Interactions between *Candida albicans* and vaginal lactobacilli

Ruth Elizabeth Haworth

Presented for the degree of Doctor of Philosophy in the Faculty of Science, University of Glasgow

Department of Microbiology

August 1993

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Preface

This thesis is the original work of the author.

Ruth Haworth

Acknowledgements

I should like to take this opportunity to thank Dr. L.J. Douglas and Mrs. Pam Hunter for their supervision, advice and encouragement throughout this work.

I wish to acknowledge the SERC Mass Spectrometry Centre, University of Swansea, for carrying out the Electron Impact Mass Spectrometry. Thanks are also due to others who worked in the 'Candida Lab' during my time at Glasgow - Simon, Donald, Audry, and also to Hugh Shannon for his assistance with the animal work carried out in Glasgow; and those from the 'Antifungals Lab' at SmithKline Beecham, especially Ian, Anne, Richard, Liz, Chris and also to Fiona Portwood for her advice on statistics.

Gratitude is expressed to Judith Firth and Martin Wheatman for their word processing expertise and to Dr. Carol Mackie for reading the manuscript. I am grateful to the Science and Engineering Council and SmithKline Beecham for funding this studentship.

Finally, I would also like to thank my friends and family for their love and support, without which this thesis would never have been completed.

SUMMARY

The aim of this project was to determine the effect of lactobacilli on *Candida* adhesion to vaginal epithelial cells *in vitro* and on the pathogenesis of vaginitis in experimental animals.

Pretreatment of exfoliated human, mouse or rat vaginal epithelial cells with *Lactobacillus acidopilus* or *L. fermentum* inhibited subsequent adhesion of *Candida albicans* to the cells. Increasing the pretreatment concentration of lactobacilli increased inhibition of *Candida* adhesion to mouse vaginal cells, up to a maximum of 60%. Fresh vaginal isolates of *Lactobacillus* species showed greater adhesion to vaginal cells than culture collection strains. They also showed greater inhibition of *Candida* adhesion. It is possible that subculture of a *Lactobacillus* isolate may cause it to lose some ability to adhere. A fresh *Lactobacillus* isolate was able to inhibit four different strains of *C. albicans* to varying extents. Inhibition was greatest with the yeast strains which had shown least adhesion when pretreatment with lactobacilli was omitted. It is postulated that in the human vagina a *C. albicans* strain which is poorly adherent may be inhibited by lactobacilli and thus be unable to cause disease. A 20-fold concentrate of *Lactobacillus* culture supernatant had no effect on yeast adhesion to epithelial cells. It would appear that inhibition of *Candida* adhesion may be due to steric hindrance by the lactobacilli of the *Candida* binding sites.

Further work was carried out to investigate the effect of vaginal pH, diabetes and steroid hormones upon *Candida* adhesion. It was observed that there was greater *Candida* adhesion when assays were carried out at pH 7.2 rather than pH 4.5 (vaginal pH). There was also more inhibition of *Candida* adhesion by lactobacilli when

adherence assays were carried out at pH 7.2. Diabetes is often cited as a predisposing factor in *Candida* infections and women with diabetes certainly have a greater incidence of vaginal candidosis. In the present study, it was found that yeasts adhered in greater numbers to cells taken from diabetic mice, with the exception of the *C. albicans* strains NCPF 3153 and 'outbreak'. Growth of yeasts in the presence of oestradiol and progesterone had no significant effect on *Candida* adhesion. A slight decrease in *Candida* adherence was observed after hormone pretreatment of either epithelial cells or yeasts. This may have been due to non-specific inhibition.

Experiments were carried out to investigate whether lactobacilli prevented *Candida* infection by inhibiting *Candida* growth and colonization. *Lactobacillus* and *Candida* strains tested were able to grow over a similar range of pH values. This suggests that they are likely to grow in the same environmental conditions and therefore interact. Lactobacilli did not inhibit *Candida* when the two organisms were grown together in mixed broth cultures. However lactobacilli did inhibit *Candida* growth in the sandwich plate assay when the plates were preincubated to allow bacterial growth prior to inoculation with *Candida*. It was found that greater numbers of lactobacilli in the base of the sandwich plate and longer preincubation of the lactobacilli gave greater yeast inhibition. Viable cells were not necessary to give inhibition of *Candida* growth. Inhibition was not due to nutrient depletion. The antimicrobial activity of lactobacilli was released into the culture supernatant. The active substance(s) passed through a cellulose membrane indicating a molecular mass of less than 10,000 Da.

Concentrated culture supernatant of *L. acidophilus* and *L. fermentum* showed activity against *C. albicans*, *Cryptococcus neoformans*, *Aspergillus niger* and *Trichopyton metagrophytes*. Butanol-extracted culture supernatants of *L. acidophilus* and *L. fermentum* were also tested against a range of bacteria; antibacterial activity was seen against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *B. megaterium*, and *Proteus vulgaris*. After purification, low resolution mass spectrometry identified three compounds produced by the lactobacilli which inhibited *Candida* growth; they had molecular masses of 419, 433 and 447 Da.

The course of *Candida* infection was followed in mice after treatment of the vagina with different concentrations of *C. albicans*. For diabetic mice the infection was seen to be much greater than for normal mice. *In vivo* interactions between *C. albicans* and lactobacilli were investigated using a rat model of *Candida* vaginitis. Establishment of a *Lactobacillus* population in the rat vagina, or pretreatment with concentrated *Lactobacillus* culture supernatant (which had shown antimicrobial activity against *C. albicans* and a range of other fungi and bacteria in plate tests) provided no significant protection against infection with *C. albicans*. However, this may reflect inadequacies of the rat model and does not preclude a role for *Lactobacillus-Candida* interactions in the pathogenesis of human infections.

CONTENTS

MATERIALS AND METHODS

RESULTS

DISCUSSION

Index of Tables

Index of Figures

Abbreviations

A	Absorbance
BiGGY	Bismuth sulphite-Glucose-Glycine-Yeast
cfu	colonty forming units
Da	Dalton
DW	Distilled Water
FIC	Fungal Inhibitory Compound
FLV	Friend Leukemia Virus
g	gravity
h	hour
IgE	Immunoglobulin E
lb/sq in	pounds per square inch pressure
LTA	Lipoteichoic acid
min	minute
MRS	De Man Rogosa Sharpe
M_r	Relative Molecular mass
OD	Optical Density
P	Probability
PBS	Phosphate Buffered Saline
PMN	Polymorphonuclear leucocyte
rev/min	revolutions per minute
R_f	Relative Mobility
s	second
SDA	Sabouraud dextrose agar
ssp.	subspecies
TLC	Thin Layer Chromatography
v	volume
VEC	Vaginal Epithelial Cells
w	weight
YNB	Yeast Nitrogen Base

INTRODUCTION

1. *Candida albicans*

1.1 Historical aspects of *Candida* infections

Candida infections of virtually every tissue of the human body have been reported. The most common manifestations are superficial lesions of mucosal surfaces, particularly in the oral cavity and the vagina (Odds, 1988). These forms are commonly known as thrush due to the similarity of the white speckled lesion to the breast of a thrush.

Thrush as a clinical condition has been recognized since time of Hippocrates, circa 460 - 377 B.C. (Odds, 1988). The disease was also mentioned in the diary of Samuel Pepys for 17th June 1665 (Winner & Hurley, 1964). However, it was not until 1839 that the organism responsible for these infections was discovered by Langenbeck. He described the yeast in buccal apthae in a case of typhus. Langenbeck incorrectly postulated the yeast as a factor contributing to the typhus and it was Berg in 1846 who became the earliest author to describe fully the relationship between the thrush fungus and mouth lesions (Odds, 1988).

Descriptions of the thrush fungus nearly all drew attention to its dimorphic nature, but it was Audry in 1887 who proved that the different morphological forms were one and the same organism and that the morphology depended on the growth environment.

The thrush fungus was the subject of confused taxonomic studies and for many years following its discovery it was linked with the genus *Monilia*. In 1923 the taxonomic position was reviewed by Berkhout, and the medical *Monilia* species were clearly distinguished from the fruit and leaf rotting moulds also called *Monilia* (Kreger-Van Rij, 1984). As the rotting fungi had been described first as *Monilia* the genus was invalid for the thrush

fungus. This led to the adoption of the name *Candida* at the Third International Congress for Microbiology in 1939.

1.2 Morphology of *C. albicans*

Depending on conditions, *Candida* can grow in different morphological forms. The different forms of *C. albicans* have been described by Odds (1979). Cells can appear as ovoid yeasts termed blastospores which multiply by a specific mode of mitotic cell division termed budding. *C. albicans* can also appear as mould-like hyphae, occasionally with large refractile spores termed chlamydospores. Hyphae are long microscopic tubes which comprise multiple fungal cell units, divided by septa. They may arise as branches of existing hyphae or from blastospores, in which case they are termed germ tubes. *Candida* can also produce pseudohyphae on certain media. Pseudohyphae are morphologically quite similar to true hyphae but they are produced by a budding process whereby the bud remains attached to the parent.

1.3 *Candida* growth and nutrition

Pathogenic yeasts are generally able to multiply in media of pH 3-8 and at temperatures between 20-40°C (Odds, 1979).

Candida species grow aerobically, but are able to grow under conditions of elevated CO_2 in air. However, an atmosphere of pure CO_2 inhibits the growth of *C. albicans* (Eklund & Jarmund, 1983).

The *Candida* species all assimilate and ferment glucose as a carbon source but none are able to assimilate nitrate as a nitrogen source. The species vary in their ability to utilise other carbon and nitrogen sources (Kreger-Van Rij, 1984). *Candida* commonly requires biotin, thiamine and other vitamins of the B complex for growth.

1.4 Occurrence of *Candida* in Nature

Yeasts are distributed ubiquitously in both terrestrial and aquatic habitats. However, Do Carmo-Sousa (1969) regarded *C. albicans* as an obligate animal saprophyte. Yeasts occur commonly as commensals in the gut, less often at other sites. *C. albicans* is the principal opportunistic yeast pathogen in most warm blooded animals and has been recovered from a far wider range of animal hosts than any other *Candida* species.

Carriers of *Candida* often contaminate their immediate environment with yeasts. This contamination does not usually spread far as *C. albicans* survives poorly on dry surfaces such as skin or glass. The survival of yeasts on moistened objects is greater and *Candida* can survive on fabrics laundered at temperatures up to 50°C. *Candida* species are frequently found in the hospital environment - in foods, in the air, on floors and other surfaces.

The most important source of *Candida* species in human disease is endogenous. Dissemination from faeces has repeatedly been cited as an important source of infection and re-infection in women predisposed to candida vaginitis.

1.5 Candidosis

Candidosis varies in severity from superficial localized infections to severe systemic disease. Superficial candidosis refers to the surface infections of the oral, aural and vaginal cavities as well as the skin and nails. Localized infections may become chronic but do not result in serious systemic involvement unless the patient is compromised.

Candidosis affecting deep organs is unusual in comparison with infections of mucous membranes. Infections of deep organs may arise as localized primary diseases with only one organ affected or as disseminated candidosis where *Candida* is spread by the blood stream to invade several organs. These severe systemic infections are often life threatening.

1.6 Pathogenic *Candida* species

There are a number of different pathogenic *Candida* species (Table 1). *C. albicans* is considered the most pathogenic. There are a number of characteristics of *C. albicans* which are thought to account for this. Adherence to host surfaces is an important factor in the virulence of the different *Candida* species as this is the initial stage of yeast infection when the fungus must gain a foothold on a target tissue. *C. albicans* demonstrates the greatest adhesion of the different *Candida* species (McCourtie & Douglas, 1984). The ability to express different phenotypic properties accounts for *C. albicans* ability to invade a wide range of host environments (Odds, 1988). Also, *C. albicans* is dimorphic. This is an important virulence factor as hyphae are able to adhere better than blastospores (Douglas, 1987); they also have the ability to grow out of phagocyte cells (Richardson & Smith, 1981). The secretion of proteinase (Kwon-Chung *et al.*, 1985) and induction of phospholipase (Barrett-Bee *et al.*,1985) have also been implicated as virulence factors. A number of authors have suggested that *C. albicans* may contain some molecules with a low but detectable biological potency i.e. toxin. However, studies so far do not unanimously confirm the occurrence of toxin(s) in *C. albicans* (Odds, 1988).

Table 1.

Different pathogenic *Candida* species

C. albicans
C. tropicalis
C. parapsilosis
C. kefyr
C. krusei
C. glabrata
C. guilliermondii

Other *Candida* species possess some of the above mentioned factors, it has been suggested that the possession of these putative virulence factors corresponds with the ranking of a pathogenic hierarchy of *Candida* species (Odds, 1987).

2. *Lactobacillus* species

2.1 *Lactobacillus* morphology

Lactobacilli are Gram-positive bacteria, although old or dying cells can give Gram-variable results. There is variability in cell morphology among different *Lactobacillus* species from long, straight or slightly crescent rods to coryneform coccobacilli (Kandler & Weiss, 1984 In :Krieg & Holt eds. Bergey's Manual of Systemic Bacteriology volume 1). The length of rods and amount of curvature can also depend on the age of the culture, the composition of the growth medium and oxygen tension. *L. acidophillus*, one of the most common vaginal strains, is a rod with rounded ends of approximate size 0.6-0.9 by 1.5-6 μm and can occur singly, in pairs or in short chains. Some species e.g. *L. fermentum* exhibit a mixture of long and short rods. The tendency of lactobacilli to form chains depends on the growth phase and the pH value of the culture medium.

Motile lactobacilli are uncommon; however, when motility is present it is by peritrichous flagella.

Internal granulation may been seen upon Gram staining, especially in homofermentative long rods. The large bipolar bodies probably contain polyphosphate (Kandler & Weiss,1984 In:Krieg & Holt eds. Bergey's Manual of Systemic Bacteriology volume 1).

2.2 Growth and nutrition of *Lactobacillus* species

Lactobacillus species grow at temperatures ranging from 2 to 53°C with an optimum generally between 30 and 40°C. Lactobacilli are microaerophilic and their growth on solid media is generally enhanced by anaerobiosis or reduced oxygen pressure and 5 to 10% CO_2. The optimum pH value for growth is usually 5.5 to 6.2 although growth generally occurs at pH values of 5.0 or less. The growth rate is often reduced at neutral pH or initially alkaline conditions.

The *Lactobacillus* species have a fermentative metabolism on the basis of which they can be split into two groups, homofermentative or heterofermentative species. Lactobacilli are extremely fastidious organisms, adapted to complex organic substrates. They require not only carbohydrates as energy and carbon sources, but also nucleotides, amino acids and vitamins. Pantothenic acid and nicotinic acid are, with only a few exceptions, required by all species; thiamine is also necessary for the growth of the heterofermentative lactobacilli. The patterns of vitamin and amino acid requirement differ among species, and among even strains, and they are often considered to be characteristic of particular species. The various requirements for essential nutrients are normally met when the medium contains fermentable carbohydrate, peptone, meat and yeast extract. Supplementations with manganese, acetate and oleic acid esters, especially Tween 80, are stimulatory or even essential for most species. MRS medium (De Man *et al.*, 1960) is widely used for the growth of lactobacilli.

Lactobacilli synthesise a variety of fermentation end-products. Homofermentative species produce principally lactate from their carbon source, while heterofermentative species also synthesise acetate, ethanol, CO_2, formate or succinate. Such end-

products of metabolism lower the pH value of the culture medium; growth of lactobacilli ceases when a pH value of 4.0 to 3.6 is reached, depending on the species and strain. (Kandler & Weiss,1984 In:Krieg & Holt eds. Bergey's Manual of Systemic Bacteriology volume 1).

2.3 Occurrence of *Lactobacillus* species in Nature

Lactobacilli appear to be ubiquitous in Nature and will grow under anaerobic conditions or under reduced oxygen tension in all habitats provided with ample carbohydrates, breakdown products of protein, nucleic acids and vitamins. Lactobacilli have been isolated from a variety of sources including humans, animals, dairy products, wines, silage and plants. Many individual species appear to have adapted to specific environments and are generally not found outside of their specialized habitat. The relative ease with which many species of lactobacilli can be reisolated from source materials shows that these niches are in fact their natural habitats (London, 1976).

Lactobacilli occur in Nature in low numbers on all plant surfaces, especially decaying fruits. Lactobacilli are important for the production of vegetable feed and food (e.g., silage, sauerkraut). Species chiefly isolated have been *L. plantarum*, *L. brevis*, *L. casei*, *L. sake* and *L. fermentum*. Lactobacilli are important in the dairy industry. They are used as starters for the production of Swiss cheese (*L. helveticus*), they are also a well known component of yoghurt flora (*L. delbrueckii* ssp. *bulgaricus*). *L. plantarum* plays an important role in the curing process of fermented sausages.

Lactobacilli, as well as being used within the food industry, may contribute to spoilage by the production of an off flavour, acid taste, gas, slime or greening.

Man and animals harbour many species of *Lactobacillus* living as commensals, usually intimately associated with the mucosal surface epithelium. *L. salivarius* can be isolated from the oral cavity. Lactobacilli are also known as important components of the normal flora of the intestine where *L. acidophilus* is the most predominant *Lactobacillus* species. Lactobacilli are the most commonly isolated organisms from the healthy adult human vagina. *L.acidophilus, L. crispatus, L. jensenii* and *L. casei* have all been identified as members of the vaginal flora (Kandler & Weiss,1984 In:Krieg & Holt eds. Bergey's Manual of Systemic Bacteriology volume 1). McGroarty *et al.* (1992) found *L. jensenii, L. acidiphilus* and *L. casei* to be the predominant *Lactobacillus* species found in the healthy vagina.

Lactobacilli are believed to exert a beneficial effect on human and animal health. Various *Lactobacillus* preparations have been produced and administered in an attempt to restore the normal flora after disturbance. This subject will be further discussed in Section 6.3.

Lactobacilli are generally considered to be apathogenic. However, there are reports that lactobacilli have been involved in human disease. *Lactobacillus* species have been found to be associated with subacute bacterial endocarditis (Bar *et al.*, 1987), bacteraemia (Manzella & Harootunian, 1982), septicaemia (Potvliege *et al.*, 1988) and abscesses (Sherman *et al.*, 1987). *L. gassei* was found in a case of urosepsis (Dickgiesser *et al.*, 1984). Haan *et al.* (1985) described a case where a metabolic acidosis and neurological

disfunction was caused by products of *Lactobacillus* metabolism in a child with a short intestine. The biochemical basis of such pathogenicity is as yet unknown.

3. The human vaginal environment

3.1 Histology of the vagina

The vagina is a musculofibrous tube lined with a mucous membrane. The epithelium, consisting of three layers of cells is of a stratified squamous type which rests on a lamina propria of dense connective tissue. The deepest stratum of the vaginal epithelium consists of a single layer of columnar cells which contain single, large, oval nuclei. The next stratum is several layers in thickness. Near the columnar layer the cells are small and closely packed together, but more superficially they are larger polygonal in shape, joined by intracellular bridges. At the epithelial surface, there are several layers of flattened cells with few nuclei and indistinct outlines; these cells have undergone keratinization. From the surface many layers of degenerated cells may be seen in the process of separation (Ham, 1969).

The more superficial layers of the epithelium tend to accumulate glycogen, found around the periphery of each individual cell. The epithelial cell walls and interstices also appear to contain glycogen granules of varying size and shape (Cruickshank & Sharman, 1934a); others regard these as fixation artefacts (Gregoire *et al.*, 1971).

At different ages variation in glycogen deposition alters the characteristic of the epithelium. At puberty, glycogen becomes abundant within a florid, thick and many layered epithelium. Post menopause, when glycogen is scanty or completely absent, the

epithelium is low, flattened and often appears atrophic and keratinized. (Cruickshank & Sharman, 1934a).

3.2 Vaginal secretions

Vaginal secretions are important in the creation of the overall vaginal environment. They are made up of several components (Preti & Huggins, 1975):

(i) vulval secretions from sebaceous, sweat, Bartholin's and Skeens glands.

(ii) mucus secretions from the cervix (Taylor *et al.*, 1986).

(iii) endometrial and oviductal fluids.

(iv) transepithelially migrating fluid comparable to the transepidermal water loss through the skin i.e., a transudate through the vaginal walls (Perl *et al.*, 1959).

(v) exfoliated cells from the mucosa.

The secretion is a complex mixture of organic compounds, the type and amount of which differ in individuals and even across menstrual cycles in certain women. The type and amount of mucus secretion from the cervix, the endometrial and oviductal fluids, and exfoliated cells from the mucosa are known to be influenced by biochemical processes that are dependent on sex steroid levels. Increases in oestrogens and combined oestrogen/progesterone have been shown to produce increases in carbohydrates in both the vaginal epithelium and secretions. Glycogen may also appear in vaginal fluid, and it is known that structural characteristics of the vaginal epithelium determine its concentration (Ayre, 1951).

Vaginal fluid is known to contain not only glycogen but also glucose, maltose, malto triose, maltotetraose and several amino

acids (Stamey & Kaufman, 1975). Fatty acids have also been found to be present in human vaginal secretions. The small aliphatic acids, lactic acid, acetic acid and 3-hydroxy-2-butanone which are found within the vagina may all be formed by the action of microorganisms (Preti & Huggins, 1975).

3.3 Human vaginal pH

The pH value of the human vagina has been measured in a number of studies. Chen *et al.* (1979) reported that the average pH of the healthy adult vagina was 4.04. Sautter & Brown (1980) found a range of vaginal pH values from 4 to 6 among 7 normal women, whilst Sjoberg *et al.* (1990) reported a slightly more restricted range of 3.8 to 4.5 for the 10 healthy volunteers in their study. Riordan *et al.* (1990) associated an elevated pH value of more than 4.5 with symptoms of vaginal infection. They noted that measurement of vaginal pH was the single most useful clinical finding for directing empirical therapy.

Vaginal acidity was found to be due mainly to lactic acid, although acetic acid and other short chain aliphatic acids could also be found (Preti & Huggins, 1975). The classical explanation of vaginal acidic pH was given by Cruickshank and Sharman (1934b). They suggested that under the influence of oestrogenic hormones the vaginal epithelium contains glycogen which can be fermented by lactobacilli. They observed glycogen, lactobacilli and an acidic pH value in the vaginas of children during the first few weeks of life - they proposed this was due to the effect of maternally derived hormones which had crossed the placenta before birth. Glycogen, lactobacilli and acidic vaginal pH reappeared with the onset of puberty. However, during childhood and after the menopause glycogen

was scant or absent, and Cruickshank & Sharman noted the bacterial flora was much reduced, lactobacilli were absent and the pH value of the limited vaginal secretions was neutral or slightly alkaline. It was Miura in 1928 (cited by Weinstein & Howard, 1939) who provided the first direct proof that the glycogen of the mucosa, bacterial flora and pH of the vagina were dependent on the ovaries. He studied vaginal secretions before and after ovarectomy. A low pH value and large numbers of lactobacilli were present before operation; this situation, accompanied by the disappearance of glycogen from the vaginal mucosa, was completely reversed after removal of the ovaries.

The close relationship between ovarian function and glycogen deposition is well established; however, some doubt has been cast on the mechanism of acid production. Weinstein & Howard (1939) demonstrated that oestrogenic hormone administration is able to increase the acidity of the vagina without the intervention of lactobacilli. Although lactobacilli are present in the vaginas of many women - 73% in a study by Gorbach *et al.* (1973) - women who do not possess a *Lactobacillus* population still have an acidic vaginal pH. The importance of the *Lactobacillus* contribution to vaginal pH can be further questioned. Wylie & Henderson (1969) demonstrated the glycogen fermenting ability of some strains of lactobacilli, but Stewart-Tull (1964) was unable to demonstrate this ability in any of the strains he tested.

On the basis of the evidence obtained, the production of acid in the vagina seems to depend mainly on the state of activity of the ovary. Ovarian function determines the amount of glycogen present in the vaginal mucosa. The action of oestrogens effect the growth and differentiation of the cells within the vagina. The glycogen

concentration per cell may be constant, however, absolute amounts of glycogen per vagina will vary, depending on the thickness of the vaginal epithelium (Gregoire *et al.*, 1967, 1971; Ayre, 1951). The glycogen may be broken down directly to acid by enzymic action or enzymes of the vaginal tissue cells may break the glycogen down to glucose which can then be attacked by bacteria present in the vagina to give acid.

3.4 The vaginal flora

It is well known that body surfaces and cavities open to the external environment are colonized by micro-organisms soon after birth. A biological succession ensues, eventually resulting in the presence of microbial communities which are characteristic of the particular body site (Tannock, 1988).

Cruickshank & Sharman (1934b) described the development of the vaginal community. They noticed that after a sterile phase lasting 12-24h the vagina was invaded by a heterogeneous bacterial flora which by the third to fourth day consisted almost entirely of Gram-positive rods; these organisms remained predominant for approximately 10 days and were then replaced by a varied bacterial flora until the onset of puberty.

Until the 1970s it was generally accepted that during the reproductive years the flora was essentially made up of *Lactobacillus* species, a concept originated by Doderlein in 1892. However, with improvements in isolation techniques, a greater awareness of the polymicrobial nature of the vaginal flora has emerged.

Quantitative studies have shown that there are, on average, 10^8 colony forming units per ml of vaginal secretions (Onderdonk *et*

al., 1977). The number of species recovered in counts exceeding 10^4 varied from 1 to 10. *Lactobacillus* was thought to be the predominant species in the vagina. In a study by Corbishley (1977) lactobacilli were found in 61% of vaginal swabs. Other studies have shown even higher incidences of lactobacilli in the vagina (82.6%, Ohm & Galask, 1976; 87.9%, Sautter & Brown, 1980). A high incidence of diptheroids (61%, Corbishley, 1977; 52.2%, Ohm & Galask, 1975) and their abundance indicate they are quite common inhabitants of the vagina. Streptococci are an important part of the vaginal flora (Levison *et al.*, 1979; Corbishley, 1977; Ohm & Galask, 1975) as are vaginal corynebacteria. In the study by Sautter & Brown (1980) *Corynebacterium vaginale* was seen to be the predominant organism in 40% of vaginal specimens. This study also showed high recovery rates from the vagina of *Ureaplasma urealyticum* and *Mycoplasma hominis*.

Corbishley (1977) noted that coagulase-negative staphylococci were frequently found in the vagina (89%) although in only one quarter of cases were they found in large numbers. Also, *Staphylococcus aureus* was isolated from 17% of swabs. The origin of all the staphylococci is probably the skin flora.

Isolation of bacteroides, faecal streptococci and coliforms from the vagina is common but isolation of *Clostridium welchii*, *Proteus* sp. or other *Enterobacteriaceae* is rare. This high incidence of faecal organisms is not surprising considering the close proximity of the anus to the female genital tract. (Corbishley 1977; Ohm & Galask, 1976).

The wide variety of organisms present, some of which are almost invariably found, indicate that the vagina does not simply contain a resident flora of lactobacilli with occasional transient

colonization by other bacteria. The vagina must be regarded as a complex microflora of interacting and competing micro-organisms.

3.5 Cyclical changes in the vaginal flora

The microbiological flora changes as the environmental conditions of the vagina changes. The effect of ovarian activity has been demonstrated (see Section 3.1, 3.2), but the effect of the normal menstrual cycle on the vaginal microbiological flora is confused.

Sautter & Brown (1980) found that the number of organisms varied greatly at different phases in the menstrual cycle for each individual in their study, whereas the types of organisms isolated from a particular subject remain relatively constant. Wilks & Tabaqchali (1987) also found significant alterations in the total vaginal flora at different stages in the menstrual cycle. However, they noticed that the mean number of species isolated per specimen declined from 4.6 in week 1 to 2.9 in week 4, which correlated with a decline in pH from 6.6 to 4.3. The observation of a decline in pH was contradicted by Mardh & Westrom (1976) who stated that pH varied little under physiological conditions. Saigh *et al.* (1978) isolated microorganisms, including lactobacilli, from the endocervix which were able to inhibit *Neisseria gonorrhoea*. They found cyclical changes in the numbers of the inhibitory lactobacilli isolated, with highest recovery during the first two weeks following menses.

Not only has the effect of the normal menstrual cycle on the recovery of the vaginal microbiological flora been studied but also the adhesion of various components of the microflora to vaginal cells exfoliated at different times in the menstrual cycle. Bibel *et al.* (1987) noted increased adherence of *S. aureus* and *C. albicans* to vaginal cells in the third and fourth weeks of the menstrual cycle.

Botta (1979) found that the adhesion of Group B streptococci varied during the cycle, being maximal near the time of ovulation. He also observed oral contraception completely abolished these observed cyclical changes. However Sobel *et al.* (1981) did not find any difference in the ability of a variety of bacteria to adhere to vaginal epithelial cells at different times of the menstrual cycle.

3.6 Changes to the normal flora in vaginitis

Alterations to the normal vaginal flora have been associated with vaginitis. However, there has been no overall concensus on the importance of the normal vaginal flora in health or disease. This is due to a lack of comparability between different studies which have used different patient populations and different methods for collecting and culturing samples of the vaginal flora (Wilks *et al.*, 1984). It has been reported that women with vaginitis have significantly higher aerobic bacterial counts, but that anaerobic counts from the vagina are not significantly different (Levison *et al.*, 1979). However, in a separate study by Auger & Joly, (1980) *Candida* vaginitis was associated with a decrease in the aerobic bacterial flora. Auger & Joly noted that Gram-negative bacteria predominated in the absence of *C. albicans* and suggested that Gram-negative bacteria were more markedly antagonistic to *C. albicans* than Gram-positive organisms. It was found that the fungus could produce, at least *in vitro*, compounds capable of lessening the bacterial population and thus decreasing the competition for environmental food supply.

Changes in the *Lactobacillus* population within the vagina have been seen in vaginitis. H_2O_2-producing facultative *Lactobacillus* species were isolated more frequently from healthy women (96%) than

from women with vaginitis (6%)(Eschenbach *et al.*, 1989). Similarly, Fredricsson *et al.* (1992) found H_2O_2-producing lactobacilli were significantly more common in healthy women than women with bacterial vaginitis. They found the species most frequently related to vaginal health were *L. jensenii* and *L. rogosae*. Investigation with a group of pregnant women showed that initial absence of H_2O_2-producing *Lactobacillus* organisms was a significant risk factor for the subsequent development of bacterial vaginitis later in pregnancy (Eschenbach *et al.*, 1989). Further investigation, with another group of pregnant women, showed the presence of H_2O_2-producing lactobacilli reduced the risk of bacterial vaginitis, symptomatic candidosis and also colonization by *Gardnerella vaginalis*, *Bacteroides*, *Peptostreptococcus*, *M. hominis*, *U. urealyticum*, *Chlamydia trachomatis* and viridans streptococci (Hillier *et al.*, 1992).

4. Vaginal candidosis

4.1 Clinical features of vaginal candidosis

Acute pruritus and vaginal discharge are the usual presenting complaints of *Candida* vaginitis. The itching may become worse when the patient is warm. Discharge can range from thin and yellowish to the more classic curdy and whitish (Jacobson, 1986). Other symptoms include painful burning and soreness in the region of the introitus and labia minora during urination, sexual intercourse or gynaecological examination. Physical findings on examination include vulvar and vaginal erythema and oedema, as well as white plaques on the epithelium (Fleury, 1986). Papular, ulcerated or granular lesions may be seen and the disease sometimes spreads to involve the perianal region and inner thighs.

4.2 Incidence of vaginal candidosis

Statistical information on the incidence of *Candida* vaginitis is not easy to obtain. It is made unreliable by the inadequacies of diagnoses.

Clinical symptoms are often used in general practice as a basis for diagnosing candidosis, sometimes without even a genital examination. However, clinical features do not, on their own, provide sufficient specific information to establish a diagnosis reliably. The opposite approach to diagnosis is one based entirely on mycological evidence. However, the most reasonable approach to diagnosis of vaginal candidosis is to consider both clinical and mycological evidence for the infection. Odds *et al.* (1988) suggested that diagnosis of vulvovaginial candidosis should be made when a patient who presents with pruritus and/or abnormal discharge can be shown, by direct microscopic examination, to harbour *Candida* in the vagina.

Many authors have expressed the opinion that the incidence of *Candida* vaginitis has risen in recent years, with the rise often ascribed to the use of antibiotics and hormonal contraceptives (Catteral, 1971; Morton & Rashid, 1977). However, the rising number of *Candida* cases reflects the rising number of women attending clinics where candidosis is diagnosed rather than a rising prevalence in the disease. In the last 10 years *Candida* has been consistently isolated from the vaginas of 20% of females attending British genitourinary medicine clinics (Odds, 1988). In the United States *Candida* vaginitis is the second commonest form of vaginal infection currently seen, occurring only slightly less frequently than non-specific vaginitis (Sobel, 1984).

4.3 Predisposing factors to *Candida* vaginitis

Candida species are strictly opportunistic pathogens that rarely or never cause clinically important disease in a host with intact antimicrobial defences. In normal circumstances an equilibrium between the host and the yeast microflora ensures the avirulent commensal state of the *Candida*. Many women can carry *Candida* in the vagina at low concentration without any symptoms of vaginitis (Berg *et al.*, 1984). However, any alteration in any antimicrobial defence can compromise the ability of the host to withstand microbial attack (Mims, 1987).

Diabetes mellitus is a condition which predisposes women to candidosis. Among diabetic women, there have been reports of incidences of vaginal thrush in excess of 50% (Segal *et al.*, 1984). Genital *Candida* infections have also been described as common presenting symptoms at the onset of diabetes (Nagesha & Ananthakrishna, 1970) with higher than normal frequencies of yeast carriage in samples from the vaginas of diabetic patients.

The mechanism or mechanisms by which diabetes increases host susceptibility to candidosis are not clear. Segal *et al.* (1984) showed that epithelial cells from diabetics had increased adhesiveness for *Candida*. It has been proposed that high blood and tissue glucose levels or low skin lactate levels favour the growth of *Candida* in diabetics (Odds *et al.*, 1987; Kandhari *et al.*, 1969). Darwazeh et al. (1991) found diabetic patients who carried *Candida* intraorally had significantly higher salivary glucose concentrations than those diabetic patients from whom *Candida* could not be isolated. Bybee & Rogers (1964) suggested that diabetics in a state of ketoacidosis had a defect in the phagocytosis of *Candida* by polymorphonuclear leukocytes.

There is a reported 30-45% incidence of vaginal candidosis during pregnancy (Segal, 1987). Vaginal carriage of yeast has almost always been found to be greater in pregnant than in non-pregnant women (Lang *et al.*, 1962). Most comparative studies have shown a higher prevalence of vaginal yeasts during the third trimester of pregnancy (Pedersen, 1964, 1969). However, it has been found that the physiological factors responsible for predisposition to *Candida* infection in pregnancy revert to normal very rapidly post partum (Gillespie *et al.*, 1960).

Segal *et al.* (1984) observed that vaginal epithelial cells from pregnant women displayed enhanced adhesiveness for *C. albicans in vitro*. This increased tendency towards adherence was correlated with a low Karyopyknotic Index (which measures the ratio of superficial to intermediate vaginal cells). Another hypothesis for the increase in *Candida* infections during pregnancy is related to a reduction in cell-mediated immunity, a condition which occurs in the third trimester of pregnancy, when *Candida* infection is most prevalent (Kalo-Klein & Witkin, 1989).

It has been suggested that the widespread administration of antibiotics can precipitate symptomatic *Candida* vaginitis (Seelig, 1966). A 25% incidence of candidal infection has been observed for women on antibiotic therapy (Segal *et al.*, 1984). It is possible that antibiotics may enhance the growth of yeasts within the vagina by direct stimulation, or, by removing organisms which normally compete for nutrients or secrete antifungal substances (Seelig, 1966). Sjoberg *et al.* (1992) suggested treatment with penicillin may interfere with the normal vaginal bacterial flora and provide an environment favourable for *Enterobacteriaceae* and fungi. Antibiotics can cause local tissue damage and may produce increased *Candida*

invasion, either as a consequence of local tissue damage or possibly by yeast conversion to a more invasive form. The effect of antibiotic therapy as a predisposing factor in *Candida* vaginitis is difficult to assess. People being treated with antibiotics are usually already ill, the illness itself may affect yeast infection and thus act as a confounding factor in the study of any causal relationship between antibiotic therapy and *Candida* vaginitis.

There is a frequently reiterated idea that hormonal contraceptives predispose women to vulvovaginal *Candida* infection. However, the majority of cross-sectional surveys provide no statistical evidence for an effect of hormonal contraceptives on the prevalence of *Candida* in the vagina (Odds, 1988). It seems that, by inducing a pregnancy-like state, the oestrogen component of hormonal contraceptives has a greater effect on *Candida* carriage than does the progesterone component. The different pharmaceutical make up of hormonal contraceptives and unreliable diagnosis in various surveys may account for the confusion as to whether hormonal contraceptives act as a predisposing factor to candidosis (Odds, 1988).

It has been suggested that dietary factors may predispose women to candidosis. Horowitz *et al.*(1984) detected elevated quantities of glucose, arabinose and ribose in urine samples from women with recurrent *Candida* vaginitis. They claimed to reduce the prevalence of candidosis among patients who reduced their intake of dietary sugar, milk products and artificial sweeteners.

The notion that the occlusive effects of nylon underwear and tights may contribute to the pathogenesis of vulvovaginal candidosis has been advanced. Elegbe & Botu (1982) and Elegbe & Elegbe (1983) provided experimental proof of this when they showed a higher prevalence and concentration of vaginal yeasts in Nigerian women who

wore tight-fitting clothes and occulsive underwear as compared with women who wore traditional loose fitting garments.

More recently a new predisposing factor has emerged. Human immunodeficiency virus (HIV) leads to a quantitative T-helper cell defect. In HIV infected women, chronic unexplained vaginal candidosis is likely to be the presenting symptom of the same quantitative T-helper cell defect (Rhoads *et al.*, 1987).

It is often found that a combination of predisposing factors can be important in candidal infection (Segal *et al.*, 1984).

4.4 Treatment of *Candida* vaginitis

From the turn of the century to the 1930s astringents and germicidal douches such as potassium permanganate solution, lactic and carbolic acid, and bichloride of mercury in various strengths were used for treating candidosis. If the patient could withstand the treatment, there was a reasonable likelihood of eradicating the condition (Brown, 1986).

The use of gentian violet began in 1935 and it remained the most popular and effective agent until 1955. In 1955 nystatin vaginal tablets came into use, and this agent dominated the treatment of vulvovaginal candidosis for the next 20 years. Nystatin is a member of the polyene group of antifungals, along with amphotericin B, and its inhibitory activity is due to cell-membrane damage and altered permeability.

The second generation of antifungal agents consists of imidazole derivatives. In 1971, clotrimazole and miconazole came into use. They were originally developed for dermatologic purposes and only secondarily applied to vulvovaginal candidosis (Henzl, 1986). Topical imidazoles cause alterations in the sterol

composition of *Candida* membranes by their action on cytochrome P-450; additionally at high concentrations most of them can exert fungicidal effects by directly damaging the membrane of *Candida* cells (Odds, 1988).

Further imidazole development gave ketoconazole with high oral activity, and butoconazole, with high local activity against *Candida* specifically developed for gynaecological purposes. These compounds do not have the direct membrane-damaging capabilities of the original imidazoles. The new derivatives have produced improved treatment results. Butoconazole gave lower relapse rates, with a 75% cure at the second follow-up examination after candidosis, whereas with miconazole therapy, 47% of the cultures were positive for *Candida* at the second evaluation (Henzl, 1986). Relapse after therapy for vaginal candidosis will be discussed further in Section 4.5.

A third generation of azoles is now in development; the triazole-derivative group includes the compounds fluconazole and itraconazole, which are orally active but do not have the associated risk of hepatoxicity seen with ketoconazole.

Antifungal preparations are available in a wide range of formulations. Tooley (1985) examined patient preference; 28% preferred topical creams, 14% hard pessaries, 10% soft pessaries and 47% oral formulations. The antifungal formulation does not have any influence on mycological cure rates, but duration of therapy is important. Poor patient compliance with long courses of vaginal antifungals has stimulated a trend towards shorter treatment courses with higher doses of antifungal.

Despite the numerous effective therapeutic agents currently available for the treatment of superficial candidosis, a search for

even better pharmacological agents is in progress. There are also, still in use, some unorthodox treatments such as garlic poultices (which contain the antimicrobial compound allicin) and yoghurt douches (which contain lactobacilli). Yoghurt therapy and the use of preparations containing lactobacilli will be discussed in more detail in Sections 6.2 and 6.3.

