiments with *C. difficile* similar high titres were achieved, despite some reports of interference between clostridia and the growth of *E. coli* in the large intestine (Itoh and Freter, 1989). Again, when antibiotic susceptibility testing was conducted in *E. coli* and *C. difficile* combination biofilms, both organisms exhibited resistance to the antibiotics (Figs. 58-61).

Another objective of this work involved screening various possible sources for an agent with antibacterial activity against *C. difficile*. An isolate of lactobacillus was identified, *L. paracasei,* and was found to have distinct activity against the clostridium as shown by the "sloppy agar" method on a solid agar base (Fig. 63). This organism and its bacteriocin-like activity had an interesting host range; in addition to *C. difficile,* other clostridial species and *S. pneumoniae* were inhibited. Previous work has showed that *C. difficile* could be inhibited by several different strains of faecal streptococci (Malamou-Ladas and Tabaqchali, 1980). Another report documented the role of an antimicrobial substance from a human *Lactobacillus* sp. strain GG which

inhibited members of the family *Enterobacteriaceae*, *Pseudomonas* spp., *Staphylococcus* spp., and *Streptococcus* spp. as well as *C. difficile* (Silva *et al.*, 1987).

It has been suggested that production of a bacteriocin would give an organism a competitive advantage when interacting with other microbes in providing a "foothold" in colonizing a new environment. Bacteriocins may also prevent the colonization of a pre-established microbial community by a competitive species (colonization resistance) (Marsh and Bowden, 2000). In their review of the ecological role of bacteriocins in bacterial planktonic cultures, Riley and Gordon (1999) stated that bacteriocin-producing and bacteriocin-sensitive strains cannot coexist; one strain will always be outcompeted. Tait and Sutherland (2002) did a study which showed that the same may not be true of biofilms. They showed that bacteriocin-producing and bacteriocin- sensitive strains can co-exist in biofilm communities. Attempts to develop a model for this activity in broth and filter biofilms were frustrating. In broth some degree of activity against *C. difficile* could be demonstrated, but this effect was very variable. In the biofilm system, a significant killing effect was difficult to define which is similar to findings by other studies (Tait and Sunderland, 2002). It is apparent however, that the bowel ecosystem is very complex and much research is warranted into understanding the concept of colonization resistance and the interplay between indigenous flora and pathogens. Naaber *et al.* (1997) suggest that lactobacilli play a significant role in the maintenance of resistance to *C. difficile* colonization in various populations and hospitalized patients. A recent meta-analysis on the role of

probiotics in prevention of AAD suggests their usefulness but their efficacy in treatment needs to be proved (D'Souza *et al.*, 2002).

In vivo, biofilms are probably very common in the human body, and the colon is likely to be no exception. Here, as elsewhere, biofilm growth will provide a protective environment that effectively prevents attack by antimicrobials, biocides, and host defence mechanisms (De Lancey Pulcini, 2001). In the work here, the Sorbarod biofilm filter system has been adapted to a novel purpose. It is reasonable to conclude that it can be used to study *C. difficile,* which is an important and expensive pathogen in medicine. Further work should continue. In order to do this more sophisticated markers of metabolism need to be used, for example RNA expression of genes for sporulation and toxin production. With these markers, more detailed work on the effects of antibiotics should be done, when the organism is on its own and in combination with other bowel bacteria. The antibacterial activity of the Lactobacillus needs to be characterized in more detail and purified for further work. Investigation of such entities may be of future clinical relevance.

Another important piece of investigative work that needs to be done is to prepare chemically defined media that grow not only *C. difficile* but other bowel organisms. The Sorbarod system is ideal for such work, as established biofilms can be left undisturbed. Using a chemically defined medium, and via the T connector upstream, individual components can be withheld, and their effect on the growth, toxin production, susceptibility to antibiotics and bacteriocins could be determined.

In conclusion, the work presented here is a novel investigation of one of the most important pathogens causing hospital-acquired infection, and lays the basis for future exciting investigation.

References

Agnifili, A., Gola, P., Marino, M., *et al.* (1994) The role and timing of surgery in the treatment of pseudomembranous colitis. A case complicated by toxic mega-colon. *Hepatogastroentrology*; **41:** 394-396.

Alonso, R., Pelaez, T., Gonzalez-Abad, M.J., Alcala, L., Munoz, P., Rodriguez, M., Bouza, E. (2001) *In vitro* activity of new quinolones against *Clostridium difficile*. *J Antimicrob Chemother*; **47:** 195-197.

An, Y.H., Dickinson, R.B., Doyle, R.J. (2000) Mechanisms of bacterial adhesion and pathogenesis of implant and tissue infections, p 1-27. in Y.H. An and R. J. friedman (ed.), Handbook of bacterial adhesion: principles, methods, and applications. Humana Press, Totowa, N.J.

Anand, A., Glatt, A.E. (1993) *Clostridium difficile* infection associated with cancer chemotherapy: a review. *Clin Infect Dis*; **17:** 109-113.

Anderl, J.N., Franklin, M.J., Stewart, P.S. (2000) Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother*; **44:** 1818-1824.

Aronsson, B., Mollby, R., Nord, C.E. (1995) Antimicrobial agents and *Clostridium difficile* in acute enteric infection: Epidemiological data from Sweden, 1980-1982. *J Hosp Infect;* **151:** 476-481.

Barbut, F., Decre, D., Burghoffer, B., Lesage, D., Delisle, F., Lalande, V., Delmee, M., Avesani, V., Sano, N., Coudert, C., Petit, J.C. (1999) Antimicrobial susceptibilities and Serogroups of Clinical Strains of *Clostridium difficile* isolated in France in 1991 and 1997. *Antimicrob Agents Chemotherm*; **43:** 2607-2611.

Barlett, J.G. (1990) *Clostridium difficile*: clinical considerations. *Rev Infect Dis*; **12 (Suppl. 2):** S243-251.

Bartlett, J.G. (1992) Antibiotic-associated diarrhea. *Clin Infect Dis;* **15:** 573-581.

Bartley, S.L. Dowell, V.R. (1991) Comparison of media for the isolation of *Clostridium difficile* from faecal specimens. *Lab Med;* **22:** 335-338.

Bettin, K., Clabots, C., Mathie, P., Willard, K., Gerding, D.N. (1994) Effectiveness of liquid soap *vs* chlorhexidine gluconate for the removal of *Clostridium difficile* from bare hands and gloved hands. *Infect Ctrl Hosp Epidemiol*; **15:** 697-702.

Bignardi, G.E. (1998) Risk factors for *Clostridium difficile* infection. *J Hosp Infect*; **40:** 1-15.