4.5 Recurrent vulvovaginal candidosis

Most women will suffer from vaginal candidosis at some time in their lives; however, a small sub-population will suffer repeated bouts of infection, sometimes presenting attacks almost every month. Women who have at least three or four clinically and mycologically proven episodes of vaginal candidosis within 12 months are classed as suffering from recurrent thrush.

It has been reported that the distribution of *C. albicans* biotypes in recurrent and non-recurrent cases of vaginitis is similar (O'Connor & Sobel, 1986), and that there is no difference in receptivity to *Candida* adherence between vaginal epithelial cells from patients who suffer from recurrent candidosis and those from healthy controls (Trumbore & Sobel, 1986).

The commonest theory for the cause of recurrent thrush is that a persistent intestinal reservoir of *Candida* results in vaginal reinfection. Miles *et al.* (1977) found virtually 100% of women with recurrent thrush had simultaneous positive rectal and vaginal cultures. However, Milne & Warnock (1979) were unable to correlate symptomatic recurrences with positive rectal cultures. When vaginal treatment of candidosis was supplemented with oral antifungals to remove any reservoir of infection from the intestine no improvement in cure rate was observed (Henzl, 1986).

The role of sexual transmission in cases of recurrent vaginal candidosis has been investigated. Studies have identified asymptomatic penile carriage in 5-25% male partners of women with candidosis (Sobel, 1985a). Recent work has expanded the concept of sexual transmission, as ejaculate specimens have been found positive for candidal organisms, suggesting an untreated reservoir of infection exists in seminal vesicles (Horowitz *et al.*, 1987). Henzl (1986) suggested that the treatment of the asymptomatic sexual partner to eradicate the possibility of sexual transmission gave no evidence that doing so improves the cure rate for women. However, Spinillo *et al.* (1992) suggested the identification and treatment of the male sexual partner's *Candida* colonization was important in the prevention of recurrent vulvovaginitis. The overall contribution of sexual transmission to vaginal yeast colonization is not fully elucidated.

Sobel (1985a) has suggested that recurrence of symptomatic vaginal infection may result from failure of conventional therapy to completely eradicate *Candida* from the vaginal lumen. The organism may remain deep in the stratum corneum, out of contact with antifungals. It was demonstrated that most recurrences within 3 months of antifungal treatment involved the same *C. albicans* biotype (O'Conner & Sobel 1986).

It has been suggested that immune status is an important factor in the susceptability of some women to recurrence. Edman *et al.* (1986) found reductions in plasma zinc concentrations in women with recurrent candidosis which might be related to variations in immune function. It has been suggested that recurrent symptoms arise as a local allergic reaction to *Candida* (Siegel, 1986). Witkin *et al.* (1989) proposed that a vaginal IgE-mediated immediate

hypersensitivity response to *C. albicans* may be a contributing factor to recurrent vaginitis. They found a significantly increased prevalence of eosinophils (which are known to exacerbate tissue damage) in symptomatic women with vaginal anti-*Candida* IgE. This adds further support for the role of an allergic response involvement in recurrent vaginitis. Defects in cell-mediated immune responses have been reported for patients with recurrent vaginal candidosis. Witkin *et al.* (1983) observed proliferation of peripheral blood lymphocytes was clearly inhibited in the presence of *C. albicans* extract. The authors concluded that women with recurrent *C. albicans* vaginitis appear to produce *Candida*-specific suppressor lymphocytes, which block the cellular immune response to this organism. Other host defects have been related to recurrent vaginitis, including decreased *Candida* killing by peripheral neutrophils (Schmid & Brune, 1974) and deficiency in the IgA secretory component in vaginal samples (Romero-Piffiguer *et al.*, 1985).

Successful management of recurrent thrush seems to require protracted antifungal therapy, with the possibility of monthly antifungal prophylaxis (Sobel, 1985b).

5. Microbial adhesion

5.1 Principles of initial adhesion

Adhesion to host surfaces is essential for both pathogenic and commensal microorganisms. Selective adhesion has been shown to be a determinant in the distribution of microbes constituting the indigenous flora of humans (Beachey, 1981). Adhesion is also widely accepted as the first event in the chain of infection (Shibl, 1985).

Microorganisms can reach a surface by:

(i) diffusive transport; bacteria exhibit Brownian motion and this may account for random contact with a surface;

(ii) convective transport which is due to liquid flow;

(iii) active movement; a motile bacterium may encounter a surface by chance or be chemotactically attracted (van Loosdrecht *et al.*, 1990).

The surfaces of bacterial and eukaryotic cells have a net negative potential that results from the ionisation of various chemical groups of their surface zones. The apparent paradox that two bodies, both with negative surface potentials, can attract and adhere can be partially explained by the theories of Derguin and Landau, and of Verwey and Overbeek (DVLO theory). This theory describes the change in Gibbs energy as a function of the distance between two bodies. Gibbs energy is obtained from the summation of the Van der Waals and the electrostatic interaction. These forces of attraction and repulsion between approaching surfaces vary with distance of separation in such a manner that at some distances there is attraction and at others repulsion.

At a distance of 1-10 nm, attractive forces allow reversible adhesion. Reversibly adhering microorganisms continue to exhibit Brownian motion and can readily be removed from a surface by mild shear or the organism's own mobility (Marshall *et al.*, 1971). A barrier of repulsion has to be overcome before greater attractive forces, at distance corresponding to molecular interactions, give irreversible adhesion. Irreversibly adhering microorganisms exhibit no Brownian motion and cannot be removed except by a strong shear force.

Extracellular polymers or pili produced by bacteria can overcome the repulsion mechanisms, provide a means for bridging to the surface and, thereby, anchor the bacteria. Different mechanisms of adhesion have been recognised. Where microorganisms are capable of adhering to many different types of surface there is an indication that adhesion of the bacterial bridging polymer to the surface is non-specific. Specific, permanent adhesion can be found in bacteria attaching to specific surfaces and involves interactions between complementary molecular structures on the bacterial surface (adhesins) and the attachment site (receptors) (Marshall, in Microbial Adhesion to Surfaces, 1980, eds. Berkley *et al.*, p187 - 196).

The specific adhesion may involve fimbriae, or pili, which are filamentous appendages that are shorter, straighter and considerably smaller than flagella. Fimbriae, or pili, have been recognised as particularly important in the process of adhesion of Gram-negative bacteria. Fimbriae (also frequently called adhesins) belong to a class of proteins called lectins, which recognise and bind to specific sugar residues in cell surface polysaccharides. The ability of certain organisms eg. *N. gonorrhoeae* and enterotoxigenic *E. coli*, to cause disease is associated with the possession of fimbriae, and mutations resulting in the loss of fimbriae are accompanied by a loss of virulence (In Basic Microbiology, 1988, eds. Volk and Wheeler).

5.2 Adhesion of *C. albicans*

Pathogenic *Candida* species have been seen to adhere to vaginal epithelial cells (Segal *et al.*, 1982), buccal epithelial cells (Calderone *et al.*, 1984; Liljemark & Gibbons, 1973), corneocytes

(Collins-Lech *et al.*, 1984; Ray *et al.*, 1984), vascular endothelium (Klotz *et al.*, 1983), fibrin-platelet matrices (Maisch & Calderone, 1980) and also to plastic surfaces (Klotz *et al.*, 1985).

The different *Candida* species show a hierarchy of relative virulence. These differences in virulence correlate closely with the ability of the organisms to adhere to epithelial cells *in vitro*. *C. albicans* is the most virulent *Candida* species and attaches in greater numbers to exfoliated mucosal cells than other *Candida* species (King *et al.*, 1980). Differences in adhesion between strains of *C. albicans* have also been observed (Segal *et al.*, 1984). Isolates of *C. albicans* from patients with vaginitis were shown to be significantly more adherent that isolates from asymptomatic carriers.

A definite relationship between *in vitro* adhesion and pathogenicity has been obtained by using known inhibitors of adhesion to block infection. Lehrer *et al.* (1983) used solutions of N-acetylglucosamine or a soluble extract of chitin (compounds which had been shown to cause inhibition of yeast adhesion *in vitro*), as vaginal rinses in mice. Mice given vaginal rinses appeared to be more resistant to subsequent vaginal infection with *C. albicans* than were untreated control animals.

5.3 The *Candida* cell wall

The cell wall composition of *C. albicans* has been analysed and found to be made up of glucan (48-60%), mannoprotein (20-23%), protein (3-6%), chitin (0.6-2.7%) and lipid (2%) (Douglas, 1985). The wall contains 5 to 8 distinct layers. The outermost layer is fibrillar, sometimes discontinuous and made up of mannoprotein. The formation of this outer layer is dependent on the growth medium. Growth in the

presence of high concentrations of galactose or sucrose promotes its formation (McCourtie & Douglas, 1981) and simultaneously enhances yeast adhesion (Douglas *et al.*, 1981).

Strains isolated from active infections synthesize the fibrillar layer when grown in medium containing a high concentration of sugar, they also show enhanced adhesion to buccal cells and increased virulence for mice. Strains isolated from asymptomatic carriers have a diminished capacity for cell surface modification when grown in high-sugar medium and exhibit little or no increase in adhesion or virulence (McCourtie & Douglas, 1984).

Yeasts from midexponential phase cultures are poorly adherent. The formation of surface fibrils in response to high sugar concentration appears to take place in the stationary phase of growth and enhances yeast adhesion (Douglas & McCourtie, 1981).

The formation of surface fibrils is not only important in adhesion *in vitro*; a fibrillar-floccular layer has been demonstrated on the surface of *C. albicans* growing *in vivo*. Electron microscopy showed fibrils to mediate yeast attachment to epithelial cells in scrapings taken from the buccal mucosa of patients with oral candidosis (Montes & Wilborn, 1968; Marrie & Costerton, 1981).

5.4 The yeast adhesin

Most of the efforts to chemically characterize the yeast adhesin indicate a role for mannoprotein in the attachment process. For example, yeast adhesion to exfoliated epithelial cells could be inhibited by pretreating *C. albicans* with Concanavalin A, a lectin which binds α-D-mannosyl residues (Sandin *et al.*, 1982). Yeast adhesion could also be blocked by preincubating epithelial cells with a crude mannoprotein preparation obtained from culture

supernatants of *C. albicans* indicating this extracellular material contained the yeast adhesin (McCourtie & Douglas, 1985). The interaction is quite specific as mannoprotein isolated from *C. albicans* GDH 2023 failed to inhibit the adhesion of strain GDH 2346 (Critchley & Douglas 1987b).

Experiments with tunicamycin gave further evidence for mannoprotein as an adhesin. Tunicamycin inhibits protein glycosylation and therefore inhibits the synthesis of yeast mannoprotein. Adhesion of tunicamycin to cultures at the end of exponential growth in a high galactose medium inhibited the formation of the fibrillar surface layer and, as a result, yeasts showed a decreased adhesion to epithelial cells (Douglas & McCourtie, 1983).

Further characterization of the mannoprotein adhesin allowed the identification of the portion of the molecule which participates in the interaction with epithelial cells. It seems that the predominant interaction between a yeast and epithelial cell involves the protein portion of the mannoprotein adhesin. Evidence for this has come from experiments in which pretreatment of crude adhesin with heat, dithiothreitol or proteolytic enzymes (with the exception of papain) partially or completely abolished the adhesin's ability to block yeast attachment to epithelial cells. Pretreatment with sodium periodate or α-mannosidase had little or no effect on inhibition. Moreover, the protein-rich fraction obtained by incubating adhesin with endoglycosidase H inhibited yeast adhesion to a greater extent than the carbohydrate fraction (Critchley & Douglas, 1987a).

The finding that the predominant interaction between a yeast and an epithelial cell involves the protein portion of a

mannoprotein adhesin would explain why yeast adhesion in not always inhibited by mannose, methyl α-D-mannoside or mannoprotein when the protein component is absent or denatured (Sobel *et al.*, 1981; Segal *et al.*, 1982; Lee & King, 1983).

Some evidence has been presented for non-mannoprotein adhesins. Segal *et al.* (1982) reported that chitin and a chitin hydrolysate could inhibit yeast adhesion. Adhesion was also inhibited by glucosamine, N-acetylglucosamine and mannosamine. This group went on to show that N-acetylglucosamine or an aqueous chitin extract could inhibit adhesion *in vivo* and block *Candida* infection in mice (Lehrer *et al.*,1983). This work suggested a possible role for chitin in yeast adhesion. Ghannoum *et al.* (1986) found that lipids extracted from *C. albicans* and *C. tropicalis* but not from the weakly adherent *C. pseudotropicalis* significantly blocked *in vitro* adherence of the respective yeast cells to buccal epithelial cells. These authors suggested phospholipids, sterols and steryl esters are involved in adherence, but in association with other unknown factors. Further clarification of both these alternative adhesion mechanisms is still required.

5.5 Epithelial cell receptors for *Candida*

Sugar inhibition tests are widely employed to characterize epithelial receptors. On the basis of such tests it has been suggested that glycosides containing L-Fucose, N-acetyl-D-glucosamine or D-mannose may all function as epithelial receptors for the yeast. Different strains of *C. albicans* bind to different glycoside receptors (Critchley & Douglas, 1987b). It appears that an L-Fucose-containing receptor may be most commonly required for *C. albicans* (Douglas, 1985). L-Fucose is only able to cause partial

inhibition of adhesion to epithelial cells for sensitive strains. This suggests that the natural mucosal receptor is larger than an L-Fucose residue and/or that a specific stereochemical configuration is required. Crude mannoprotein adhesins have been shown to contain different lectin-like proteins capable of binding all the suggested glycoside receptors. The proportion of each of the different proteins varied for different strains of *C. albicans* (Critchley & Douglas, 1987b). Thus, the relative abundance of these proteins and their accessibility on the yeast surface may determine the receptor specificity of different strains of *C. albicans*.

Candida adhesion may be affected by fibronectin, a large trypsin-sensitive glycoprotein present on the epithelial cell surface. Both *C. albicans* and *C. tropicalis* have been reported to bind to fibronectin-coated wells of tissue culture plates suggesting that fibronectin could act as a yeast receptor (Skerl *et al.*, 1984). However there is also evidence against such a role for fibronectin (Douglas, 1987) and importance of this host protein in yeast adhesion is still to be fully elucidated. The possible ability of fibronectin and other host protiens to mediate the adherence of *C. albicans* to vascular structures has been recently reviewed (Klotz, 1992).

5.6 Adhesion of *Lactobacillus* species

Man and animals harbour many species of *Lactobacillus* living as commensals. They are usually found associated with the epithelial cell surface. Fuller & Turvey (1971) showed lactobacilli were attached *in situ* in the chicken crop. *Lactobacillus* attachment to the pig stomach wall has also been observed (Dubos *et al.*, 1965). Lactobacilli have also been found associated with the squamous

epithelium of the rat and mouse stomach (Savage, 1972). They are an important component of the normal flora of humans, both in the gastrointestinal tract and the vagina.

There is also considerable specificity involved in *Lactobacillus* adhesion. *Lactobacillus* strains isolated from rats will only adhere to epithelial cells collected from the non secretory epithelium of the rat stomach (Suegara *et al.*, 1975). In a similar manner, lactobacilli isolated from poultry will only adhere *in vitro* to crop epithelial cells (Fuller, 1973). Adhesion in this case is not only specific for the host species but also specific for attachment to crop cells; no adhesion was observed to cells obtained from the small intestine or caecum.

5.7 Mechanism of *Lactobacillus* adhesion

The mechanism of *Lactobacillus* adhesion has been investigated and different authors have proposed alternative mechanisms. Savage (1972) suggested that adhesion of indigenous *Lactobacillus* species to the keratinised squamous epithelial cells of the digestive tract of mice was mediated by extracellular polysaccharide. This proposal was supported by Brooker & Fuller (1975) who demonstrated a capsule composed of acidic carbohydrate on lactobacilli isolated from the chicken crop. Costerton *et al.* (1985) also demonstrated a fuzzy, fibrillar-like surface layer, which they referred to as the bacterial glycocalyx, on selected strains of lactobacilli isolated from pigs. More recently, Hood & Zottola (1987) investigated the presence of polysaccharide material on the cell surface of various strains of *L. acidophilus*. In their study, polysaccharide was found in *L. acidophilus* strain BG2F04 which had previously been shown to adhere strongly to human intestinal cells. Strain Lac 12 which

adhered weakly to human intestinal cells had a variable polysaccharide layer, whereas strain NCFM, which was unable to adhere, had no polysaccharide layer. This suggests the polysaccharide layer may be involved in adhesion. Further unpublished work by Fuller (cited in Berkley *et al.*, 1980, p.499) supported this hypothesis by showing that neutralization of the carbohydrate eliminated adhesion.

By contrast with these findings, Wadstrom *et al.* (1987) proposed that carbohydrate may not be involved in the adhesion of lactobacilli to the secretory epithelium of porcine ileum and suggested that a different or multiple adhesion mechanisms may be involved. They demonstrated that lactobacilli with high surface hydrophobicity adhered in high numbers to isolated epithelial cells. Heat and pronase treatment produced a drastic reduction in this property, suggesting that hydophobic proteins in the cell wall are important in adhesion. Wadstrom *et al.* (1987) found that hydrophilic strains could also adhere to epithelial cells, but not as well as hydrophobic strains, indicating the possible existence of several adhesion mechanisms. Savage (1992) also found lactobacilli could adhere by either hydrophilic or hydrophobic molecules. Suegara *et al.* (1975) showed no clear correlation between extracellular polysaccharide and adhesion of lactobacilli to the keratinised epithelial cells of the rat stomach *in vitro*, and proposed that hydrogen bonding might mediate adhesion via bacterial surface components. More recently, Conway & Adams (1989) demonstrated that addition of the red food colour erothrosine to the growth medium of *L. fermentum* strain 737 inhibited the production of the bacterial component responsible for adhesion, without affecting the production of extracellular polysaccharide. Further work by Conway & Kjelieberg

(1989) suggested that adhesion of *L. fermentum* strain 737 may be mediated by a protein. This conclusion is supported by reports that adhesion to epithelia can be inhibited following exposure of lactobacilli to proteolytic enzymes (Barrow *et al.*, 1980) or by treatment with heat or SDS (Suegara *et al.*, 1975; Barrow *et al.*, 1980). Henriksson *et al.* (1991) found protease treatment dramatically decreased the adhesion of *L. fermentum* 104-R and the variant strain 104-S to porcine gastric squamous epithelium, thus suggesting that the determinants responsible for adhesion were proteinaceous. However, a suggestion for the existance of several adhesion mechanisms was supported by the work of Henriksson *et al.* (1991) who found that, in addition to proteinaceous adhesins, carbohydrate may also be involved in the adhesion of *L. fermentum* strain 104-R.

Regular protein arrays have been observed in the cell walls of *L. bulgaricus, L. helveticus, L. acidophilus, L. fermentum, L. brevis* and *L. buchneri* (Masuda & Kawata, 1983)

Lipoteichoic acids (LTA) have also been implicated in adhesion by some authors. Sherman & Savage (1986) demonstrated that the adherent lactobacilli included in their survey all had LTA present in their cell walls. Wadstrom *et al.* (1987) observed that greatest adherence was obtained with highly hydrophobic bacteria. They showed that these bacteria contained LTA in their cell walls and suggested that LTA-protein complexes, which would confer a high degree of hydrophobicity, might be involved in adhesion.

6. Microbial interactions

6.1 Interactions between *C. albicans* and other organisms

The effect of bacteria on *Candida* growth and adhesion has been investigated by a number of authors (Isenberg *et al.*, 1960; Liljemark & Gibbons,1973: Paine, 1958). Several investigators have suggested that the resident bacterial flora of e.g. the gut, mouth, vagina suppresses fungal colonization, whereas alteration and reduction in surface bacteria, such as occurs with antibiotic therapy, are associated with fungal colonization and often symptomatic disease (Kennedy & Volz,1985).

Paine (1958) demonstrated the inhibition of *Candida* growth by bacteria representative of the usual human flora. He did not specify the origin of the species he tested, however, they were typical of organisms which may be isolated from the mouth, gut, vagina or skin. He suggested a number of reasons for yeast inhibition by bacteria: changes in the hydrogen ion concentration or the oxidation-reduction potential, competition for growth factors, or the possible production of anticandidal substances. In 1960 Isenberg *et al.* examined the inter-relationship between *C. albicans* and the normal intestinal bacteria. Their results suggest that the bacterial residents of the gastrointestinal tract may be categorized as inhibitory, stimulatory or indifferent with respect to yeast growth.

Kennedy & Volz (1985) showed the importance of bacteria in the gut ecology. They found that antibiotic treatment decreased the total population of indigenous bacterial flora within the gut of hamsters. Intragastric challenge with *Candida* led to opportunistic colonization of the gut, gastrointestinal overgrowth and subsequent systemic dissemination of *C. albicans*. The feeding of *C. albicans* to animals, not given antibiotics, did not lead to *Candida*

dissemination from the gastrointestinal tract. This suggests that members of the indigenous flora suppress growth of *C. albicans* within the gut, thereby preventing systemic invasion.

Liljemark & Gibbons (1973) also provided evidence for the protective effect of the bacterial flora against candidosis. They found that mixed salivary bacteria - strains of *Streptococcus salivarius* and *S. mitior* - suppressed colonization by *C. albicans* in gnotobiotic mice. They showed that twice as many cells of *C. albicans* became attached to tongue and cheek epithelial cells from germ free rats as compared with those from conventional animals. It appears that the presence of an indigenous bacterial flora can interfere with the adherence of *C. albicans* to the epithelial surface. This could be achieved by competing for, or otherwise modifying, epithelial receptor sites required for *Candida* attachment, or by enzymatically altering the surface of the yeast cells.

Makrides & MacFarlane (1982) carried out an *in vitro* study of the effect of commensal bacteria on the adherence of *C. albicans* to epithelial cells. They found that cell suspensions and supernatants from cell suspensions of *E. coli* and *Klebsiella aerogenes* significantly enhanced candidal adherence to HeLa cells, while those of *S. aureus* , *S. mitior* and *S. sanguis* significantly reduced candidal adherence. Cell suspensions of *S. mutans*, *L. casei* and *S. salivarius* significantly reduced candidal adherence while their supernatants had no significant effect. These results suggest that in addition to intact bacterial cells, surface or other components released from the bacteria could be involved in the stimulation or inhibition of adherence. In the case of *E. coli*, increased adherence could be due to the presence of fimbriae on the bacterial cells

acting as a bridging mechanism between *C. albicans* and the epithelial cells. Centeno *et al.* (1983) confirmed that the number of *C. albicans* attaching to epithelial cells was enhanced by preincubation of epithelial cells with piliated strains of *E. coli* and also *K. pneumoniae;* preincubation with non-piliated strains of bacteria had no effect on yeast attachment. They also hypothesized that the increase in yeast attachment was due to the attachment of yeasts to pili on bacteria already attached to epithelial cells.

Purohit *et al.* (1977) studied the incubation of *C. albicans,* in human serum, in the presence of various concentrations of *S. pyogenes, E. coli, K. pneumoniae, Proteus vulgaris* and *L. acidophilus.* The percentage of germ-tube formation diminished proportionally with increasing concentrations of bacteria. Purohit *et al.* (1977) suggested that inhibition of blastospore-germ-tube transformation by commensal bacteria may be responsible for prevention of clinical infection by *Candida.*

Candida species are frequently co-pathogens with bacteria in cases of systemic candidosis. In the mouse model, *C. albicans* has been shown to interact synergistically with *S. aureus.* Dual infection with the two organisms, at doses which separately caused no animal deaths, resulted in 100% mortality (Carlson, 1982, 1983b). *C. albicans* has also been shown to interact synergistically, in animal models, with murine cytomegalovirus (Hamilton *et al.,* 1976), *Serratia marcescens* (Carlson, 1983a) *S. faecalis* (Carlson, 1983a) and *E. coli* (Burd *et al.,* 1992).

Viral infections are seen to increase the host's susceptibility to *Candida* infection. Infection with the human immunodeficiency virus (HIV) predisposes sufferers to *Candida*

infection. Vaginal candidosis is common among women with AIDS (Rhoads *et al.*, 1987).

Increased susceptibility to *Candida* infection is seen in animals infected with Friend Leukemia Virus (FLV) which induces profound immunosuppression in susceptible mice. FLV gives a reduction in the number of neutrophils, and the neutrophils present are deficient in their ability to kill *C. albicans* (Moors *et al.*, 1990).

6.2 Interactions between *Lactobacillus* species and other organisms

Lactobacilli have been found to inhibit the growth of other vaginal microorganisms including *E. coli* (Tramer, 1966), *C.albicans* (Collins & Hardt, 1980), *N. gonorrhoeae* (Saigh *et al.*, 1978), *G. vaginalis* and *Mobiluncus* species (Skarin & Sylwan, 1986). Inhibition was thought to be due to the lactobacilli decreasing the vaginal pH or producing H_2O_2 and other inhibitory metabolites.

The end products of *Lactobacillus* metabolism, such as H_2O_2, lactic acid and acetic acid, are well known for their antimicrobial activities. Wheater *et al.*(1952) demonstrated that H_2O_2-producing lactobacilli inhibited the growth of other organisms. Eschenbach *et al.* (1989) extended these studies to show that women who harboured H_2O_2-producing lactobacilli were less likely to suffer from bacterial vaginitis. Further work by this group suggested H_2O_2-producing lactobacilli may contribute to the control of the vaginal flora, particularly in the presence of peroxidase and a halide (Klebanoff et al., 1991; Klebanoff & Coombs, 1991). A study by Tramer (1966) showed conclusively that *L. acidophilus* inhibition of *E. coli* was due to the strong germicidal action of lactic acid at low pH.

Antimicrobial activities associated with lactobacilli, but unrelated to the normal end products of metabolism, have also been reported. The production of bacteriocins and bacteriocin-like substances by a number of *Lactobacillus* species has been detected *in vitro* (Table 2). In general, the *Lactobacillus* bacteriocins which have been characterized are proteinaceous, exhibit a bactericidal mode of action and usually display a narrow range of inhibitory activity, affecting only closely related species within the *Lactobacilliaceae* (Klaenhammer, 1988). It is bacteriocins which are often cited as important effectors of the suspected probiotic effects of lactobacilli in the intestinal tract of man and animals.

A number of *Lactobacillus* species have also been reported to produce other antimicrobial substances (Table 3). These substances are distinct from the bacteriocins in that they are of low molecular weight (< 1000). They have a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria and also some fungi. The compounds are heat stable, being able to withstand a variety of rigorous heat treatments. These substances are acidic in nature and, with the exception of fungal inhibitory compound (FIC), not particularly stable above pH 5/6.

Lactobacilli have been seen not only to inhibit the growth of other organisms, but also to inhibit adhesion. They attach readily to epithelial cells. When attached to uroepithelial cells they have been seen to block the adhesion of mannose-sensitive, mannose-resistant and encapsulated *E. coli* strains, strains of *K. pneumoniae* and mucoid and non mucoid *P. aeruginosa* (Chan *et al.*, 1984). Lactobacilli have also been found to interact with *E. coli* by coaggregation. This finding may have a bearing on the importance of

Table 2.

High molecular weight antimicrobial substances produced by lactic acid bacteria

SPECIES	COMPOUND	REFERENCE
L. acidophilus	Lactocidin	Vincent *et al.* (1959)
	Lactacin B	Barefoot & Klaenhammer (1983)
	Lactacin F	Muriana & Klaenhammer (1987)
		Muriana & Klaenhammer (1991)
	100 - 37	McCormick & Savage (1983)
L. fermenti	not named	DeKlerk & Smit(1967)
L. helveticus	Lactocin 27	Upreti & Hinsdill (1975)
	Helveticin J	Joerger & Klaenhammer (1986)
	Helveticin V-1829	Vaughan *et al.* (1992)
L. plantarum	Lactolin	Kodama (1952)
	L. plantarum 83	Andersson (1986)
	Plantaricin A	Daeschel *et al.* (1986)
	Plantacin B	West & Warner (1988)
L. sake	Sakacin A	Schillinger & Lucke (1989)
	Lactocin S	Mortvedt *et al.* (1991)
	Sakacin M	Sobrino *et al.* (1992)

Table 3.

Low molecular weight antimicrobial substances produced by lactic acid bacteria

SPECIES	COMPOUND	REFERENCE
L. acidophilus	Acidolin Acidophilin	Hamdan & Mikolajcik (1974) Shahani *et al.* (1972)
L. casei var. *rhamnosus*	Lactobacillus 99 F I C	Silva *et al.* (1987) Vandenbergh & King (1989)
L. reuteri	Reuterin	Talarico *et al.* (1988) Talarico & Dobrogosz (1989)

lactobacilli *in vivo* particularly if inhibitor production accompanies coaggregation (Reid *et al.*, 1988).

Lactobacilli have long been thought to have a beneficial effect on health. Metchnikoff early in the 19th century proposed that harmful putrefying bacteria residing in the intestinal tract could be suppressed by acid producing lactobacilli (Klaenhammer, 1982). Since this time *Lactobacillus* products have been used in man and animals. Lactobacilli have been administered directly or in lyophilized form as a powder, capsules or tablets; yoghurt and acidophilus milk have also been used in treatments.

Survival of mice after intraperitoneal infection with *P. aeruginosa* was augmented in mice that had been pretreated intraperitonealy with heat-killed *L. casei* 5 days previously. This treatment also inhibited the growth of *P. aeruginosa* and *Listeria monocytogenes* within the peritoneal cavity (Maike *et al.*, 1985).

Lactobacilli were seen to be of benefit in treatment of the monkey vagina persistently colonized with *E. coli*. However, vaginal fluid from a healthy vagina was found to be a more effective treatment than the *Lactobacillus* suspension (Herthelius *et al.*, 1989).

Kvasnikov *et al.*, (1983) suggested *L. salivarius* may be used to form a normal microflora in the chicken intestinal tract where it could exert an antagonistic effect on colibacteriosis agents and thus prevent disease. When Carlste dt-Duke *et al.*(1987), using rats, investigated whether lactobacilli could reduce antibiotic related ecological disturbance of the normal intestinal microflora, they found that they had no effect. Jonsson (1986) found lactobacilli fed to pigs survived passage through the digestive tract, but did not become established within the gut. No benefits to health or

performance were seen in either pigs (Jonsonn, 1986) or calves (Jonsonn & Olsson, 1985) treated with lactobacilli.

Nader de Macias *et al.* (1992) found milk fermented by *L. casei* and *L. acidophilus* had a protective effect against subsequent gastrointestinal infection of mice with *Shigella sonnei*. Animal experiments have been carried out to investigate aspects of yoghurt therapy. De Simone *et al.*(1988) found mice given a diet supplemented with yoghurt had increased defence mechanisms and reduced mortality to *S. typhimurium*. However, in another study, feeding yoghurt to rats was associated with increased counts of coliforms and reduced lactobacilli (Garvie *et al.*, 1984).

6.3 *Lactobacillus* preparations used in treatment of disease

Lactobacilli and *Lactobacillus* products have been administered to man in an attempt to control various gastrointestinal infections. Variable results in the success of therapy have been obtained. Ambartsumian *et al.* (1989) found the cultured milk mixture "Narine" gave a positive effect on inflammation of the colon. Rafskey & Rafskey (1955) studied the effect of "viacil" tablets containing viable *L. acidophilus* on cases of irritable bowel and diverticulosis. They found clinical improvement in 32 of 39 cases within 1-3 weeks of treatment. This clinical improvement was not necessarily associated with evidence of *Lactobacillus* implantation. Studies carried out in more recent years have looked more closely for increases in faecal *Lactobacillus* numbers associated with therapy. A spray-dried *L. acidophilus* product fed to humans has been seen to result in an increase in lactobacilli and a suppression of coliforms (Prajapti & Dave, 1984). Lidbeck *et al.* (1987) obtained similar results using a fermented milk product; an increase in

lactobacilli was seen in 9 out of 10 subjects and a reduction in *E. coli* in 6 out of 10 subjects. They noted that there was a need to take the fermented milk preparation continuously to maintain high levels of lactobacilli.

Isolauri *et al.* (1991) found that *L. casei* strain GG, in the form of fermented milk or freeze-dried powder, was effective in shortening the course of acute diarrhoea. Luckey *et al.* (1983) saw conflicting results as to whether lactobacilli were beneficial in the treatment of diarrhoea. "Lactinex", a commercial preparation of *L. acidophilus* and *L. bulgaricus,* did not prevent or alter the course of enterotoxigenic *E. coli* diarrhoea. However, Lactobacillus therapy with one batch of "Lactinex" reduced the volume and duration of neomycin-associated diarrhoea, whereas another batch of "Lactinex" had no therapeutic effect suggesting batch to batch variation in *Lactobacillus* preparations. In another study, Steffan *et al.* (1986) saw no effect against travellers diarrhoea with lactobacilli.

Lactobacillus preparations have also been used in the treatment of vaginitis. It has been noted that when vaginitis occurs, lactobacilli are often absent (Fredricsson *et al.*, 1992). Attempts have been made, over a number of years, to treat vaginitis by restoring a population of vaginal lactobacilli. Mohler & Brown (1933) noted that 6 out of 21 patients with symptomatic vaginitis and white discharge were cured of symptoms after treatment with a culture of Doderleins bacillus. In cases of vaginitis where lactobacilli were absent from the vagina Butler & Beakley (1960) also noted a good cure on reimplanting Doderlein bacillus; they also found a lyophilized preparation effective. Hallen *et al.* (1992) treated women suffering from bacterial vaginitis with lyophilized *L.*

acidophilus. In a double blind, placebo-controlled treatment trial 16 out of the 28 women who were treated with lactobacilli had normal vaginal wet smear results immediately after completion of treatment in comparison to none of the 29 women treated with placebo. Fredricsson *et al.* (1989) found only 1 case in 14 treated with a fermented milk product was cured of bacterial vaginitis.

Intravaginal installation of lactobacilli has been used for the prevention of recurrent urinary tract infections. In a limited study, 5 females suffering from recurrent urinary tract infections were treated twice weekly with *L. casei*. Each of the 5 patients had infection-free periods ranging from 4 weeks to 6 months (Bruce & Reid, 1988). A recent study by Reid *et al.* (1992) showed treatment with *Lactobacillus* vaginal suppositories reduced the recurrence of urinary tract infection following antimicrobial therapy.

6.4 Interactions between *Lactobacillus* species and *C. albicans*

As far back as 1956, Young *et al.* demonstrated a marked decrease in the number of *Candida* cells when grown with *L. acidophilus*, whereas the lactobacilli showed increased numbers. These authors found inhibition of *Candida* comparable to that produced by the lactobacilli was obtained following the addition of lactic acid to reduce the pH to levels reached in mixed cultures. They also found that lactobacilli, which were unable to grow in vitamin-deficient culture media, grew well in the same medium as a mixed culture with *C. albicans*. Young *et al.* (1956) suggested a counterbalance existed between the two organisms, with *Candida* providing nutritional stimulation for the lactobacilli and the latter producing lactic acid which prevented excessive *Candida* colonization.

Koser *et al.*(1960) also found that *L. casei*, *L. plantarum* and *L. fermenti* were capable of growing in association with *C. albicans* in a medium originally deficient in vitamins for the lactobacilli. These authors found a reduced growth of *C. albicans* when grown in association with lactobacilli compared with when *Candida* was grown in pure culture.

Guillot (1958) reported that *L. acidophilus* produced a substance that was active against *C. albicans* but he failed to isolate the substance. In 1980 Collins & Hardt found that *C. albicans*, grown at pH 7.7 in nutrient broth containing 5% glucose, was retarded by filtrates of *L. acidophilus* grown in 1.5% Bacto-casitone broth. Again, the active factor was not isolated. More recently, *L. acidophilus* was found to inhibit *C. albicans* when grown on MRS agar plates. Addition of sodium thiocyanate to the agar increased the inhibition by *Lactobacillus*. The authors suggested that H_2O_2 produced by the *Lactobacillus* was being used to convert the thiocyanate to the more toxic hypothiocyanate (Jack *et al.*,1990).

Savage (1969) demonstrated strong microbial interference between indigenous yeasts and lactobacilli in the gastrointestinal tract of mice. He noted that yeasts grew as layers on the secreting epithelium within the stomach of rodents, whereas lactobacilli appeared on the non-secreting epithelium. With penicillin treatment, lactobacilli disappeared, and the yeast from the secreting epithelium colonized the non-secreting epithelium within 24h. When penicillin treatment was discontinued lactobacilli again colonized the non-secreting epithelium within 5 to 8 days. The author suggested that the lactobacilli displaced the yeast from the non-secreting epithelium by interfering with the multiplication of the yeast, or the attachment of the yeast to the keratin layer. This

interference must occur continuously during normal life. In studies of the vaginal flora, Saigh *et al.*(1978) noted reduced counts of lactobacilli during and immediately after menstruation, during which times vaginal candidosis occurs with peak frequency.

There have been reports that *Lactobacillus* interactions with *Candida* could be important in combating *Candida* vaginitis. Pinotti *et al.*(1981) reported 100% cure of leukorrheas, due to *Candida,* after oral and topical treatment with a live culture of *L. acidophilus*. Observations that lactobacilli are of clinical value have been noted by general practitioners (Will, 1979; Sandler, 1979) but such observations have not been backed up with microbiological evidence. The possible importance of *Lactobacillus* therapy in the treatment of vaginitis is still unknown. *Lactobacillus* products commercially available are often of dairy origin and may contain strains unsuitable for human colonization. Another problem in assessing the use of these products is that most of the *Lactobacillus*-containing products currently available either do not contain the *Lactobacillus* species advertised and/or contain other bacteria of questionable benefit (Hughes & Hillier, 1990).

Objects of Research

Candida vaginitis plagues millions of women worldwide, and often proves refractory to treatment. The effect of the normal vaginal flora on *Candida* adhesion and colonization has received little attention although it is widely assumed that a flourising population of lactobacilli is important in combating infection.

The primary objective of this study was to determine the effect of lactobacilli on *Candida* adhesion to vaginal epithelial cells. Such investigation would include *in vitro* assays of *Candida* adhesion using exfoliated vaginal epithelial cells (both human and animal) pretreated with bacterial suspesions. A variety of bacterial and yeast strains were to be screened in these experiments.

The second objective of this study was to investigate the effect of lactobacilli on *Candida* growth *in vitro*. Such an investigation would be carried out by growth experiments and plate bioassay methods.

Finally, having investigated the interactions between lactobacilli and *Candida in vitro*, it was important to determine the effect of lactobacilli on the pathogenesis of vaginitis in experimental animals. Such an investigation would include assessing the ability of a vaginal inoculation of lactobacilli to inhibit *Candida* infection.

MATERIALS AND METHODS

1. Origin and maintenance of organisms

The strain numbers and, where known, the origin of microorganisms used in this study are presented in Tables 4, 5 and 6. *Bacillus subtilis, Bacillus megaterium, Escherichia coli, Proteus vulgaris, Staphylococcus aureus,* all *Lactobacillus* species, plus the *C. albicans* strains were stored as freeze-dried cultures. Fresh ampoules were opened every 2 months. Bacterial strains (with the exception of *Lactobacillus* species) were reconstituted in nutrient broth, plated out on nutrient agar and incubated aerobically for 24h at 37°C. A single bacterial colony was used to inoculate a nutrient agar slope; after growth for 48h at 37°C, this acted as a stock culture. *Lactobacillus* species were reconstituted in MRS broth, and plated out on MRS agar. Plates of lactobacilli were incubated microaerophilically, in a candle jar, for 48h at 37°C.

Freeze-dried *C. albicans* strains were reconstituted in Sabouraud dextrose broth. They were then plated out on Sabouraud dextrose agar and incubated aerobically for 24h at 37°C. A single colony was used to inoculate a Sabouraud dextrose agar slope and the slope was incubated for 48h at 37°C to give a stock culture.

Aspergillus niger, Cryptococcus neoformans and *Trichophyton mentagrophytes,* from overnight cultures in nutrient broth, were stored as 10% glycerol stocks in liquid N_2.

Table 4.