Bolton, R.P, Culshaw, M.A. (1986) Faecal metronidazole concentrations during oral and intravenous therapy for antibiotic associated colitis due to *Clostridium difficile*. *Gut* **27:** 1169-1172.

Borriello, S.P. (1998) Pathogenesis of *Clostridium difficile* infection. *J Antimicrob Chemo*; **41 (Suppl. C):** 13-19.

Borriello, S.P., Barclay, F.E. (1986) An *in-vitro* model of colonisation resistance to *Clostridium difficile* infection. *J Med Micro*; **21:** 299-309.

Borriello, S.P., Davies, H.A., Kamiya, S., Reed, P. J., Seddon, S. (1990) Virulence factors of *Clostridium difficile. Rev Infect Dis*; **12(Suppl 2):** S185-191.

Borriello, S.P., Ketley, J.M., Mitchell, T.J., Barclay, F.E., Welch, A.R., Price, A.B., Stephen, J. (1987) *Clostridium difficile*- a spectrum of virulence and analysis of putative virulence determinants in the hamster model of antibiotic-associated colitis. *J Med Micro;* **25:** 53-64.

Bouza, E., Pelaez, T., Alonso, R., Catalan, P., Munoz, P., Rodriguez Creixems, M. (2001) 'Second look' cytoxoicity: an evaluation of culture plus cytotoxin assay of *Clostridium difficile* isolates in the laboratory diagnosis of CDAD. *J Hosp Infect;* **48:** 233-237.

Brazier, J.S. (1998) The diagnosis of *Clostridium difficile*-associated disease. *J Antimicrob Chemo*; **41 (Suppl. C):** 29-40.

Brazier, J.S. (1998) The epidemiology and typing of *Clostridium difficile. J Antimicrob Chemo*; **41(Suppl):** 47-57.

Brazier, J.S., Warren, F., Freeman, J., Wilcox, M.H. (2001) Reduced susceptibility of *Clostridium difficile* to metronidazole. *J Antimicrob Chemother*; **48:** 741-742.

Brooks, S.E., Veal, R.O., Kramer, M., Dore, L., Schupf, N., Adachi, M. (1992) Reduction in the incidence of *Clostridium difficile*-associated diarrhea in an acute care hospital and a skilled nursing facility following replacement of electronic thermometers with single-used disposables. *Infect Ctrl Hosp Epidemiol*; **13:** 98-103.

Brooun, A., Liu, S., Lewis, K. (2000) A dose-response study of antibiotic resistance in *Pseudomonas aeruginoas* biofilms. *Antimicrob Agents Chemother*; **44:** 640-646.

Brown, E., Talbot, G.H., Axelrod, P., Provender, M., Hoegg, C. (1990) Risk factors for *Clostridium difficile* toxin-associated diarrhoea. *Infect Ctrl Hosp Epidemiol*; **11:** 283-290.

Budhani, R.K., Struthers, J.K. (1997) The use of Sorbarod biofilms to study the antimicrobial susceptibility of a strain of *Streptoccous pneumoniae. J Antimicrob Chemother*; **40:** 601-602.

Budhani, R.K., Struthers, J.K. (1998) Interaction of The role of beta-lactamase in biofilm cultures of *Streptococcus pneumoniae* and *Moraxella catarrhalis*: investigation of the indirect pathogen role of β-lactamase producing moraxellae by use of a continuous culture biofilm system. *Antimicrob Agents Chemother*; **42:** 2521-2526.

Buggy, B.P. (1993) *Clostridium difficile* colitis: causes, cures. *J Am Med Assoc*; **269:** 2088.

Bulusu, M., Narayan, S., Shelter, K., Triadafilopoulos, G. (2000) Leukocytosis as a Harbinger and Surrogate Marker of *Clostridium difficile* Infection in Hospitalized Patients With Diarrhea. *Am J Gastroenterol;* **95(11):** 3137-3141.

Buswell, C.M., Nicholl, H.S., Walker, J.T. (2001) Use of continuous culture bioreactors for the study of pathogens such as *Campylobacter jejuni* and *Escherichia coli* in biofilms. *Methods Enzymology*; **337:** 70-78.

Caldwell, D.E., Lawrence, J.R. (1988) CRC Hanbook of Laboratory Model Systems for Microbial Ecosystems (J.W.T. Wimpenny, ed), p117. CRC Press, Boca Raton, Fl.

Carpentier, B., Cerf, O. (1993) Biofilms and their consequences, with particular reference to hygiene in the food industry. *J Appl Bacteriol*; **75:** 499-511.

Castagliuolo, I., Riegler, M.F., Valenick, L., *et al.* (1999) *Saccharomyces boulardii* protease inhibits the effects of *Clostridium difficile* toxins A and B in human colonic mucosa. *Infect Immunol*; **67:** 302-307.

Ceri, H., Olson, M.E., Stremick, C., Read, R.R., Morck, D., Buret, A. (1999) The Calgary Biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol*; **37:** 1771-1776.

Characklis, W.G., McFeters, G.A., Marshall, K.C. (1990) Physiological ecology in biofilm systems, p. 341-394. In W.G. Characklis and K.C. Marshall (ed.) Biofilms. John Wiley and Sons, New York, N.Y.

Chen, W., Palmer, R.J., Kuramitsu, H.K. (2002) Role of polyphosphate kinase in biofilm formation by *Porphyromonas gingivalis*. *Infect Immun*; **70(8):** 4708-15.

Clabots, C.R., Johnson, S., Olson, M.M., Peterson, L.R., Gerding, D.N. (1992) Acquistion of *Clostridium difficile* by hospitalised patients: evidence for colonised new admissions as a source of infection. *J Infect Dis*; **166:** 561-567.

Cochrane, W.L., McFeters, G.A., Stewart, P.S. (2000) Reduced susceptibility of thin *Pseudomonas aeruginosa* biofilms to hydrogen peroxide and monochloramine. *J Appl Microbiol*; **88:** 22-30.

Cochrane, D.M.G., Brown, M.R.W., Anwar, H., Weller, P.H., Lam, K., Costerton, J.W. (1988) Antibody response to *Pseudomonas aeruginosa* surface protein antigens in a rat model of chronic lung infection. *J Med Microbiol*; **27:** 255-261.

Cohen, S.H., Tang, Y.J., Silva Jnr., J. (2000) Analysis of the pathogenicity locus in *Clostridium difficile* strains. *J Infect Dis*; **181:** 659-663.

Collie, R.E., McClane, B.A. (1998) Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastroenteral diseases. *J Clin Microbiol*; **36:** 30-36.