Origin of *Candida albicans* strains

STRAIN	SOURCE OF STRAIN	TYPE OF INFECTION	SITE OF INFECTION/ ISOLATION
GDH 2346	Glasgow Dental Hospital	Denture stomatitis	oral cavity
GDH 2023	Glasgow Dental Hospital	Denture stomatitis	oral cavity
NCPF 3153	National Collection of Pathogenic Fungi	unknown	unknown
GRI 681	Glasgow Royal Infirmary	none[a]	cervix
GRI 682	Glasgow Royal Infirmary	none[a]	cervix
GRI 272	Glasgow Royal Infirmary	Vaginitis	vagina
'outbreak' strain (Serotype A)	London Hospital Medical College	Systemic candidosis	oral cavity
B2630	Janssen	not known	not known
B/079	SmithKline Beecham	not known	not known

[a] These strains were isolated from routine cervical smears taken from symptomless women.

Table 5.

Origin of *Lactobacillus* species

SPECIES	STRAIN NO.	SOURCE OF STRAIN	SITE OF ISOLATION
L. acidophilus	NCTC 4504	National Collection of Type Cultures	Vagina
L. fermentum	NCIB 2797	National Collection of Industrial Bacteria	not known
L. jenseni	LMG 6414	LMG Culture Collection	Human vaginal dicharge
L. casei ssp. *rhamnosus*	LMG 8153	LMG Culture Collection	Urethra
L. casei	Unknown	National Collection of Type Cultures	unknown
L. acidophilus	629706A	Glasgow Maternity Hospital	Vagina
L. crispatus	632697M	Glasgow Maternity Hospital	Vagina
L. acidophilus	631575L	Glasgow Maternity Hospital	Vagina
L. acidophilus	630360E	Glasgow Maternity Hospital	Vagina
L. crispatus	629425X	Glasgow Maternity Hospital	Vagina

Table 6.

Bacterial and fungal strains used in plate inhibition assays

SPECIES	STRAIN	SOURCE OF STRAIN
Bacillus subtilis	NCTC 3610	National Collection of Type Cultures
Bacillus megaterium	NCTC 10342	National Collection of Type Cultures
Escherichia coli	Lily	unknown
Proteus vulgaris	NCTC 4175	National Collection of Type Cultures
Staphylococcus aureus	'Oxford' NCTC 6571	National Collection of Type Cultures
Aspergillus niger	-	SmithKline Beecham
Candida albicans	B079	SmithKline Beecham
Cryptococcus neoformans	-	SmithKline Beecham
Trichophyton mentagrophytes	-	SmithKline Beecham

2. Growth conditions

2.1 *Candida* growth conditions

In all experiments, organisms were exclusively in the budding yeast phase.

2.1.1 For freeze-drying

For freeze-drying, yeasts were grown in yeast nitrogen base (Difco) containing 500mM sucrose. Batches of medium (50ml, in 250ml Erlenmeyer flasks) were inoculated with overnight yeast cultures (5ml) and incubated at 37°C on an oribital shaker operating at 150 rev/min.

After 24h, cells were harvested (MSE bench centrifuge: 5 min, 1200 x g) and washed in sterile 0.15M phosphate-buffered saline (pH 7.2; PBS). Yeast cells were resuspended in a small volume of 2% (w/v) skimmed milk and a drop of suspension was added to a sterile glass ampoule. Ampoules were dried in a centrifugal freeze-drier (Edwards High Vacuum Ltd., Sussex).

2.1.2 For adherence assays

Batches of medium (50ml, in 250ml Erlenmeyer flasks) containing yeast nitrogen base (YNB) and 50mM glucose or 500mM galactose were inoculated from stock cultures and incubated overnight at 37°C on an orbital shaker operating at 150 rev/min. The overnight culture (5ml) was used to inoculate YNB medium (50ml) containing the same carbon source as before. This culture was incubated at 37°C on an oribital shaker at 150 rev/min for 24h so that yeast cells reached the stationary phase of growth. Cells were harvested by centrifugation (MSE bench centrifuge: 5 min, 1200 x g) and washed twice in 0.15M PBS (pH 7.2)

2.1.3 For infection in the rat vaginitis model

A Sabouraud dextrose agar (Difco) slope was inoculated, using a sterile loop, from a stock culture and incubated at 37°C for 48h. After *Candida* growth, sterile PBS (10ml) was added to the agar slope and a sterile loop used to scrape the yeasts from the surface and suspend them in the PBS. The PBS was removed from the slope and the cells were harvested by centrifugation (MSE bench centrifuge: 5 min, 1200 x g), washed twice in 0.15M PBS (pH 7.2), and diluted in gum arabic.

2.1.4 Growth of yeasts in hormone-containing media

β-oestradiol and progesterone, obtained from Sigma, were dissolved in methanol and diluted in PBS to give 1mM solutions (1% methanol, v/v). The solutions were filter sterilized (Acrodisc; 0.22μm) and 50μl of hormone solution added to batches of medium (50ml, in 250ml Erlenmeyer flasks) containing yeast nitrogen base (YNB) and 50mM glucose or 500mM galactose. The batches of medium were inoculated from stock cultures and incubated overnight at 37°C on an orbital shaker at 150 rev/min. The overnight cultures (5ml) were used to inoculate YNB medium (50ml) containing the same hormone and carbon source as before. These cultures were incubated for 24h at 37°C with shaking.

A 1% (v/v) solution of methanol in PBS was filter sterilised (Arodisc; 0.22μm) and 50μl was added to batches of medium (50ml) containing YNB and 50mM glucose or 500mM galactose. Yeasts grown in this medium (final methanol concentration 0.001%) were used as control organisms in adhesion assays testing the effect of yeast growth in the presence of steroid hormones, on adhesion.

2.2 *Lactobacillus* growth conditions

2.2.1 For freeze-drying

For freeze-drying, lactobacilli were grown in De Man Rogosa Sharpe (MRS) broth (Oxoid). Batches of medium (50ml, in 55ml screw-capped glass bottles) were inoculated with 24h *Lactobacillus* cultures (5ml) and incubated at 37°C without shaking. After 24h, cells were harvested (MSE bench centrifuge: 5 min, 1200 x g), washed in PBS and resuspended in a small volume of 2% (w/v) skimmed milk. The *Lactobacillus* suspension was dispensed in glass ampoules and dried in a centrifugal freeze-drier (Edwards High Vacuum Ltd., Sussex).

2.2.2 For *in vitro* and *in vivo* experiments

Batches of MRS broth (50ml, in 55ml screw-capped glass bottles) were inoculated with 24h *Lactobacillus* cultures (5ml) grown in MRS broth and incubated statically for a further 24h, or as specified, at 37°C. Cells were harvested by centrifugation (MSE bench centrifuge: 5min, 1200 x g) and washed twice in 0.15M PBS (pH 7.2).

3. Adherence experiments

3.1 Collection and preparation of epithelial cells

3.1.1 Human vaginal cells

Vaginal epithelial cells were obtained from a healthy female volunteer by gentle swabbing. Swabs were agitated in a universal containing PBS. Epithelial cells were harvested by centrifugation (MSE bench centrifuge; 5 min, 1200 x g) and washed twice in PBS to remove unattached microorganisms. After the second washing the epithelial cells were standardized using an improved Neubauer haemocytometer before suspending in PBS at a concentration of 5 x

10^5 cells/ml. Vaginal cells were always collected at the same time of day, but at different stages of the menstrual cycle.

3.1.2 Mouse vaginal epithelial cells

Twenty, female mice aged 6 weeks (CD-1 strain) were injected subcutaneously with a suspension of β-oestradiol-3-benzoate.

To prepare this suspension, β-oestradiol-3-benzoate (Sigma) was made up to a concentration of 10mg/ml in 0.5% Tween 80 and ball-milled overnight. This concentration was then diluted 1 in 5 with 0.5% Tween 80 giving a final β-oestradiol-3-benzoate concentration of 2mg/ml. Each mouse was injected with 0.25ml (ie 0.5mg β-oestradiol-3-benzoate) to induce pseudoestrous.

Two days after oestradiol treatment, mice were swabbed for vaginal epithelial cells using small sterile cotton wool swabs. The swabs were gently agitated in a universal containing PBS. The pooled vaginal epithelial cells were harvested by centrifugation (MSE bench centrifuge; 5 min, 1200 x g) and resuspended in PBS. Vaginal epithelial cells were washed twice in PBS and standardized using an improved Neubauer haemocytometer so that a final epithelial cell concentration of 5 x 10^5 cells/ml was obtained.

3.1.3 Diabetic mouse epithelial cells

Twenty female mice, aged 6 weeks were injected intravenously with Alloxan (Sigma; 8mg/kg mouse) to produce a chemically induced diabetes mellitus. The diabetic status of the mice was confirmed 2 days later by the detection of glucose in the mouse urine using Clinistick indicators. Diabetic mice were induced into pseudoestrous with a subcutaneous injection of β-oestradiol-3-benzoate (0.5mg in 0.25ml; see Section 3.1.2). Two days after oestradiol treatment mice

were swabbed to obtain vaginal epithelial cells. The swabs were gently agitated in PBS. The pooled epithelial cells were harvested by centrifugation, washed and standardized as described previously.

3.1.4 Rat vaginal epithelial cells

Twelve female, ovarectomised Sprague Dawley rats (200g; Charles River) were injected subcutaneously with 0.25ml β-oestradiol-3-benzoate (2mg/ml;see Section 3.1.2) to induce pseudoestrous. Three days after oestradiol treatment, rats were swabbed for vaginal epithelial cells. The swabs were gently agitated in a universal containing PBS (pH 7.2). Vaginal epithelial cells were harvested (MSE bench centrifuge; 5 min, 1200 x g) and washed twice in PBS. Washed rat vaginal epithelial cells were standardized, using an improved Neubauer haemocytometer, to a final concentration of 5 x 10^5 cells/ml.

3.2 Preparation of yeast cells

After the second washing of the yeasts in PBS, yeast suspensions were standardized using an improved Neubauer haemocytometer. The amount of PBS added was adjusted so a final yeast concentration of 5 x 10^7 cells/ml (unless otherwise stated) was obtained.

3.3 Pretreatment conditions for adherence experiments

3.3.1 Pretreatment of vaginal epithelial cells with steroid hormones

β-oestradiol and progesterone, obtained from Sigma, were prepared as 1μM solutions. The steriod hormones were dissolved in methanol and diluted in PBS such that the final concentration of methanol in the 1μM solution was 0.001%. The control solution was a 0.001% solution of methanol in PBS (v/v).

Standardized suspensions of rat vaginal epithelial cells (0.5ml; 5 x 10^5 cells/ml) were treated with a 1μM solution of either progesterone or β-oestradiol (0.5ml) for 30 min at 37^{0}C with shaking. Control vaginal epithelial cells (0.5ml; 5 x 10^5 cells/ml) were treated with a 0.001% solution of methanol in PBS (0.5ml).

After incubation the vaginal epithelial cells were collected on a filter (12μm; 25mm; Nuclepore, Pleaston, LA) and washed with PBS (25ml), after which they were resuspended to a concentration of 5 x 10^5 cells/ml.

3.3.2 Pretreatment of *Candida* with steroid hormones

C. albicans GDH 2346, grown in either 50mM glucose or 500mM galactose, was washed twice in PBS and then resuspended to a concentration of 5 x 10^7 *Candida*/ml in a 1μm solution of steroid hormone. Control *Candida* were resuspended in 0.001% methanol in PBS (v/v). After incubation for 30 min at 37^{0}C with shaking, the yeasts were harvested by centrifugation (MSE bench centrifuge; 5 min, 1200 x g), washed twice in PBS and resuspended to a concentration of 5 x 10^7 cells/ml.

3.3.3 Pretreatment of epithelial cells with lactobacilli

A 24h culture of *Lactobacillus* was harvested by centrifugation (MSE bench centrifuge; 5 min, 1200 x g) and washed twice in PBS. The optical density (OD_{600}) of the washed suspension of lactobacilli was measured (Shimadzu spectrophotometer) and the amount of PBS adjusted to give an OD_{600} of 5.0 which was equivalent to 5 x 10^8 cells/ml.

The standardized suspension of lactobacilli (0.5ml) was mixed with a standarized suspension of vaginal epithelial cells (5 x 10^5 cells/ml; 0.5ml) in glass bijou bottles. The mixtures were incubated

for 30 min at 37°C with gentle shaking. The reaction was stopped by adding 4ml of sterile PBS.

The epithelial cells were collected on a filter (12μm pore size; 25mm diameter: Nuclepore) and the unattached lactobacilli gently washed away using sterile PBS (10ml). Epithelial cells on the filter were resuspended in 1ml of sterile PBS by agitating the filter, using a whirlimixer, in a glass bijou containing the PBS. The resuspended epithelial cells were standardized using an improved Neubauer haemocytometer and the amount of PBS adjusted to give a concentration of 5 x 10^5 cells/ml.

Control epithelial cells were prepared by mixing vaginal epithelial cells (5 x 10^5 cells/ml; 0.5ml) with PBS (0.5ml) and incubating with gentle shaking for 30 min at 37°C. Epithelial cells were collected and resuspended as described above.

Pretreated and restandardized epithelial cells were used in the adhesion assay.

3.3.4 Pretreatment of epithelial cells with concentrated *Lactobacillus* culture supernatant

Lactobacillus species were grown for 24h at 37°C in MRS broth (200ml; in 200ml screw capped bottle) without shaking. Cells were harvested by centrifugation (Sorvall; 10 min, 10,000 x g) and the culture supernatant retained. The supernatant was filtered (0.45μm pore size) to remove any bacterial debris. Supernatant was divided into 2x 100ml volumes.

The first volume of culture supernatant (100ml) was freeze-dried to give a 20-fold concentration. This gave concentrated culture supernatant.

The remaining culture supernatant (100ml) was extensively dialysed against 8 x 4l distilled water before 20-fold concentration by freeze-drying. After concentration the dialysed culture supernatant was passed down a G25 (Pharmacia Fine Chemicals PD10) prepacked column to give treated concentrated culture supernatant.

The concentrated culture supernatant (0.5ml) or treated concentrated culture supernatant (0.5ml) was mixed with a standardized suspension of vaginal epithelial cells ($5x10^5$ cells/ml; 0.5ml) in glass bijou bottles. The mixtures were incubated for 30 min at 37°C with gentle shaking. The reaction was stopped by adding 4ml of sterile PBS.

The epithelial cells were collected on a filter (12 μm pore size; 25mm diameter; Nuclepore) and washed using sterile PBS (10ml). The epithelial cells on the filter were resuspended and standardized as described in Section 3.3.3. Control cells were prepared as described in Section 3.3.3.

Pretreated and restandardized epithelial cells were used in the adhesion assay.

3.4 Adherence assay

Epithelial and yeast cell suspensions were vortexed using a whirlimixer before being used in adherence assays. Standardized suspensions of vaginal epithelial cells (5×10^5 cells/ml in PBS; 0.1ml) and yeasts (5×10^7 cells/ml in PBS; 0.1ml) were mixed together and incubated at 37°C, with gentle shaking, for 45 min. Control mixtures were set up containing epithelial cells and PBS. After incubation, 4.8ml of PBS was added to each bijou bottle to stop any further yeast attachment. The epithelial cells were collected on polycarbonate filters (12μm pore size, 25mm diameter;

Nuclepore) and washed with PBS (25ml) to remove unattached yeasts. The washed filters were placed on labelled slides and left to dry. Epithelial cells on the filters were fixed with 50% acetone/alcohol (v/v) and stained using the Gram procedure. After drying, filters were mounted under coverslips using DPX mountant (BDH Chemicals Ltd.). The numbers of adherent yeasts on each of 100 epithelial cells were counted on every filter. Each adhesion assay was carried out three times in triplicate.

3.5 Adhesion assays at different pH values

Candida and lactobacilli were grown up as described previously (Section 2.1.2 and section 2.2.2 respectively). Cells were harvested by centrifugation (MSE bench centrifuge;5 min, 1200 x g) and then washed twice in either PBS (pH 7.2) or 0.1M citric acid-sodium citrate buffer (pH 4.5). The cells were then resuspended and standardized in the appropriate buffer.

Human vaginal epithelial cells were obtained from a single donor by gently swabbing. Swabs were agitated in either PBS (pH 7.2) or 0.1M citric-acid sodium citrate buffer (pH 4.5) to obtain a suspension of epithelial cells which were washed twice with the appropriate buffer and resuspended to a concentration of 5×10^5 cells/ml using an improved Neubauer haemocytometer.

Vaginal epithelial cells (5×10^5 cells/ml; 0.5ml) suspended in PBS (pH 7.2) were pretreated with lactobacilli (5×10^8 cells/ml; 0.5ml) also suspended in PBS (pH 7.2). Epithelial cells suspended in citrate buffer were pretreated with lactobacilli suspended in the same citrate buffer (pH 4.5).

After pretreatment for 30 min, epithelial cells were washed and resuspended to 5×10^5 cells/ml in the appropriate buffer.

Control epithelial cells were pretreated with either PBS or citrate buffer.

After pretreatment, restandardized epithelial cells were treated with *Candida* cells ($5x10^7$ cells/ml) suspended in PBS (pH 7.2) or citrate buffer (pH 4.5).

4. Growth experiments

4.1 Growth of *Candida* at different pH values

Liquid culture medium (50ml, in 250ml Erlenmeyer flasks) composed of yeast nitrogen base (6.7g/l) with glucose as the carbon source (9g/l) was made up in 0.1M citric acid-0.2M disodium hydrogen phosphate buffer (to give pH values of 3.0, 4.0, 5.0, 6.0, 7.0), or in 0.2M Tris maleate-sodium hydroxide buffer (for pH 8.0). The liquid medium was sterilized through a 0.2μm filter (Sartorius). Batches of medium (25ml), each at a different pH value were inoculated with an overnight culture of *C. albicans*, to give an initial concentration of 1.8 x 10^6 yeasts/ml. Cultures were then incubated at 37°C on an orbital shaker (150 revs/min). Measurements were made of cell numbers using an improved Neubauer haemocytometer at 2.5, 5, 7.5, 10, 29, 96 and 190h. At the same intervals, the pH value of a sample of medium was determined.

4.2 Growth of *Lactobacillus* species at different pH values

Lactobacilli were grown in MRS broth (50ml) made up in 0.2M sodium acetate-acetic acid buffer (to give pH values 3.7, 4.0, 5.0), or 0.2M disodium hydrogen phosphate -sodium dihydrogen phosphate buffer (to give pH values 6.0, 7.0, 8.0). The liquid medium was sterilized through a 0.2μm Sartorius filter. Each broth was inoculated from an overnight culture of *Lactobacillus* in MRS broth

(5ml) to give an initial concentration of 7 x 10^6 lactobacilli/ml. Cultures were then incubated statically at 37°C. Growth measurements (determined by OD_{600}) were made at 2.5, 5, 7.5, 13.5, 16, 18.5, 21.5, 40 and 96h after inoculation. At the same time intervals, the pH value of a sample of culture was determined.

4.3 Growth of *Lactobacillus* and *Candida* in mixed broth cultures

Lactobacilli, (3 x 10^8 cells/ml; 1ml) from a 24h culture in MRS, were used to inoculate, in duplicate, 50mM glucose YNB medium (50ml) and MRS broth (50ml). *Candida*, (3 x 10^8 cells/ml; 1ml) from a 24h culture in 50mM glucose YNB medium, were used to inoculate, in duplicate, 50mM glucose YNB medium (50ml) and MRS broth (50ml). These acted as controls to show growth of the single organism in each medium. Mixed broth cultures were set up by adding lactobacilli (3 x 10^8 cells/ml; 1ml) and *Candida* (3 x 10^8 cells/ml; 1ml) to 50mM glucose YNB medium and also to MRS medium, in duplicate.

Growth within the single and mixed cultures was measured by OD_{600} readings. Samples of cultures were taken and diluted 1/100 in sterile PBS. Viable *Candida* counts were obtained by plating out the 1/100 dilution (0.1ml) on to Sabouraud dextrose agar and incubating plates aerobically at 37°C for 24h. Viable counts of lactobacilli were obtained by plating out a 1/100 dilution (0.1ml) of cultures onto Rogosa agar (Oxoid Ltd.). The Rogosa agar plates were incubated microaerophilically, in a candle jar for 48h, before *Lactobacillus* colonies were counted.

5. Plate inhibition assays

5.1 Sandwich plate assay

The sandwich plate technique used was a modification of a method described by Reid *et al.* (1988). Lactobacilli from a 24h MRS broth culture were harvested by centrifugation (MSE bench centifuge; 5 min, 1200 x g) and washed twice with sterile PBS. Lactobacilli were resuspended to an OD_{600} value of 0.05 or 5 (Shimadzu Spectrophotometer) equivalent to 5 x 10^6 or 5 x 10^8 cells/ml respectively. In one experiment, different concentrations of lactobacilli prepared in a similar manner were also tested in the base of the sandwich plate. The standardized *Lactobacillus* suspensions (0.5ml) were spread on to MRS agar plates and incubated microaerophilically, in a candle jar, for 48h (unless otherwise specified) at 37°C.

Following incubation, replicate plates were treated with ultraviolet radiation (30 min) or chloroform (10ml; 15 min) which killed the lactobacilli, or remained untreated. Plates were then overlaid with either MRS (10ml) or Sabouraud dextrose agar (10ml), which was allowed to set. This created the sandwich plate.

C. albicans GDH 2346 was used as an indicator organism. Standardized suspensions of glucose-grown *C. albicans* GDH 2346 were prepared by harvesting the yeasts by centrifugation (MSE bench centrifuge; 5 min, 1200 x g), washing twice in sterile PBS and then standardizing to give suspensions of 10^5 cells/ml, 10^4 cells/ml or 10^3 cells/ml.

The indicator organism *C. albicans* GDH 2346 (0.1ml, unless otherwise specified; standardized suspension) was spread on to the surface of the sandwich plates. Following aerobic incubation for 24h (unless otherwise specified), growth of the indicator organism was

assessed, and compared to controls where no lactobacilli were present in the base of the sandwich plates.

5.2 Lawn growth inhibition assay

Assay plates (400ml volume) were placed on a level surface and sterilized by flaming with absolute alcohol. Cultures of *T. mentagrophytes* (2ml), *C. neoformans* (2ml) and *A. niger* (0.5ml) were removed from storage in liquid nitrogen as 10% glycerol stocks before mixing with Sabouraud dextrose agar (400ml) and pouring into the assay plates. *C. albicans* B079, inoculated from a stock culture, was grown up in static culture overnight at 37°C in Sabouraud dextrose broth (10ml). This overnight culture (0.75ml) was used to inoculate Sabouraud dextrose agar (400ml) and also as an inoculum (1.0ml) for Yeast Nitrogen Base agar (400ml) before pouring into the assay plates. The agar was allowed to set and the required number of holes for samples were cut in the agar using a sterile punch.

Test substances were pipetted into the wells, 80μl into each 8mm diameter well. The *T. mentagrophytes* plate was then incubated at 30°C for 48h, while the *C. neoformans, A. niger* and *C. albicans* plates were incubated at 37°C for 24h. After incubation the diameter of any zones of growth inhibition were measured with a ruler.

Bacterial strains, *S. aureus* 'Oxford' NCTC 6571, *B. subtilis* NCTC 3610, *E. coli* Lily, *B. megaterium* NCTC 10342 and *P. vulgaris* NCTC 4175, inoculated from stock cultures, were grown in static culture overnight in nutrient broth (Oxoid; 10ml) at 37°C. The overnight cultures (0.5ml) were used to inoculate nutrient agar (Oxoid; 15ml) to form a pour plate in a sterile petri dish. The agar was allowed to set and the required number of holes were cut in the agar using a sterile punch. Test substances were introduced into the

wells, 80μl into each 4mm diameter well. The bacterial plates were then incubated at 37°C for 24h and after incubation the diameter of any zones of growth inhibition were measured with a ruler.

5.3 Cellulose covered agar plate assay

A cellulose membrane square was rolled up and placed in a measure cylinder containing PBS and sterilized by autoclaving for 15 min at a pressure of 15lb/sq. in. The sterile membrane was used to completely cover a large square petri dish (Nunc Bioassay dish 14x14 cm^2) containing 400ml of MRS agar. The cellulose membrane was not allowed to dry out. The surface of the cellulose membrane was then inoculated with 1ml of a 24h culture of *Lactobacillus* grown in MRS broth as a static culture at 37°C. The inoculum was spread across the surface of the cellulose membrane using a sterile glass spreader. The plate was incubated microaerophilically, in a candle jar, for 5 days at 37°C.

After incubation, the bacterial growth from the surface of the cellulose membrane was recovered and centrifuged (Microfuge maximum speed; 2 min) and the supernatant collected. The supernatant was bioassayed against *C. albicans* B079 in a lawn growth inhibition assay, as described previously in section 5.2.

The cellulose membrane was aseptically removed from the surface of the MRS agar. The agar was then inoculated with 1ml of an overnight culture of *C. albicans* B079 (1.8 x 10^8 cells/ml) which had been grown in static culture in Sabouraud dextrose (10ml) at 37°C. The inoculated plate was incubated aerobically for 24h at 37°C.

6. Preparation and purification of antimicrobial substance from lactobacilli

6.1 Preliminary preparation

Batches of MRS medium (50ml, in 55ml screw-capped glass bottles) were inoculated with 24h *Lactobacillus* cultures (5ml) grown in MRS broth and incubated statically for 5 days, or as specified, at 37°C. The cultures were centrifuged (MSE bench centrifuge: 5min, 1200 x g) and the cell pellet and culture supernatant for each organism treated separately. The culture supernatant was concentrated 20-fold by the removal of water using a rotary evaporator (Buchi; 40^0C). Concentrated culture supernatant (80μl) was tested for activity in the lawn growth inhibition assay. MRS broth was concentrated in a similar manner to act as a control. The pH of 20-fold concentrated culture supernatant was adjusted with concentrated acid (1M HCl) or alkali (1M NaOH) and maintained at room temperature for 1h prior to being introduced (80μl) into the wells in a lawn growth inhibition plate inoculated with *C.albicans* B079.

The cell pellet of the *Lactobacillus* culture was washed in 0.15M PBS (pH 7.2) and resuspended to a concentration of 5 x 10^9 cells/ml. A sample of cell pellet was subject to ultrasonic vibration (ultrasonicating bath; 5x10s)to disintegrate the cells and release intracellular materials.

6.2 Solvent extractions with water-immiscible solvents

Supernatants from 5-day *Lactobacillus* cultures grown in MRS broth, as described in Section 6.1, were concentrated 20-fold by the removal of water using a rotary evaporator (Buchi; 40°C). Concentrated culture supernatant (0.7ml) was mixed with solvent (0.7ml) and mixed vigourously using a whirlimixer. The solvents used

for culture supernatant extraction were butanol, chloroform, and ethyl acetate. The aqueous and solvent phases were allowed to separate completely and then a sample from each phase was tested in the lawn growth inhibition bioassay with *C. albicans* B079 as the test organism, as described previously (Section 5.2).

6.3 Solvent extractions with water-miscible solvents

Culture supernatants (100ml) from 5-day *Lactobacillus* cultures grown in MRS broth were freeze-dried and mixed with solvent (100ml). The solvents used were methanol, ethanol, chloroform/ methanol/ H_2O (5:2:1), chloroform/methanol (4:1) and butanol (to act as a comparison for the previous method of solvent extraction). The freeze-dried culture supernatants were extracted, with stirring, for 6h at room temperature then 18h at 4°C. The supernatant from the solvent extraction was collected after centrifugation (Sorvall; 10 min, 10,000 x g) and concentrated 50-fold by rotary evaporator (Buchi; 40°C).

6.4 Preparation of a butanol extract of *Lactobacillus* culture supernatant

Lactobacilli inoculated from stock cultures were grown for 24h at 37°C in MRS broth. The 24h culture (25ml) was used to inoculate MRS broth (500ml, in a 550ml screw capped glass bottle). Incubation was for 5 days at 37°C without shaking. The bacteria were harvested by centrifugation (Sorvall; 10 min, 10,000 x g) and the culture supernatant filtered (0.45μm filter) to remove any bacterial debris. The culture supernatant was mixed with an equal volume of butanol (500ml) in a separating funnel. After vigo rous mixing, the solvent and aqueous phases were allowed to separate at room temperature for

2h. The aqueous phase was removed and discarded; the butanol phase was concentrated using a rotary evaporator (Buchi;40°C).

6.5 Column chromatography of a butanol extract of *Lactobacillus* culture supernatant

6.5.1 Cation-exchange

A column of IR 120 cation-exchange resin (bed volume 10ml; 8mm internal diameter) was prepared in distilled water. The column was washed twice with 1M NaOH (10ml), three times with distilled water (10ml) and three times with 1M HCl (10ml). It was finally rinsed six times with distilled water (10ml). A sample of butanol extract of *Lactobacillus* culture supernatant (50-fold concentrate; 5ml) was applied to the column. The percolate from the column was collected. The column was then eluted with 20ml distilled water, followed by 20ml 0.2M NaCl and 20ml 2M NaCl and 5ml fractions were collected. The unfractionated sample and all fractions from the column were bioassayed against *C. albicans* B079 in the lawn growth inhibition assay described previously (section 5.2). Cation-exchange column chromatography of a 50-fold concentrated butanol extract of MRS broth was carried out and fractions were tested as controls in the bioassay.

6.5.2 Anion-exchange

A column of IRA 548 anion-exchange resin (bed volume 10ml; 8mm internal diameter) was prepared in distilled water. The column was washed twice with 1M HCl (10ml), three times with distilled water (10ml) and three times with 1M NaOH (10ml). It was finally rinsed six times with distilled water (10ml).

A sample of butanol extract of *Lactobacillus* culture supernatant (50-fold concentrate; 5ml) was applied to the column and the percolate collected. The column was then eluted with 20ml distilled water, followed by 20ml 0.2M NaCl and 20ml 2M NaCl; 5ml fractions were collected.

All fractions and also an unfractionated sample were bioassayed in the lawn growth inhibition assay against *C. albicans* B079.

Anion-exchange column chromatography of a 50-fold concentrated butanol extract of MRS broth was carried out in the same way and column fractions were tested as controls in the lawn growth inhibition assay.

6.5.3 Adsorption resin

Adsorption resin HP 20, suspended in acetone, was used to pour a column of internal diameter 8mm and bed volume 10ml. The column was washed with acetone (20ml) and distilled water (200ml).

Butanol extract of *Lactobacillus* culture supernatant (50-fold concentrate; 5ml) was applied to the column which was then eluted with distilled water (20ml), 40% propanol (20ml) and acetone (20ml). These washes were pumped through the column using a syringe in order to avoid airlocks within the adsorption resin.

Fractions (5ml) were collected manually. All fractions were tested in the lawn growth inhibition bioassay against *C. albicans* B079. A concentrated butanol extract of MRS broth was chromatographed similarly and the fractions obtained were used as controls in the bioassay.

6.6 Fractional distillation of a butanol extract of *Lactobacillus* culture supernatant

Fractional distillation was investigated as an alternative purification procedure for the antimicrobial component. A 50-fold concentrated butanol extract of *L. acidophilus* was fractionally distilled under reduced pressure and samples collected at distillation temperatures of 75, 100, 125, 150, 175, 200, 225 and 250°C. Samples were tested in the lawn growth inhibition assay.

6.7 Preparation of an ethanol extract of *Lactobacillus* culture supernatant

Lactobacilli, inoculated from stock cultures, were grown for 24h at 37°C in MRS broth. The 24h culture (25ml) was used as an inoculum for fresh MRS broth (500ml, in a 550ml screw capped glass bottle). Incubation was for 5 days at 37°C without shaking. The bacteria were harvested by centrifugation (Sorvall; 10min, 10,000 x g) and the culture supernatant filtered (0.45μm filter). The culture supernatant was then frozen as a thin shell, using liquid N_2, and freeze-dried (24h). The dried material was broken up and extracted with 500ml ethanol, with constant stirring for 6h at room temperature, and then for a further 18h at 4°C. The extract was centrifuged (Sorvall; 10min, 10,000 x g) and the supernatant concentrated 50-fold using a rotary evaporator.

6.8 Sephadex LH 20 chromatography of an ethanol extract of *Lactobacillus* culture supernatant

Sephadex LH 20 beads (25g) were allowed to swell in ethanol and used to pour a column (bed volume 100ml; 20mm internal diameter) which was washed with more ethanol (300ml). A sample of 50-fold

concentrated ethanol extract of *Lactobacillus* culture supernatant (2ml) was applied and the column was eluted with ethanol at a flow rate of 1ml/min; and 15ml fractions were collected. The absorbance of the fractions was measured at 280nm and samples were tested against *C. albicans* B079 in the lawn growth inhibition bioassay. Fractions were then pooled, concentrated 50-fold using a rotary evaporator and retested in the bioassay. Ethanol was used as a control in the bioassay.

6.9 Thin layer chromatography

6.9.1 Purification of biological activity from ethanol extracts of *Lactobacillus* culture supernatant by TLC using butanol/acetic acid/water as solvent

Biological activity was eluted from the LH 20 Sephadex column along with a peptide fraction (as measured by A_{280}); for this reason TLC separation methods for peptides were carried out.

Precoated plastic TLC plates (Polygram SilG; 0.25mm layer Silica gel) were loaded with the biologically active fraction from the LH 20 Sephadex column. A 15cm wide streak chromatogram (load 200μl) was prepared on the same TLC plate as a spot chromatogram (5μl). Plates were run for 6.5h in a solvent system consisting of butanol/ glacial acetic acid/ distilled water (60:15:25, v/v). After drying, components of the spot chromatogram were visualised by spraying with ninhydrin and heating to allow a colour reaction.

The streak chromatogram, which had been run at the same time, was cut up according to the elution profile of the spot chromatogram. Silica gel was scraped from the different sections of the TLC plate, resuspended in ethanol (5ml), and extracted overnight with shaking at 21°C. The silica was removed by centrifugation (MSE bench centrifuge; 10 min, 1200 x g) and the ethanol extract

evaporated to dryness by bubbling through N_2 gas. The dried material was then redissolved in 0.1ml ethanol.

6.9.2 TLC using chloroform/methanol/water as solvent

Biologically active fractions (R_f value 0.63 - 1.0) eluted from TLC plates run in butanol/acetic acid/water (Section 6.9.1) were further separated by TLC in a second solvent system.

A streak chromatogram of the biologically active fraction was prepared on a precoated plastic TLC plate (Polygram Sil G; 100μl sample volume). A spot chromatogram (5μl sample volume) was prepared on the same plate. The plate was run for 2h in a solvent system consisting of chloroform/ methanol/ water (75:22:3, v/v).

The plate was allowed to dry, and then separated into two portions corresponding to the streak chromatogram and spot chromatogram. The spot chromatogram was exposed to iodine vapour for 15 min to visualise lipids. The spot chromatogram was then stained with ninhydrin, as described previously, to locate peptides.

The streak chromatogram, which had been run at the same time, was separated into different portions according to the elution profile of the spot chromatogram. Silica gel was scraped from the different sections of the plate, suspended in ethanol (5ml) and extracted overnight with shaking at 21^{0}C. The silica was removed by centrifugation (MSE bench centrifuge; 10 min, 1200 x g) and the ethanol extracts were evaporated to dryness under a stream of N_2 gas. The dried material was then redissolved in 0.1ml ethanol.

A chromatogram of lactic acid was run in the chloroform/methanol/water solvent system. Extracts from the chromatogram were prepared as above. The extracts were tested in the lawn growth inhibition assay.

6.10 Low resolution Electron Impact Mass Spectrometry

The molecular mass of antifungal substance purified by ethanol extraction (Section 6.7), followed by LH 20 column chromatography (Section 6.8), TLC in a butanol/glacial acetic acid/water solvent (Section 6.9.1) and then TLC in a chloroform/methanol/water solvent (section 6.9.2) was measured by low resolution Electron Impact Mass Spectrometry. This work was carried out by the SERC Mass Spectrometry Centre, University of Swansea.

7. Animal studies

7.1 Vaginal infections in healthy mice

For vaginal infections yeasts were grown in YNB medium containing 500mM galactose as described for *in vitro* adherence assays (Section 2.1.2). Female mice (5 per group; CD-1 strain), aged 6 weeks were injected subcutaneously with 0.5mg β-oestradiol-3-benzoate (Sigma) prepared as described in Section 3.1.2 to maintain the oestrous stage of the oestrous cycle. Two days later, the mice were inoculated intravaginally using a washed suspension of a 24h culture of *C. albicans* GDH 2346 at a concentration of 10^7, 10^8 or 10^9 yeasts/ml in saline; the yeast suspension was squirted into the vagina using a blunt-ended 1ml syringe until liquid was seen to run out from the vagina. Control mice were similarly treated with PBS. On day 7 after inoculation the animals were killed, and their vaginas were removed and added to sterile saline (5ml) in a universal. The vaginas were homogenised (Silverson homogeniser; 4x10s at maximum speed) and samples diluted 1/10 and 1/100 with sterile saline. Portions (50μl) of the undiluted and diluted homogenate were plated out on Sabouraud dextrose agar, containing

penicillin (100μg/ml) and streptomycin (100μg/ml). All plates were incubated at 37°C for 24h. The total number of yeast colonies on each plate was counted to assess the extent of vaginal infection.

7.2 Time course of vaginal infections in healthy mice

Female mice (15 per group; CD-1 strain) were inoculated, as described in Section 7.1, with *C. albicans* GDH 2346 at a concentration of 10^7, 10^8, or 10^9 yeasts/ml in saline. Control mice were similarly treated with PBS. On days 2, 4 and 7, after inoculation , 5 animals from each treatment group were killed and vaginal infection quantified as described in 7.1.

7.3 Vaginal infections in diabetic mice

Female mice, (15 per group; CD-1 strain) aged 6 weeks, were injected intravenously with alloxan (Sigma; 8mg/kg mouse) to produce a chemically induced diabetes mellitus. Two days later, to confirm the diabetic status of the mice, the presence of glucose in the urine was tested for using Clinistick indicators.

Diabetic mice were then put into pseudooestrous by a subcutaneous injection of 0.5mg β-oestradiol-3-benzoate. Two days later, the mice were inoculated intravaginally with a washed suspension of a 24h galactose-grown culture of *C. albicans* GDH 2346 containing 10^7, 10^8 or 10^9 yeasts/ml in saline as described in section 7.1. Control diabetic mice were treated similarly with PBS. On days 2, 5 and 7 after infection, 5 animals from each treatment group were killed, and the level of vaginal infection was assessed as described in Section 7.1.

7.4 Colonization of the rat vagina with *L. acidophilus* NCTC 4504

Three female, ovarectomised rats (Sprague Dawley; Charles River; 250g) were injected subcutaneously with 0.2ml β-oestradiol (Benztrone 5mg; Paines and Byrne Ltd., Greenford, England) 7 days before infection and weekly thereafter throughout the experiment to induce pseudooestrous.

Lactobacilli were cultured as described in Section 2.2.2. After their second wash bacteria were resuspended in PBS and suspensions standardized by measurement of OD_{600} (Pye Unicam SP600 spectrophotometer). The amount of PBS added to the undiluted *Lactobacillus* suspension was adjusted so a concentration of $5x10^9$ cells/ml was obtained. (This corresponded to a measurement of OD_{600} 0.5 for a 1/100 dilution of the *Lactobacillus* suspension). This washed *Lactobacillus* suspension was diluted 1/10 in gum arabic to give an inoculum containing $5x10^8$ cells/ml.

Before infection, the rats were injected intravenously with Hypnorm (0.4ml/kg) for sedation to allow measurement of the pH value of the vagina with a portable glass electrode previously calibrated to pH 7 at 35°C. The electrode was inserted to a depth of approximately 5mm into the vagina and equilibriated with the internal pH.

The rat vaginas were each swabbed with a sterile swab, moistened in PBS which was touched on to a labelled slide and broken off into a small labelled test tube containing 1.5ml sterile PBS. The slides were fixed, stained by the Giemsa method and the number of adherent bacteria/vaginal epithelial cell counted. Each swab was agitated in 1.5ml sterile PBS using a whirlimixer and a 0.1ml sample plated out on MRS agar using a spiral plater to obtain a background count of lactobacilli within the vagina. The rats were then infected

intravaginally with 0.1ml of a suspension of lactobacilli (5 x 10^8 cells/ml) in gum arabic using a 1ml sterile blunt-ended syringe.

Each day after infection the rats were sampled by swabbing. The swabs were touched on a labelled slide which was fixed and then stained by the Giemsa method. The swab was broken off into a small test tube containing 1.5ml PBS, agitated using a whirlimixer and a 0.1ml sample plated out on MRS agar using a spiral plater. Plates were incubated microaerophilically in a candle jar at 37°C for 48h.

On days 1, 3 and 7 samples were also plated out on modified MRS agar, the pH value of which had been lowered to 5.4 by the addition of 20g/l sodium acetate and 1.32ml/l glacial acetic acid. This medium is similar to the selective acetate medium described by Rogosa *et al.* (1951).

On days 2 and 4 the pH value of the vagina was measured after sedation as described earlier.

7.5 Repeated colonization of the rat vagina with *L. acidophilus*

Six female, ovarectomised rats (Sprague Dawley; Charles River; 250g) were induced to a state of permanent pseudooestrous, as described in Section 7.4.

On days 1, 2, 5, 9 and 16 the rats were infected intravaginally with an inoculum of $5x10^8$ lactobacilli/ml (0.1ml) suspended in gum arabic (Section 7.4), using a 1ml sterile blunt-ended syringe.

The rats were sampled by swabbing before infection and on days 1, 2, 5, 6, 7, 8, 9, 13, 14, 15, 16, 20, 21, 23 and 26 after the initial infection. Each swab was touched on to a labelled slide which was fixed and then stained by the Giemsa method. The swab was broken off into a small test tube containing 1.5ml PBS, agitated

using a whirlimixer and a 0.1ml sample plated out on Rogosa agar (Oxoid Ltd.) using a spiral plater. Plates were incubated microaerophilically in a candle jar at 37°C for 48h and the number of *Lactobacillus* colonies counted.

7.6 Effect of colonization by *L. acidophilus* on subsequent *Candida* infection of the rat vagina

Fifteen female, ovarectomised rats (Sprague Dawley; 250g) were injected subcutaneously with 0.25ml β-oestradiol-3-benzoate (2mg/ml Sigma) as a ball-milled suspension in 0.5% Tween 80, 5 days before the first infection with lactobacilli, and weekly thereafter throughout the experiment to induce pseudooestrous.

On day 0, the rats were each swabbed with a sterile moistened swab. Each swab was broken off into a small test tube containing 1.5ml PBS, agitated using a whirlimixer and a 0.1ml sample plated out on Rogosa agar (Oxoid Ltd.). Plates were incubated microaerophilically in a candle jar at 37°C for 48h and the number of *Lactobacillus* colonies counted to obtain a background count of lactobacilli within the vagina. The rats were assigned to 3 groups: a *Lactobacillus* control, a *Candida* control and a test group. Each group comprised 5 animals. Rats in the *Lactobacillus* control group and test group were infected with 0.1ml of a $5x10^9$ lactobacilli/ml suspension in 10% gum arabic on days 0, 2 and 4. On day 5, the rats were swabbed with a sterile swab moistened in PBS which was broken off in a small labelled test tube containing 1.5ml PBS. Samples were plated out on to Sabouraud dextrose agar containing penicillin G (100μg/ml; Sigma) and streptomycin (100μg/ml; Sigma). The plates were incubated aerobically at 37°C for 24h to obtain a background count of *Candida* within the vagina.

A 48h culture of *C. albicans* B2630, grown on a Sabouraud dextrose agar slope at 37°C, was harvested and washed with 10ml sterile PBS. The cells were resuspended in PBS at a concentration of $5x10^7$ cells/ml as described in Section 2.1.3. The yeast suspension was diluted 1/10 in 10% gum arabic to give an inoculum containing $5x10^6$ cells/ml.

On day 5, the *Candida* control group and test group were inoculated with 0.1ml of the yeast suspension (5 x 10^6 cells/ml) using a 1ml blunt-ended syringe.

On day 7, 10 and 16 the rats were sampled by swabbing. The swab was broken off into a small test tube containing 1.5ml PBS, agitated using a whirlimixer and 0.1ml samples were plated out on Rogosa agar and Sabouraud dextrose agar.

The Rogosa agar plates were incubated microaerophilically in a candle jar at 37°C for 48h, to assess the numbers of lactobacilli within the rat vaginas. The Sabouraud dextrose agar plates were incubated aerobically at 37°C for 24h to assess *Candida* infection within the vagina.

7.7 Effect of butanol extract of *Lactobacillus* culture supernatant on *Candida* infection of the rat vagina

Female, ovarectomised rats (Sprague Dawley; 250g) were injected subcutaneously with 0.25ml β-oestradiol-3-benzoate (2mg/ml; Sigma) as a ball-milled suspension in 0.5% Tween 80. The animals were injected weekly throughout the experiment to induce pseudooestrous.

Rats were assigned to three groups each comprising 5 animals. Group 1 was inoculated intravaginally with 0.1ml of a butanol extract of *L. acidophilus* culture supernatant (prepared as described in Section 6.4), Group 2 was inoculated intravaginally with 0.1ml of

a butanol extract of MRS broth (prepared as described in Section 6.4), and Group 3 was treated with 0.1ml 0.1M citric acid-sodium citrate buffer (pH 4.5).

Rats were dosed as above on Days 1, 2, 3, 4, 5, 8, 10, and 12. A 48h culture of *C. albicans* B2630, grown on a Sabouraud dextrose agar slope at 37°C, was washed with sterile PBS. The *Candida* cells were harvested by centrifugation (MSE bench centrifuge: 5 min, 1200 x g) and washed twice in sterile PBS. The yeast suspension was standarized using an improved Neubauer haemocytometer and adjusted so that a final yeast concentration of $5x10^6$ cells/ml was obtained. On Day 3, each rat was infected intravaginally with 5×10^5 *Candida* B2630 cells suspended in 0.1ml PBS.

The *Candida* infection was assessed by swabbing. A background *Candida* count was obtained on day 3, before infection, and further swabs were taken on days 4, 5, 8, 10, 12 and 15 of the experiment. Each swab was broken off into a bijou bottle containing 1.5ml PBS and agitated by a whirlimixer. The samples were diluted 1/10 and 1/100 with sterile saline and plated out (100μl) on Sabouraud dextrose agar containing penicillin G (100μg/ml) and streptomycin (100μg/ml). The plates were incubated aerobically at 37°C for 24h. The number of *Candida* colonies on each plate was counted to assess the level of vaginal infection.

RESULTS

For convenience of presentation, the results of this investigation are divided into 3 main sections. The first describes some basic characteristics of the adhesion of *Candida albicans* to vaginal epithelial cells (VEC); the second explores the influence of *Lactobacillus* species on the growth of *C. albicans*; while the third makes use of the information in the first two sections to define the *in vivo* interactions of *Candida* and *Lactobacillus* species in the vagina of the mouse and rat. To avoid excessive repetition the terms *Candida*, *C. albicans* and yeast are used interchangeably.

1. Adhesion of *C. albicans* to vaginal epithelial cells

1.1 Adhesion to epithelial cells from different animal species

Preliminary experiments were carried out to investigate some basic characteristics of the adhesion of *C. albicans* to vaginal epithelial cells and to establish conditions for use in further adhesion studies. Experiments were done using *C. albicans* and mouse, rat and human vaginal epithelial cells.

1.1.1 Mouse VEC

1.1.1.1 Effect of yeast and epithelial cell concentrations on adhesion

Different concentrations of epithelial and yeast cells were used to assess their effect on *Candida* adhesion.

Vaginal epithelial cells were obtained from mice brought into pseudoestrous by hormone treatment with 17 β-oestradiol-3-benzoate. Epithelial cells from different animals were pooled before use in adhesion assays. Three different concentrations of vaginal cells were used: 5×10^4, 1×10^5 and 5×10^5 cells/ml. Epithelial cells were incubated with yeasts at yeast concentrations ranging from 5×10^6 to 5×10^8 cells/ml. Yeasts had been grown to stationary phase

in YNB medium containing 500mM galactose. Figure 1 illustrates the results obtained from these experiments.

At low yeast concentrations little difference was seen in the number of adherent *C. albicans* for different epithelial cell concentrations. However, with increasing yeast concentration, differences in the numbers of adherent yeasts per 100 vaginal epithelial cells were observed. Greater numbers of yeasts attached when greater numbers of epithelial cells were present. Higher concentrations of vaginal epithelial cells increased the probability of an epithelial cell and yeast coming into contact and binding.

The ratio of yeast concentration: epithelial cell concentration was important; an increase in this ratio gave greater binding. With an epithelial cell concentration of 5×10^4 cells/ml; the highest ratio of yeasts: vaginal epithelial cell was seen (10^4:1), at this point the yeasts seemed to have saturated all the available binding sites on the epithelial cells.

An epithelial cell concentration of 5×10^5 cells/ml was used in later adherence assays; this concentration gave the greatest number of yeasts binding to mouse vaginal epithelial cells.

1.1.1.2 Effect of yeast concentration on adhesion after growth on two different carbon sources

Yeasts were grown to stationary phase in YNB medium supplemented with 50mM glucose or 500mM galactose. Yeast cell concentrations ranging from 1×10^6 to 5×10^9 cells/ml were used in adherence assays with mouse vaginal cells (5×10^5 cells/ml). Results are illustrated in Figure 2. Yeasts harvested from medium containing 500mM galactose showed greater adherence to vaginal cells than glucose-grown organisms at concentrations up to 1×10^9 cells/ml.

Figure 1.

Effect of epithelial cell concentration on number of adherent *C. albicans* GDH 2346 to mouse vaginal epithelial cells (VEC) after growth of yeasts in YNB medium containing 500mM galactose

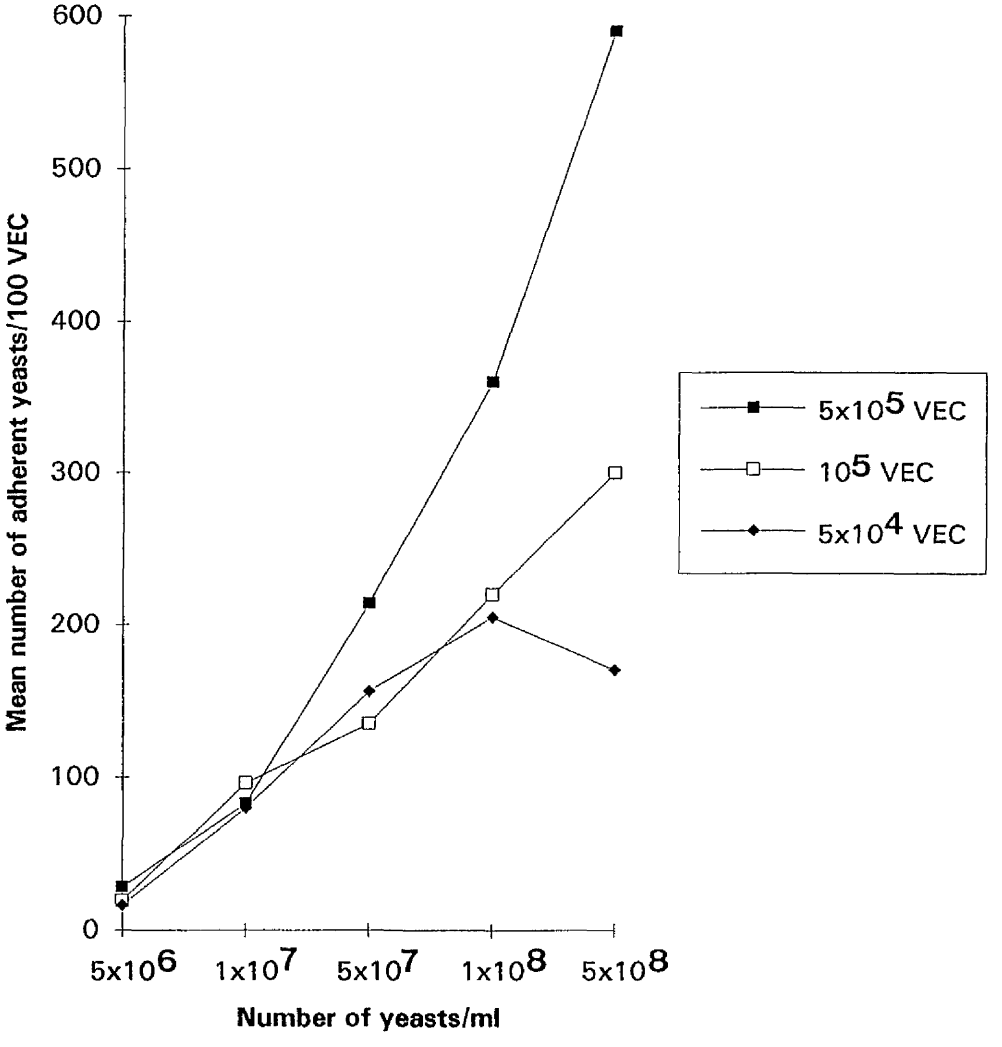
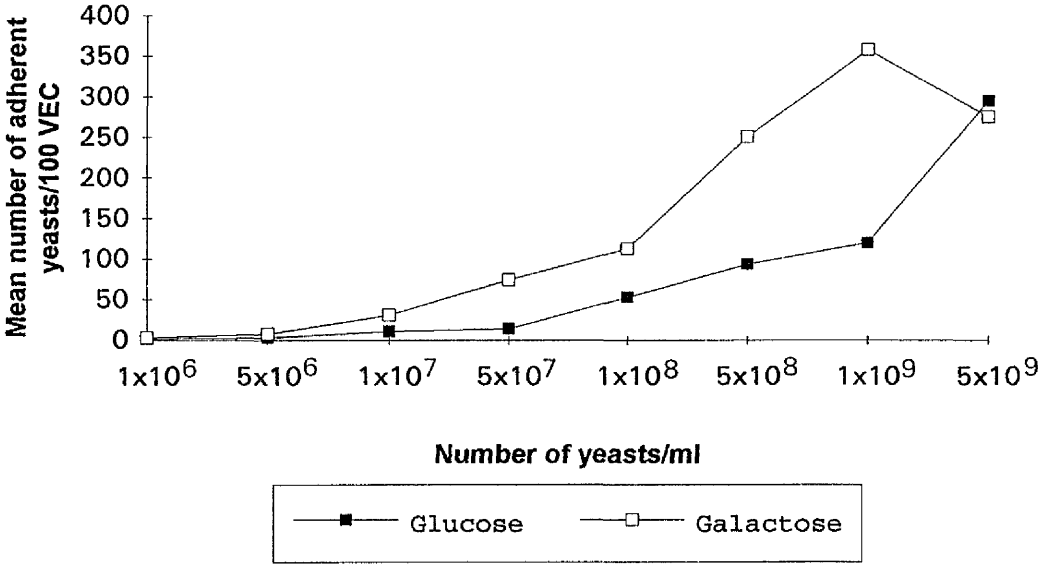


Figure 2.

Effect of yeast cell concentration on adherence of *C. albicans* GDH 2346 to mouse vaginal epithelial cells (VEC) after growth in defined medium containing 50mM glucose or 500mM galactose



Increasing the concentration of galactose-grown *Candida* from 1 x 10^6 to 1 x 10^9 cells/ml gave an increase in the mean number of adherent yeasts per 100 vaginal epithelial cells. At yeast cell concentrations greater than 1 x 10^9 cells/ml a decrease in galactose-grown yeast adherence was observed.

At concentrations lower than 5 x 10^9 cells/ml, glucose-grown yeasts adhered in lower numbers than galactose-grown cells. Increasing the concentration from 1 x 10^6 to 5 x 10^9 cells/ml gave an increase in the mean number of adherent yeasts per 100 vaginal epithelial cells. No saturation of binding sites, similar to that observed with galactose-grown yeasts, was seen at these treatment concentrations.

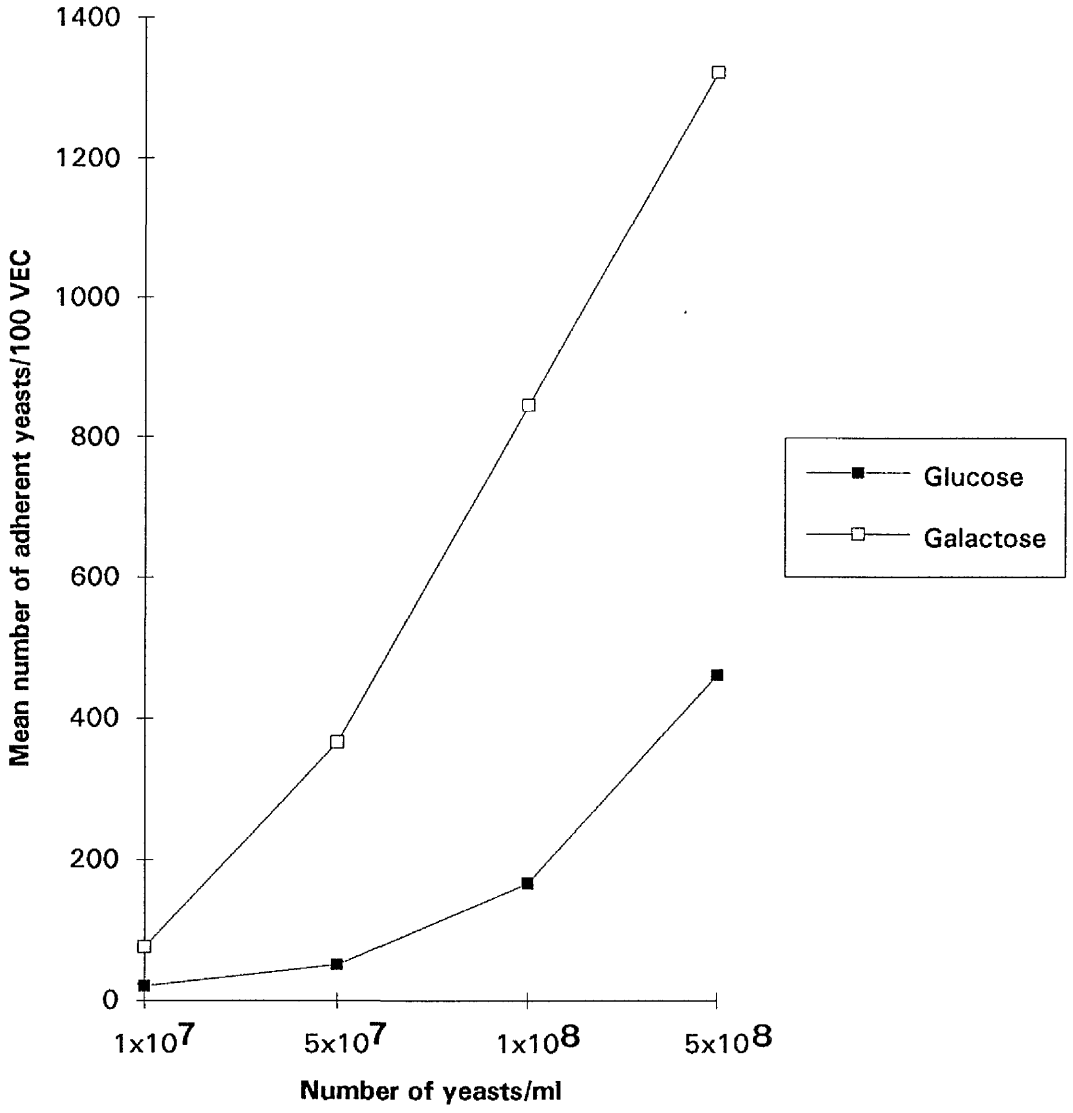
1.1.2 Human VEC

The effect of yeast cell concentration on the adherence of *C. albicans* GDH 2346 to human vaginal epithelial cells was investigated. Yeasts were grown to stationary phase in YNB medium containing 50mM glucose or 500mM galactose. Yeast cell concentrations ranging from 1 x 10^7 to 5 x 10^8 cells/ml were used in adherence assays with human vaginal cells collected from one healthy donor on three different days of the menstrual cycle.

Galactose-grown yeasts showed greater adherence to human vaginal epithelial cells than yeasts harvested from the 50mM glucose medium (Figure 3). Increasing the yeast cell concentration used in the adherence assay gave an increase in the number of adherent yeasts per 100 vaginal epithelial cells.

Figure 3.

Effect of yeast cell concentration on adherence of *C. albicans* GDH 2346 to human vaginal epithelial cells (VEC) after growth in defined medium containing 50mM glucose or 500mM galactose



1.1.3 Rat VEC

The adherence of *C. albicans* B2630 to rat vaginal epithelial cells after growth on Sabouraud dextrose agar or YNB medium containing 50mM glucose was investigated.

C. albicans B2630 was used in animal experiments with the rat model of vaginitis. Preparation of the yeast inoculum to infect rats was by growth on a Sabouraud dextrose agar slope for 48h. The yeasts were then scraped from the surface of the agar, suspended in PBS, washed, and diluted in gum arabic. In this experiment, the adherence of *C. albicans* B2630 to rat epithelial cells after growth on Sabouraud dextrose agar was compared with that of the same strain after growth in 50mM glucose YNB medium.

No significant difference was seen in the adherence properties of *C. albicans* B2630 to rat vaginal epithelial cells due to growth on the different media (Table 7).

1.1.4 Comparison of VEC from different species

Sandin *et al.* (1987α), using an *in vitro* adherence assay, observed that collecting mucosal cells from various anatomical sites, varying the date of collection and varying the cell donor had significant effects on the adhesion of *C. albicans* to human epithelial cells. In this experiment vaginal cells were obtained from three different species.

Vaginal epithelial cells were obtained from mice which had been brought into pseudoestrous by hormone treatment with 17 β-oestradiol-3-benzoate. Rat cells were obtained from animals which had been ovarectomised prior to the hormone treatment. The epithelial cells from different animals of the same species were pooled before use in adhesion assays. Human vaginal epithelial cells

Table 7.

Adherence of *C. albicans* B2630 to rat vaginal epithelial cells (VEC) after growth on Sabouraud dextrose agar or YNB medium containing 50mM glucose

GROWTH CONDITIONS	MEAN NO. (± SEM[a]) ADHERENT YEASTS PER 100 VEC	P[b]
Sabouraud dextrose agar	83.9 ± 9.6	NS[c]
50mM glucose YNB	75.1 ± 18.5	

[a] SEM, standard error of the mean.

[b] Probability value comparing adherence of yeast after growth on Sabouraud dextrose agar and after growth in 50mM glucose YNB.

[c] NS, not significant.

were obtained from a single healthy donor on different days in the menstrual cycle.

Vaginal epithelial cells were treated with yeasts (5×10^7 cells/ml) which had been grown to stationary phase in YNB medium supplemented with 50mM glucose or 500mM galactose.

Yeasts harvested from medium containing 500mM galactose showed greater adherence to vaginal epithelial cells of all species compared with yeasts harvested from the medium with a relatively low concentration of glucose (Table 8). Glucose- and galactose-grown *Candida* adhered in greatest numbers to vaginal epithelial cells of human origin; this is not surprising as *C. albicans* GDH 2346 was isolated from an active human infection and *Candida* is a much more common pathogen of humans than of mouse or rat.

The number of glucose-grown yeasts adherent to mouse and rat vaginal epithelial cells was similar. However, the rat vaginal epithelial cells were able to bind galactose-grown *Candida* in significantly higher numbers than mouse epithelial cells ($P < 0.001$), a fact reflected by the relative adherence value which is greatest for rat vaginal epithelial cells.

Using the *in vitro* adherence assay it was observed that the number of *C. albicans* cells that attached to individual vaginal epithelial cells varied greatly. The data from experiments with 1800 vaginal cells were analysed according to the number of yeasts adhering per cell. Results for epithelial cells incubated with glucose-grown yeasts were treated separately from results for epithelial cells incubated with galactose-grown yeasts. Figure 4 shows the distribution of glucose-grown *Candida albicans* GDH 2346 to mouse, rat and human vaginal epithelial cells according to the number of adhering yeasts per cell. Figure 5 shows distribution of

Table 8.

Adherence of *C. albicans* GDH 2346 to mouse, rat and human vaginal epithelial cells (VEC) after growth in YNB medium containing 50mM glucose or 500mM galactose as the carbon source

EPITHELIAL CELL TYPE	CARBON SOURCE	MEAN NO. (±SEM[a]) ADHERENT YEASTS PER 100 VEC	RELATIVE ADHERENCE[b]
Mouse	Glucose	68.2 ± 16.1	1.00
	Galactose	199.7 ± 22.8	2.93
Rat	Glucose	48.9 ± 9.9	1.00
	Galactose	391.7 ± 37.6	8.01
Human	Glucose	107.9 ± 16.0	1.00
	Galactose	443.1 ± 32.7	4.11

[a] SEM, standard error of the mean.

[b] Adherence relative to that of 50mM glucose-grown yeasts to the same epithelial cell type.

Figure 4.

Distribution of glucose-grown *C. albicans* GDH 2346 to mouse, rat and human vaginal epithelial cells (VEC) by number of adhering yeasts/cell

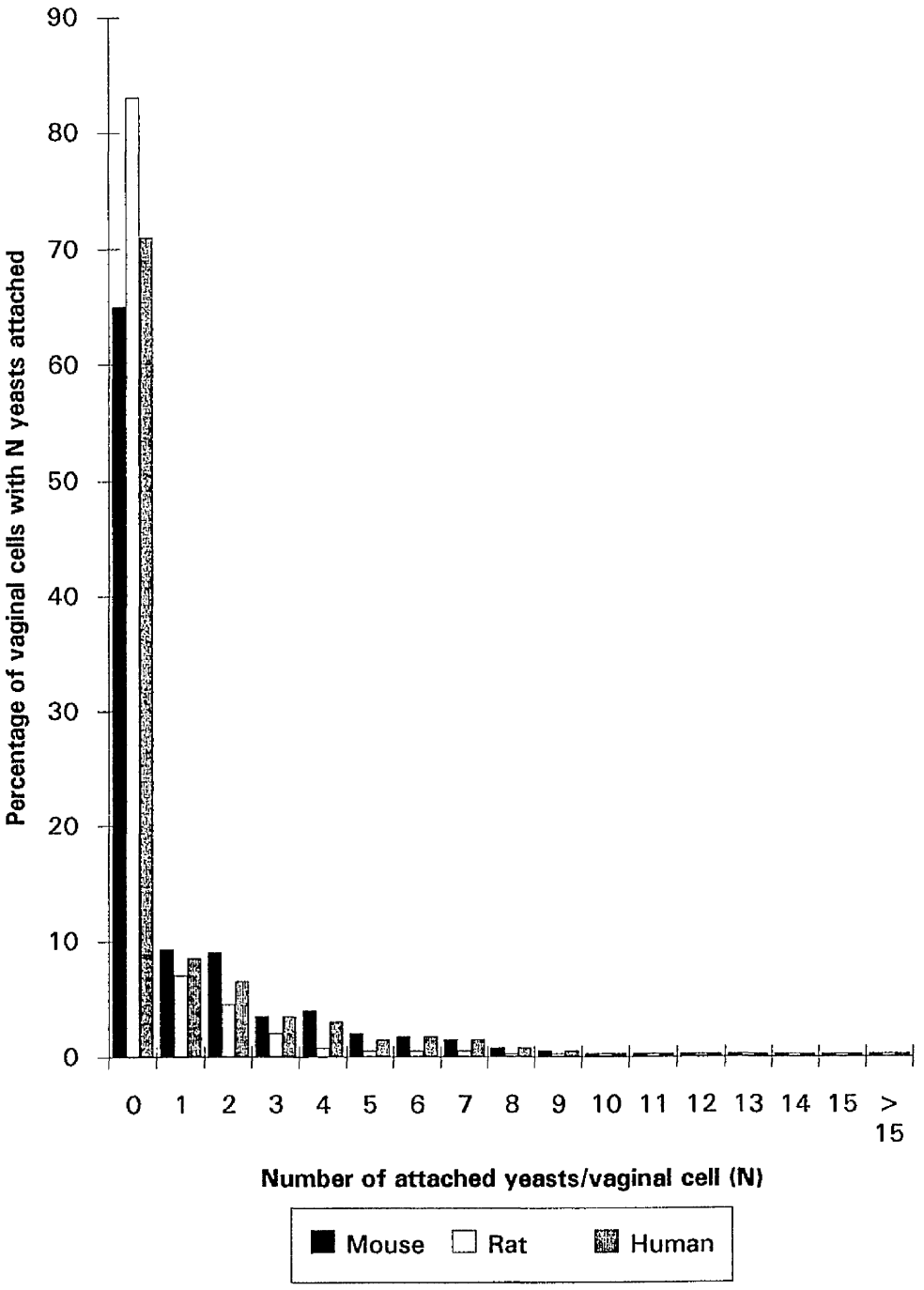
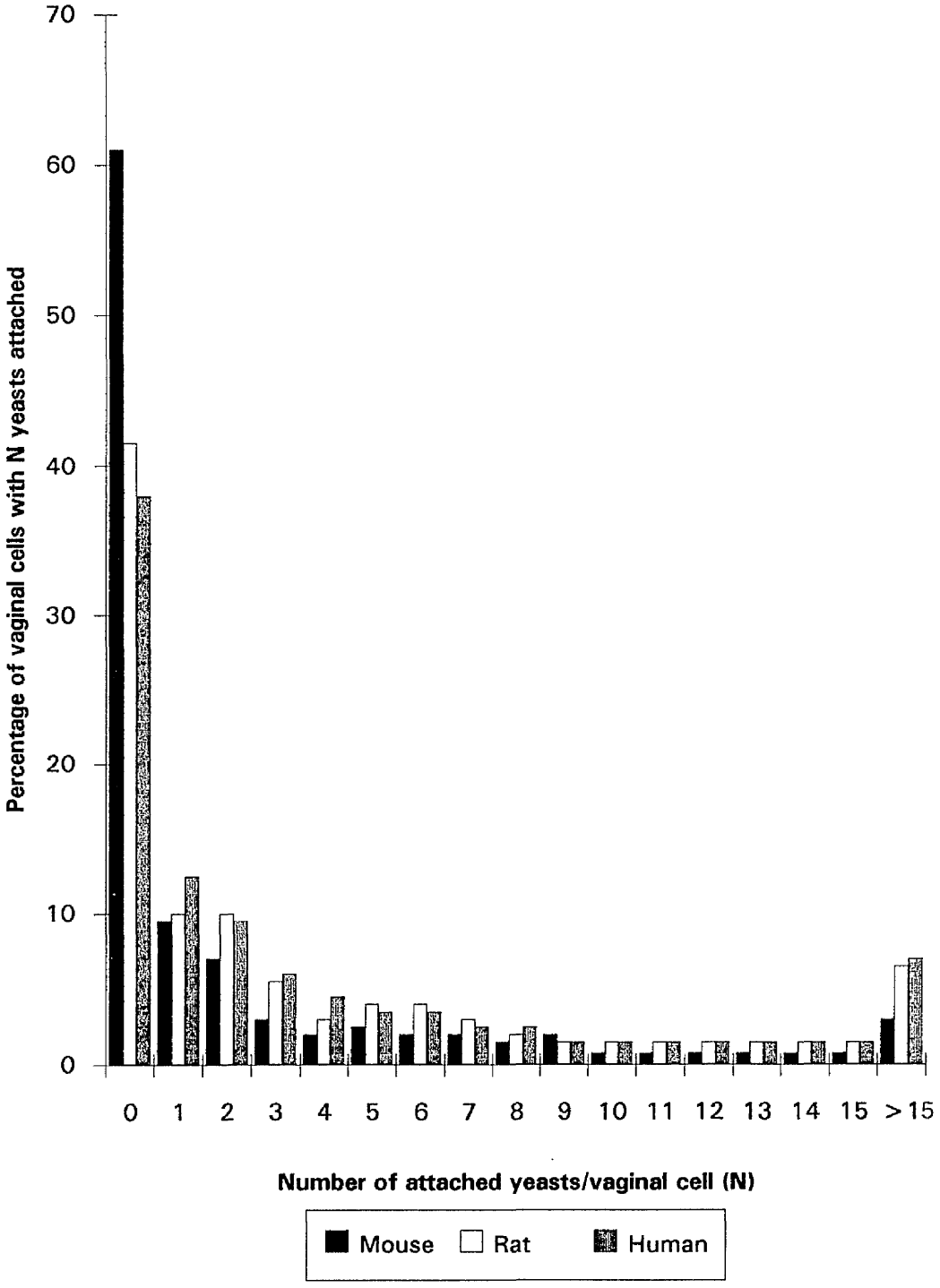


Figure 5.

Distribution of galactose-grown *C. albicans* GDH 2346 to mouse, rat and human vaginal epithelial cells (VEC) by number of adhering yeasts/cell



galactose-grown yeasts. It appeared that sub-populations of vaginal epithelial cells varied in their ability to bind *C. albicans*. To test this hypothesis the vaginal cells were segregated into two arbitrary sub-groups, those cells with 0 to 5 attached yeasts per cell (Group A) and those with 6 or more yeasts each (Group B). With galactose-grown *Candida* only 13.8%, 23.5% and 28.3% respectively of mouse, rat and human epithelial cells had 6 or more yeasts attached but to this small fraction of vaginal cells were bound 65.7%, 73.7% and 75.8% of all the yeasts that had adhered to each particular epithelial cell type. Thus, for galactose-grown *Candida* most of the yeasts were attached to a small group of highly receptive vaginal cells.

Yeasts grown in YNB medium supplemented with 50mM glucose adhered to epithelial cells in lower numbers than yeasts which had been grown in galactose medium. Only 6.3%, 3.5% and 6.3% respectively of mouse, rat and human epithelial cells had 6 or more yeasts attached. To these small fractions of mouse, rat and human vaginal epithelial cells were bound 52.1%, 37.6% and 43.6% of the total of adherent glucose-grown yeasts. Thus, for glucose-grown yeasts too, most of the *Candida* were attached to a small group of highly receptive vaginal cells.

A further comparison of the adhesion of yeasts to vaginal epithelial cells from different species was made. The adherence of 3 strains of *C. albicans* (yeast cell concentration 5×10^7 cells/ml) to mouse, rat and human vaginal epithelial cells was determined after growth of the yeasts in either 50mM glucose or 500mM galactose YNB medium. The three strains were used in different aspects of this investigation into the interactions between *C. albicans* and lactobacilli. Strain GDH 2346 was used in adhesion assays. Its

adherence properties had been previously well characterized (McCourtie & Douglas,1984). Strain B2630, originally from Janssen, was used in animal work. The strain was of unknown origin but had previously been used in animal work involving the mouse model (ICI Pharmaceuticals) and the rat model (SmithKline Beecham) and so its pathogenicity was well characterized. Strain B079, of unknown origin, was obtained from SmithKline Beecham and was used as a test organism in plate bioassays.

Results in Table 9 show that the three strains vary slightly in their ability to adhere. Yeasts harvested from medium containing 500mM galactose adhered in greater numbers than glucose-grown yeasts. Strain B2630 showed the greatest modification of adherence after growth in galactose medium, giving the highest relative adherence values and also the highest absolute adherence value. Strain B079 showed least modification of adherence after growth in galactose medium. However, this strain gave the highest absolute adherence value when grown in glucose medium

Glucose- and galactose-grown *Candida* adhered in greatest numbers to vaginal epithelial cells of human origin. Adhesion of yeasts to rat cells showed the greatest increase after growth in high sugar medium.

1.2 Comparison of VEC from normal and diabetic mice

1.2.1 Adherence of 6 strains of *C. albicans* to normal mouse vaginal epithelial cells

The adherence of 6 strains of *C. albicans* (yeast cell concentration 5×10^8 cells/ml) to mouse vaginal epithelial cells was compared after growth of the yeasts in either 50mM glucose or 500mM galactose YNB medium. Four of the strains had been isolated from active

Table 9.

Adherence of three strains of *C. albicans* to mouse, rat and human vaginal epithelial cells (VEC) after growth in YNB medium containing 50mM glucose or 500mM galactose as the carbon source

EPITHELIAL CELL TYPE	YEAST STRAIN	CARBON SOURCE	MEAN NO. (±SEM[a]) OF ADHERENT YEASTS PER 100 VEC	RELATIVE ADHERENCE[b]
Mouse	GDH 2346	Glucose	129.0 ± 15.3	1.00
		Galactose	330.9 ± 37.6	2.60
Rat	GDH 2346	Glucose	79.9 ± 17.5	1.00
		Galactose	359.8 ± 76.8	4.50
Human	GDH 2346	Glucose	158.7 ± 17.0	1.00
		Galactose	511.6 ± 42.1	3.22
Mouse	B 2630	Glucose	105.3 ± 21.4	1.00
		Galactose	445.3 ± 28.7	4.23
Rat	B 2630	Glucose	60.7 ± 5.7	1.00
		Galactose	311.7 ± 64.5	5.14
Human	B 2630	Glucose	162.2 ± 22.5	1.00
		Galactose	564.9 ± 60.8	3.48
Mouse	B 079	Glucose	169.8 ± 48.6	1.00
		Galactose	347.5 ± 34.7	2.05
Rat	B 079	Glucose	75.0 ± 20.2	1.00
		Galactose	239.8 ± 39.2	3.20
Human	B 079	Glucose	272.7 ± 101.8	1.00
		Galactose	430.0 ± 56.7	1.60

[a] SEM, standard error of the mean.

[b] Adherence relative to that of 50mM glucose-grown yeasts of the same strain to the same epithelial cell type.

infections; GDH 2346, GDH 2023, NCPF 3153 and 'outbreak' strain, strains GRI 681 and GRI 682 were isolated from symptomless carriers.

Yeasts originally isolated from active infections after growth in either glucose or galactose medium adhered to mouse vaginal epithelial cells in greater numbers than the carrier strains. For each strain the adherence of organisms grown in YNB medium containing 500mM galactose was compared with that of yeasts harvested from medium containing 50mM glucose (Table 10). Four of the 6 strains showed significantly increased adherence when 500mM galactose was present in the growth medium, but to slightly different extents; *C. albicans* GDH 2023 gave the highest relative adherence value. The 'outbreak' strain demonstrated increased adherence after growth on galactose but the increase was not significant by the Student t-test. With strain GRI 681 growth of yeasts in galactose medium produced no significant effect on adhesion.

This confirms that some strains have a potential for cell-surface modification when grown in high galactose containing medium.

The distribution of adherent yeasts on vaginal epithelial cells was investigated for the different strains of *C. albicans* after growth in 50mM glucose or 500mM galactose (Appendix 1). Data analysed by the Chi-squared statistical test showed growth in 500mM galactose modified the distribution of yeasts on vaginal epithelial cells to various extents for all the strains tested except GRI 681. Modification of the distribution of yeasts on vaginal epithelial cells by growth of *Candida* in galactose medium was shown as a decreased number of epithelial cells with adherent yeasts absent and increased numbers of vaginal cells with more than 16 adherent *C. albicans*.

Table 10.

Adherence of six strains of *C. albicans* to mouse vaginal epithelial cells (VEC) after growth in YNB medium containing 50mM glucose or 500mM galactose as the carbon source

STRAIN	CARBON SOURCE	MEAN NO. (± SEM[a]) OF ADHERENT YEAST PER 100 VEC	RELATIVE ADHERENCE[b]	P[c]
GDH 2346	Glucose	93.2 ± 39.9	1.0	
	Galactose	384.4 ± 62.5	4.2	<0.001
GDH 2023	Glucose	213.8 ± 39.8	1.0	
	Galactose	918.4 ± 123.8	4.3	<0.001
NCPF 3153	Glucose	248.7 ± 65.9	1.0	
	Galactose	541.4 ± 67.6	2.2	0.005
'Outbreak' Strain	Glucose	284.8 ± 39.5	1.0	
	Galactose	392.9 ± 70.6	1.4	NS[d]
GRI 681	Glucose	86.4 ± 18.9	1.0	
	Galactose	78.2 ± 12.4	0.9	NS
GRI 682	Glucose	83.9 ± 15.7	1.0	
	Galactose	168.4 ± 28.2	2.0	0.010

[a] SEM, standard error of the mean.

[b] Adherence relative to that of 50mM glucose-grown cells of the same strain.

[c] Probability values comparing adherence of glucose- and galactose-grown yeasts.

[d] NS, not significant.

1.2.2 Adherence of 6 strains of *C. albicans* to vaginal epithelial cells from diabetic mice

Mice were injected intravenously with alloxan to produce a condition of chemically induced diabetes mellitus. Alloxan gives a state of permanent hyperglycaemia by means of its cytotoxicity to the pancreatic islet B cells. To confirm that mice had become diabetic the presence of glucose in the urine was tested. Vaginal epithelial cells were obtained from diabetic mice brought into pseudoestrous by hormone treatment with 17 β-oestradiol-3-benzoate. Epithelial cells from different animals were pooled before use in adhesion assays.