Communicable Disease Surveillance Centre. (2000) *Clostridium difficile* in England and Wales: 1999. *Commun Dis Rep CDR weekly*; **10:** 135.

Conkie, D. (1988) A novel orchid micropropagation matrix. *Orchid Review*; **6:** 390.

Cooperstock, M., Riegle, L., Woodruff, C.W., Onderdonk, A. (1983) Influence of age, sex, and diet on asymptomatic colonization of infants with *Clostridium difficile. J Clin Microbiol*; **17:** 830-8333.

Corona, M.L., Peters S.G., Narr, B.J., Thompson, R.L. (1990) Subspeciality clinics: critical care medicine. Infections related to central venous catheters. *Mayo Clin Proc*; **65:** 979-986.

Costerton, J.W. (1999) Introduction to biofilm. *Int J Antimicrob Agents*; **11:** 217-222.

Costerton, J.W., Cheng, K.J., Geesey, G., Ladd, T.I., Nickel, J.C., Dasgupta, M., Marrie, T.J. (1987) Bacterial biofilms in nature and disease. *Annu Rev Microbiol*; **41:** 435-464.

Costerton, J.W., Irwin, R.J., Cheng, K.J. (1981) The bacterial glycocalyx in nature and disease. *Ann Rev Microbiol*; **35:** 399-424.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., Lappin-Scott, H.M. (1995) Microbial biofilms. *Annu Rev Microbiol*. **49:** 711-745.

Costerton, J.W., Stewart, P.S., Greenberg, E.P. (1999) Bacterial biofilms: a common cause of persistent infections. *Science*; **284:** 1318-1322.

Cozart, J.C., Kalangi, S.S., Clench, M.H., Taylor, D.R., Borucki, M.J., Pollard, R.B. *et al.* (1993) *Clostridium difficile* diarrhoea in patients with AIDS veruses non-AIDS controls. Methods of treatment and clinical response to treatment. *J Clin Gastroenterol*; **16:** 192-194.

Das, J.R., Bhakoo, M., Jones, M.V., Gilbert, P. (1998) Changes in the biocide susceptibility of *Staphylococcal epidermidis* and *Escherichia coli* cells associated with rapid attachment to plastic surfaces. *J Appl Microbiol*, **84:** 852-858.

Davies, H.A., Borriello, S.P. (1990) Detection of capsule in strains of *Clostridium difficile* of varying virulence and toxigenicity. *Microbial Pathogen*; **9:** 141-146.

De Beer, D., Stoodley, P., Roe, F., Lewandowski, Z. (1994) Effects of biofilm structure on oxygen distribution and mass transport. *Biotechnol Bioeng*; **43:** 1131-1138.

De Girolami, P.C., Hanff, P.A., Eichelberger, K, *et al.* (1992) Multicenter evaluation of a new enzyme immunoassay for detection of *Clostridium difficile* enterotoxin A. *J Clin Microbiol*; **30:** 1085-1088.

De Lancey Pulcini, E. (2001) Bacterial biofilms: a review of current research. *Nephrologie*; **22(8):** 439-41.

D' Souza, A.L., Rajkumar, C., Cooke, J Bulpitt, C.J. (2002) Probiotics in prevention of antibiotic associated diarrhoea: meta-analysis. *Br Med J*; **324:** 1361-1364.

Dearing, W.H., Bagenstoss, A.H., Weed, L.A. (1960) Studies on the relationship of *Staphylococcus aureus* to pseudomembranous enteritis and to postantibiotic enteritis. *Gastroenterology*; **38:** 441-451.

Delmee, M., Melin, P., Peetermans, W., Verbist, L., Verschraegen, G. (1995) Treatment of *Clostridium difficile* colitis. *Acta Clin Belg*; **50 2:** 114-116.

Delmee, M., Avesani, V. (1990) Virulence of ten serogroups of *Clostridium difficile* in hamsters. *J Med Micro;* **33:** 85-90.

Department of Health and Public Health Laboratory Service Joint Working Group. (1994) *Clostridium difficile Infection. Prevention and Management.* BAPS Health Publication Unit, Heywood, Lancs.

Donkie, M.E., Price, D.N. (1989) In vitro growth of pea seeds after removal of the pod wall. *J Plant Physio*; **134:** 382-384.

Donlan, R.M. (2001) Biofilm formation. *Clin Infect Dis*; **33:** 1387-1392.

Donlan, R.M., Costerton, J.W. (2002) Biofilms: Survival Mechanisms of Clinically relevant Microorganisms. *Clin Microbiol Rev*; **15:** 167-193.

Donlan, R.M., Murga, R., Carson, L. (1999) Growing biofilms in intravenous fluids, p. 23- 29. In J. Wimpenny, P. Gilbert, J.Walker, M. Brading, and R. Bayston (ed.), Biofilms: the good, the bad, and the ugly. Bioline, Cardiff, Wales.

Dunne, W.M.Jnr. (2002) Bacterial adhesions: Seen any good biofilms lately? *Clin Microbiol Rev;* **15:** 155-166.

Dzink, J., Bartlett, J.G. (1980) *In vitro* susceptibility of *Clostridium difficile* isolates from patients with antibiotic-associated diarrhea or colitis. *Antimicrob Agents Chemother*; **17(4):** 695-698.

Ehlers, L.J., Bouwer, E.J. (1999) RP4 plasmid transfer among species of *pseudomonas* in a biofilm reactor. *Water Sci Technol*; **7:** 163-171.

Embil, J., Al-barrack, A., Dyek, B., *et al.* (1999) The one year experience following an outbreak of toxin A (-) and toxin B (+) *Clostridium difficile* associated disease in a Canadian tertiary care hospital. *Clin Infect Dis*; **29:** 1041.

Evans, E., Brown, M.R.W., Gilbert, P. (1994) Iron chelator, exopolysacchardie and protease production in *Staphylococcus epidermidis*: a comparative study of the effects of specific growth rate in biofilm and planktonic culture. *Microbiol;* **140:** 153-157.

Falla, T.J., Karunaratne, D.N., Hancock, R.E.W. (1996) Mode of action of the antimicrobial peptide indolicidin. *J Biol Chem*; **271:** 19298-19303.

Fekety, R. (1997) Guidelines for the diagnosis and management of *Clostridium difficile*-associated diarrhea and colitis. American College of Gastroenterology, Practice Parameters Committee. *Am J Gastroenterol;* **92:** 739-750.

Fekety, R., Shah, A.B. (1993) Diagnosis and treatment of *Clostridium difficile* colitis. *J Am Med Assoc*; **269:** 71-75.

Finney, S.M.T. (1893) Gastroenterostomy for cicatrizing ulcer of the pyloris. *Bull. Johns Hopkins Hosp*; **4:** 53-64.

Gander, S. (1996) Bacterial biofilms: resistance to antimicrobial agents. *J Antimicrob Chemother*; **37:** 1047-1050.

George, W.L., Sutter, V.L., Citron, D., Finegold, S.M. (1979) Selective and differential medium for isolation of *Clostridium difficile*. *J Clin Micro*; **9:** 214-9.

Gerding, D.N., Brazier, J.S. (1993) Optimal methods for identifying *Clostridium difficile* infections. *Clin Infect Dis*; **16(Suppl 4):** S439-442.

Gerding, D.N., Olson, M.M., Peterson, L.R., Teasley, D.G., Gebhard, R.L., Schwartz, M.L. *et al.* (1986) *Clostridium difficile*-associated diarrhea and colitis in adults. A prospective case-controlled epidemiologic study. *Arch Int Med;* **146:** 95-100.

Gerding, G.N., Johnson, S., Peterson, L.R., Mulligan, M.E., Sliva, J. Jr. (1995) *Clostridium difficile*-associated diarrhea and colitis. *Infect Ctrl Hosp Epidemiol*; **16:** 459-77.

Gillies, R.R., Govan, J.R.W. (1966) Typing of Pseudomonas pyocyanea by pyocine production. *J Path Bact;* **91:** 339-345.

Gorbach, S.L. (1999) Antibiotics and *Clostridium difficile. N Eng J Med;* **341:** 1690-1691.

Gorbach, S.L., Chang, T.W., Goldin, B. (1987) Successful treatment of relapsing *Clostridium difficile* colitis with *Lactobacillus GG. Lancet*; **2:** 1519.

Gravet, A., Colin, D.A., Keller, D., *et al.* (1998) Characterisation of a novel structural member, LukE-LukD, of the bicomponent *staphylococcal* leucotoxins family. *FEBS Letters*; **436:** 202-8.

Gravet, A., Rondeau, M., Harf-Monteil, C. *et al.* (1999) Predominant *Staphylococcus aureus* isolated from antibiotic-associated diarrhoea is clinically relevant and produces enterotoxin A and the bicomponent toxin LukE-LukD. *J Clin Microbiol*; **37:** 4012-19.

Groschel, D.H. (1996) *Clostridium difficile* infection. *Crit Rev Clin Lab Sci*; **33(3):** 203-45.

Guzman, R., Kirkpatrick, J., Forward, K., Lim, F. (1988) Failure of parenteral metronidazole in the treatment of pseudomembranous colitis. *J Infect Dis*; **158:** 1146-1147.

Hafiz, S., Oakley, A. (1976) *Clostridium difficile*: isolation and characterisitics. *J Med Microbiol*; **9:** 129-136.

Hall, K., O'Toole, E. (1935) Intestinal flora in new-born infants with a description of a new anaerobe, *Bacillus difficilis*. *Am J Dis Child;* **49:** 390-402.

Hancock, P. (1997) Antibiotic-associated diarrhoea: *C. difficile* or *C. perfringens*? *Rev Med Microbiol*; **8(Suppl 1):** 566-7.

Hancock, R.E.W. (2001) Cationic peptides: effectors in innate immunity and novel antimicrobials. *Lancet*; **1:** 156-164.

Hancock, R.E.W., Chapple, D.S. (1999) Peptide antibiotics. *Antimicrob Agents Chemother*; **43:** 1317.

Hancock, R.E.W., Lehrer, R.I. (1998) Cationic peptides: a new source of antibiotics. *Trends biotechol*; **16:** 82-88.

Hathaway, C.L., Johnson, E.A. (1998) *Clostridium*: The Spore-Bearing Anaerobes. Topley and Wilson's Microbiology and Microbial Infections. 9th Edn. Oxford University Press Inc., New York, p731-782.

Hodgson, A.E., Nelson, S.M., Brown, M.R.W., Gilbert, P. (1995) A simple *in vitro* model for growth control of bacterial biofilms. *J Appl Bacteriol*; **79:** 87-93.

Holdeman, L.V., Cato, E.P., Moore, W.E.C. (1977) Anaerobe Laboratory Manual, 4th edn, Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg.

Hoyle, B.D., Alcantara, J., Costerton, J.W. (1992) *Pseudomonas aeruginosa* biofilm as a diffusion barrier to piperacillin. *Antimicrob Agents Chemother*; **36:** 2054-2056.

Hughes, C.E., Gebhard, R.L., Peterson, L.R., Gerding, D.N. (1986) Efficacy of routine fiberoptic endoscope cleaning and disinfection for killing *Clostridium difficile*. *Gastrointest Endosc*; **32:** 7-9.

Hummel, R.P., Altemeier, W.A., Hill E.O. (1964) Iatrogenic *Staphylococcal* enterocolitis. *Ann Surg*; **160:** 551-562.

Itoh, K., Freter, R. (1989) Control of *Escherichia coli* populations by a combination of indigenous clostridia and lactobacilli in gnotobiotic mice and continuous flow cultures. *Infect Immunol*; **57:** 559-564.

James, R., Lazdunski, C., Pattus, F. (1991) Bacteriocins, Microcins and Lantibiotics (NATO ASI series) (Vol. 65), Springer-Verlag.

James, R., Pattus, F., Lazdunski, C. (1992) Plasmid encoded toxins. Springer-Verlag, Heidelberg.

Johnson, S., Samore, M., Farrow, K.A., Killgore, G.E., Tenover, F.C., Lyras, D., Rood, J.I., DeGirolami, P., Balton, A.L.,Rafferty, M.E., Pear, S.M., Gerding, D.N. (1999) Epidemics of diarrhea caused by a clindamycin-resistant strain of *Clostridium difficile* in four hospitals. *N Engl J Med*; **341:** 1645-51.

Johnson, S.J., Gerding, D.N., Olson, M.M., Weiler, M.D., Hughes, R.A., Clabots, C.R., *et al.* (1990) Prospective controlled study of vinyl glove use to interrupt *Clostridium difficile* nosocomial transmission. *Am J Med*; **88:** 137-40.

Joklik, W.K., Willett, H.P, Amos, D.B. and Wilfret, C.M. Eds (1988) Zinsser Microbiology, 19th edition, Prentice-Hall Inc, 1053 pp.

Jones, E.M., Kirkpatrick, B.L., Feeney, R., Reeves, D.S., MacGowan, A.P. (1997) Hospital-acquired *Clostridium difficile* diarrhoea. *Lancet;* **349:** 1176-7.