The adherence of 6 strains of *C. albicans* (yeast cell concentration 5 x 10^8 cells/ml) to diabetic mouse vaginal cells was compared after growth of the yeasts in either 50mM glucose or 500mM galactose YNB medium. Four of the strains, GDH 2346, GDH 2023, NCPF 3153 and 'outbreak' were isolated from active infections while the other two, GRI 681 and GRI 682, were from symptomless carriers.

Yeasts originally isolated from active infections after growth in either glucose or galactose medium adhered to diabetic mouse vaginal epithelial cells in greater numbers than carrier strains (Table 11). All the strains showed increased adherence when 500mM galactose was present in the growth medium, but to slightly different extents. *C. albicans* GDH 2346 gave the highest relative adherence value; however, absolute adherence values were highest with strain GDH 2023. The 'outbreak' strain and strain GRI 681 both demonstrated increased adherence value for galactose-grown yeasts compared with glucose-grown yeasts but these values were not significant by the Student t-test.

Table 11.

Adherence of six strains of *C. albicans* to vaginal epithelial cells (VEC) from diabetic mice after growth of the yeasts in YNB medium containing 50mM glucose or 500mM galactose as the carbon source

STRAIN	CARBON SOURCE	MEAN NO. (± SEM[a]) OF ADHERENT YEASTS PER 100 VEC	RELATIVE ADHERENCE[b]	P[c]
GDH 2346	Glucose	223.3 ± 29.1	1.0	
	Galactose	691.0 ± 124.9	3.1	<0.001
GDH 2023	Glucose	495.9 ± 83.7	1.0	
	Galactose	1028.5 ± 165.9	2.1	<0.005
NCPF 3153	Glucose	231.0 ± 41.8	1.0	
	Galactose	621.0 ± 86.8	2.7	<0.001
'Outbreak' Strain	Glucose	287.3 ± 64.4	1.0	
	Galactose	516.7 ± 108.0	1.8	NS[d]
GRI 681	Glucose	139.4 ± 27.2	1.0	
	Galactose	254.0 ± 37.3	1.8	NS
GRI 682	Glucose	132.5 ± 26.9	1.0	
	Galactose	367.0 ± 65.7	2.8	<0.001

[a] SEM, standard error of the mean.

[b] Adherence relative to that of 50mM glucose-grown cells of the same strain.

[c] Probability values comparing adherence of glucose- and galactose-grown yeasts.

[d] NS, not significant.

The distribution of adherent yeasts on diabetic mouse vaginal epithelial cells was investigated for the different strains of *C. albicans* after growth in 50mM glucose or 500mM galactose (Appendix 2); data analysed by the Chi-square test showed growth of *Candida* in galactose medium modified the distribution of yeasts on vaginal epithelial cells to various extents for all of the strains tested. Modification of the distribution of yeasts on vaginal epithelial cells by growth of *Candida* in galactose medium was shown by increased numbers of vaginal cells with more than 16 adherent *C. albicans*.

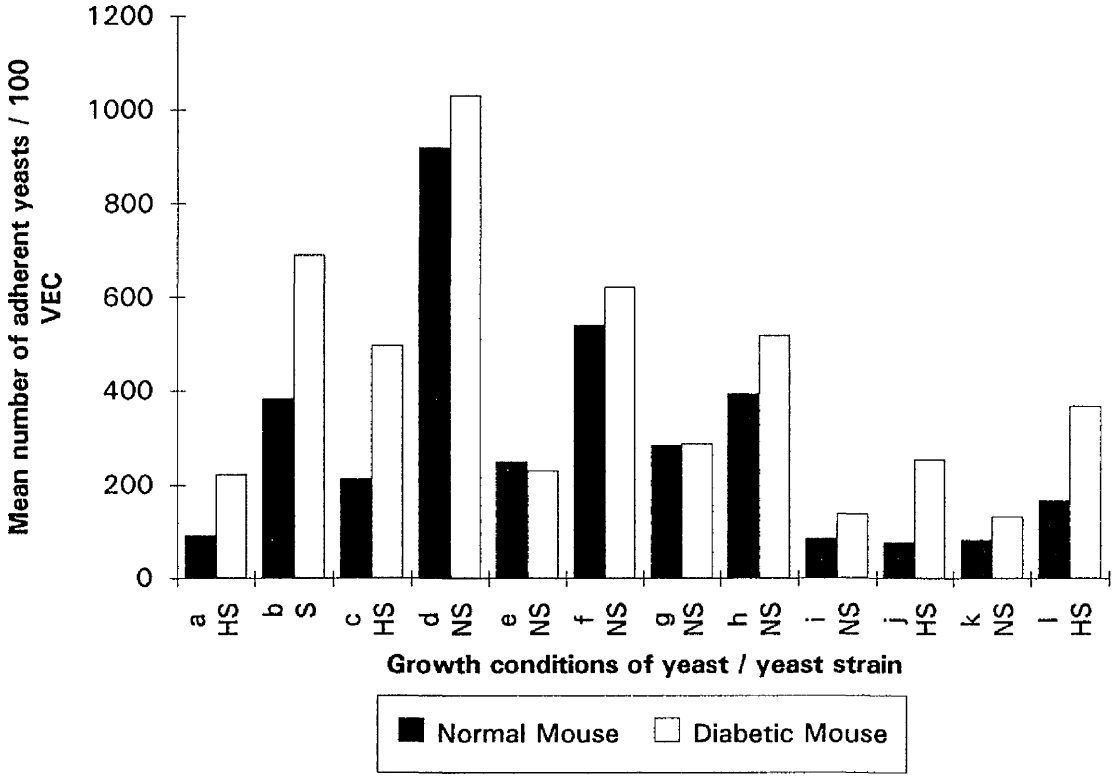
1.2.3 Comparison of yeast adhesion to normal and diabetic vaginal epithelial cells

Figure 6 compares the adherence of six strains of *C. albicans* to vaginal epithelial cells from normal or diabetic mice. Adhesion to diabetic mouse vaginal cells compared with cells from normal mice was increased for all yeast strains with the exception of the NCPF 3153 and 'outbreak' strains.

Adherence of glucose-grown strains GDH 2346 and GDH 2023 to diabetic mouse cells was enhanced by over 50% (58.1 and 56.8% respectively) compared with their adherence to normal epithelial cells. For these strains, the increase with galactose-grown yeasts was not as great; however, absolute adherence values were highest with these strains suggesting adherent cell numbers may have reached a maximum. Adherence of glucose-grown carrier strains, GRI 681 and 682, to diabetic mouse epithelial cells was enhanced by 38.0 and 36.6%, an increase not significant by the Student t-test; for galactose-grown carrier strains adhesion was increased by 69.2 and 54.1% respectively, a highly significant increase by the Student t-test.

Figure 6.

Adherence of six strains of *C. albicans* to vaginal epithelial cells (VEC) from normal or diabetic mice



Key:

a	Glucose-grown GDH 2346
b	Galactose-grown GDH 2346
c	Glucose-grown GDH 2023
d	Galactose-grown GDH 2023
e	Glucose-grown NCPF 3153
f	Galactose-grown NCPF 3153
g	Glucose-grown 'outbreak' strain
h	Galactose-grown 'outbreak' strain
i	Glucose-grown GRI 681
j	Galactose-grown GRI 681
k	Glucose-grown GRI 682
l	Galactose-grown GRI 682

HS	Highly Significant, by the Student t-Test
S	Significant, by the Student t-Test
NS	Not Significant, by the Student t-Test

These results suggest that vaginal epithelial cells from diabetic animals are modified in some way which allows increased adhesion of some *Candida* strains.

1.3 Effect of steroid hormone pretreatment

1.3.1 Growth of *Candida* in the presence of steroids

The adherence of *C. albicans* to mouse, rat and human vaginal epithelial cells was determined after growth of yeasts to stationary phase in defined media containing different steroid hormones (1μM). The adherence of organisms grown in medium containing hormones was compared with that of yeasts harvested from a control medium without hormones. The effect of hormone treatment on the adherence of organisms grown in medium with a relatively low concentration of glucose (50mM; 0.9% w/v) was compared with that on yeasts harvested from media containing a high concentration (500mM; 9.0% w/v) of galactose.

Adherence of *C. albicans* GDH 2346, a denture stomatitis isolate, was slightly enhanced after growth in 500mM galactose medium containing oestradiol or progesterone. Yeasts grown in 50mM glucose medium containing hormone showed slight decrease in adherence to vaginal epithelial cells compared to control organisms (Table 12). These differences in mean numbers of adherent yeasts per 100 vaginal epithelial cells were not found to be significant by the Student t-test.

From the data collected, the effect of steroid hormones on the distribution of adherent *Candida* to mouse, rat and human vaginal epithelial cells was evaluated (Appendix 3). With *Candida* grown in 50mM glucose medium, approximately 80% of the vaginal epithelial cells had no adherent *Candida*. Yeasts grown in 500mM galactose

Table 12.

Adherence of *C. albicans* GDH 2346 to mouse, rat and human vaginal epithelial cells (VEC) after growth in the presence of steroid hormones

EPITHELIAL CELL TYPE	TREATMENT[a]	CARBON SOURCE[b]	MEAN NO. (±SEM[c]) OF ADHERENT YEASTS PER 100 VEC	RELATIVE ADHERENCE[d]
Mouse	Oestradiol	Glucose	66.8 ± 10.5	0.93
		Galactose	291.6 ± 22.5	1.04
	Progesterone	Glucose	57.2 ± 13.9	0.79
		Galactose	356.2 ± 33.0	1.27
	Control	Glucose	72.0 ± 11.1	1.00
		Galactose	279.8 ± 21.0	1.00
Rat	Oestradiol	Glucose	45.4 ± 12.8	0.74
		Galactose	359.4 ± 35.1	1.24
	Progesterone	Glucose	57.3 ± 6.8	0.94
		Galactose	345.6 ± 43.6	1.19
	Control	Glucose	61.0 ± 7.6	1.00
		Galactose	289.9 ± 28.4	1.00
Human	Oestradiol	Glucose	46.8 ± 4.8	0.93
		Galactose	281.6 ± 22.4	1.29
	Progesterone	Glucose	49.8 ± 5.0	0.99
		Galactose	263.9 ± 26.7	1.21
	Control	Glucose	50.1 ± 7.1	1.00
		Galactose	218.1 ± 15.9	1.00

[a] Yeasts were grown to stationary phase in medium which contained a 1μM concentration of steroid hormone which had been originally solubilized in methanol. Control yeasts were grown in medium containing an equivalent concentration of methanol without the presence of steroid hormone.

[b] Yeasts were grown in YNB medium containing either 50mM glucose or 500mM galactose as the carbon source.

[c] SEM, standard error of the mean.

[d] Adherence relative to that of control cells grown in YNB medium containing the same carbon source.

attached in greater numbers than 50mM glucose-grown organisms and only 44% of vaginal epithelial cells were without *Candida*. However, yeasts grown with different sugar sources showed no significant change in distribution on the vaginal epithelial cells due to the presence of steroid hormones.

1.3.2 Pretreatment of VEC

Steroid hormones were examined for their effect on the adherence of *C. albicans* GDH 2346 to rat vaginal epithelial cells. Rat cells were pretreated with a solution of steroid hormone (1μM) for 30 min at 37°C and then washed and resuspended in PBS for use in adherence assays. Such pretreatment gave a reduction in the mean number of adherent *Candida* per 100 vaginal epithelial cells compared to control values (Table 13). These reductions were not significant by the Student t-Test.

The effect of steroid hormones on the distribution of adherent *Candida* to hormone pretreated rat vaginal epithelial cells was investigated (Appendix 4). Hormone treatment of epithelial cells had no significant effect on the distribution of adherent glucose-grown *Candida* compared with the control. On average, with *Candida* grown in 50mM glucose medium, 71% of the vaginal epithelial cells had no adherent organisms.

Yeasts grown in 500mM galactose YNB attached in greater numbers than 50mM glucose-grown *Candida*, and, on average, only 12% of vaginal epithelial cells had *Candida* absent. The distribution of galactose-grown *Candida* on hormone treated rat vaginal epithelial cells was significantly different from the distribution on control epithelial cells ($P < 0.01$).

Table 13.

Effect of pretreatment of rat vaginal epithelial cells (VEC) with steroid hormones on *Candida* adhesion

PRETREATMENT[a]	CARBON SOURCE[b]	MEAN NO. (± SEM[c]) OF ADHERENT YEASTS PER 100 VEC	PERCENTAGE INHIBITION OF ADHERENCE[d]
Oestradiol	Glucose	128.1 ± 34.3	16.7
	Galactose	1146.1 ± 151.5	25.5
Progesterone	Glucose	106.3 ± 26.1	30.9
	Galactose	1389.9 ± 136.1	9.7
Control	Glucose	153.8 ± 30.0	
	Galactose	1538.8 ± 116.5	

[a] Prior to adhesion assays, vaginal epithelial cells were incubated for 30 min at 37°C in PBS which contained a 1μM concentration of steroid hormone which had been originally solubilized in methanol. Control cells were incubated in PBS containing an equivalent concentration of methanol without the steroid hormone. After this pretreatment, vaginal cells were washed, resuspended in PBS and used in adhesion assays.

[b] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[c] SEM, standard error of the mean.

[d] Calculated as the percentage of yeasts not adhering to hormone pretreated epithelial cells when compared with adherence to control epithelial cells.

Oestradiol pretreatment gave an increase in the number of epithelial cells with no adherent yeasts or less than 6 attached *Candida* per epithelial cell. Progesterone was also seen to affect the *Candida* distribution, with an increased number of epithelial cells containing 1 to 5 adherent yeasts and a decrease in the number of epithelial cells with more than 16 yeasts. The effect on *Candida* distribution was not as marked with progesterone as with oestradiol.

1.3.3 Pretreatment of *Candida*

Steroid hormones were examined for their effect on the adherence of *C. albicans* GDH 2346 to rat vaginal epithelial cells. The yeasts were suspended in a solution of steroid hormone (1μM) for 30 min at 37°C then washed and resuspended in PBS. Treated yeasts were used in the adherence assay.

Hormone treatment gave a reduction in the mean number of adherent *Candida* per 100 vaginal epithelial cells compared to the control values (Table 14). Again, the difference was not significant by the Student t-test.

However, steroid hormone pretreatment of the yeasts did have an effect on their distribution over the epithelial cells (Appendix 5). The distribution of both 50mM glucose-grown and 500mM galactose-grown *Candida* was affected. A similar effect was seen with progesterone and oestradiol. In all cases there was an increase in the number of epithelial cells with no adherent yeasts and fewer epithelial cells attached 16+ *Candida*. The Chi-square statistic showed these differences to be significant.

Table 14.

Effect of pretreatment of *Candida* with steroid hormones on yeast adhesion to rat vaginal epithelial cells

PRETREATMENT[a]	CARBON SOURCE[b]	MEAN NO. (±SEM[c]) OF ADHERENT YEASTS PER 100 VEC	PERCENTAGE INHIBITION OF ADHERENCE[d]
Oestradiol	Glucose	157.1 ± 33.7	32.6
	Galactose	893.3 ± 110.1	13.9
Progesterone	Glucose	154.0 ± 37.5	33.9
	Galactose	789.1 ± 88.9	23.9
Control	Glucose	233.1 ± 53.6	
	Galactose	1037.0 ± 132.1	

[a] Prior to adhesion assays, yeasts were incubated for 30 min at 37°C in PBS which contained a 1μM concentration of steroid hormone which had been originally solubilized in methanol. Control cells were incubated in PBS containing an equivalent concentration of methanol without the steroid hormone. After this pretreatment, yeasts were washed, resuspended in PBS and used in adhesion assays.

[b] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[c] SEM, standard error of the mean.

[d] Calculated as the percentage of hormone pretreated yeasts not adhering to epithelial cells when compared with adherence of control yeasts.

1.4 Effect of Lactobacilli

1.4.1 Preliminary experiments

1.4.1.1 Pretreating vaginal epithelial cells with *L. acidophilus*

Prior to adhesion assays, vaginal epithelial cells were incubated for 30 min in PBS or in a suspension of *L. acidophilus* ($5x10^8$ cells/ml). After this pretreatment, vaginal cells were washed to remove any non-adherent lactobacilli, resuspended in PBS and used in adhesion assays (see Materials and Methods Section 3.3.3.). *L. acidophilus* was used to pretreat mouse (Table 15), rat (Table 16) and human (Table 17) vaginal epithelial cells. *L. acidophilus* inhibited *Candida* adhesion to vaginal epithelial cells from different sources to different extents. Inhibition was greatest with mouse VEC, followed by rat cells, with least inhibition of yeast adhesion to human vaginal epithelial cells. Only the inhibition of adhesion to mouse vaginal epithelial cells was significant by the Student t-Test ($P < 0.02$).

The Chi-square test showed that the distribution of adherent yeasts on *Lactobacillus*-pretreated epithelial cells was significantly different from that of yeasts on control vaginal cells. This was the case for all the epithelial cell types (Appendix 6).

For all epithelial cell types, inhibition of galactose-grown yeast adhesion by lactobacilli (mouse 38.3%; rat 27.6%; human 23.7%) was less than the inhibition of glucose-grown yeast adhesion (mouse 48.1%; rat 36.7%; human 29.2%).

1.4.1.2 Pretreating vaginal epithelial cells with *L. fermentum*

In another series of experiments different vaginal epithelial cell types - mouse (Table 18), rat (Table 19) and human (Table 20) were pretreated with *L. fermentum* ($5x10^8$ cells/ml) prior to use in

Table 15.

Inhibition by *L. acidophilus* of *Candida* adhesion to mouse vaginal epithelial cells (VEC)

PRETREATMENT[a]	CARBON SOURCE[b]	MEAN NO. (± SEM[c]) OF ADHERENT YEASTS PER 100 VEC	PERCENTAGE INHIBITION OF ADHERENCE[d]
Lactobacilli	Glucose	63.3 ± 4.1	48.1
PBS	Glucose	108.8 ± 16.4	
Lactobacilli	Galactose	152.2 ± 25.4	38.3
PBS	Galactose	246.8 ± 19.6	

[a] Prior to adhesion assays, vaginal epithelial cells were incubated for 30 min at 37°C in PBS or in a suspension of *L. acidophilus* NCTC 4504 (5×10^8 cells/ml). After this pretreatment, vaginal cells were washed to remove any non-adherent lactobacilli, resuspended in PBS and used in adhesion assays.

[b] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[c] SEM, standard error of mean.

[d] Calculated as the percentage inhibition compared with adherence to PBS-pretreated epithelial cells.

Table 16.

Inhibition by *L. acidophilus* of *Candida* adhesion to rat vaginal epithelial cells (VEC)

PRETREATMENT[a]	CARBON SOURCE[b]	MEAN NO. (± SEM[c]) OF ADHERENT YEASTS PER 100 VEC	PERCENTAGE INHIBITION OF ADHERENCE[d]
Lactobacilli	Glucose	49.1 ± 13.8	36.7
PBS	Glucose	77.6 ± 17.3	
Lactobacilli	Galactose	183.5 ± 33.3	27.6
PBS	Galactose	253.4 ± 56.7	

[a] Prior to adhesion assays, vaginal epithelial cells were incubated for 30 min at 37°C in PBS or in a suspension of *L. acidophilus* NCTC 4504 (5×10^8 cells/ml). After this pretreatment, vaginal cells were washed to remove any non-adherent lactobacilli, resuspended in PBS and used in adhesion assays.

[b] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[c] SEM, standard error of the mean.

[d] Calculated as the percentage inhibition when compared with adherence to PBS-pretreated epithelial cells.

Table 17.

Inhibition by *L. acidophilus* of *Candida* adhesion to human vaginal epithelial cells (VEC)

PRETREATMENT[a]	CARBON SOURCE[b]	MEAN NO. (± SEM[c]) OF ADHERENT YEASTS PER 100 VEC	PERCENTAGE INHIBITION OF ADHERENCE[d]
Lactobacilli	Glucose	143.1 ± 22.9	29.2
PBS	Glucose	202.0 ± 32.9	
Lactobacilli	Galactose	491.9 ± 32.6	23.7
PBS	Galactose	644.6 ± 74.6	

[a] Prior to adhesion assays, vaginal epithelial cells were incubated for 30 min at 37°C in PBS or in a suspension of *L. acidophilus* NCTC 4504 (5×10^8 cells/ml). After this pretreatment, vaginal cells were washed to remove any non-adherent lactobacilli, resuspended in PBS and used in adhesion assays.

[b] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[c] SEM, standard error of the mean.

[d] Calculated as the percentage inhibition when compared with adherence to PBS-pretreated epithelial cells.

Table 18.

Inhibition by *L. fermentum* of *Candida* adhesion to mouse vaginal epithelial cells (VEC)

PRETREATMENT[a]	CARBON SOURCE[b]	MEAN NO. (± SEM[c]) OF ADHERENT YEASTS PER 100 VEC	PERCENTAGE INHIBITION OF ADHERENCE[d]
Lactobacilli	Glucose	60.5 ± 19.5	34.9
PBS	Glucose	92.9 ± 31.8	
Lactobacilli	Galactose	244.1 ± 44.1	36.9
PBS	Galactose	386.6 ± 67.6	

[a] Prior to adhesion assays, vaginal epithelial cells were incubated for 30 min at 37°C in PBS or in a suspension of *L. fermentum* (5 x 10^8 cells/ml). After this pretreatment, vaginal cells were washed to remove any non-adherent lactobacilli, resuspended in PBS and used in adhesion assays.

[b] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[c] SEM, standard error of the mean.

[d] Calculated as the percentage inhibition when compared with adherence to PBS-pretreated epithelial cells.

Table 19.

Inhibition by *L. fermentum* of *Candida* adhesion to rat vaginal epithelial cells (VEC)

PRETREATMENT[a]	CARBON SOURCE[b]	MEAN NO. (± SEM[c]) OF ADHERENT YEASTS PER 100 VEC	PERCENTAGE INHIBITION OF ADHERENCE[d]
Lactobacilli	Glucose	43.2 ± 11.9	37.3
PBS	Glucose	68.9 ± 20.8	
Lactobacilli	Galactose	325.6 ± 42.1	24.5
PBS	Galactose	430.3 ± 67.9	

[a] Prior to adhesion assays, vaginal epithelial cells were incubated for 30 min at 37°C in PBS or in a suspension of *L. fermentum* (5 x 10^8 cells/ml). After this pretreatment, vaginal cells were washed to remove any non-adherent lactobacilli, resuspended in PBS and used in adhesion assays.

[b] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[c] SEM, standard error of the mean.

[d] Calculated as the percentage inhibition when compared with adherence to PBS-pretreated epithelial cells.

Table 20.

Inhibition by *L. fermentum* of *Candida* adhesion to human vaginal epithelial cells (VEC)

PRETREATMENT[a]	CARBON SOURCE[b]	MEAN NO. (± SEM[c]) OF ADHERENT YEASTS PER 100 VEC	PERCENTAGE INHIBITION OF ADHERENCE[d]
Lactobacilli	Glucose	39.5 ± 10.9	61.0
PBS	Glucose	101.3 ± 23.6	
Lactobacilli	Galactose	342.1 ± 32.2	33.7
PBS	Galactose	516.1 ± 64.2	

[a] Prior to adhesion assays, vaginal epithelial cells were incubated for 30 min at 37°C in PBS or in a suspension of *L. fermentum* (5 x 10^8 cells/ml). After this pretreatment, vaginal cells were washed to remove any non-adherent lactobacilli, resuspended in PBS and used in adhesion assays.

[b] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[c] SEM, standard error of the mean.

[d] Calculated as the percentage inhibition when compared with adherence to PBS-pretreated epithelial cells.

adhesion assays and the percentage inhibition of adherence due to lactobacilli was calculated.

L. fermentum inhibited *Candida* adhesion to the different vaginal epithelial cell types to varying extents. The lactobacilli inhibited adhesion of glucose-grown yeasts to human VEC to the greatest extent (61.0%), followed by rat VEC (37.3%), with least inhibition of glucose-grown yeast adhesion to mouse vaginal epithelial cells (34.9%).

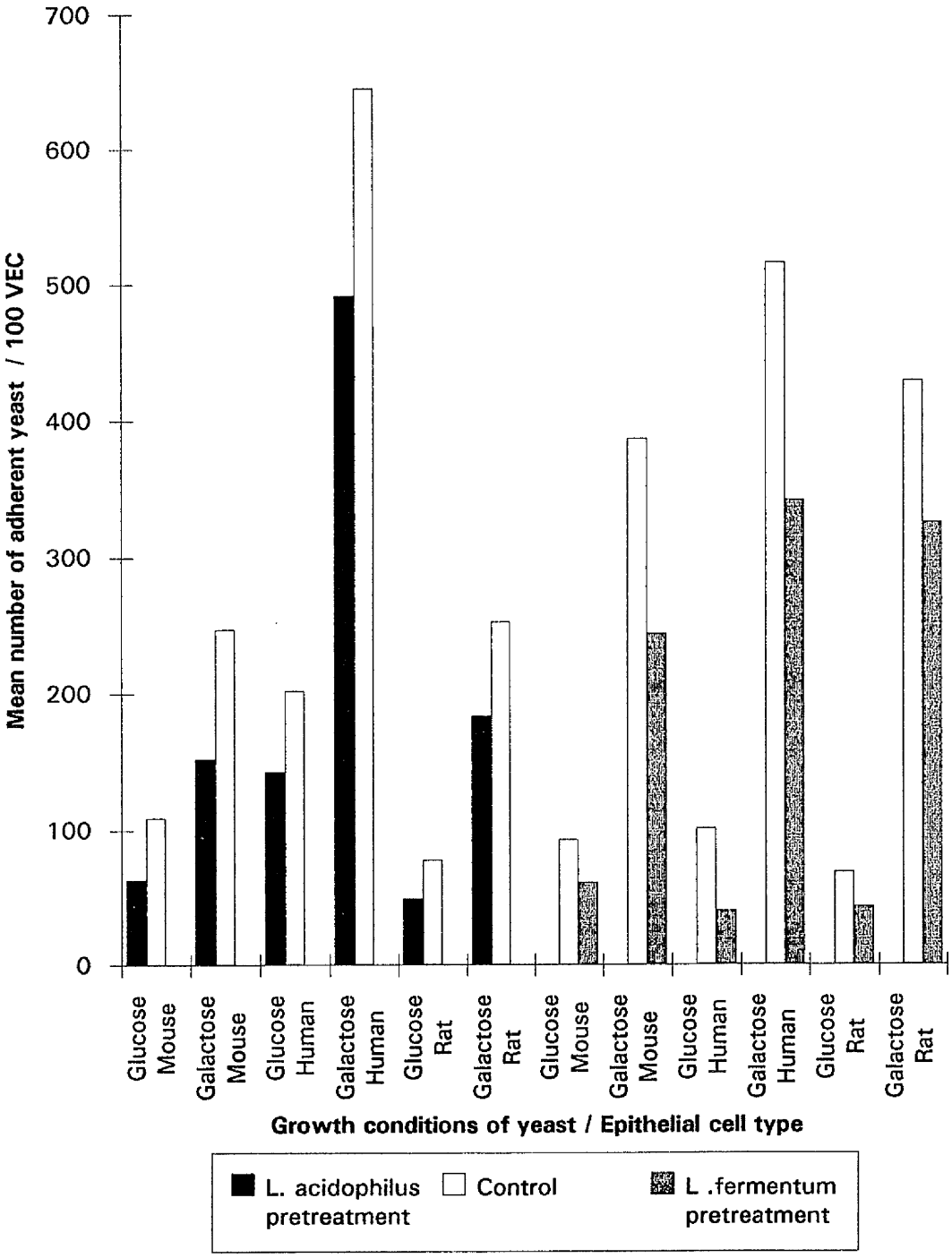
The greatest inhibition of galactose-grown yeasts was to mouse VEC (36.9%) and was similar to the inhibition of glucose-grown yeasts to mouse vaginal cells (34.9%). The inhibition of galactose-grown yeast adhesion to human VEC (33.7%) and rat vaginal cells (24.5%) was less than that seen for glucose-grown yeasts.

Only inhibition of *Candida* adhesion to human VEC was significant by the Student t-Test ($P < 0.05$). The Chi-square test showed the distribution of adherent yeasts on *Lactobacillus*-pretreated epithelial cells was significantly different from the distribution of yeast on control vaginal cells. This was the case for all the epithelial cell types (Appendix 7) except the adhesion of glucose-grown *Candida* to rat vaginal epithelial cells.

Results showing inhibition by lactobacilli of *Candida* adhesion to vaginal epithelial cells are summarised in Figure 7. From the histograms it can be seen that pretreatment of all epithelial cell types with either species of *Lactobacillus* reduces the mean number of adherent *Candida* per 100 VEC compared with control values. The different epithelial cell types have varying abilities to attach *Candida*. Human vaginal epithelial cells are able to bind *Candida* in greatest numbers. The histograms also show the ability of galactose-grown yeasts to attach in greater numbers than glucose-grown yeasts.

Figure 7.

Inhibition by lactobacilli of *Candida* adhesion to vaginal epithelial cells (VEC)



Despite these differences, in each case *Lactobacillus*-pretreatment reduces the mean number of adherent *Candida* per 100 VEC.

1.4.2 Influence of pH

Previous studies have shown that adherence of *C. albicans* to vaginal mucosal cells *in vitro* can be affected by the pH value of the suspending buffer (Persi *et al.*, 1985; King *et al.*, 1980; Sobel *et al.*, 1981). These studies gave an optimal pH value for adherence of 6-8. Mehentee & Hay (1989) suggested that the effect of pH on *C. albicans* adherence varied according to the source of the mucosal cell. Human vaginal cells come from an environment which is normally acidic (pH 4.5) and so the effect of low pH on *Candida* adhesion was investigated.

Suspensions of lactobacilli, vaginal epithelial cells and *Candida* were washed and standardized in either PBS (pH 7.2) or citrate buffer (pH 4.5). Pretreatment of vaginal cells with lactobacilli and adherence assays were carried out at pH 4.5 or 7.2. *Candida* adhered to human vaginal epithelial cells in greater numbers when the adhesion assay was carried out at pH 7.2 (Table 21). Increased adhesion at pH 7.2 was not significant by the Student t-Test, however the Chi-square test showed that the distribution of adherent yeasts was significantly different when the adhesion assay was carried out at pH 7.2, compared with pH 4.5 (Appendix 8).

Inhibition of galactose-grown yeast adhesion at pH 7.2 by *L. fermentum* and *L. acidophilus* was significant by the Student t-Test ($P < 0.02$). When the assay was carried out at pH 4.5 only the inhibition of galactose-grown yeast adhesion by *L. fermentum* was significant ($P < 0.05$). Inhibition of glucose-grown yeast adhesion was observed at pH 7.2, but not at pH 4.5.

Table 21.

Effect of pH on the inhibition by lactobacilli of *Candida* adhesion to human vaginal epithelial cells (VEC)

pH OF ASSAY[a]	PRETREATMENT[b]	CARBON SOURCE[c]	MEAN NO. (± SEM[d]) OF ADHERENT YEASTS PER 100 VEC	PERCENTAGE INHIBITION OF ADHERENCE[e]
4.5	*L. acidophilus*	Glucose	78.8 ± 8.2	0
		Galactose	506.7 ± 45.7	26.3
4.5	*L. fermentum*	Glucose	67.6 ± 11.2	6.6
		Galactose	471.4 ± 31.1	31.4
4.5	citrate buffer	Glucose	72.4 ± 8.1	-
		Galactose	687.6 ± 85.0	-
7.2	*L. acidophilus*	Glucose	97.1 ± 19.6	23.7
		Galactose	628.8 ± 27.5	29.9
7.2	*L. fermentum*	Glucose	76.4 ± 14.1	39.9
		Galactose	584.3 ± 50.0	34.8
7.2	PBS	Glucose	127.2 ± 26.3	-
		Galactose	896.6 ± 95.5	-

[a] Suspensions of lactobacilli, vaginal epithelial cells and *Candida* were washed and standardized in either PBS (pH 7.2) or citrate buffer (pH 4.5) before use in the adhesion assay.

[b] Prior to adhesion assays, vaginal epithelial cells were incubated for 30 min at 37°C in buffer (pH 7.2 or pH 4.5) or in a suspension of *Lactobacillus* (pH 7.2 or pH 4.5)(5×10^8 cells/ml). After this pretreatment, vaginal cells were washed to remove any non-adherent lactobacilli, resuspended in buffer (pH 7.2 or pH 4.5) and used in adhesion assays.

[c] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[d] SEM, Standard error of the mean.

[e] Calculated as the percentage of inhibition when compared with adherence to buffer (pH 7.2 or pH 4.5) pretreated epithelial cells.

Mehentee & Hay (1989) suggested that the effect of pH on *C. albicans* adherence was strain dependent. This experiment shows that adherence of *C. albicans* GDH 2346 to human vaginal cells is greater at pH 7.2 than at pH 4.5 and that the ability of lactobacilli to inhibit *Candida* adherence is also greater at pH 7.2. However, other yeast strains were not tested.

1.4.3 Influence of bacterial concentration

The inhibition of *Candida* adhesion to mouse VEC was investigated for different pretreatment concentrations in order to find the number of lactobacilli which would give the maximum inhibition of adhesion. Vaginal epithelial cells were treated with different concentrations of *L. acidophilus* or *L. fermentum*. It was found that increasing concentrations of lactobacilli gave increasing numbers of adherent lactobacilli per vaginal cell. *L. acidophilus* adhered in greater numbers than *L. fermentum* (Table 22).

Vaginal epithelial cells were treated with different concentrations of *L. acidophilus* or *L. fermentum* prior to incubation with *C. albicans* grown in medium with 50mM glucose or 500mM galactose (Figure 8). Maximum inhibition of glucose-grown *Candida* adhesion was obtained with *L. acidophilus* at the lowest pretreatment concentration. Any greater concentration gave inhibition at a similar level. At the lower pretreatment concentrations of *L. fermentum* (2.5×10^8 - 1×10^9 cells/ml) less inhibition of glucose-grown *Candida* adhesion was observed compared with that obtained with *L. acidophilus*. At the highest *Lactobacillus* pretreatment concentration (2×10^9 cells/ml), inhibition of *Candida* adhesion by *L. fermentum* reached a maximum (61%), at a level similar to that seen with *L. acidophilus* (58%).

Table 22.

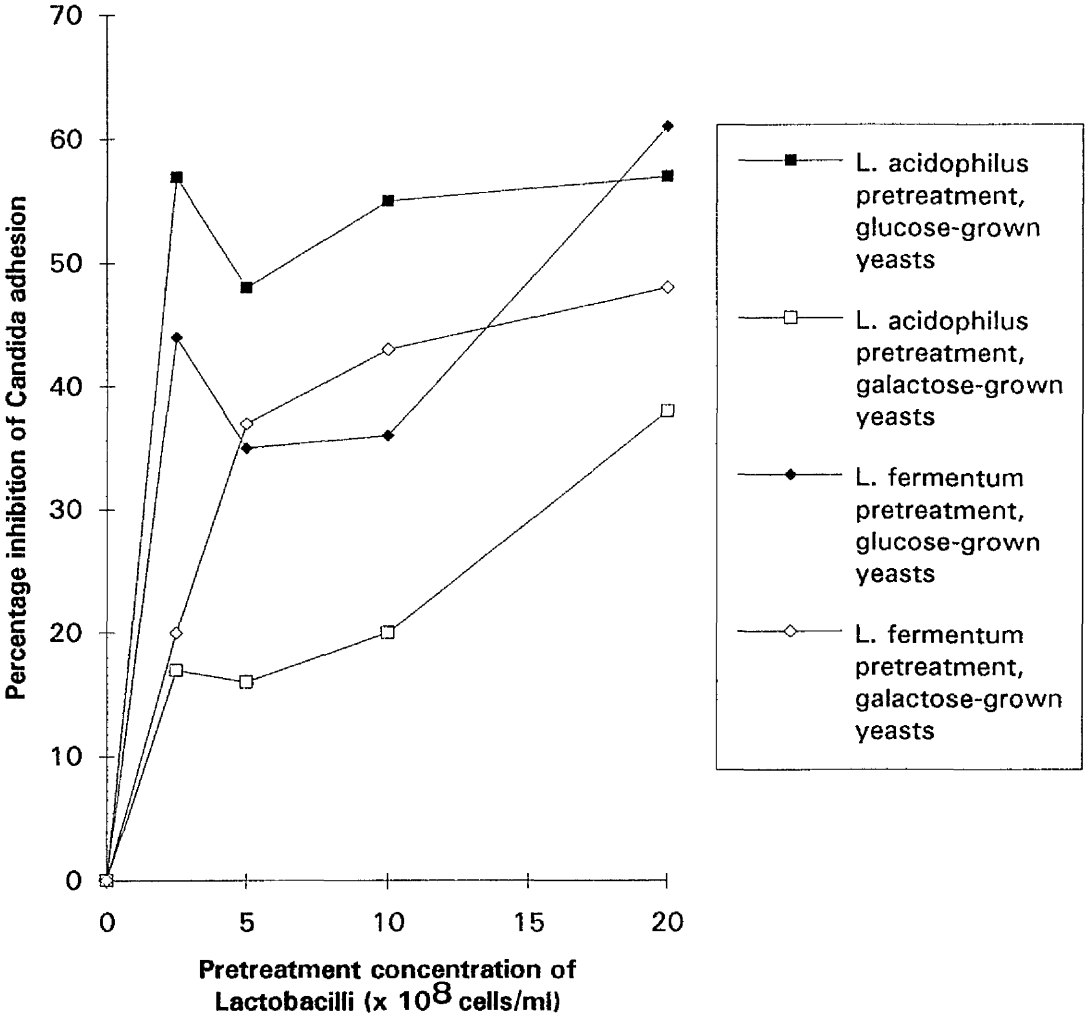
Effect of bacterial concentration on adhesion of lactobacilli to mouse vaginal epithelial cells (VEC)

LACTOBACILLUS SPECIES	TREATMENT CONCENTRATION (10^9 cells/ml)	MEAN NO. ADHERENT LACTOBACILLI/CELL±SEM[a]
L. acidophilus	2	11.25 ± 1.9
	1	10.47 ± 0.9
	0.5	9.14 ± 0.9
	0.25	6.97 ± 0.7
L. fermentum	2	10.45 ± 1.2
	1	5.88 ± 0.7
	0.5	4.80 ± 0.7
	0.25	3.67 ± 0.3
PBS Control	-	1.81 ± 0.3

[a] SEM, standard error of the mean.

Figure 8.

Pretreatment of mouse vaginal epithelial cells with different concentrations of lactobacilli



Vaginal epithelial cells were treated with different concentrations of *L. acidophilus* or *L. fermentum* prior to incubaction with *C. albicans* grown in medium with 50mM glucose or 500mM galactose. Experiments were carried out 3 times in triplicate. Each point represents the percentage inhibition of adherence when compared with adherence to PBS-pretreated epithelial cells.

For galactose-grown yeasts, an increase in the inhibition of *Candida* adhesion was observed with increasing pretreatment concentrations of lactobacilli. The maximum inhibition of galactose-grown yeast adhesion was seen with the highest concentration of lactobacilli. The maximum inhibition of galactose-grown *Candida* adhesion by *L. fermentum* (43%) was greater than the maximum inhibition obtained for *L. acidophilus* (33%). The maximum inhibition of adhesion by *L. fermentum* was 43% for galactose-grown yeasts compared with 61% for glucose-grown yeasts. For *L. acidophilus*, the maximum inhibition for galactose-grown yeasts was 33%, again lower than the figure of 58% inhibition observed with glucose-grown organisms.

1.4.4 Activity of culture supernatant

L. acidophilus and *L. fermentum* were grown to stationary phase in MRS broth and culture supernatants were concentrated 20-fold by freeze-drying. The concentrates were then used to pretreat mouse vaginal epithelial cells prior to their incubation with yeasts which had been grown in either 50mM glucose or 500mM galactose medium. In some experiments, an attempt was made to remove components from the culture supernatants which might interfere with the adhesion assay. This involved dialysis of the supernatants prior to concentration and then passage down a G25 desalting column.