Kaatz, G.W., Gitlin, S.D., Schaberg, D.R, Wilson, K.H., Kauffman, C.A., Seo, S.M. (1988.) Acquistion of *Clostridium difficile* from the hospital environment. *Am J Hosp Epidemiol*; **127:** 1289-93.

Kabins, S.A., Spira, T.J. (1975) Outbreak of clindamycin-associated colitis. *Ann Int Med*; **83:** 830-1.

Kay, D. (1967) Techniques for electron microscopy 2nd edition page 560. Blackwell Scientific Publications, Oxford and Edinburgh.

Karlstrom, O., Fryklund, B., Tulus, K., *et al.* (1998) A prospective nationwide study of *Clostridium difficile* – associated diarrhoea in Sweden. The Swedish *C. difficile* Study group. *Clin Infect Dis*; **26:**141-5.

Kelly, C.P. (1996) Immune response to *Clostridium difficile* infection. *Eur J Gastroenterol Hepatol*; **8(11):** 1048-53.

Kelly, C.P., Pothoulakis, C., LaMont, J.T. (1994) *Clostridium difficile* colitis. *N Eng J Med* **330:** 257-62.

Kelly, C.P., Pothoulakis, C., Vavva, F., Castagliuolo, I., Bostwick, E.F., O'Keane, J.C., *et al.* (1996) Anti-*Clostridium difficile* bovine immunoglobulin concentrate inhibits cytotoxicity and enterotoxicity of C. difficile toxins. *Antimicrob Agent Chemother*; **40:** 373-9.

Knoop, F.C., Owens, M., Crooker, I.C. (1993) *Clostridium difficile*: Clinical Disease and Diagnosis. *Clin Micro Rev*; **6:** 251-265.

Kreisel, D., Savel, T.C., Silver, A.L., Cunningham, J.D. (1995) Surgical antibiotic prophylaxis and *Clostridium difficile* toxin positivity. *Arch Surg;* **130:** 989-93.

Kucers, A., Bennett, N. McK. (1987) The Use of Antibiotics. 4th Edn Heinemann Medical Books, Oxford.

Kyne, L., Farrell, R.J., Kelly, C.P. (2001) *Clostridium difficile. Gastro Clinic N Am*; **30:** 751-77.

Kyne, L., Merry, C., O'Connell. B., Harrington, P., Keane, C., O'Neill, D. (1998) Simultaneous outbreaks of two strains of toxigenic *Clostridium difficile* in a general hospital. *J Hosp Infect*; **38:** 101-112.

Kyne, L., Warny, M., Qamar, A., Kelly, C.P. (2000) Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. *N Engl J Med*; **342:** 390-397.

Lambe, D.W., Fergeson, K.P., Mayberry-Carson, K.J., Tober-Meyer, B., Costerton, J.W. (1991) Foreign-body-associated experimental osteomyelitis induced by *Bacteroides fragilis* and *Staphylococcal epidermidis* in rabbits. *Clin Orthop*; **266:** 285.

Lambert, H., O'Grady, F., Greenwood, D. (1997) Antibiotic and Chemotherapy: Anti-Infective Agents and Their Use in Therapy. Churchill Livingstone, UK.

Larson, H.E., Barclay, F.E., Honour, P., Hill, D. (1982) Epidemiology of *Clostridium difficile* in infants. *J Infect Dis*; **146:** 727-33.

Larson, H.E., Parry, J.V., Price, A.B., *et al.* (1977) Undescribed toxin pseudomembranous colitis. *Br Med J*; **i:** 1246-1248.

Larson, H.E., Price, A.B., Honour, P., Borriello, S.P. (1978) *Clostridium difficile* and the aetiology of pseudomembranous colitis. *Lancet*; **1(8073):** 1063-1066.

Leung, D.Y., Neville, L.M., Wilkins, T.D., *et al.* (1991) Treatment with intravenously administered gamma globulin of chronic relapsing colitis induced by *Clostridium difficile* toxin. *J Paediatr*; **118:** 633-637.

Lewis, K. (2001) Riddle of biofilm resistance. *Antimicrob Agents Chemother*; **45:** 99-1007.

Lienhardt, J., Schripsema, J., Qureshi, N., Blaschek, H.P. (2002) Butanol production by *Clostridium beijerinckii* BA101 in an immobilized cell biofilm reactor: increase in sugar utilization. *Appl Biochem Biotechnol;* **98-100:** 591-598.

Lillie, R.D. (1928) The Gram stain. I. A quick method for staining Gram-positive organisms in the tissues. *Arch Path;* **5:** 828.

Lyerly, D.M., Neville, L.M., Evans, D.T., *et al.* (1998) Multicenter evaluation of the *Clostridium difficile* TOX A/B TEST. *J Clin Microbiol*; **36:** 184-90.

MacFarlane, S., McBain, A.J., and MacFarlane, G.T. (1997) Consequences of biofilm and sessile growth in the large intestine. *Advances Dental Research*; **11:** 59-68.

Malamou-Ladas, H., Tabaqchali, S. (1980) Inhibition of *Clostridium difficile* by faecal streptococci. *J Med Microbiol*; **15:** 569-574.

Marrie, T.W., Costerton, J.W. (1984) Scanning and transmission electron microscopy of *in situ* bacterial colonisation of intravenous and intraarterial catheters. *J Clin Microbiol;* **19:** 687-693.

Marsh, P.D., Bowden, G.H.W. (2000) Microbial community interactions in biofilms. In Community Structure and Co-operation in Biofilms Socitey for General Microbiology Symposium no. 59. pp 167-198. ed. Allison, D.G., Gilbert, P., Lappin-Scott, H.M. and Wilson, M. Cambridge: Cambridge University Press.

Marshall, K.C. (1976) Interfaces in microbial ecology. Harvard University Press, Cambridge, Mass. p44-47.

McCoy, W.F., Bryers, J.D., Robbins, J., Costerton, J.W. (1981) Observations in fouling biofilm formation. *Canad J Microbiol*; **27:** 910.

McFarland, L.V., Mulligan, M.E., Kwok, R.Y.Y., Stamm, W.E. (1989) Nosocomial acquistion of *Clostridium difficile* infection. *New Engl J Med*; **320:** 204-210.

McFarland, L.V., Surawicz, C.M., Greenberg, R.N., Fekety, R., Elmer, G.W., Moyer, K.A., *et al.* (1994) A randomizied placebo-controlled trial of *Saccharomyces boulardii* in combination with standard antibiotics for *Clostridium difficile* disease. *J Am Med Assoc*; **271:** 1913-8.