The results (Table 23) showed that pretreatment of VEC with concentrated culture supernatant had no significant effect on the adhesion of *C. albicans* when compared with pretreatment with concentrated MRS broth. Dialysed, concentrated culture supernatants which had been passaged down a desalting column also had no significant effect on adhesion when compared with dialysed, desalted

Table 23.

Effect of concentrated *Lactobacillus* culture supernatant on *Candida* adhesion to mouse vaginal epithelial cells (VEC)

PRETREATMENT[a]	CARBON SOURCE[b]	MEAN NO. (± SEM[c]) OF ADHERENT YEASTS PER 100 VEC
L. acidophilus supernatant	Glucose	195.6 ± 37.4
	Galactose	673.1 ± 110.8
Dialysed, desalted *L. acidophilus* supernatant	Glucose	127.7 ± 30.6
	Galactose	609.2 ± 88.6
L. fermentum supernatant	Glucose	145.8 ± 31.9
	Galactose	642.6 ± 68.4
Dialysed, desalted *L. fermentum* supernatant	Glucose	137.9 ± 35.8
	Galactose	469.4 ± 58.9
MRS broth	Glucose	196.8 ± 40.5
	Galactose	580.6 ± 99.7
Dialysed, desalted MRS broth	Glucose	130.8 ± 46.4
	Galactose	509.4 ± 74.6
PBS	Glucose	115.9 ± 28.0
	Galactose	598.3 ± 80.3

[a] *L. acidophilus* and *L. fermentum* were grown to stationary phase in MRS broth and the culture supernatants were concentrated 20-fold by freeze-drying. The concentrates were then used to pretreat mouse vaginal epithelial cells prior to incubation with *C. albicans*. In some experiments an attempt was made to remove components from culture supernatants which might interfere with adhesion assays. The supernatants were dialysed before being concentrated, and passaged down a G25 desalting column.

[b] Yeasts (*C. albicans* GDH 2346) were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[c] SEM, standard error of the mean.

MRS broth control. However, pretreatment with MRS broth did have an effect on adhesion of glucose-grown yeasts when compared to pretreatment with PBS ($P < 0.05$). Components within the broth appeared to promote *Candida* adhesion to mouse vaginal epithelial cells. Dialysis of MRS and passage down a G25 column reduced this effect (Table 23). MRS broth had no effect on adhesion of galactose-grown yeasts although dialysed and desalted MRS gave a slight reduction in the number of adherent *Candida*.

1.4.5 Influence of different bacterial isolates

The ability of eight different *Lactobacillus* isolates to inhibit the adhesion of *Candida* to mouse vaginal epithelial cells was investigated. *L. jensenii*, *L. casei* ssp. *rhamnosus* and *L. casei* were culture collection strains. The strains of *L. acidophilus* and *L. crispatus* were fresh vaginal isolates obtained from Glasgow Royal Maternity Hospital. These isolates were identified by Gram's stain and the API 50CHL identification kit.

The different strains (5×10^8 cells/ml) were used to pretreat mouse vaginal epithelial cells. After pretreatment, vaginal cells were washed to remove any non-adherent lactobacilli, resuspended in PBS and used in adhesion assays. The number of yeasts adhering to *Lactobacillus*-pretreated epithelial cells was compared with adherence to control epithelial cells which had been pretreated with PBS. The percentage inhibition of adherence was calculated (Table 24). Different *Lactobacillus* isolates gave a different percentage inhibition of *Candida* adhesion. The three culture collection isolates showed less inhibition of *Candida* adhesion than the fresh vaginal isolates. The vaginal isolates also attached to epithelial cells in greater numbers than the culture collection isolates (Table

Table 24.

Inhibition by eight different *Lactobacillus* isolates of *Candida* adhesion to mouse vaginal epithelial cells (VEC)

PRETREATMENT[a]	CARBON SOURCE[b]	MEAN NO. OF ADHERENT YEASTS/100 EPITHELIAL CELLS ± SEM[c]	PERCENTAGE INHIBITION OF ADHERENCE[d]	P[e]
L. jensenii	Glucose	25.4 ± 6.2	33.3	NS[f]
	Galactose	233.2 ± 32.4	18.3	NS
L. casei ssp.*rhamnosus*	Glucose	22.2 ± 2.9	41.7	0.01
	Galactose	173.7 ± 24.1	39.1	0.01
L. casei	Glucose	22.0 ± 3.5	42.3	0.01
	Galactose	235.6 ± 31.7	17.5	NS
L. acidophilus 629706A	Glucose	7.0 ± 1.6	81.6	0.001
	Galactose	128.4 ± 25.1	55.0	<0.001
L. crispatus 632697M	Glucose	8.7 ± 3.6	77.2	<0.001
	Galactose	78.0 ± 17.1	72.7	<0.001
L. acidophilus 631575L	Glucose	11.1 ± 4.0	70.9	<0.001
	Galactose	115.6 ± 25.8	59.5	<0.001
L. acidophilus 630360E	Glucose	11.3 ± 3.5	70.3	0.01
	Galactose	128.3 ± 32.0	55.0	<0.001
L. crispatus 629425X	Glucose	19.3 ± 3.7	49.3	0.05
	Galactose	193.2 ± 3.8	32.3	0.01
PBS	Glucose	38.1 ± 3.9		
	Galactose	285.4 ± 23.8		

[a] Prior to adhesion assays, VEC were incubated for 30 min at 37°C in PBS or in a suspension of one of the *Lactobacillus* isolates (5 x 10^8 cells/ml). After this pretreatment, vaginal cells were washed to remove any non-adherent lactobacilli, resuspended in PBS and used in adhesion assays.

[b] Yeasts (*C. albicans* GDH 2346) were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[c] SEM, standard error of the mean.

[d] Calculated as the percentage of inhibition when compared with adherence to PBS-pretreated epithelial cells.

[e] Probability values comparing adherence of yeasts to *Lactobacillus*-pretreated epithelial cells with adherence of yeasts to PBS-pretreated epithelial cells.

[f] NS, not significant.

Table 25.

Adhesion of eight different *Lactobacillus* isolates to mouse vaginal epithelial cells (VEC)

LACTOBACILLUS ISOLATE	MEAN NO. ADHERENT LACTOBACILLI/CELL ± SEM[a]
L. jensenii	4.5 ± 0.7
L. casei ssp. rhamnosus	3.9 ± 0.5
L. casei	4.6 ± 1.1
L. acidophilus 629706A	29.7 ± 3.1
L. crispatus 632697M	45.9 ± 4.8
L. acidophilus 631575L	21.4 ± 2.0
L. acidophilus 630360E	13.6 ± 1.7
L. crispatus 629425X	5.8 ± 0.5
PBS	3.7 ± 0.6

[a] SEM, standard error of the mean.

25). Greatest overall inhibition of *Candida* adhesion was seen with the isolate able to attach to the mouse vaginal epithelial cells in highest numbers, *L. crispatus* 632697M. The adhesion of culture collection isolates was little above the background level of bacterial adhesion to PBS-pretreated epithelial cells. However, *L. casei* ssp. *rhamnosus*, the *Lactobacillus* isolate least able to attach to vaginal epithelial cells, still gave significant inhibition of *Candida* adherence.

All of the isolates inhibited adhesion of glucose-grown yeasts (33.3-81.6%; Table 24) more than that of galactose-grown yeasts. (17.5-72.7%; Table 24).

1.4.6 Influence of different *Candida* strains

In previous experiments a single strain of *C. albicans* (GDH 2346) was used in adhesion assays. Here four different strains were compared. Strains GDH 2023 and GDH 2346 were denture stomatitis isolates, GRI 272 was isolated from an active infection of the human vagina and GRI 682 was obtained from the cervix of a symptomless carrier.

Adhesion assays showed that the isolate from the vaginal infection, GRI 272, adhered to mouse vaginal epithelial cells in greatest numbers (Table 26). The denture stomatitis isolate, GDH 2023, adhered in numbers similar to GRI 272 and in greater numbers than the other denture stomatitis isolate, GDH 2346. Strain GRI 682 adhered least well to the mouse cells of the four strains tested (Table 26).

Growth of yeasts in galactose medium increased the adherence of *C. albicans* compared to the adhesion of glucose-grown *Candida* (Table 26). Galactose-grown *C. albicans* strains GDH 2023 and GRI 272

Table 26.

Effect of *L. crispatus* 632697M on adherence of four strains of *C. albicans* to mouse vaginal epithelial cells (VEC)

YEAST STRAIN USED IN ASSAY	CARBON SOURCE[a]	PRETREATMENT OF VAGINAL CELLS[b]	MEAN NO. OF ADHERENT YEASTS/100 VEC ± SEM[c]	PERCENTAGE INHIBITION OF ADHERENCE[d]
	Glucose	Lactobacilli	25.8 ± 4.8	43.7
	Glucose	PBS	45.8 ± 12.9	
GDH 2346	Galactose	Lactobacilli	106.1 ± 18.3	52.3
	Galactose	PBS	222.4 ± 71.0	
	Glucose	Lactobacilli	40.6 ± 7.5	48.1
	Glucose	PBS	78.3 ± 18.6	
GDH 2023	Galactose	Lactobacilli	235.3 ± 64.3	47.7
	Galactose	PBS	449.6 ± 56.5	
	Glucose	Lactobacilli	15.3 ± 5.6	38.2
	Glucose	PBS	24.8 ± 4.7	
GRI 682	Galactose	Lactobacilli	27.8 ± 8.1	67.5
	Galactose	PBS	85.6 ± 34.7	
	Glucose	Lactobacilli	66.9 ± 16.4	18.3
	Glucose	PBS	81.9 ± 17.3	
GRI 272	Galactose	Lactobacilli	275.6 ± 52.7	40.7
	Galactose	PBS	464.6 ± 55.8	

[a] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[b] Prior to adhesion assays, vaginal epithelial cells were incubated for 30 min at 37°C in PBS or in a suspension of *L. crispatus* (5 x 10^8 cells/ml). After this pretreatment, vaginal cells were washed to remove any non-adherent lactobacilli, resuspended in PBS and used in adhesion assays.

[c] SEM, standard error of the mean.

[d] Calculated as the percentage inhibition when compared with adherence to PBS-pretreated epithelial cells.

were 5.7 times more adherent than the glucose-grown yeasts. Strain GDH 2346 was 4.9 times more adherent after growth in galactose. Least modification of adhesion was obtained after growing strain GRI 682 in galactose; these yeasts were 3.5 times more adherent compared with glucose-grown organisms.

Mouse vaginal epithelial cells were pretreated with *L. crispatus* 632697M prior to use in adhesion assays. This *Lactobacillus* isolate had given the greatest inhibition of *Candida* adhesion of all the lactobacilli tested (Table 24). When the percentage inhibition of adherence was calculated for each strain it was observed that *L. crispatus* 632697M gave greatest inhibition of galactose-grown *C. albicans* GRI 682, the strain isolated from the cervix of a symtomless carrier. The *Lactobacillus* isolate inhibited the adherence of denture stomatitis isolates GDH 2023 and GDH 2346 to a similar extent; inhibition of galactose-grown yeasts was less than that observed with strain GRI 682. Least inhibition was obtained with glucose-grown strain GRI 272, the strain originally isolated from an active vaginal infection.

Inhibition of adhesion for galactose-grown strains GDH 2023 and GRI 272 were significant by the Student t-Test. The Chi-square test showed distribution of adherent yeasts on *Lactobacillus* pretreated epithelial cells was significantly different from the distribution of yeasts on control vaginal cells. Only the distribution of glucose-grown *C. albicans* GRI 272 was not significantly affected by the *Lactobacillus* pretreatment (Appendix 9).

2. Effect of lactobacilli on the growth of *Candida*

If microorganisms are to establish in a particular environment they must not only have the ability to adhere but also to grow. When two microorganisms are present in the same location they may influence each other's capacity for growth and multiplication.

2.1 Preliminary observations

Experiments were carried out to establish whether *C.albicans* and lactobacilli could grow under similar conditions, and therefore in close proximity *in vivo*, with the possibility of interactions between them.

2.1.1 Growth of *C. albicans* over a wide pH range

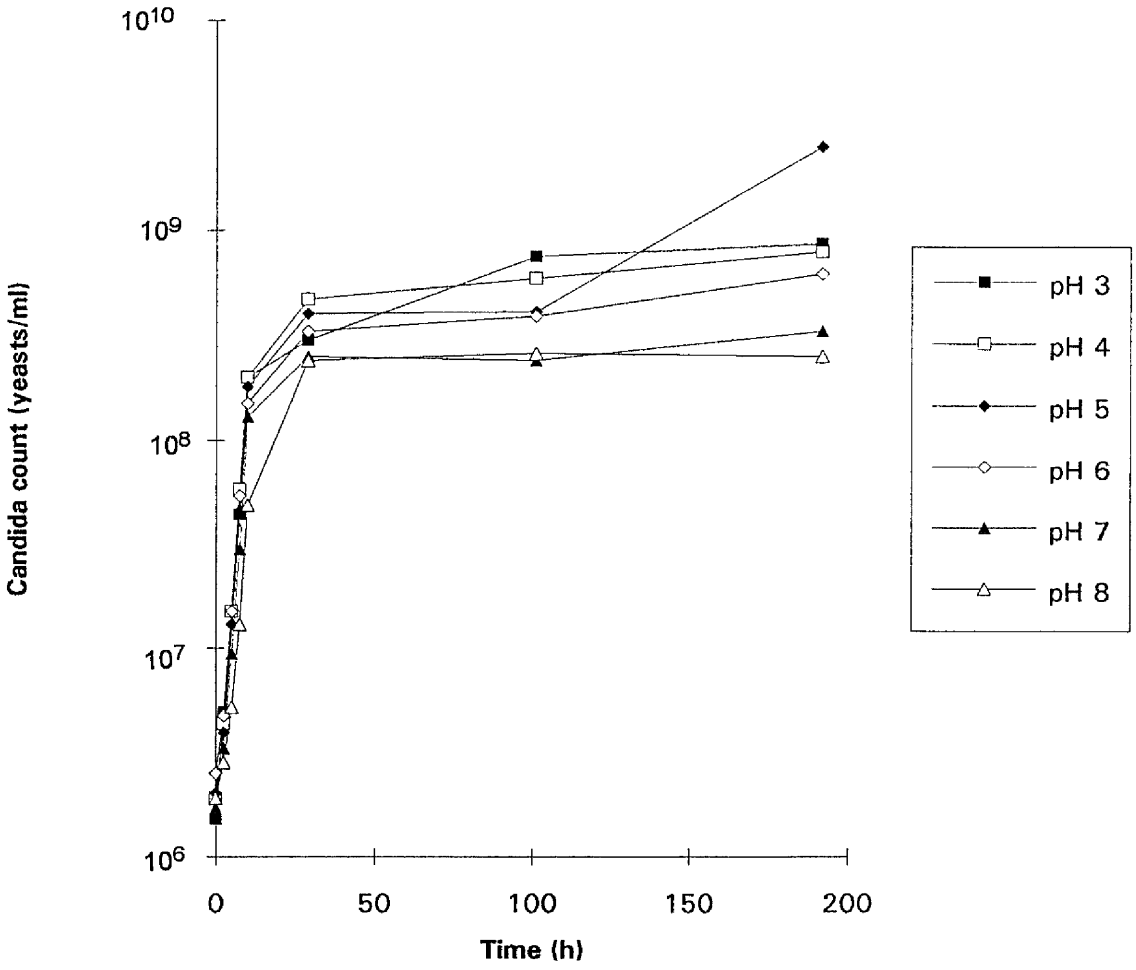
C.albicans GDH 2346 was grown in defined medium with 50mM glucose as a carbon source. The medium was buffered in the range of pH 3-8. Each batch of medium, at a different pH value, was inoculated with *Candida* grown overnight in 50mM glucose YNB to give an initial concentration of 1.8 x 10^6 yeasts/ml. Cultures were incubated at 37°C with shaking. Samples were removed as eptically throughout the incubation period to measure growth.

The results are shown in Figure 9. Yeasts were able to grow well in 50mM glucose YNB at all pH values between 3 and 8. No lag phase was seen at the start of the experiment. The exponential phase lasted 10h after which the cultures entered stationary phase. On day 4 (96h) greatest growth of *Candida* was observed at pH 3; there was least growth at pH 7.

Despite the presence of buffer, as the yeasts grew they altered the pH value of the growth medium. At day 8 of this experiment the average pH value (for all batches of medium) was 6.82

Figure 9.

Growth of *C. albicans* GDH 2346 at different pH values



The medium was buffered with citric acid-disodium hydrogen phosphate or tris-maleate buffers in the range of pH 3-8. Each point represents the mean value of 6 determinations. Duplicate determinations were carried out in an experiment which was performed in triplicate.

± 0.13. A preliminary experiment, in which the pH value of the medium was adjusted using concentrated acid or alkali had shown that no growth of *C.albicans* was possible above pH 8.

2.1.2 Growth of *L. acidophilus* over a wide pH range

L. acidophilus was grown in MRS broth. Preliminary experiments in which the pH value was adjusted using concentrated acid or alkali showed that *L. acidophilus* was unable to grow at pH 9, 10 or 11. The growth of the organism was further investigated by buffering MRS medium in the range pH 3.7-8. Each broth was inoculated with *L. acidophilus* taken from an overnight culture to give an initial concentration of 7 x 10^6 lactobacilli/ml.

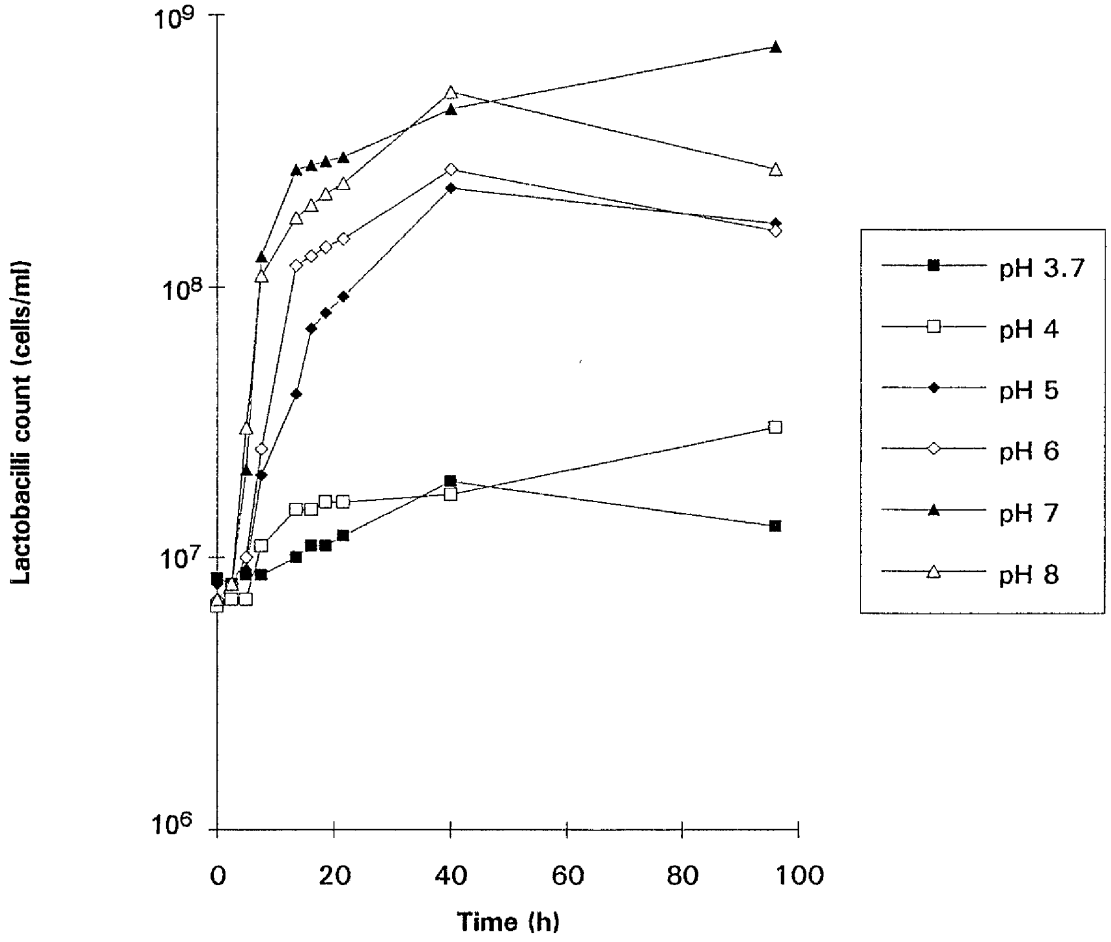
L. acidophilus was able to grow well at pH values between 5 and 8 (Figure 10). At pH 3.7 and 4 growth was restricted. Growth was greatest in media buffered at pH 7 and 8. Cultures grown at these pH values had the shortest lag phase (3h) and were the first to reach stationary phase. Cultures grown at pH 5 and 6 had a longer lag phase (6h) and a slower growth rate than cultures grown at pH 7 or 8. Despite the presence of buffer, as the lactobacilli grew they altered the pH value of the growth medium. At day 4 of this experiment the average pH value (for all batches of media) was 4.2 ± 0.4.

2.2 Mixed broth cultures

MRS broth and YNB medium containing 50mM glucose were inoculated, in duplicate, with *L. acidophilus* (3 x 10^8 cells/ml; 1ml), *C.albicans* GDH 2346 (3 x 10^8 cells/ml; 1ml), or equivalent numbers of lactobacilli and *Candida* together. Cultures were incubated, without shaking at 37°C for 24h. After incubation the optical density of

Figure 10.

Growth of *L. acidophilus* over a wide range of pH values



The medium was buffered with sodium acetate-acetic acid and disodium hydrogen phosphate-sodium dihydrogen phosphate buffers in the range of pH 3.7-8. Each point represents the mean value of 6 determinations. Duplicate determinations were carried out in an experiment which was performed in triplicate.

each culture was measured at 600nm. Viable *Candida* counts were obtained by plating out samples of broth culture on Sabouraud dextrose agar and incubating aerobically. Samples of broth were also plated out on Rogosa agar and incubated microaerophilically in order to obtain viable counts of lactobacilli.

The results obtained are shown in Table 27. Growth of the lactobacilli and yeasts together in mixed culture was compared to their growth alone in control cultures. MRS broth is a medium recommended for growth of lactobacilli. In this medium the OD_{600} of the *Lactobacillus* control was greater than that of the *Candida* control. The OD_{600} of the mixed culture was slightly greater than that of the *Lactobacillus* control. Viable counts showed a slight reduction in the number of *Candida* and also in the number of lactobacilli in the mixed culture compared with the controls.

Glucose YNB is a defined medium used for the cultivation of yeasts. In this medium *Candida* grew better than lactobacilli, although yeast cell numbers were similar to those in MRS broth. The OD_{600} of the mixed culture was greater than that of the controls. Viable counts showed a slight increase in the number of *Candida* and a slight reduction in the number of lactobacilli in the mixed culture compared with the controls.

Overall, the results indicate that growth of *L. acidophilus* and *C. albicans* in mixed broth culture had no significant effect on either organism.

2.3 Sandwich plate test

A sandwich plate technique was used which was a modification of a method described by Reid *et al.* (1988). Lactobacilli from a 24h broth culture were washed in sterile PBS and resuspended to a

Table 27.

Growth of *L. acidophilus* and *C. albicans* GDH 2346 in mixed broth culture[a]

GROWTH MEDIUM[b]	CULTURE	OD_{600}[c]	*CANDIDA* COUNTS[d] (10^6 cfu/ml)	*LACTOBACILLUS* COUNTS[e] (10^6 cfu/ml)
MRS	*Candida* control	1.7	2.6	0.00
	Mixed culture	4.4	2.5	35.00
	Lactobacillus control	4.3	0.0	40.00
50mM glucose YNB	*Candida* control	1.7	2.3	0.00
	Mixed culture	1.9	2.8	0.26
	Lactobacillus control	0.025	0.0	0.90

[a] Values represent the mean of duplicate determinations. The experiment was repeated twice.

[b] Lactobacilli and yeasts were grown together in either MRS broth or YNB medium containing 50 mM glucose.

[c] Growth was determined by measuring the optical density at 600nm.

[d] Viable *Candida* counts were obtained by plating out samples of broth culture onto Sabouraud dextrose agar.

[e] Viable *Lactobacillus* counts were obtained by plating out samples of broth culture onto Rogosa agar.

concentration of 5 x 10^8 cells/ml, unless otherwise specified. The suspension (0.5ml) was spread on to MRS agar and incubated microaerophilically for 48h. The lactobacilli were then either treated with UV radiation for 30 min, chloroform (10ml) for 15 min or were untreated prior to overlay with MRS agar. After overlay the indicator organism, *C. albicans* (0.1ml; 10^5 cells/ml, unless otherwise specified), was inoculated on to the surface of the plate. The plates were then incubated aerobically, for a further 24h.

2.3.1 Inhibition of *Candida* growth

Table 28 shows the percentage inhibition of *Candida* growth at 24h when growth on the test plates was compared to that found on control plates. Controls had been inoculated with the indicator organism but contained no lactobacilli in the base of the sandwich plate. When lactobacilli were grown in the base of the plate there was complete inhibition of *Candida* growth. This effect was seen with both *L.acidophilus* and *L. fermentum*. Complete inhibition of *Candida* growth also was observed on sandwich plates which had been treated with UV radiation or chloroform prior to overlay and inoculation with the indicator organism. The UV and chloroform treatments killed all the lactobacilli before overlay thus showing that live cells were not necessary for inhibition of *Candida* growth and that growth inhibition was not solely due to competition for nutrients. From these results it seems possible that lactobacilli may release some factor which can diffuse through the agar and inhibit *Candida* growth.

Table 28.

Effect of lactobacilli on the growth of *C. albicans* GDH 2346 in sandwich plate tests

TREATMENT[a]	% INHIBITION OF *CANDIDA* GROWTH[b]
L. acidophilus NCTC 4504	100
L. fermentum NCIB 2797	100
UV killed *L. acidophilus* NCTC 4504	100
UV killed *L. fermentum* NCIB 2797	100
$CHCl_3$ killed *L. acidophilus* NCTC 4504	100
$CHCl_3$ killed *L. fermentum* NCIB 2797	100

[a] Lactobacilli (0.5ml; 5 x 10^8 cells/ml) on MRS agar were incubated microaerophilically for 48h. Plates were either treated with UV radiation for 30 min, $CHCl_3$ (10ml) for 15 min or were untreated prior to overlay with MRS agar. The overlay was inoculated with *C. albicans* (0.1 ml; 10^5 cells/ml) and incubated for 24h.

[b] At 24h, growth on the test plates was compared to that found on control plates.

2.3.2 Inhibition of *C. albicans* GDH 2346 by different concentrations of *Lactobacillus fermentum* NCIB 2797

L. fermentum from a 24h broth culture was washed and a range of dilutions prepared in sterile PBS. Dilutions (0.5ml) were inoculated on to MRS agar and incubated microaerophilically for 48h. After incubation plates were overlaid with MRS agar and seeded with a *C. albicans* suspension (0.5ml) of 10^4 cells/ml or 10^7 cells/ml. The plates were reincubated aerobically for a further 24h.

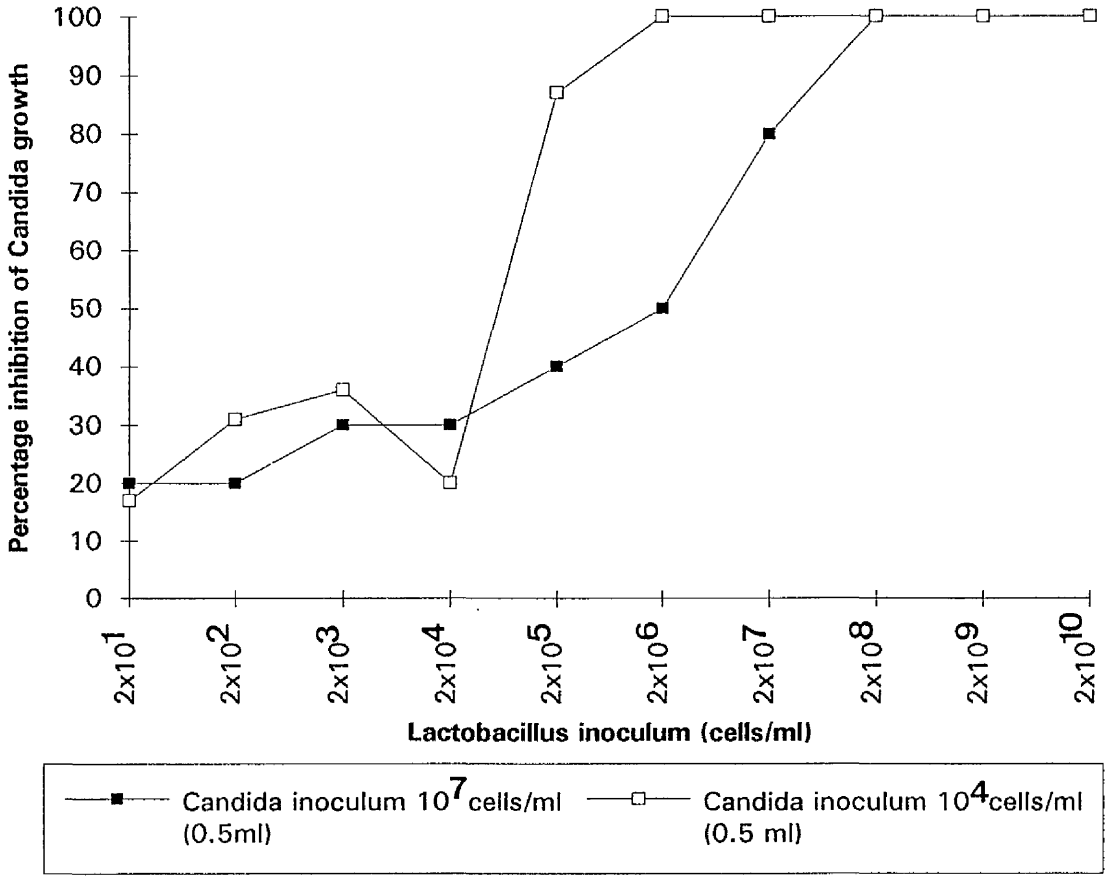
Sandwich plates prepared using any *Lactobacillus* inoculum >2 x 10^6 cells/ml gave a confluent covering of the MRS agar with *L. fermentum*. Decreasing growth of lactobacilli was seen with increasing dilution.

Figure 11 shows the effect of different concentrations of lactobacilli on the growth of *Candida*. Sandwich plates inoculated with 10^7 *Candida*/ml (0.5ml) were scored (an estimate of plate cover/ density of growth) for growth when compared to control plates which had confluent *Candida* growth. There was an increasing inhibition of *Candida* growth with increasing numbers of lactobacilli in the base of the sandwich plates. Total inhibition of *Candida* growth was observed on plates with a *Lactobacillus* inoculum of 2 x 10^8 cells/ml or greater.

When plates were seeded with fewer yeasts (0.5ml; 10^4 yeasts/ml) the percentage inhibition of *Candida* growth was obtained by comparing colony forming units on test plates with those on control plates. Again, the trend of increasing inhibition of *Candida* growth corresponded to increasing numbers of lactobacilli in the base of the sandwich plates. Plates seeded with 10^4 *Candida*/ml (0.5ml) showed total inhibition of *Candida* growth if the lactobacillus inoculum was 2 x 10^6 cells/ml or greater. A lower

Figure 11.

Effect of different concentrations of *L. fermentum* on the growth of *C. albicans* in a sandwich plate test



Suspensions of lactobacilli (0.5ml) at different concentrations were inoculated onto MRS agar plates and incubated microaerophilically. Sandwich plates were prepared. Growth of *Candida* on test sandwich plates was compared to that found on control plates.

concentration of lactobacilli in the sandwich plate totally inhibited the growth of plates seeded with 10^4 *Candida*/ml (0.5ml) compared with the concentration of lactobacilli that totally inhibited the growth of plates seeded with 10^7 yeasts/ml (0.5ml).

2.3.3 Inhibition of different concentrations of *C. albicans*

Sandwich plates inoculated with either 5 x 10^6 or 5 x 10^8 lactobacilli/ml (0.5ml) were incubated then treated with chloroform or left untreated prior to overlay with MRS agar or Sabouraud dextrose agar. The overlays were seeded with *C. albicans* suspensions (0.1 ml) of different concentrations and incubated for 72h. After incubation colony forming units of *Candida* on test plates were compared with those on control plates and results expressed as a percentage inhibition of yeast growth.

Less inhibition of *Candida* growth was seen in MRS sandwich plates which had a lower inoculum of lactobacilli (Table 29). Killed lactobacilli were able to inhibit yeast growth.

At 72h, there was no distinguishable trend of changing inhibition of *Candida* growth for the different yeast concentrations. It was seen that changes in lactobacilli numbers in the base of the plate were more important in altering the inhibition of *Candida* growth. Variation in the growth of lactobacilli between plates of the same treatment could be responsible for different inhibition values which would make interpretation of results difficult. Counting colony forming units of *Candida* as a measure of percentage inhibition of *Candida* growth did not take account of the different colony sizes seen. On treatment plates the onset of *Candida* growth

Table 29.

Effect of *L. fermentum* on the growth of different concentrations of *C. albicans* GDH 2346 in a sandwich plate test with MRS agar overlay

TREATMENT[a]	*CANDIDA* INOCULUM (yeasts/ml)	% INHIBITION OF *CANDIDA* GROWTH[c]
2.5×10^8	10^3	100
L. fermentum	10^4	96
untreated	10^5	96
2.5×10^8	10^3	84
L. fermentum	10^4	98
$CHCl_3$ treated	10^5	54
2.5×10^6	10^3	58
L. fermentum	10^4	50
untreated	10^5	48
2.5×10^6	10^3	56
L. fermentum	10^4	48
$CHCl_3$ treated	10^5	64

[a] Lactobacilli (0.5ml; 5×10^8 cells/ml or 5×10^6 cells/ml) on MRS agar were incubated microaerophilically for 48h. Plates were either treated with $CHCl_3$ for 15 min or were untreated prior to overlay with MRS agar.

[b] The MRS overlays were inoculated with *C. albicans* (0.1ml) of different concentrations and incubated for 72h.

[c] Growth on test plates was compared to that found on control plates.

was markedly delayed compared with the controls and thus different colony sizes were observed.

When plates were overlaid with Sabouraud dextrose agar rather than MRS agar inhibition of Candida growth was seen (Table 30). Sabouraud dextrose agar is the usual medium for the cultivation of yeasts. Overlaying with Sabouraud dextrose agar showed inhibition of *Candida* growth was not due to an insufficient provision of nutrients by MRS agar.

2.3.4 Effect of age and viability of *L. fermentum* on inhibition

Sandwich plates were inoculated with 5 x 10^6 or 5 x 10^8 lactobacilli (0.5ml) and incubated microaerophilically for 2 or 4 days, after which time they were treated with chloroform or left untreated prior to overlay with MRS agar. The MRS overlays were inoculated with *C. albicans* and incubated aerobically at 37°C for 72h. After incubation the percentage inhibition of yeast growth was calculated.

Longer preincubation of lactobacilli, prior to MRS overlay, gave greater inhibition of *Candida* growth (Table 31). Overall, greater inhibition of *Candida* growth was seen in sandwich plates which had a larger *Lactobacillus* inoculum. In this experiment, sandwich plates which had been treated with chloroform to kill the lactobacilli, prior to overlay with MRS agar, showed greater inhibition of *Candida* growth than plates which had remained untreated prior to overlay.

Table 30.

Effect of *L. fermentum* on the growth of different concentrations of *C. albicans* GDH 2346 in a sandwich plate test with Sabouraud dextrose agar overlay

TREATMENT[a]	*CANDIDA* INOCULUM[b] (yeasts/ml)	% INHIBITION OF *CANDIDA* GROWTH[c]
2.5×10^8	10^3	94
L. fermentum	10^4	66
untreated	10^5	99
2.5×10^8	10^3	86
L. fermentum	10^4	13
$CHCl_3$ treated	10^5	99
2.5×10^6	10^3	95
L. fermentum	10^4	18
untreated	10^5	100
2.5×10^6	10^3	95
L. fermentum	10^4	17
$CHCl_3$ treated	10^5	99

[a] Lactobacilli (0.5ml; 5×10^8 cells/ml or 5×10^6 cells/ml) on MRS agar were incubated microaerophilically for 48h. Plates were either treated with $CHCl_3$ for 15 min or were untreated prior to overlay with Sabouraud dextrose agar (SDA) and incubated for 72h.

[b] The SDA overlays were inoculated with *C. albicans* (0.1ml) of different concentrations.

[c] Growth on test plates were compared to that found on control plates.

Table 31.

Effect of age and viability of *L. fermentum* on the growth of *C. albicans* GDH 2346 in a sandwich plate test

TREATMENT[a]	PREINCUBATION TIME FOR LACTOBACILLI (DAYS)[b]	% INHIBITION OF *CANDIDA* GROWTH[c]
2.5×10^8 *L. fermentum* untreated	2	39
	4	75
2.5×10^8 *L. fermentum* $CHCl_3$ treated	2	75
	4	92
2.5×10^6 *L. fermentum* untreated	2	46
	4	62
2.5×10^6 *L. fermentum* $CHCl_3$ treated	2	39
	4	79

[a] Lactobacilli (0.5ml; 5×10^8 cells/ml or 5×10^6 cells/ml) were incubated microaerophilically on MRS agar. Plates were either treated with $CHCl_3$ for 15 min or untreated prior to overlay with MRS agar. The overlay was inoculated with *C. albicans* (0.1ml; 10^5 yeasts/ml) and incubated for 72h.

[b] Lactobacilli were incubated for 2 or 4 days prior to *Candida* overlay.

[c] Growth on test plates were compared to that found on control plates.

2.4 Plate bioassay

2.4.1 Production of antimicrobial substances by lactobacilli

Inhibition of *Candida* growth by lactobacilli in a sandwich plate assay had been demonstrated. To further investigate the antimicrobial activity of lactobacilli an alternative assay system was used.

L. acidophilus and *L. fermentum* were grown for 5 days in MRS broth. The cultures were centrifuged and the cell pellet and culture supernatant for each organism treated separately. The culture supernatant was concentrated 20-fold. MRS broth was concentrated in a similar manner to act as a control. The cell pellet was washed in PBS and resuspended to a concentration of 5 x 10^9 cells/ml. A sample of cell pellet was subject to ultrasonic vibration to disintegrate the cells and release intracellular materials.

Sabouraud dextrose agar inoculated with 0.75ml of overnight broth culture of *C. albicans* B079 was poured into a levelled assay plate. Wells, diameter 8mm, were cut into the agar and 80μl of test substance introduced into the wells. Diameters of zones of growth inhibition around the wells were measured after the test plates had been incubated at 37^0C for 24h. Results are shown in Table 32.

Zones of growth inhibition were seen around the wells containing concentrated culture supernatant of *L. acidophilus* and *L. fermentum*. This effect was not due to an inhibitory action of the concentrated medium as the MRS control gave no zone of inhibition. Washed cells of *L. acidophilus* and *L. fermentum* did not inhibit *Candida* growth. Treatment of the cells by ultrasonication released no intracellular component with antimicrobial activity.

Table 32.

Production of an antimicrobial substance by lactobacilli grown in MRS broth

SAMPLE[a]	ZONE DIAMETER (mm)[b]		
	L. acidophilus	*L. fermentum*	MRS broth
Concentrated supernatant	9	12	0
Unbroken cellular material	0	0	-
Sonicated cellular material	0	0	-

[a] *L. acidophilus* and *L. fermentum* were grown for 5 days in MRS broth. The cultures were centrifuged and the cell pellet and culture supernatant for each organism treated separately

[b] Samples (80µl) were added to wells (8mm diameter) in plates seeded with *C. albicans* B079. Zones of growth inhibition were measured after incubation at 37°C for 24h.

From these results, it appears that the antimicrobial activity of the lactobacillus cultures is due to a substance produced extracellularly.

2.4.2 pH stability of antimicrobial substance produced by lactobacilli

The pH of 20-fold concentrated culture supernatant was adjusted with concentrated acid or alkali and maintained at room temperature for 1h prior to being introduced (80µl) into the wells in a bioassay plate inoculated with *C. albicans* B079. It was observed that antimicrobial activity was unstable at neutral or alkaline pH (Table 33).

2.4.3 Antimicrobial substances from *Lactobacillus* cultures incubated for different time periods

The effect of culture age upon the production of antimicrobial substances by lactobacilli was investigated. *L. acidophilus* and *L. fermentum* were grown for 1, 2, 4, 5 and 6 days in MRS broth. Each culture supernatant was concentrated 20-fold. MRS broth was concentrated by the same method to act as a control. The concentrates were added to wells in plates seeded with *C. albicans*, *C. neoformans*, *A. niger* or *T. mentagrophytes*. Zones of growth inhibition were measured after incubation.

The concentrated culture supernatant of *L. acidophilus* had antimicrobial activity against *C. albicans* (Table 34), *C. neoformans* (Table 35) and *A. niger* (Table 36) which varied with the age of the culture. Antimicrobial activity increased to a maximum on day 4, a value which was maintained on day 5 but which started to decline by day 6. The inhibition of *T. mentagrophytes* (Table 37) due to *L. acidophilus* culture supernatant also varied with the age of the

Table 33.

pH stability of an antimicrobial substance produced in *Lactobacillus* culture supernatant

TEST pH[a]	ZONE DIAMETER (mm)	
	L. acidophillus	*L. fermentum*
3	11	10
7	0	0
9.5	0	0

[a] pH of concentrated culture supernatant was adjusted to the test pH with concentrated acid or alkali, maintained at room temperature for 1h prior to introduction into wells (8mm diameter) in a test plate seeded with *C. albicans* B079.

Table 34.

Inhibition of *Candida* growth in plate tests by concentrated *Lactobacillus* supernatant from cultures of different ages

AGE OF *LACTOBACILLUS* CULTURE[a] (days)	ZONE DIAMETER (mm)[b] *L. acidophilus* supernatant	*L. fermentum* supernatant	MRS broth
1	9	10	0
2	10	11	0
4	11	10	0
5	11	10	0
6	9	10	0

[a] *L. acidophilus* and *L. fermentum* were grown for different time periods in MRS broth and the culture supernatants concentrated 20-fold.

[b] Samples of concentrated culture supernatant (80µl) were added to wells (8mm diameter)in plates seeded with *C. albicans* B079. Zones of growth inhibition were measured after incubation at 37°C for 24h.

Table 35.

Inhibition of *Cryptococcus* growth in plate tests by concentrated *Lactobacillus* supernatant from cultures of different ages

AGE OF *LACTOBACILLUS* CULTURE[a] (days)	ZONE DIAMETER (mm)[b] *L. acidophilus* supernatant	*L. fermentum* supernatant	MRS broth
1	+[c]	10	0
2	12	11	0
4	13	12	0
5	13	13	0
6	11	13	0

[a] *L. acidophilus* and *L. fermentum* were grown for different time periods in MRS broth and the culture supernatants concentrated 20-fold.

[b] Samples of concentrated culture supernatant (80μl) were added to wells (8mm diameter) in plates seeded with *C. neoformans*. Zones of growth inhibition were measured after incubation at 37°C for 24h.

[c] + indicates a visible halo of growth inhibition around the well < 9mm zone diameter.

Table 36.

Inhibition of *Aspergillus* growth in plate tests by concentrated *Lactobacillus* supernatant from cultures of different ages

AGE OF *LACTOBACILLUS* CULTURE[a] (days)	ZONE DIAMETER (mm)[b] *L. acidophilus* supernatant	*L. fermentum* supernatant	MRS broth
1	9	+[c]	0
2	11	11	0
4	13	11	0
5	13	12	0
6	11	11	0

[a] *L. acidophilus* and *L. fermentum* were grown for different time periods in MRS broth and the culture supernatants concentrated 20-fold.

[b] Samples of concentrated culture supernatant (80μl) were added to wells (8mm diameter) in plates seeded with *A. niger*. Zones of growth inhibition were measured after incubation at 37°C for 24h.

[c] + indicates a visible halo of growth inhibition around the well < 9mm zone diameter.

Table 37.

Inhibition of *Trichophyton* growth in plate tests by concentrated *Lactobacillus* supernatant from cultures of different ages

AGE OF *LACTOBACILLUS* CULTURE[a] (days)	ZONE DIAMETER (mm)[b] *L. acidophilus* supernatant	*L. fermentum* supernatant	MRS broth
1	40	40	36
2	50	42	36
4	52	42	36
5	46	44	36
6	44	40	36

[a] *L. acidophilus* and *L. fermentum* were grown for different time periods in MRS broth and the culture supernatants concentrated 20-fold.

[b] Samples of concentrated culture supernatant (80µl) were added to wells (8mm diameter) in plates seeded with *T. mentagrophytes*. Zones of growth inhibition were measured after incubation at 30°C for 48h.

culture. Antimicrobial activity reached a maximum on day 4 and began to decline on days 5 & 6.

With *L. fermentum*, antimicrobial activity of concentrated culture supernatant reached a maximum on day 5 for *C. neoformans* (Table 35), *A. niger* (Table 36) and *T. mentagrophytes* (Table 37). The concentrate gave maximum inhibition of *C. albicans* growth on day 2; activity then declined on day 4 but remained constant at this level for the rest of the experiment (Table 34).

The antimicrobial activity of concentrated 5-day *Lactobacillus* culture supernatant is shown in Table 38. *L. acidophilus* and *L. fermentum* both produced an antimicrobial substance and the culture concentrates from both species had similar activities. The activity had a broad spectrum, as it inhibited growth of all the fungi tested. The zones of inhibition for *T. mentagrophytes* were much greater than those seen for the other test organisms. However, a large proportion of this activity was due to the inhibitory action of the MRS broth. MRS broth had no activity against *C. albicans*, *C. neoformans* or *A. niger*.

Concentrated culture supernatants inhibited the growth of *A. niger*. In addition to this activity an inhibition of sporulation was observed for the *Aspergillus* growing closest to the wells.

2.4.4 Antimicrobial activity of culture supernatant from different *Lactobacillus* isolates

Different *Lactobacillus* isolates were grown for 5 days in MRS broth. Each culture supernatant was freeze-dried and concentrated 20-fold. The concentrates were added to wells in plates seeded with *C. albicans* B079. Zones of growth inhibition were measured after incubation.

Table 38.

Antimicrobial activity of concentrated *Lactobacillus* culture supernatant in plate tests

TEST ORGANISM	ZONE DIAMETER (mm)		
	L. acidophilus supernatant	*L. fermentum* supernatant	MRS broth
C. albicans	11	10	0
C. neoformans	13	13	0
A. niger	13	12	0
T. mentagrophytes	46	44	36

L. acidophilus and *L. fermentum* were grown for 5 days in MRS broth and the culture supernatants concentrated 20-fold. The concentrates (80μl) were added to wells (8mm diameter) in plates seeded with the test organisms indicated. Zones of growth inhibition were measured after 24 or 48h.

Table 39 shows that all the isolates tested gave antimicrobial activity, to varying degrees. Greatest activity was seen with *L. acidophilus* 629706A, a fresh vaginal isolate obtained from the Glasgow Royal Maternity Hospital.

2.5 Cellulose-covered agar plate assay

This procedure was carried out with the aim of isolating the antimicrobial substance(s) uncontaminated by media components. MRS agar plates were totally covered with a cellulose membrane. The covered plates were then inoculated with *L. acidophilus* or *L. fermentum* and incubated microaerophilically for 5 days. After this time, growth was scraped from the top of the membrane, collected and centrifuged. This method allowed lactobacilli to absorb nutrients through the membrane, but any extracellular substances produced with a molecular weight greater than 10,000 Da were retained on top of the cellulose membrane and could be recovered.

To test for antimicrobial activity, supernatants obtained by this method were added to wells in plates seeded with *C. albicans* B079 (see Materials and Methods Section 5.2). No zones of inhibition were seen for supernatants from either *L. acidophilus* or *L. fermentum*. In further tests, the agar from beneath the cellulose membrane was seeded with *C. albicans* and incubated. After 24h, 100% inhibition of *Candida* growth was seen for test plates when compared to controls which had not previously supported *Lactobacillus* growth.

Recovery of antimicrobial substances uncontaminated by media components was therefore not achieved using this procedure. However, the method demonstrated that the antimicrobial substance(s) produced by lactobacilli has a molecular weight of less than 10,000 Da.

Table 39.

Antimicrobial activity in plate tests of concentrated culture supernatant from different *Lactobacillus* isolates

LACTOBACILLUS ISOLATE[a]	ZONE DIAMETER (mm)[b]
L. jensenii	9
L. casei ssp. *rhamnosus*	7
L. casei	7
L. acidophilus 629706A	10
L. crispatus 632697M	7
L. acidophilus 63157SL	9
L. acidophilus 630360E	7
L. crispatus 629425X	9
L. acidophilus NCTC 4504	9
L. fermentum NCIB 2797	7

[a] The *Lactobacillus* isolates were grown for 5 days in MRS broth. The culture supernatants were freeze-dried and concentrated 20-fold and used in the bioassay.

[b] Samples (80µl) were added to wells (4mm diameter) in plates seeded with *C. albicans* B079. Zones of growth inhibition were measured after incubation at 37°C for 24h.

2.6 Purification of antimicrobial substance(s)

2.6.1 Solvent extraction of concentrated *Lactobacillus* culture supernatant

Attempts were made to find a solvent into which the antimicrobial activity of *Lactobacillus* culture supernatant could be extracted. Solvent extraction would allow concentration of antimicrobial activity by removal of some media components which would be left behind in the aqueous phase.

Extractions were carried out by mixing equal volumes of solvent and 20-fold concentrate of *Lactobacillus* culture supernatant. The solvents used were chloroform, ethyl acetate and butanol. After mixing, the aqueous and solvent phases were allowed to separate and samples of each added to wells in a plate seeded with *C. albicans* B079. After incubation for 24h at 37^0C zones of growth inhibition were measured (Table 40).

For each extraction, zones of growth inhibition were obtained with both aqueous and solvent phases. When chloroform was used as the solvent the antimicrobial activity remained in the aqueous phase. The small zone of inhibition which was seen with the solvent phase was due to the inhibitory action of the chloroform itself as an equivalent zone was seen with the chloroform phase of the MRS control extraction. With the ethyl acetate extraction, antimicrobial activity was divided between the aqueous and solvent phases. More activity remained in the aqueous phase than was extracted into ethyl acetate. Some of the activity demonstrated for the solvent phase was due to the antimicrobial activity of the solvent itself. The butanol extracts of concentrated culture supernatants of both *L. acidophilus* and *L. fermentum* gave the largest zones of growth inhibition. However, the butanol extract of MRS also gave the greatest zone of inhibition of any of the controls; this was clearly a factor in the

Table 40.

Plate bioassay of solvent extracts of concentrated culture supernatant of *L. acidophilus* and *L. fermentum*

SOLVENT EXTRACTION[a]	EXTRACTION PHASE[b]	ZONE DIAMETER (mm)[c] *L. acidophilus*	*L. fermentum*	MRS broth
$CHCl_3$	solvent	+[d]	+	+
	aqueous	13	12	0
Ethyl acetate	solvent	10	10	+
	aqueous	11	12	0
Butanol	solvent	22	21	13
	aqueous	9	10	0

[a] Solvent extraction was carried out using equal volumes of either chloroform, ethyl acetate or butanol and 20-fold concentrated *Lactobacillus* culture supernatant.

[b] Samples from each phase of the solvent extraction were bioassayed.

[c] Samples (80μl) were added to wells (8mm diameter) in plates seeded with *C. albicans* B079. Zones of growth inhibition were measured after incubation at 37°C for 24h.

[d] + indicates a visible halo of growth inhibition around the well < 9mm zone diameter

large zone sizes obtained for the supernatant extracts. Some antimicrobial activity remained in the aqueous phase after butanol extraction but this activity was less than that seen with the other solvents.

2.6.2 Antimicrobial activity of butanol extracts

The antimicrobial activities of butanol extracts of *Lactobacillus* culture supernatants were tested against a spectrum of microorganisms. *L. acidophilus* and *L. fermentum* were grown for 5 days in MRS broth and the culture supernatants extracted with an equal volume of butanol. MRS broth extracted with an equal volume of butanol acted as a control. The butanol extracts were concentrated 100-fold and then added to wells in plates seeded with test organisms. Test organisms included the Gram-positive bacteria *S. aureus*, *B. subtilis* and *B. megaterium*, the Gram-negative bacteria *E. coli* and *P. vulgaris* and the yeast *C. albicans*. Zones of growth inhibition were measured after incubation of the plates for 24h (Table 41).

Butanol extracts of culture supernatants of *L. acidophilus* and *L. fermentum* showed very similar antimicrobial activity. The zone diameter for inhibition of Candida growth showed that butanol extraction had concentrated the antimicrobial activity as zones were greater than those obtained for a 20-fold concentrate of culture supernatant (Table 38).

The butanol extracts of culture supernatants were not only antifungal but also inhibited the growth of both Gram-positive and Gram-negative bacteria. The inhibition varied slightly for the different test organisms. The MRS control extract showed activity against some of the test organisms. This inhibitory activity may

Table 41.

Antimicrobial activity of butanol extracts of *Lactobacillus* culture supernatants in plate tests

TEST ORGANISM	ZONE DIAMETER (mm)		
	L. acidophilus extract	*L.fermentum* extract	MRS extract
C. albicans B079	28	28	0
S. aureus	28	28	12
E. coli	28	28	0
B. subtilis	33	32	7
B. megaterium	28	28	7
P. vulgaris	30	30	8

L. acidophilus NCTC 4504 and *L. fermentum* were grown for 5 days in MRS broth and the culture supernatants extracted with an equal volume of butanol. The butanol extracts were concentrated 100-fold and then added (80 μl) to wells (4mm diameter) in plates seeded with the test organisms indicated. Zones of growth inhibition were measured after incubation for 24h.

have been due either to media components or residual butanol from the extraction process which may not have been totally removed. When background growth inhibition due to MRS was taken into account, antimicrobial activity was greatest against *C. albicans* and *E. coli*. The butanol extracts gave least growth inhibition with *S. aureus*.

2.6.3 Antimicrobial activity of lactic acid

As growth occurs, *Lactobacillus* species are known to produce lactic acid which has antimicrobial activity. The antimicrobial activity of different dilutions of a butanol extract of *L. acidophilus* culture supernatant was compared to the antimicrobial activity of different dilutions of lactic acid (Table 42).

The culture extract had greater antimicrobial activity against *C. albicans* than lactic acid. The butanol solvent also had antimicrobial activity which may have contributed to the effect of the culture extract against *C. albicans*. However, the antimicrobial activity of the lactic acid may simply be a pH effect. It was thought the lactic acid may lower the pH of the agar medium and hence prevent *Candida* growth. To investigate this, the pH value of lactic acid was adjusted to 4.5 (the same pH value as that of the *Lactobacillus* extract), and the antimicrobial activity measured. At pH 4.5, the antimicrobial activity of the lactic acid was much lower than the activity of the culture extract, even taking account of any activity due to remaining traces of solvent. Therefore, antimicrobial activity is not due to the presence of lactic acid alone in the culture extract.

Table 42.

Comparison of antimicrobial activity of a butanol extract of *L. acidophilus* culture supernatant and lactic acid

SAMPLE[a]	ZONE DIAMETER (mm)[b]	
	culture extract	lactic acid
undiluted	28	20
1/2 diluted	20	16
1/4 diluted	12	9
1/8 diluted	7	+[d]
1/16 diluted	+[d]	0
1/32 diluted	0	0
pH 4.5[c]	28	6
butanol control	10	-
citrate buffer	0	0

[a] *L. acidophilus* was grown in MRS broth for 5 days and the culture supernatant extracted with an equal volume of butanol. The butanol extract was concentrated 100-fold. Dilutions of the concentrated extract were made with citrate buffer (pH 4.5). Lactic acid (13.3M, pH 1) was diluted with citrate buffer (pH 4.5).

[b] Samples (80µl) were added to wells (4mm diameter) in plates seeded with *C. albicans* B079. Zones of growth inhibition were measured after incubation at 37°C for 24h.

[c] pH 4.5, the pH value of the concentrated culture extract, the lactic acid was adjusted by addition of concentrated alkali to a value of 4.5 .

[d] +, indicates a visible halo of growth inhibition around the well <5mm zone diameter.

2.6.4 Column chromatography of a butanol extract of *Lactobacillus* culture supernatant

To purify the antimicrobial component found in a butanol extract of *Lactobacillus* culture supernatant, column chromatography was attempted. Chromatography with a cation-exchange, anion-exchange and adsorption resin was carried out as the properties of the antimicrobial substance were unknown. It was hoped that the antimicrobial substance would bind to one of these column resins and allow other components from the culture supernatant to be eluted from the column before elution of the antimicrobial substance.

Columns of IR 120, IRA 548 and HP 20 resins were prepared. Samples (5ml) of 50-fold concentrated butanol extract were loaded and the percolate collected. The columns were then eluted. Fractions (5ml) from the columns were collected and bioassayed. A MRS butanol extract was run down the columns and assayed as a control.

The unfractionated samples showed antimicrobial activity in the plate test. The MRS control also showed some antimicrobial activity due to residual butanol after the extraction process.

For columns IR 120 (Table 43) and IR 548 (Table 44), the plate test showed that the antimicrobial activity came off the column in the percolate and first distilled water wash. The ion-exchange resins did not bind the antimicrobial activity which suggests the substance is uncharged. The antimicrobial activity of fractions from the HP 20 adsorption-resin were compared for the concentrated butanol extract and the MRS control (Table 45). The concentrated butanol extract and the control both showed some activity in the 2nd-4th 40% propanol washes and the acetone washes. Activity may have been due to some component of the MRS or solvent system. Antimicrobial activity for the concentrated butanol extract appeared

Table 43.

Antimicrobial activity of a butanol extract of *Lactobacillus* culture supernatant fractionated on an IR 120 cation-exchange column

SAMPLE[a]	ZONE DIAMETER (mm)[b]		
	L. acidophilus extract	*L. fermentum* extract	MRS broth extract
Unfractionated	14	14	9
Percolate	10	9	0
DW wash 1	11	11	0
DW wash 2-4	0	0	0
0.2M NaCl 1-4	0	0	0
2M NaCl 1-4	0	0	0

[a] 5ml of concentrated butanol extract was loaded onto an IR 120 cation-exchange column (bed volume 10ml, 8mm internal diameter) and the percolate collected. The column was then washed with 4 x 5ml distilled water (DW), 4 x 5ml 0.2M NaCl and 4 x 5ml 2M NaCl. 5ml fractions from the column were collected and bioassayed.

[b] Samples (80μl) were added to wells (8mm diameter) in plates seeded with *C. albicans* B079. Zones of growth inhibition were measured after incubation at 37°C for 24h.

Table 44.

Antimicrobial activity of a butanol extract of *Lactobacillus* culture supernatant fractionated on an IRA 548 anion-exchange column

SAMPLE[a]	ZONE DIAMETER (mm)[b]		
	L. acidophilus extract	*L. fermentum* extract	MRS broth extract
Unfractionated	14	14	9
percolate	9	9	0
DW wash 1	10	10	0
DW wash 2-4	0	0	0
0.2M NaCl 1-4	0	0	0
2M NaCl 1-4	0	0	0

[a] 5ml of concentrated butanol extract was loaded onto an IRA 548 anion-exchange column (bed volume 10ml, internal diameter 8mm) and the percolate collected. The column was then washed with 4 x 5ml distilled water (DW), 4 x 5 ml 0.2 M NaCl and 4 x 5ml 2M NaCl. 5ml fractions from the column were collected and bioassayed.

[b] Samples (80μl) were added to wells (8mm diameter) in plates seeded with *C. albicans* B079. Zones of growth inhibition were measured after incubation at 37°C for 24h.

Table 45.

Antimicrobial activity of a butanol extract of *Lactobacillus* culture supernatant fractionated on a HP 20 adsorption resin

SAMPLE[a]	ZONE DIAMETER (mm)[b]		
	L. acidophilus extract	*L. fermentum* extract	MRS broth extract
Unfractionated	14	14	9
percolate	+[c]	+	0
DW wash 1	+	+	0
DW wash 2-4	0	0	0
40% propanol 1	0	0	0
40% propanol 2-4	+	+	+
Acetone 1-4	+	+	9

[a] 5ml of concentrated butanol extract was loaded onto a HP 20 adsorption resin column (bed volume 10ml, internal diameter 8mm) and the percolate collected. The column was then washed with 4 x 5ml distilled water (DW), 4 x 5ml 40% propanol followed by 4 x 5ml acetone. 5ml fractions from the column were collected and bioassayed.

[b] Samples (80μl) were added to wells (8mm diameter) in plates seeded with *C. albicans* B079. Zones of growth inhibition were measured after incubation at 37°C for 24h.

[c] + indicates a visible halo of growth inhibition around the well < 9mm zone diameter.

in the percolate and first distilled water wash. However most of the activity seemed to remain bound to the column and could not be recovered in this system.

Column chromatography with either cation-exchange, anion-exchange or adsorption resin was therefore an unsatisfactory method of purification of the antimicrobial substance produced in *Lactobacillus* culture supernatant.

2.6.5 Fractional distillation of a butanol extract of *Lactobacillus* culture supernatant

Fractional distillation was investigated as an alternative purification procedure for the antimicrobial component. A concentrated butanol extract of *L. acidophilus* was fractionally distilled under reduced pressure and samples collected at distillation temperatures of 75, 100, 125, 150, 175, 200, 225 and 250°C. Samples were added to wells on plates seeded with *C. albicans* B079. Zones of growth inhibition were measured after incubation at 37°C for 24h. Increasing distillation of active component was seen with increase in temperature. Greatest antimicrobial activity was observed with the fraction distilled at 250°C (Table 46).

2.6.6 Solvent extraction of freeze-dried culture supernatant of *Lactobacillus*

Due to difficulties in purifying butanol extracts and problems with the antimicrobial activity of butanol itself in plate tests, alternative solvents were investigated. The use of freeze-dried culture supernatant allowed extraction with solvents miscible with water. Samples of 50-fold concentrated solvent extracts were bioassayed (Table 47). The zones of growth inhibition obtained with the solvent extracts were very similar for *L. acidophilus* and *L.*

Table 46.

Antimicrobial activity of fractions produced by fractional distillation of a butanol extract of *Lactobacillus* culture supernatant

DISTILLATION FRACTION (°C)[a]	ZONE DIAMETER[b] (mm)
75	+[c]
100	9
125	+
150	+
175	11
200	11
225	22
250	24

[a] Concentrated butanol extract of *L. acidophilus* was fractionally distilled under reduced pressure and samples collected at different temperatures.

[b] Samples (80µl) were added to wells (8mm diameter) in plates seeded with *C. albicans* B079. Zones of growth inhibition were measured after incubation at 37°C for 24h.

[c] + indicates a visible halo of growth inhibition around the well < 9mm zone diameter.

Table 47.

Antimicrobial activity of solvent extracts of freeze-dried culture supernatant of *Lactobacillus*

SOLVENT EXTRACTION[a]	ZONE DIAMETER (mm)[b] *L. acidophilus*	*L. fermentum*	MRS broth
methanol	8	6	0
ethanol	11	11	+[c]
butanol	14	14	7
chloroform/methanol	9	9	+[c]
chloroform/methanol/H_2O	7	7	0

[a] Culture supernatant (100ml) was freeze-dried. The resultant powder was extracted by stirring in 100ml solvent for 6h at room temperature and 18h at 4°C. The solvent extracts were then concentrated 50-fold.

[b] Samples (80µl) were added to wells (4mm diameter) in plates seeded with *C. albicans* B079. Zones of growth inhibition were measured after incubation at 37°C for 24h.

[c] +, indicates a visible halo of growth inhibition around the well <5mm zone diameter.

fermentum culture supernatants. Greatest inhibition was observed with the butanol extracts but part of this activity was due to growth inhibition by the solvent. Ethanol gave the second largest zone size after butanol. The ethanol control showed only a trace of activity, rather than a definite zone of growth inhibition which was obtained with the butanol control. The methanol, chloroform/ methanol and chloroform/ methanol/ water solvent systems did not extract antimicrobial activity as efficiently as ethanol. Ethanol extraction gave some purification of the antimicrobial substance as some media components were insoluble and could be removed from the solvent phase.

2.6.7 Column chromatography of an ethanol extract of *Lactobacillus* culture supernatant

The concentrated ethanol extract from a 5-day culture of *L. acidophilus* was applied to a column of Sephadex LH 20. Fractions from the column were pooled, concentrated and bioassayed in plate tests (Table 48). Fractions 1-7 and 8-20 inhibited growth of *C. albicans*. The elution was monitored by measuring absorbance of fractions at 280nm (Figure 12). This showed most of the extract was eluted from the column in a single large peak corresponding to fractions 8-20 which showed greatest antimicrobial activity. Therefore this method did not result in significant purification of the antimicrobial substance.

Table 48.

Bioassay of pooled, concentrated fractions from LH 20 Sephadex column chromatography of *L. acidophilus* culture supernatant

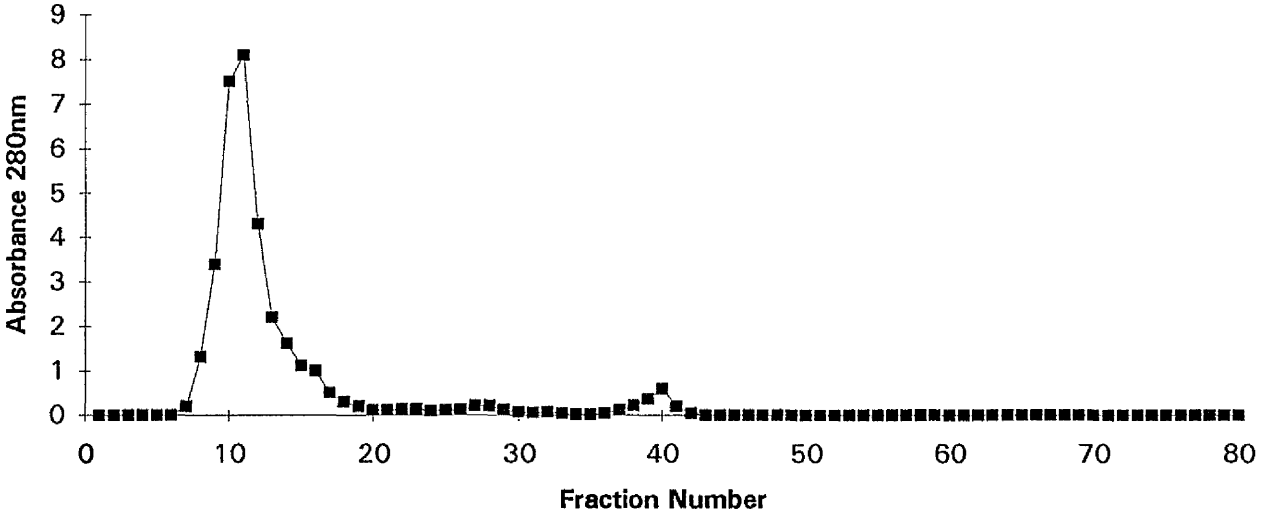
SAMPLE[a]	ZONE DIAMETER (mm)[b]
Unfractionated	11
Fractions 1-7	7
Fractions 8-20	10
Fractions 21-36	0
Fractions 37-42	0
Fractions 43-57	0
Fractions 58-72	0
Fractions 73-88	0
Fractions 89-104	0
Fractions 105-120	0

[a] Ethanol extract of *Lactobacillus* culture supernatant was applied to an LH 20 Sephadex column. The column was eluted with ethanol and 15ml fractions collected. Fractions were pooled and concentrated 50-fold using rotovapour apparatus.

[b] Samples (80μl) were added to wells (4mm diameter) in plates seeded with *C. albicans* B079. Zones of growth inhibition were measured after incubation at 37°C for 24h.

Figure 12.

Column chromatography of an ethanol extract of *L. acidophilus* culture supernatant on LH 20 Sephadex



L. acidophilus was grown for 5 days in MRS broth. Culture supernatant was freeze-dried and extracted with ethanol. The ethanol extract was concentrated 50-fold using a rotary evaporator and applied to a column (bed volume 100ml; 200mm internal diameter) of Sephadex LH 20. The column was eluted with ethanol and fractions (15ml) collected automatically. Elution was monitored by measuring absorbance of fractions at 280nm.

2.6.8 Thin Layer Chromotography

2.6.8.1 Butanol/glacial acetic acid/water solvent

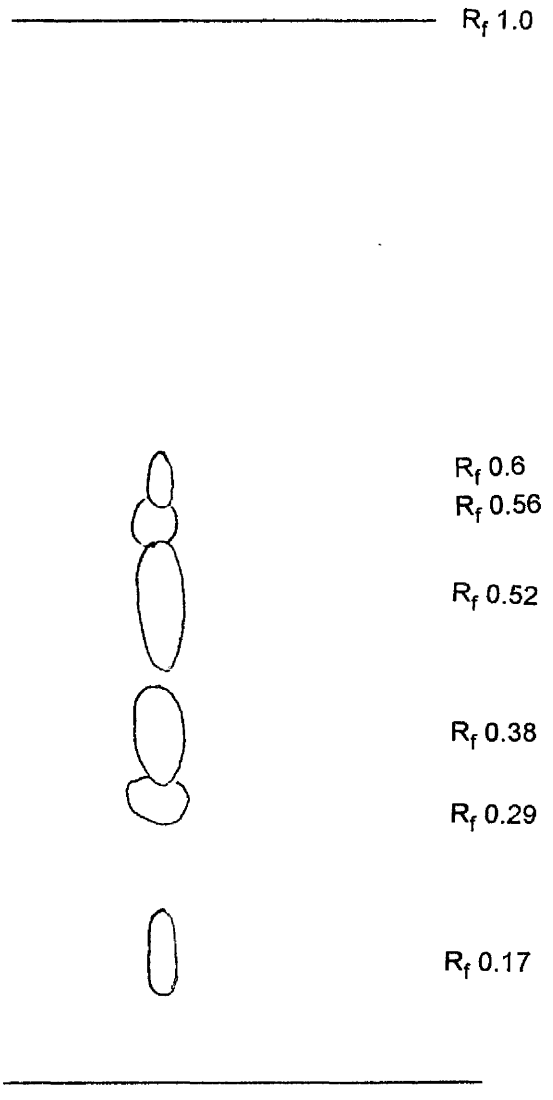
Preparative TLC was carried out in an attempt to separate the antimicrobial agent in Sephadex LH 20 column fractions from peptide components of the growth medium. Fractions 8-20 from the LH 20 column were pooled, concentrated applied to a TLC plate and run in a butanol/ glacial acetic acid/ water solvent system. After chromatography, six components were revealed with ninhydrin, a locating reagent for amino acids (Figure 13).

A preparative chromatogram was also run in this solvent system. The plate was then split into sections corresponding to R_f values of 0.25, 0.38, 0.56, 0.60 and 1.00. The silica from each section was scraped from the plate and extracted with ethanol. The ethanol extracts were concentrated and samples were tested in plate assays with *C. albicans* B079. Zones of growth inhibition corresponded to fractions with R_f values of 0.6 and 1.00 (Table 49).

An analytical TLC plate was run in the same solvent to check the purity of each sample used in the bioassay (Figure 14). The sample corresponding to an R_f value of 0.25 on the preparative plate gave no spot with ninhydrin probably due either to insufficient loading or ineffective elution from the original TLC plate. One sample which gave bioactivity showed up as a spot with a R_f value of 0.63. The sample corresponding to R_f 1.00 showed bioactivity but gave no visible spot with ninhydrin.

Figure 13.

TLC separation of the biologically active fraction from Sephadex LH 20 column



Solvent system, butanol / glacial acetic acid / H_2O (60 : 15 : 25, by volume)
Locating reagent , Ninhydrin

Table 49.

Antimicrobial activity of ethanol extracts fractionated by TLC (butanol / glacial acetic acid / water solvent)

SAMPLE[a]	ZONE DIAMETER (mm)[b]
Unfractionated	10
R_f 0.25	+[c]
R_f 0.38	+
R_f 0.56	0
R_f 0.60	9
R_f 1.00	9
ethanol control	+

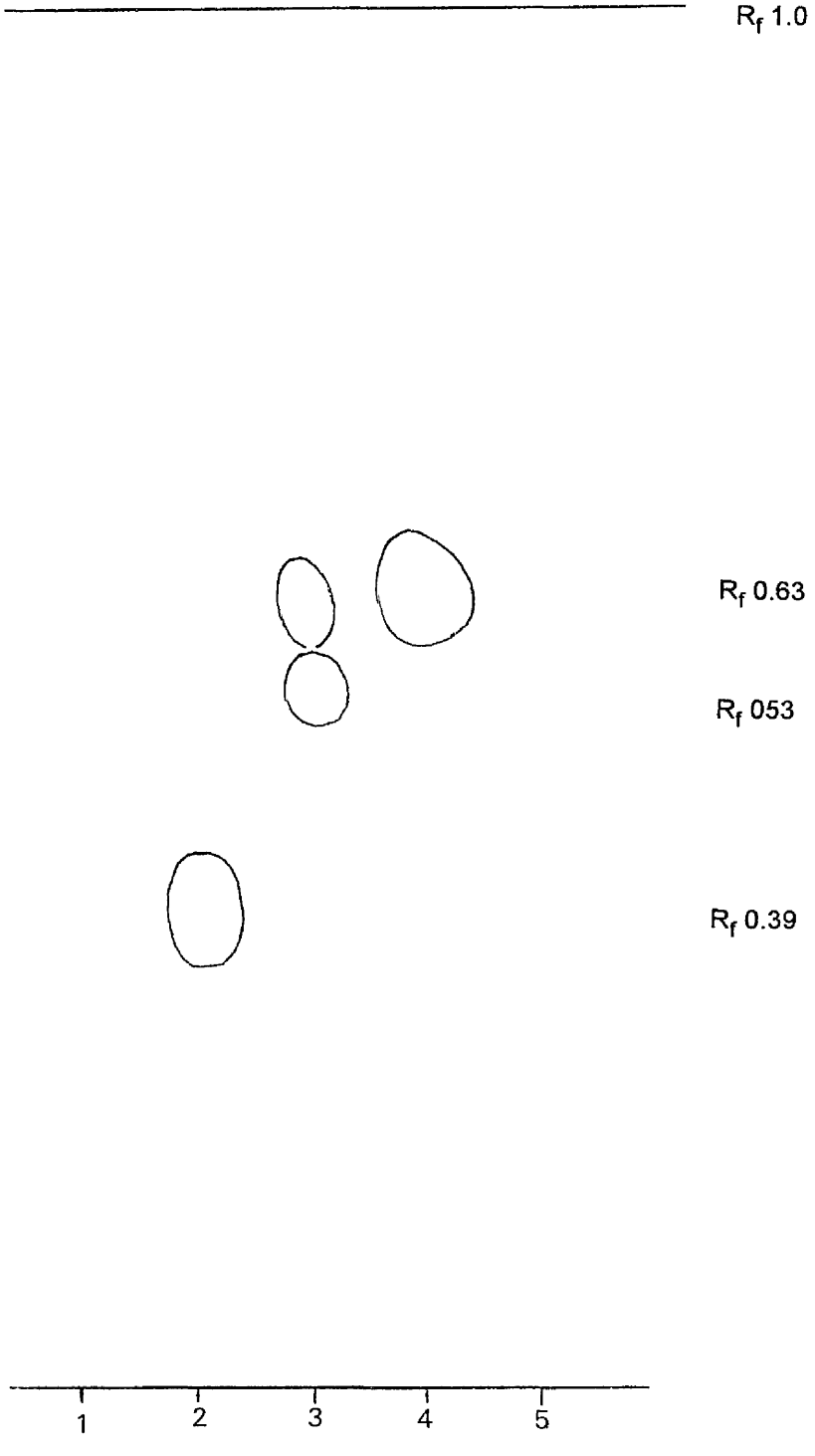
[a] A streak chromatogram was prepared on a silica gel G TLC plate with the biologically active fraction from the Sephadex LH 20 column. The TLC plate was run in butanol/ glacial acetic acid/ H_2O. The plate was split into sections corresponding to the indicated R_f values, the silica scraped from the plate and extracted overnight with ethanol. The ethanol extracts were concentrated and tested for bioactivity in a plate test.

[b] Samples (80µl)were put into wells (4mm diameter)in an agar plate seeded with *C. albicans* B079. After incubation for 24h, the zone diameter around each well was measured.

[c] +, indicates a visible halo of growth inhibition around the well <5mm zone diameter.

Figure 14.

Purity check of fractions separated by preparative TLC and bioassayed in a plate test



Fractions obtained by preparative chromatography of the biologically-active material from a Sephadex LH 20 column were run on TLC plates to check the purity of the samples. The solvent system was butanol/ glacial acetic acid/ H_2O with ninhydrin as the locating reagent.

Track 1, R_f 0.25 component; Track 2, R_f 0.38 component; Track 3, R_f 0.56 component; Track 4, R_f 0.60 component; Track 5, R_f 1.00.

2.6.8.2 Chloroform/methanol/water solvent

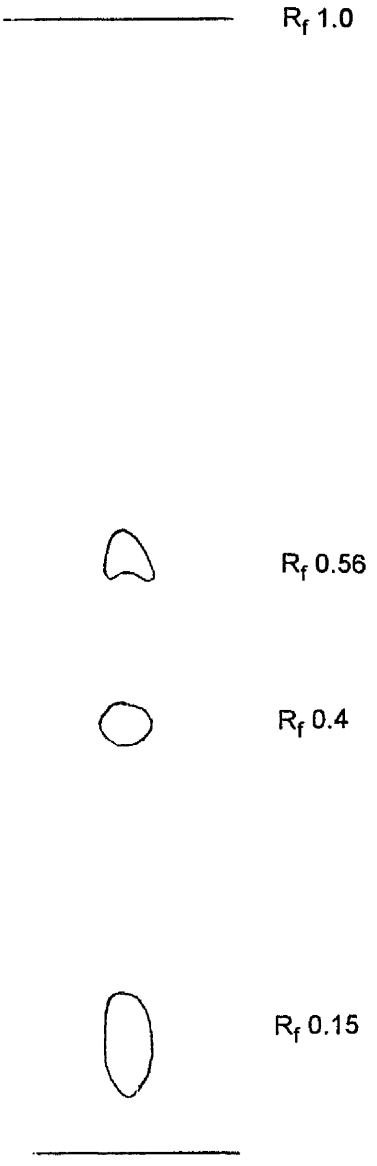
Concentrated extract, which corresponded to R_f values between 1.0 and 0.6 in the butanol/glacial acetic acid/water solvent, was then applied to a separate analytical TLC plate and run in a chloroform/ methanol/water solvent system. Iodine vapour was used as a locating reagent. This system located a faint spot with an R_f value of 0.56. Subsequent spraying of the chromatogram with ninhydrin revealed two spots with R_f values of 0.4 and 0.15, respectively (Figure 15). This showed that concentrated extract which corresponded to R_f values between 1.0 and 0.6 in the butanol/glacial acetic acid/water solvent system still contained some peptide components of the growth medium.

A preparative chromatogram which had been run in the chloroform/methanol/water solvent system was split into two sections. Silica was scraped from the section corresponding to R_f 0.00-0.56 and eluted with ethanol. The second section of the chromatogram corresponded to R_f values between 1.00 and 0.56; again the silica was scraped from the section and eluted with ethanol. The ethanol extracts were concentrated and put into wells in agar seeded with *C. albicans* B079. Both components (R_f 0.56 and R_f 1.00) showed antimicrobial activity (Table 50). This demonstrated that antimicrobial activity could not be attributed to either a peptide or a lipid as activity was found in the fraction corresponding to R_f 0.56-1.00 in the chloroform/methanol/water solvent and this fraction had been shown to contain neither peptide nor lipid.

It was postulated that antimicrobial activity might be due to a low molecular weight acid. The antimicrobial substance from the culture extract was therefore compared with lactic acid. Lactic acid was applied to a TLC plate and run in the chloroform/methanol/water solvent system. The preparative TLC plate was separated into 2

Figure 15.

Further TLC separation of the biologically active fraction from Sephadex LH 20 column



Biologically active fractions from a Sephadex LH 20 column were pooled, concentrated and run in a butanol/ glacial acetic acid / water solvent system. The plate was split into sections which correspnded to R_f values between 1.0 and 0.6 and the silica was scraped from the plate and extracted with ethanol. This concentrated extract was then applied to a second analytical TLC plate and run in chloroform / methanol /water solvent system.