McFarland, L.V., Surawicz, C.M., Stamm, W.E. (1990) Risk factors for *Clostridium difficile* carriage and *C. difficile*-associated diarrhoea in a cohort of hospitalised patients. *J Infect Dis*; **162:** 678-684.

McVay, C.S., Rolfe, R.D. (2000) *In vitro* and *in vivo* activities of nitazoxanide against *Clostridium difficile*. *Antimicrob Agents Chemother*; **44(9):** 2754-2758.

Mermel, M.L., Osborn, T.G. (1989) *Clostridium difficile*-associated reactive arthritis in an HLA-27 positive female: Report and literature review. *J Rheumatol;* **16:** 133-135.

Miles, A.A., Misra, S.S., Irwin, J.D. (1938) The estimation of the bacteriocidal power of blood. *J Hyg*; **38:** 732-749.

Mittelmann, M.W., Kohring, L.L., White, D.C. (1992) Multipurpose laminar-flow adhesion cells for the study of bacterial colonisation and biofilm formation. *Biofouling*; **6:** 39-51.

Mogg, G.A.G., Keighley, M.R.B., Burdon, D.W., Alexander-Williams, J., Youngs, D., Johnson, M., Bentley, S., George, R.H. (1979) Antibiotic- associated colitis- a review of 66 cases. *Br J Surg*; **66:** 738-742.

Morris, J.B., Zollinger, R, Jnr., Stellato, T.A. (1990) Role of surgery in antibiotic-induced pseudomembranous enterocolitis. *Am J Surg*; **160:** 535-539.

Muli, F.M., Struthers, J.K. (1998) The growth of *Gardnerella vaginalis* and *Lactobacillus acidophilus*. *Antimicrob Agents Chemother*; **47:** 401-405.

Muli, F.M., Struthers, J.K. (1998) The use of a continuous culture biofilm system to study the antimicrobial susceptibilities of *Gardnerella vaginalis* and *Lactobacillus acidophilus*. *Antimicrob Agents Chemother*; **42:** 1428-1432.

Mulligan, M.E. (1988) General epidemiology, potential reservoirs and typing procedures. in *Clostridium difficile- its Role in Intestinal disease* (Rolfe, R.D. & Finegold, S.M., Eds), pp. 229-56. Academic Press, London.

Mulligan, M.E., Rolfe, R.D., Finegold, S.M., George, W.M. (1979) Contamination of a hospital environment by *Clostridium difficile*. *Curr Micro*; **13:** 173-175.

Mundy, L.S., Shanholtzer, C.J., Willard, K.E., Gerding, D.N., Peterson, L.R. (1994) Laboratory detection of *Clostridium difficile*: a comparison of media and incubation conditions. *Am J Clin Pathol*; **103:** 52-56.

Murga, R., Miller, J.M., Donlan, R.M. (2001) Biofilm formation by gram negative bacteria on central venous catheter connectors: effect of conditioning films in a laboratory model. *J Clin Microbiol*; **39:** 2294-2297.

Mylonakis, E., Ryan, E.T., Calderwood, S.B. (2001) *Clostridium difficile*-Associated Diarrhea. *Arch Int Med*; **161:** 525-533.

Naaber P, Klaus K, Sepp E, Bjorksten B, Mikelsaar M. (1997) Colonization of infants and hospitalized patients with *Clostridium difficile* and lactobacilli. *Clin Infect Dis*; **25 Suppl 2:** S189-90.

National Committee for Clinical Laboratory Standards. (1997) *Methods for antimicrobial susceptibility testing of anaerobic bacteria*, 4th edn. Document M11-A4. Villanova, Pa: National Committee for Clinical Laboratory Standards.

Niault, M., Thomas, F., Prost, J., *et al.* (1999) Fungemia due to *Saccharomyces* species in a patient treated with enteral *Saccharomyces boulardii*. *Clin Infect Dis*; **28:** 930.

Nickel, J.C., Costerton, J.W., McLean, R.J.C., Olson, M. (1994) Bacterial biofilms: influence on the pathogenesisi, diagnosis, and treatment of urinary tract infections. *J Antimicrob Chemother*; **33:** 31-41.

Nickel, J.C., Ruseska, I., Wright, J.B., Costerton, J.W. (1985) .Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrob Agents Chemother*; **27:** 619-624.

Niyogi, S.K. (1992) Antimicrobial susceptibility of *Clostridium difficile* strains isolated from hospitalised with acute diarrhoea. *J Diarrhoeal Dis Res*; **10:** 156-158.

O'Connell, N.H., Struthers, J.K., Dowson, C., Sturgess, I. (2003) The growth of *Clostridium difficile* in biofilm, using the Sorbarod Filter System. *J Infect;* FIS Abstracts A54 **In Press.**

O'Neill, G.L., Brazier, J.S., Magee, J.T., Duerden, B.I. (1996) A comparison of PCR ribotyping and pyrolysis mass spectrometry for typing clinical isolates of *Clostridium difficile. Anaer;* **2:** 211-215.

Olson, M.M., Shanholtzer, C.J., Lee Jnr., J.T., *et al.* (1994) Ten years of prospective *Clostridium difficile*-associated disease surveillance and treatment at the Minneapolis VA Medical Center, 1982-1991. *Infect Control Hosp Epidemiol*; **15:** 371-381.

Oren, Z., Shai, Y. (1998) Mode of action of linear amphipathic alpha-helical antimicrobial peptides. *Biopolymers*; **47:** 451-63.

Pasic, M., Jost, R., Carrel, T., *et al.* (1993) Intracolonic vancomycin for pseudomembranous colitis. *N Engl J Med*; **329:** 585.

Pasteur, L., Joubert, J.F. (1877) Charbon et septicemie. *Comptes Rendus hebdomadaires des Seances de l'Academie des Sciences (Paris),* **85:** 101-115.

Pelaez, T., Alcala, L., Martinez-Sanchez,L., Munoz, P., Garcia-Lechuz, J.M., Rodriguez-Creixems, M., *et al.* (1998) Metronidazole resistance in *Clostridium difficile*: an emerging problem? In Abstracts of the Thirt-eight Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, CA. Abstract E173. American Society for Microbiology, Washington, DC.

Pelaez, T., Gijon, P., Martinez, L., Catalan, P., Rivera, M.L., Bouza, E. (1997) *Clostridium difficile* associated diarrhoea in the AIDs era. In Abstracts of the 37th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto. Abstract C28.

Pelaez, T., Alcala, L., Alonso, R., Rodriguez-Creixems, M., Garcia-Lechuz, J.M., Bouza, E. (2002). Reassessment of *Clostridium difficile* susceptibility to metronidazole and vancomycin. *Antimicrob Agents Chemother*; **46:** 1647-1650.

Pelaez, T., Alonso, R., Perez, C., Alcala, L., Cuevas, O., Bouza, E. (2002*) In vitro* activitiy of linezolid against *Clostridium difficile. Antimicrob Agents Chemother*; **46:** 1617-1618.

Peterson, L.R., Holter, J.J., Shanholtzer, C.J., *et al.* (1986)Detection of *Clostridium difficile* toxins A (enterotoxin) and B (cytotoxin) in clinical specimens. Evaluation of a latex agglutination test. *Am J Clin Pathol*; **86:** 208-211.

Peterson, L.R., Olson, M.M., Shanholtzer, C.J. *et al.* (1988) Results of a prospective, 18-month clinical evaluation of cutlure, cytotoxicity testing, and culturette brand (CDT) latex testing in the diagnosis of *Clostridium difficile*-associated diarrhea. *Diagn Microbiol Infect Dis*; **10:** 85-91.

Pettet, J.D., Baggenstoss, A.H., Dearing, W.H., Judd, E.S.Jnr. (1954) Postoperative pseudomembranous enterocolitis. *Surg Gynecol Obstet*; **8:** 546-552.

PHLS. (2002) Antimicrobial resistance in 2000: England and Wales. London: Public Health Laboratory Service.

Piers, K.L., Brown, M.H., Hancock, R.E.W. (1993) Recombinant DNA procedures for producing small antimicrobial cationic peptides in bacteria. *Gene*; **134:** 7-13.

Poilane, I., Cruaud, P., Torlotin, J.C., Collignon, A. (1999) Comparison of the E test to the reference agar dilution method antibiotic susceptibility testing of *Clostridium difficile*. *Clin Micro Infect*; **6:** 154-156.

Pothoulakis, C. (1996) Pathogenesis of *Clostridium difficile*-associated diarrhoea. *Eur J Gastroentrol Hepatol*; **8:** 1041-1047.

Pothoulakis, C., Barone, L.M., Ely, R. *et al.* (1986) Purification and properties of *Clostridium difficile* cytotoxin B. *J Biol Chem*; **261:** 1316-1321.

Privitera, G., Scarpellin, P., Ortsi, G., Nicastro, G., Nicolin, R., deLatta, F. (1991) Prospective study of *Clostridium difficile* intestinal colonization and disease following single dose antibiotic prophylaxis in surgery. *Antimicrob Agents Chemother;* **35:** 208-10.

Reece, R.E., Betts, R.F. (1996) In : A Practical Approach to Infectious Diseases. Little and Brown, 1043 pages.

Reid, G., Bruce, A.W., McGroarty, J.A., Cheng, K.J., Costerton, J.W. (1990) Is there a role for Lactobacilli in prevention of urogenital and intestinal infections. *Clin Microbiol Rev*; **3:** 335-344.

Reynolds, E.S. (1963) The use of lead citrate at high pH as an electron-opaque in electron microscopy. *J Cell Biology*; **17:** 208-212.

Riederer, K.M., Lawson, P., Held, M.S., Petrylka, K., Briski, L. E., Khatib, R. (1995) Diagnosis of *Clostridium difficile* associated diarrhoea: comparison of three rapid methods employing different markers for detection. *Canad J Micro;* **41:** 81-91.

Riley, M.A., Gordon, D.M. (1999) The ecological role of bacteriocins in bacterial competition. *Trends Microbiol*; **7:** 129-133.

Riley, T.V., Bowman, R.A., Golledge, C.L. (1995) Usefulness of culture in the diagnosis of *Clostridium difficile* infection. *Eur J Clin Microbiol Infect Dis*; **14:** 1109-11.

Roffe, C. (1996) Biotherapy for antibiotic-associated and other diarrhoeas. *J Infect*; **32:** 1-10.

Rolfe, R.D. (1984) Interactions among microorganisms of the indigenous intestinal flora and their influence on the host. *Rev Infect Dis*; **6(Suppl. 1):** 873-879.

Rogers, L.A. (1928) The inhibiting effect of *Streptococcus lactis* on *Lactobacillus bulgaricus*. *J Bacteriol*; **16:** 321-325.

Saginur, R., Hawley, C.R., Bartlett, J.C. (1980) Colitis associated with metronidazole therapy. *J Infect Dis*; **141:** 772-774.

Salcedo, J., Keates, S., Pothoulakis *et al.* (1997) Intravenous immunoglobulin therapy for severe *Clostridium difficile* colitis. *Gut*; **41:** 366-370.

Samore, M.H. (1999) Epidemiology of nosocomial *Clostridium difficile. J Hosp Infect;* **43(Suppl):** S183-190.

Savage, A.M., Alford, R.H. (1983) Nosocomial spread of *Clostridium difficile. Infect Ctrl*; **4:** 31-33.

Seal, D., Borriello, S.P., Barclay, F., *et al.* (1987) Treatment of relapsing *Clostridium difficile* diarrhoea by administration of a nontoxigenic strain. *Eur J Clin Microbiol*; **6:** 51-53.

Schaeffer, A.B., Fulton, M. (1933) A simplified method of staining endospores. *Science*; **77:** 194.

Shanholtzer, C.J., Willard, K.E., Holter, J.J. *et al.* (1992) Comparison of VIDAS *C difficile* toxin A immunoassay (CDA) with *C difficile* culture, cytotoxin, and latex test. *J Clin Microbiol*; **30:** 1837-1840.

Siitonen, S., Vapaatalo, H., Salminen, S., Gordin, A., Saxelin, M, Wikberg, R. *et al.* (1990) Effect of Lactobacillus GG yoghurt in prevention of antibiotic associated diarrhoea. *Ann Med*; **22:** 57-59.

Silva, M., Jacobus, N.V., Deneke, C., Gorbach, S.L. (1987) Antimicrobial substance from a human Lactobacillus strain. *Antimicrobial Agents Chemother*; **31:** 1231-1233.

Spencer, R. (1998) The role of antimicrobial agents in the aetiology of *Clostridium difficile*-associated disease. *J Antimicro Chemo;* **41:** 21-27.

Stewart, P.S., Costerton, J.W. (2001) Antibiotic resistance of bacteria in biofilms. *Lancet*; **358:** 135-138.

Stickler, D., Hughes, G. (1999) Abiltiy of *Proteus mirabilis* to swarm over urethral catheters. *Eur J Clin Microbiol Dis*; **18:** 206-208.

Strimburg, M.O., Sacho, H. and Berkowitzl, I. (1989) *Clostridium difficile* infection in healthcare workers. *Lancet*; **2:** 866-867.

Struthers, J.K. (2000) The use of a continuous culture system to study the antimicrobial susceptibility of bacteria in biofilm. Methods in Molecluar Medicine; Antibiotic Resistance Methods and Protocols. **Vol 48:** 215-225. Humana Press Inc., Tetowa, NJ.

Surawicz, C.M., Elmer, G.M., Speelman, P., McFarland, L.V., Chinn, J., VanBelle, G. (1989) Prevention of antibiotic-associated diarrhoea by *Saccharomyces boulardii*: a prospective study. *Gastroenterol*; **96:** 981-8.

Synott, K., Mealy, K., Merry, C., *et al.* (1998) Timing of surgery for fulminant pseudomembranous colitis. *Br J Surg*; **85:** 229-231.

Tait, K., Sutherland, I.W. (2002) Antagonistic interactions amongst bacteriocin-producing enteric bacteria in dual species biofilms. *J Appl Microbiol;* **93:** 345-353.

Tabaqchali, S., Jumaa, P. (1995) Diagnosis and management of *Clostridium difficile* infection. *Br Med J;* **310:** 1375-80.

Tedesco, F.J., Barton, R.W., Alpers, D.H. (1974) Clindamycin-associated colitis. A prospective study. *Ann Intern Med*; **81:** 429-433.

Tedesco, F.J., Corless, J.K., Brownstein, R.E. (1982) Rectal sparing in antibiotic-associated pseudomembranous colitis: a prospective study. *Gastroenterol;* **16:** 1259-1260.

Tedesco, F.J., Gordon, D., Fortson, W.C. (1985) Approach to patients with multiple relapses of antibiotic associated pseudomembranous colitis. *Am J Gastroenterol*; **80:** 867-8.

Tedesco, F.J., Markham, R., Gurwith M, *et al.* (1978) Oral vancomycin for antibiotic-associated pseudomembranous colitis. *Lancet;* **2:** 226-228.

Theilman, (2000) Antibiotic-Associated Colitis, in *Principles and Practice of Infectious Diseases* (Mandell, Bennett and Dolin., eds.), Churchill Livingstone, Phileadelphia, Pennsylvania, p1111-1126.

Thelestam, M., Bronnegard, M. (1980) Interaction of cytopathogenic toxin from cells in tissue culture. *Scand J Infect Dis*; **22(Suppl.):** 16-29.

Tvede, M., Rask-Maden, J. (1989) Bacteriotherapy for chronic relapsing *Clostridium difficile* diarrhoea in six patients. *Lancet*; **i:** 1156-1160.

Verity, P., Wilcox, M.H., Parnell, P. (2001) Prospective evaluation of environmental contamination by *Clostridium difficile* in isolation side rooms. *J Hosp Infect*; **49:** 204-209.

Vorachit, M., Lam, K., Jayanetra, P., Costerton, J.W. (1993) Resistance of *Pseudomonas pseudomallei* growing as a biofilm on sialstic disks to ceftazidime and cotrimoxazole. *Antimicrob Agents Chemother*; **37:** 2000-2002.

Waite, R.D., Struthers, J.K., Dowson, C.G. (2001) Spontaneous sequence duplication within an open reading frame of the pneumococcal type 3 capsule locus causes high-frequency phase variation. *Molecular Med*; **42(5):** 1223-1232.

Walker, R.C., Ruane, P.J., Rosenblatt, J.E., *et al.* (1986) Comparison of culture, cytotoxicity assays, and enzyme-linked immunosorbent assay for toxin A and toxin B in the diagnosis of *Clostridium difficile*-related enteric disease. *Diagn Microbiol Infect Dis*; **5:** 61-69.

Wilcox, M.H. (1998) Treatment of *Clostridium difficile* infection. *J Antimicrob Chemother*; **41(Suppl C):** 41-46.

Wilcox, M.H., Cunniffe, J.G., Trundle, C., Redpath, C. (1996) Financial burden of hospital-acquired *Clostridium difficile* infection. *J Hosp Infect*; **34:** 23-30.

Wilcox, M.H., Fawley, W.N. (2000) Hospital disinfectants and spore formation by *Clostridium difficile*. *Lancet*; **356(9247):** 2098-2099.

Wilcox, M.H., Fawley, W.N., Settle, C.D., Davidson, A. (1998) Recurrences of symptoms in *Clostridium difficile* infection- relapse or reinfection? *J Hosp Infect*; **38:** 93-100.

Wilcox, M.H., Spencer, R.C. (1992) *Clostridium difficile* infection: responses, relapses and reinfections. *J Hosp Infect*; **22:** 85-92.

Wong, S.S., Woo, P.C., Luk, W.K., Yuen, K.Y. (1999) Susceptibility testing of *Clostridium difficile* against metronidazole and vancomycin by disk diffusion and E-test. *Diagnostic Microbiol Infect Dis*. **34:** 1-6.

Worsley, M.A. (1998) Infection control and prevention of *Clostridium difficile* infection. *J Antimicro Chemo*; **41(Suppl. C):** 59-66.

Wren, B.W., Clayton, C.L., Tabaqchali, S. (1990) Rapid.identification of toxigenic *Clostridium difficile* by polymerase chain reaction. *Lancet* **335:** 423.

Wu, M., Maier, F., Benz, R., Hancock, R.E.W. (1999) Mechanisms of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with cytoplasmic membrane of *Escherichia coli*. *Biochem*; **38:** 7232-42.

www.bsac.uk Antimicrobial susceptibility testing.

Zhang, T.C., Bishop, P.L. (1996) Evaluation of substrate and pH effects in a nitrifying biofilm. *Wat Environ Res*; **68:** 1107-15.

Zimmermann M.J., Bak, A., Sutherland, L.R. (1997) Treatment of *Clostridium difficile* infection. *Aliment Pharmacol Ther*; **11:** 1003-1012.

Publications

O'Connell, N.H., Struthers, J.K., Dowson, C., Sturgess, I. (2003) The growth of *Clostridium difficile* in biofilm, using the Sorbarod Filter System. *J. Infect;* : FIS Abstracts A54 **In Press**

O'Connell, N.H., Struthers, J.K., Dowson, C., Sturgess, I. (2003) An Investigation of the Growth Characteristics and Antibiotic Susceptibility Profile of *Clostridium difficile* using the Sorbarod Biofilm Filter System. *Anaerobe;* **Submitted**