Solvent system/ chloroform/ methanol/ H_2O (75: 22: 3, by volume)

Locating reagent, Iodine vapour located the component with R_f value of 0.56
Ninhydin located the components with R_f values of 0.4 and R_f 0.15

Table 50.

Antimicrobial activity of ethanol extracts fractionated by TLC (chloroform / methanol / water solvent)

SAMPLE[a]	ZONE DIAMETER (mm)[b]
Concentrated extract[c]	9
R_f 0.56	6
R_f 1.00	8

[a] Concentrated extract, which correspond to R_f values between 1.0 and 0.6 in the butanol / glacial acetic acid / water solvent was then applied to a separate TLC plate. A streak chromatogram was prepared on a silica gel G TLC plate. The chromatogram was run in chloroform / methanol / H_2O solvent system. The plate was split into sections corresponding to the indicated R_f values, the silica scraped from the plate and extracted with ethanol. The ethanol extracts were concentrated and tested for activity.

[b] Samples (80µl) were added to wells (4mm diameter) in plates seeded with *C. albicans* B079. Zones of growth inhibition were measured after incubation at 37°C for 24h.

[c] Concentrated extract, purified antimicrobial agent from *L. acidophilus*, which corresponded to R_f values between 1.0 and 0.6 in the butanol / glacial acetic acid / water solvent system.

sections corresponding to R_f 0.00-0.56 and R_f 0.56-1.00 and the silica extracted with ethanol. Lactic acid in this solvent system showed a similar separation of antimicrobial activity in plate tests as the *Lactobacillus* extract (Table 51).

2.6.9 Low resolution Electron Impact Mass Spectrometry

The molecular mass of antifungal substance purified by ethanol extraction, LH 20 column chromatography, followed by TLC in a butanol/glacial acetic acid/water solvent and then TLC in a chloroform/methanol/water solvent was measured by low resolution Electron Impact Mass Spectrometry. Three homologues, with molecular mass 419, 433 and 447Da, were found in the sample.

These experiments showed antimicrobial activity was not due to an antimicrobial peptide or lipid. It was also not due to the lactic acid which has a relative molecular mass of 90.

3. Interactions between *Candida* and Lactobacilli *in vivo*

3.1 Mouse model for *Candida* vaginitis

3.1.1 Treatment with different concentrations of *Candida*

Female mice were injected subcutaneously with 17 β—oestradiol-3-benzoate to bring them into a state of pseudoestrous. Animals in groups of five were treated with suspensions containing either 10^7, 10^8 or 10^9 galactose-grown *C. albicans* GDH 2346/ml in PBS (pH 7.2). After 7 days, infection was quantified by removal and homogenization of the vagina followed by dilution and viable plate counts. Samples were plated out on Sabouraud dextrose agar containing penicillin and streptomycin to obtain the *Candida* count per vagina.

Results, expressed as the average vaginal count for each group

Table 51.

Antimicrobial activity of lactic acid fractionated by TLC (chloroform / methanol / water solvent)

SAMPLE[a]	ZONE DIAMETER (mm)[b]
Lactic acid	ND[c]
R_f 0.56	7
R_f 1.00	9

[a] A streak chromatogram of lactic acid was prepared on a silica gel G TLC plate. The chromatogram was run in chloroform/ methanol/ H_2O solvent system. The plate was split into sections corresponding to the indicated R_f values, the silica scraped from the sections and extracted with ethanol. The ethanol extracts were concentrated and tested for activity.

[b] Samples (80µl) were added to wells (4mm diameter) in plates seeded with *C. albicans* B079. Zones of growth inhibition were measured after incubation at 37°C for 24h.

[c] ND, not done.

of 5 animals, are shown in Table 52. Increases in total vaginal counts were observed with increasing treatment concentrations of *Candida*. The average total vaginal count doubled for each log increase in treatment concentration. For the 10^7 *Candida*/ml treatment concentration only 60% of the mice were infected on day 7 after inoculation. Increasing the yeast treatment concentration increased the percentage of infected animals in a treatment group. Increasing the yeast treatment concentration to 10^8 cells/ml gave an 80% infection rate; with a concentration of 10^9 cells/ml all animals within the group were infected by day 7.

3.1.2 Time course of infection

Oestradiol-treated mice were inoculated with galactose-grown *C. albicans* GDH 2346, of concentration 10^7, 10^8 or 10^9 *Candida*/ml suspended in PBS. On days 2, 4 and 7 after inoculation five animals from each treatment group were sacrificed so the course of infection could be followed (Table 53).

Infection of mice inoculated with 10^8 and 10^9 yeasts/ml was maximal on day 2. However, with a treatment concentration of 10^7 yeasts/ml, infection was maximal on day 4. After peak infection had occurred there was a decline in *Candida* numbers. With a treatment concentration of 10^9 yeasts/ml infection had completely cleared by day 7.

The highest vaginal counts were observed on days 2 and 4 for a yeast treatment concentration of 10^8 cells/ml. By day 7, the highest count was observed with 10^7 yeasts/ml, as more effective clearing appeared to occur with concentrations of 10^8 and 10^9 cells/ml.

Table 52.

Treatment of the mouse vagina with different concentrations of *Candida*

YEAST CONCENTRATION (cells/ml)[a]	TOTAL VAGINAL COUNT[b] (10^3 cfu/ml) ± SEM[c] DAY 7	PERCENTAGE INFECTION[d]
10^7	0.605 ± 0.3	60
10^8	1.275 ± 0.5	80
10^9	2.880 ± 1.7	100

[a] Oestradiol-treated mice were inoculated by squirting the suspension of *C. albicans* GDH 2346 into the vagina with a 1ml syringe until the suspension began to run out of the vagina.

[b] After 7 days, infection was quantified by removal and homogenization of the vagina followed by dilution and plate counting. Results are expressed as the average *Candida* count for 5 animals.

[c] SEM, standard error of the mean.

[d] The number of mice showing infection was expressed as a percentage of the total number of mice treated with *Candida*.

Table 53.

Time course of infection after treatment of the mouse vagina with different concentrations of *Candida*

YEAST CONCENTRATION[a] (cells/ml)	TOTAL VAGINAL COUNT (10^3 cfu/ml)[b] ± SEM[c]		
	Day 2	Day 4	Day 7
10^7	1.13 ± 0.46	5.12 ± 3.08	2.43 ± 1.13
10^8	42.38 ± 22.63	24.28 ± 15.64	0.24 ± 0.10
10^9	14.06 ± 4.25	10.22 ± 6.49	0.0 ± 0

[a] Oestradiol-treated mice were inoculated by squirting the suspension of *C. albicans* GDH 2346 into the vagina with a 1ml syringe until the suspension began to run out of the vagina.

[b] At different times after infection 5 animals were taken from each treatment group. Infection was quantified by removal and homogenization of the vagina followed by dilution and plate counting. Results for each time interval are expressed as the average *Candida* count for 5 animals.

[c] SEM, standard error of the mean.

3.1.3 Time course of infection in diabetic mice

Female mice were injected intravenously with alloxan to produce a chemically induced diabetes mellitus. To confirm that the mice had become diabetic the presence of glucose in the urine was tested. Diabetic mice were then put into pseudoestrous by a subcutaneous injection of 17 β-oestradiol-3-benzoate. Two days later mice were infected intravaginally with suspensions of 10^7, 10^8 or 10^9 galactose-grown *Candida*/ml. Mice were sacrificed at different time intervals to follow the course of *Candida* infection (Table 54).

The total vaginal counts for diabetic mice were much greater than those seen for normal mice (Table 53). Infection of mice inoculated with 10^9 yeasts/ml was maximal on day 2. On day 5, higher yeast concentrations gave higher total vaginal counts. Clearing of the infection was most marked with a yeast treatment concentration of 10^9 cells/ml. Clearing of infection was not as effective as that seen in normal mice where by day 7, no *Candida* were recovered from vaginas of mice treated with 10^9 cells/ml (Table 53). With a yeast concentration of 10^7 cells/ml infection was maximal on day 7 and no sign of clearing was observed. Indeed, the group of mice on day 7 gave the highest vaginal count observed in the experiment.

3.2 Rat model for *Candida* vaginitis

3.2.1 Colonization of the rat vagina with *L. acidophilus* NCTC 4504

This experiment was designed to determine whether *L. acidophilus* could colonize the rat vagina. Lactobacilli have previously been isolated as part of the normal rat vaginal flora but in lower numbers than are present in the human vagina (Larsen *et al.*, 1976)α.

Table 54.

Time course of infection in diabetic mice after treatment of the vagina with different concentrations of *Candida*

YEAST CONCENTRATION[a] (cells/ml)	TOTAL VAGINAL COUNT (10^3 cfu/ml)[b] ± SEM[c] Day 2	Day 5	Day 7
10^7	7165.6 ± 52.7	16.3 ± 4.7	7300.8 ± 155.7
10^8	218.7 ± 60.1	58.4 ± 22.3	789.2 ± 51.5
10^9	7230.8 ± 69.8	755.0 ± 25.0	58.7 ± 27.8

[a] Oestradiol-treated diabetic mice were innoulated by squirting the suspension of *C. albicans* GDH 2346 into the vagina with a 1ml syringe until the suspension began to run out of the vagina.

[b] At different times after infection, animals were taken from each treatment group and infection was quantified by removal and homogenization of the vagina followed by dilution and plate counting. Results for each time interval are expressed as the average *Candida* count for 5 animals.

[c] SEM, standard error of the mean.

Female ovarectomised rats were injected with oestradiol to induce pseudoestrous. A group of 3 rats was used in this experiment. The rats were swabbed to obtain a background bacterial count. The rats were then infected with 0.1ml lactobacilli (5×10^8 cells/ml) in gum arabic using a 1ml sterile blunt-ended syringe. On days 1, 2, 3 and 7 after infection *Lactobacillus* colonization was assessed by swabbing and preparation of a vaginal smear and plate counts. On days 0, 2 and 4 the pH value of the vagina was measured using a portable glass electrode.

Results are shown in Table 55. Samples were plated out on MRS agar and incubated microaerophilically. MRS is a non-selective medium allowing profuse growth of lactobacilli and other organisms from the vaginal flora. The viable counts on MRS agar gave some indication of changes in total numbers of bacteria over the course of the experiment. An increase in bacterial numbers was greatest on day 1 after infection. Numbers declined on days 2 and 3, with numbers on day 7 being lower than at the start of the experiment.

On days 1, 3 and 7 samples were also plated out on modified MRS agar, the pH value of which had been lowered to 5.4 by the addition of 20 g/l sodium acetate and 1.32 ml/l glacial acetic acid. Total vaginal counts on this medium were lower than those obtained on MRS agar; lowering the pH value of the medium made growth conditions unsuitable for a proportion of the vaginal flora. With this modified medium, no comment can be made on the colonization of the vagina with lactobacilli as the background count before colonization was initiated was not determined.

On MRS agar four different colony types were observed. Lactobacilli had a distinctive rough colony morphology. Consequently, counts of colonies fitting with this description on

Table 55.

Colonization of the rat vagina by *L. acidophilus* NCTC 4504

DAY	TOTAL VAGINAL COUNT (10^5 cfu/ml)[a] ± SEM[e]			MEAN NUMBER OF ADHERENT LACTOBACILLI/ EPITHELIAL CELL ± SEM[e]	AVERAGE VAGINAL pH
	MRS[b]	Modified MRS[c]	Lactobacilli[d]		
0	11.0 ± 1.3	ND[f]	0.04 ± 0.04	25.8 ± 4.8	7.0 ± 0.1
1	17.7 ± 2.5	8.9 ± 6.4	0.8 ± 0.3	66.7 ± 7.4	ND
2	13.1 ± 0.2	ND	4.0 ± 0.4	69.2 ± 8.8	6.9 ± 0.1
3	13.3 ± 0.9	6.5 ± 2.7	3.4 ± 1.1	58.6 ± 8.5	7.0 ± 0.0
7	7.6 ± 2.0	3.8 ± 2.1	0.2 ± 0.1	31.7 ± 4.9	ND

[a] Rats were sampled by swabbing. The swab was broken off into a small test tube containing 1.5ml PBS, agitated using a whirlimixer and a 0.1ml sample plated out using a spiral plater. Plates were incubated microaerophilically at 37°C.

[b] A 0.1ml sample was plated out onto MRS agar.

[c] A 0.1ml sample was plated out onto modified MRS agar containing 20g/l sodium acetate and 1.32ml/l glacial acetic acid.

[d] From MRS plates, counts of bacteria with a rough colony morphology were taken.

[e] SEM, standard error of the mean.

[f] ND, not done.

the MRS plates were made. Much lower counts were obtained when using this method for distinguishing lactobacilli (Table 55). Prior to inoculation few lactobacilli were seen, a situation which is not unusual (Larsen *et al.*, 1976)**a**. This method showed that colonization began on day 1, with increases in numbers to a maximum at days 2 and 3. The numbers of lactobacilli decreased by day 7.

The numbers of adherent bacterial rods per cell were counted for 50 epithelial cells examined on a Giemsa-stained vaginal smear taken from each rat on every day of the experiment. The mean count/cell was calculated and the standard error of the mean determined. Numbers of adherent bacteria increased on day 1 after colonization. Maximum numbers of adherent bacteria occurred on day 2. Numbers decreased to background level by day 7. This picture of colonization was similar to that obtained when lactobacilli were distinguished by colony morphology on MRS plates. Colonization of the rat vagina with *L. acidophilus* had no significant effect on the pH value of the vagina.

3.2.2 Repeated colonization of the rat vagina with *L. acidophilus*

Previous results showed *L. acidophilus* was able to colonize the rat vagina. However, this colonization was short-term and numbers of lactobacilli returned to a background level by day 7 (Table 55). In this experiment repeated inoculation of the rat vagina with lactobacilli was carried out to establish whether a longer term vaginal colonization could be achieved. Female, ovarectomised rats, induced to pseudoestrous, were swabbed in order to obtain a background bacterial count. Rats were infected with 0.1ml lactobacilli (5×10^8 cells/ml) in gum arabic on days 0, 2, 5, 9 and 16. The course of *Lactobacillus* colonization was followed over 26

days. Colonization was assessed by swabbing and preparation of plate counts on Rogosa agar, a selective medium for lactobacilli.

Viable counts on Rogosa agar gave an indication of changes in the total numbers of lactobacilli. Results are illustrated in Figure 16. The *Lactobacillus* count was calculated as the average colony forming units/ml (cfu/ml) for a group of 6 rats. The numbers of lactobacilli fluctuated over the course of the experiment; however, repeated inoculation maintained the increased *Lactobacillus* population in the rat vagina. Ten days after the final inoculation, numbers of lactobacilli returned to the background level. Repeated inoculation maintained lactobacilli numbers but could not establish a permanently elevated *Lactobacillus* population.

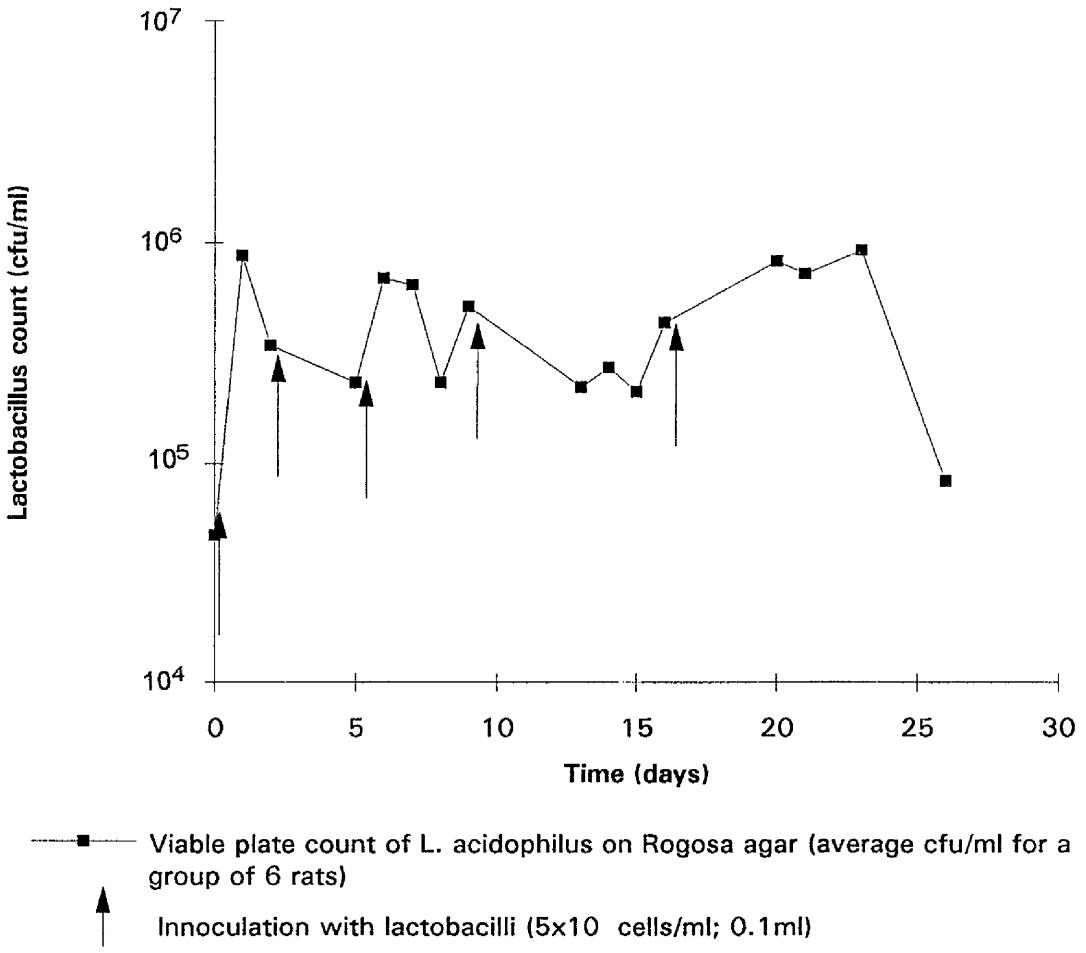
3.2.3 Effect of colonization by *L. acidophilus* on subsequent *Candida* infection of the rat vagina

This experiment was designed to determine whether colonization of the rat vagina with a population of *L. acidophilus* NCTC 4504 would protect against vaginal infection with *C. albicans* B079.

Female, ovarectomised rats, induced to pseudoestrous, were assigned to three groups: a *Lactobacillus* control, a *Candida* control and a test group. Each group comprised five animals. The *Lactobacillus* control and test group were inoculated intravaginally with *L. acidophilus* (0.1ml; 5×10^9 cells/ml) on days 0, 2 and 4. On day 5, *C. albicans* (0.1ml; 5×10^6 cells/ml) was used to infect the test and *Candida* control groups. *Lactobacillus* colonization was assessed by swabbing and plate counts on Rogosa agar, a selective medium for lactobacilli. *Candida* infection was determined by swabbing and plate counts on Sabouraud dextrose agar containing penicillin and streptomycin. A background count of lactobacilli was

Figure 16.

Repeated colonization of the rat vagina with *L. acidophilus*



taken on day 0 and of *Candida* on day 5. Results were expressed as average cfu/ml for a treatment group of rats (Table 56).

Inoculation of rats with lactobacilli gave colonization and an increase in numbers of *L. acidophilus* within the vagina. Colonization was not long-term and by day 10 numbers of lactobacilli had returned to the background level. A background count of *Candida* on day 5 was obtained before the test group and *Candida* control groups were inoculated with *Candida*. The numbers of *Candida* after the establishment of infection fluctuated from day to day.

In this experiment, inoculation of the rat vagina with *L. acidophilus*, resulting in an increased *Lactobacillus* population, had no protective effect against subsequent *Candida* infection. This is shown in Table 56 where numbers of *Candida* are not significantly different in the control group and test group.

3.2.4 Effect of butanol extract of *Lactobacillus* culture supernatant on *Candida* infection of the rat vagina

A butanol extract of *L. acidophilus* culture supernatant had shown anticandidal activity in plate assays (Table 41). This experiment was carried out to determine whether the same extract would show anticandidal activity *in vivo* and give a protective effect against infection with *C. albicans* B079.

Female ovarectomised rats, induced to pseudoestrous, were assigned to three groups each comprising five animals. Group 1 was inoculated intravaginally with a butanol extract (pH 4.5; 0.1ml) of *L. acidophilus* culture supernatant. Group 2 received a butanol extract of MRS broth. Group 3 was treated with citrate buffer, pH 4.5. Rats were dosed as above on days 1, 2, 3, 4, 5, 8, 10, and 12. On day 3 all animals were inoculated with a suspension of *C.*

Table 56.

Effect of colonization by *L. acidophilus* on subsequent *Candida* infection in the rat vagina

DAY	*LACTOBACILLUS* COUNT (10^6 cfu/ml)		*CANDIDA* COUNT (10^3 cfu/ml)	
	LACTOBACILLUS CONTROL GROUP	TEST GROUP	*CANDIDA* CONTROL GROUP	TEST GROUP
0	0.64 ±0.2	0.63 ±0.3	-	-
5	3.8 ±2.1	11.0 ±1.3	0.0	0.3 ± 0.1
7	4.7 ±2.0	1.6 ±0.3	24.8 ±16.7	27.8 ±10.5
10	0.78 ±2.0	0.43 ±1.1	34.0 ±13.5	19.0 ± 7.8
16	1.2 ±0.13	0.41 ±0.2	11.0 ± 5.2	38.0 ±14.4

[a] *Lactobacillus* colonization was assessed by swabbing and plate counts on Rogosa agar, a selective medium for lactobacilli.

[b] *Candida* infection was determined by swabbing and plate counts on Sabouraud dextrose agar containing penicillin and streptomycin.

[c] The *Lactobacillus* control group was inoculated intravaginally with *L. acidophilus* (0.1ml; 5 x 10^9 cells/ml) on days 0, 2 and 4.

[d] The test group was inoculated intravaginally with *L. acidophilus* (0.1ml; 5 x 10^9 cells/ml) on days 0, 2 and 4. On day 5, *C. albicans* (0.1ml; 5 x 10^6 cells/ml) was used to infect the test group.

[e] On day 5, *C. albicans* (0.1ml; 5 x 10^6 cells/ml) was used to infect the *Candida* control group.

albicans (0.1ml; 5 x 10^6 cells/ml). *Candida* infection was assessed by swabbing and plate counts on Sabouraud dextrose agar containing penicillin and streptomycin.

Results (Table 57) showed that after the establishment of infection numbers of *Candida* fluctuated from day to day. Prior to day 10, rats treated with *Lactobacillus* extract showed greater numbers of *Candida* within the vagina than control rats treated with an MRS extract. On days 12 and 15 numbers of *Candida* within the vaginas of rats treated with *Lactobacillus* extract declined and were lower than numbers within the vagina of control rats treated with MRS extract. Rats from the *Candida* control group showed counts higher than those obtained for rats treated with *Lactobacillus* extract on days 5, 10 and 15, and counts lower than those observed for *Lactobacillus* extract-treated rats on days 4, 8 and 12.

These results indicate that treatment of the rat vagina with a butanol extract of *L. acidophilus* culture supernatant (which showed anticandidal activity in plate assays) had no significant protective effect against subsequent *Candida* infection.

Table 57.

Effect of butanol extract of *Lactobacillus* culture supernatant on *Candida* infection in the rat vagina

DAY	*CANDIDA* COUNT[a] (10^3 cfu/ml)		
	GROUP 1[b] (*LACTOBACILLUS* EXTRACT)	GROUP 2[c] (MRS EXTRACT CONTROL)	GROUP 3[d] (*CANDIDA* CONTROL)
1	-	-	-
2	-	-	-
3	0	0	0
4	9.6 ± 4.6	6.9 ± 2.6	5.5 ± 2.1
5	7.5 ± 2.5	4.4 ± 1.2	66.3 ± 44.4
8	83.1 ± 46.3	36.2 ± 14.3	58.0 ± 50.4
10	6.9 ± 2.3	6.2 ± 2.1	7.7 ± 3.1
12	5.1 ± 0.8	8.6 ± 3.8	3.5 ± 0.6
15	3.6 ± 1.6	6.9 ± 3.0	4.5 ± 1.0

[a] *Candida* infection was assessed by swabbing and plate counts on Sabouraud dextrose agar containing penicillin and streptomycin.

[b] Five female, ovarectomised rats, induced to pseudoestrous were inoculated intravaginally with butanol extract (pH 4.5; 0.1ml) of *L. acidophilus* culture supernatant on days 1, 2, 3, 4, 5, 8, 10 and 12. On day 3, animals were inoculated with a suspension of *C. albicans* (0.1ml; 5 x 10^6 cells/ml)

[c] Five female, ovarectomised rats, induced to pseudoestrous, were inoculated intravaginally with a butanol extract of MRS broth on days 1, 2, 3, 4, 5, 8, 10 and 12. On day 3, animals were inoculated with a suspension of *C. albicans* (0.1 ml; 5 x 10^6 cells/ml)

[d] Five female, ovarectomised rats, induced to psuedoestrous were inoculated intravaginally with citrate buffer (pH 4.5) on days 1, 2, 3, 4, 5, 8, 10 and 12. On day 3, animals were inoculated with a suspension of *C. albicans* (0.1ml; 5 x 10^6 cells/ml)

DISCUSSION

1. Adhesion of *C. albicans* to vaginal epithelial cells

Various methods have been used to measure adherence of *C. albicans* to mucosal epithelial cells. The method used in this study was that of Kimura & Pearsall (1978) as modified by Douglas *et al.* (1981). This involved mixing standardized yeast and epithelial suspensions under controlled conditions, filtering the assay mixtures on polycarbonate filters and counting the numbers of yeasts adhering to epithelial cells by light microscopy.

1.1 Effect of concentration of assay components on adhesion

Increasing the concentration of *Candida* in the assay mixture gave increased adhesion of yeast to epithelial cells. Kimura & Pearsall (1978) showed adherence of *C. albicans* was dependent on yeast cell concentration.

Anderson & Odds (1985), using a yeast concentration of 10^8 cells/ml, reported a mean number of approximately four *C. albicans* cells attached per epithelial cell; this was of the same order as the adhesion described by Collins-Lech *et al.* (1984), Kearns *et al.* (1983), Kimura & Pearsall (1980), Ray *et al.* (1984) and also that of this study, but was much lower than the range of 42-174 *C. albicans*/epithelial cell found by King *et al.* (1980). These workers suggested that the large numbers of adherent blastospores observed by them were a reflection not only of yeast cell-to-epithelial cell adhesion, but also of blastospore co-adherence. Some co-adherence was seen in the present study; however, the problem was not as severe as that observed by King *et al.* (1980) and, for the purpose of our adherence assays, only the yeasts directly attached to the epithelial cells were counted.

When high concentrations of *C. albicans* were used in the assay mixture (1 x 10^8 cells/ml) the concentration of epithelial cells also had an effect on yeast adherence. More yeasts adhered when greater numbers of epithelial cells were present. This was probably due to the increased likelihood of *Candida* making contact with an epithelial cell and therefore binding. Vaginal epithelial cells were used at 5 x 10^5 cells/ml in most assays as this concentration gave maximal values for *Candida* adhesion.

In all assays, the ratio of yeast concentration to epithelial cell concentration was important. When this ratio was greater than 10^4:1 there was a decrease in yeast binding. A possible explanation for this is the saturation of available *Candida* binding sites on the epithelial cell. Another possibility is that increasing the yeast concentration may cause *Candida* co-adherence which, by clumping yeasts together would effectively reduce the number available to attach to binding sites.

1.2 Adhesion to epithelial cells from different animal species

Adherence is influenced not only by the yeasts; the origin of the epithelial cells also plays a critical role. When different epithelial cell types (i.e. rat, mouse or human) were used in the adhesion assay, the yeasts adhered to different extents. Human epithelial cells were able to bind more *Candida*. This is perhaps not surprising as *Candida* species are most commonly found as human pathogens. The differences in binding may also partly depend on the fact that human cells are slightly bigger than mouse or rat cells. Mouse and rat cells are approximately the same size.

Within a single animal species, the origin of the epithelial cells can influence yeast adhesion and mucosal cells from various

sources are not equivalent (King *et al.*, 1980; Sobel *et al.*, 1981). It seems reasonable to conclude that the inherent mucosal cell receptiveness for *Candida*, coupled with other factors in their microenvironment, make some body sites more prone to *Candida* infections than others.

1.3 Day-to-day variation in adhesion values

It might have been expected that use of epithelial cells from the same donor would result in consistent adherence values from day-to-day. However, in this study considerable variations were observed. Kearns *et al.* (1983) also found substantial day-to-day variance compared with variance between replicates. Sandin *et al.* (1987a) and Bibel *et al.* (1987) similarly noted that numbers of yeasts adhering to buccal mucosal cells collected on different dates varied significantly. The day-to-day variation could be due to small differences in growth conditions of the fungus, or day-to-day variation in the surface properties of the epithelial cells due to environmental factors which might lead to differences in the expression of *Candida* receptors. Hormonal status, for example, is likely to affect the nature and availability of vaginal cell receptors at different stages in the menstrual cycle. It has been suggested that acquisition of adherence-interfering or adherence-enhancing host secretions may also have an important role (Bibel *et al.*, 1987).

Day-to-day variations observed in experiments with mouse or rat cells may have been due to the use of pooled cells, with differences between individual animals from whom epithelial cells were collected. A number of authors (Sandin *et al.*, 1987a; King *et al.*, 1980; Kearns *et al.*, 1983; Sobel *et al.*, 1981; Cox, 1986) found

donor-to-donor variations when using human epithelial cells; there is no reason to suppose that there would not be similar donor-to-donor variations between mouse or rat vaginal epithelial cells from different animals. The effect of artificial oestrous on *Candida* receptors is not fully elucidated. It is known that *Candida* infections of the rat or mouse vagina cannot be induced if the animals are not in the oestrous stage of their oestrous cycle. This may be because yeasts are less able to attach to the vaginal epithelial cells at the other stages of the oestrous cycle and are thus unable to cause infection.

1.4 Cell-to-cell variation in adhesion values

In assessing adherence by light microscopy it is possible to observe the distribution of yeasts over the epithelial cell surface. Certain epithelial cells have larger numbers of adherent yeasts than others. This is probably due to non-uniformity in the population of epithelial cells. Human, mouse and rat vaginal epithelial cells all showed great variation in the number of *C. albicans* that could attach to each individual cell. To explain these findings consideration was given to the possibility that there might be sub-populations of vaginal epithelial cells with high and low receptivity for *C. albicans*. It was found that the majority of the vaginal epithelial cells had few or no *C. albicans* attached to their surfaces, whereas a minority of the epithelial cells had greater numbers of attached yeasts.

One factor which could contribute to the non-normal distribution of *Candida* is the presence of the indigenous bacterial microflora. Epithelial cells have been shown to exhibit large cell-to-cell variations in the number of bacteria that adhere to their

surfaces (Bartelt & Duncan, 1978; Rosenstein *et al.*, 1985). Bacteria which are present on the mucosal cell surface may block or compete for *Candida* receptor sites and thus inhibit yeast adhesion. For instance, epithelial cells from conventional rats were found to have lower numbers of attached *C. albicans* than epithelial cells from germ-free rats (Liljemark & Gibbons, 1973). Epithelial cells pretreated with lactobacilli also showed decreased numbers of adherent *C. albicans* (Sobel *et al.*, 1981). Conversely, antibiotic treatment of hamsters reduced the number of indigenous bacteria in the gut and promoted yeast adhesion, colonization and dissemination (Kennedy & Voltz,1985).

Another factor which may contribute to the non-normal distribution of *Candida* is differences in the membrane receptor population on the mucosal cell surface. The population of epithelial cell receptors for *C. albicans* may vary from cell-to-cell. Sandin *et al.* (1987a) suggested that environmental factors such as host diet and nutritional components of the body fluids could cause differences in the expression and number of receptors for yeast cells. Sandin, *et al.* (1987b) also investigated other mucosal cell traits that might influence yeast adhesion. These were size, viability, and state of aggregation. The size of buccal cells and the numbers of yeasts adhering to each buccal cell varied considerably. Cells in the 36 to 70μm size range showed the greatest yeast attachment (3 yeasts/cell), whereas very large cells showed the lowest (2.1 yeasts/cell). Buccal cell viability appeared not to be necessary for *Candida* attachment (Sandin *et al.*, 1987b). Vudhichamnong *et al.* (1982) also found *C. albicans* adhered equally well to viable and non-viable epithelial cells. Whether buccal cells

occurred in sheets or as single cells did not appear to influence results (Sandin *et al.*, 1987b).

1.5 Alternative experimental approaches used to investigate yeast adhesion to epithelial cells

The method utilizing exfoliated epithelial cells, used in this investigation, has certain limitations. This method fails to allow investigation of effects such as the influence of hormonal and nutritional factors upon epithelial cell receptor sites. Other limitations include the loss of viablilty of the cells used in the assay and also the non-uniformity of the population of epithelial cells obtained during cell collection. A method described by Sobel *et al.* (1982) used multilayered tissue explants. There was functional similarity between the multilayered explant cells and the exfoliated epithelial cells. Epithelial cells in the process of desquamating from the explant had large numbers of adherent bacteria. These data suggest either the presence of unique surface constituents on the more superficial keratinized cells or that certain cell constituents which affect adhesion may have been lost.

Other methods may be able to overcome or reduce the problems found with the exfoliated epithelial cell method. Samaranayake & MacFarlane (1981) devised an *in vitro* technique to quantitate the adhesion of *C. albicans* to monolayers of human epithelial cells (HeLa cell monolayers). The advantages of using monolayer assays are (i) the ability to perform adherence studies with living monolayers and living yeasts (ii) the capacity to avoid adherence to the ablumenal surface. Tissue culture monolayers provide uniform populations of cells, which are not subject to many of the difficulties encountered with exfoliated epithelial cells including

bacterial contamination as well as day-to-day and donor-to-donor variation (McGroarty, 1990). The HeLa cell system of Samaranayake & MacFarlane (1982a) gave a significantly lower percentage coefficient of variation than an epithelial system similar to the one used in this study. Further, standardized, common sources of mucosal cells with which to perform adhesion assays would be a step forward in the process of standardizing the methodology used in the study of yeast attachment.

1.6 Statistical analysis of adherence assay data

Histograms of count data (Figures 4 & 5) clearly show that the data are not normally distributed, and so traditional statistical techniques such as t tests or analysis of variance are not applicable, and the mean is probably not the best descriptor to use. Therefore alternative methods have been employed. The cells were firstly classified into groups according to the numbers of yeasts which had adhered to them. Just two groups seemed insufficient to express the information collected, so four groups were chosen and a frequency table was constructed. The groups into which buccal cells were classified were (i) zero adherence (ii) 1-5 *Candida* adhered (iii) 6-15 *Candida* adhered (iv) 16 or more *Candida* adhered. The resulting frequency tables, which show the number of epithelial cells in each group for each treatment in each experiment are given in the appendices. The chi-squared test was applied to test for differences between the treatments. Alternative statistical methods were also applied. These included fitting a binomial model to the number of cells to which yeasts adhered, and fitting a Poisson model to the number of *Candida* which adhered to each cell. However, these methods were not felt to be appropriate and were much more difficult

to interpret than the chi-squared test. Results are presented both as frequency tables and also as tables showing the mean number of adherent yeasts per 100 vaginal epithelial cells. Most authors present data as the mean number of adherent yeasts and therefore it is useful to present data in this way, even if it is not the most appropriate descriptor, so comparisons between work in this study and work carried out by others can be made. In some experiments in this study when the Student t-Test was used, no statistically significant differences between the treatments were seen. This is probably due to the large day-to-day variations obtained in this study masking differences between treatments. In most instances the chi-square test showed significant differences between treatments.

1.7 Effect of growth on different carbon sources

The adherence of different strains of *C. albicans* to mouse vaginal epithelial cells was determined after growth in defined medium containing different carbon sources. The results confirmed those of Douglas *et al.*, (1981) and McCourtie & Douglas (1984) which indicated that strains isolated from active infections (I strains) showed enhanced adherence after growth in a medium with high sugar content. Strains of *C. albicans* isolated from asymptomatic carriers (C strains) showed much smaller or no increases in adherence after growth in high sugar medium. I strains are capable of synthesizing surface components, in response to high sugar concentrations, which enhance adhesion to vaginal epithelial cells whereas C strains have a diminished capacity for cell surface modification. Kearns *et al.*, (1983) did not notice any difference in adherence between yeasts isolated from active infections and asymptomatic patients, although they used Lee's medium rather than high sugar medium and therefore

the yeasts would not synthesize the modifying components. It has been suggested that cell surface modification represents increased production of an outer fibrillar layer (McCourtie & Douglas, 1981).

Enhanced adhesion may play a role in pathogenesis since a high sugar environment is frequently found during the last trimester of pregnancy and among diabetic women. Pregnancy and diabetes are known as predisposing factors to *Candida* vaginitis. These conditions are associated with occurrence of high concentrations of vaginal glycogen, which may be broken down to glucose by microbial enzymes (Samaranayake & MacFarlane, 1982a). High sugar concentrations may lead to cell surface modification, enhanced adhesion and hence enhanced pathogenesis.

1.8 Comparison of VEC from normal and diabetic mice

Diabetes is often cited as a predisposing factor in *Candida* infections and women with diabetes certainly have a greater incidence of vaginal candidosis. Fromtling *et al.* (1987) demonstrated that diabetic mice infected with *C. tropicalis* are more susceptible to organ colonization and have a higher mortality rate than normal mice. They failed to discuss possible reasons for these differences. Knight & Fletcher (1971) suggested that increased levels of glucose in the saliva from diabetics were associated with increased candidal growth in the saliva and that this explained the greater incidence of infections cited for diabetics. In the present study, adhesion of *C. albicans* to epithelial cells from diabetic mice was investigated. Yeasts adhered in greater numbers to cells taken from diabetic mice, with the exception of the *C. albicans* NCPF 3153 and 'outbreak' strains.

Segal *et al.* (1984) suggested diabetes is a condition which contributes to an increased adhesiveness of vaginal epithelial cells for *Candida*. They showed that 51% of the vaginal epithelial cells from diabetic women had >20 adherent *C. albicans*, this corresponded to a total count of 1900 adherent yeasts; the equivalent values for the control group were 37% and 1400 respectively. Segal *et al.* (1984) assayed the epithelial cells cytologically using Karyopyknotic and Maturation Indices (KPI) to determine the ratios of superficial, intermediate and parabasal vaginal epithelial cells. They suggested increased *C. albicans* adherence occurred when there was an increase in the number of intermediate epithelial cells indicated by a low Karyopyknotic and Maturation Index. They found increased numbers of intermediate epithelial cells in diabetic women.

1.9 Effect of steroid hormones

The presence of hormones plays an important part in many infections. Animal experiments with *Mycocoplasma hominis* (Furr & Taylor-Robinson, 1989) and *C. albicans* (Sobel *et al.*, 1985) have shown that infection can depend directly on hormonal status. Hormonal changes may influence microbial adherence. Studies have been carried out using cells exfoliated at different times during the menstrual cycle and therefore under different hormonal influences. Botta (1979, 1981) observed that adhesion of Group B streptococci, *E. coli* and *C. albicans* to vaginal and uroepithelial cells fluctuated throughout the menstrual cycle; however, adhesion of Group A streptococi did not. Botta (1981) found that adhesion of *C. albicans*, *E. coli* and Group B streptococci was greatest to vaginal epithelial cells collected during the third week of the menstrual cycle. On the other

hand, Segal *et al.* (1984) found that adherence of *C. albicans* to vaginal epithelial cells was greatest during the last week of the menstrual cycle.

Hormones have been seen to have an effect on the attachment of various microorganisms to tissue culture cells. The hormones α and β oestradiol, progesterone and estrone increased the adherence of *Trichomonas vaginalis* to human amnios epithelial (WISH) cells (Martinotti *et al.*, 1986). Bose & Goswami (1986) noted β oestradiol at 10^{-10} M enhanced inclusion body formation of *Chlamydia trachomatis* within HeLa 229 culture cells. The hormones β oestradiol, estriol and estrone promoted the adhesion of *E. coli* and *S. aureus* to HeLa cells (Sugarman & Epps, 1982). In all of these studies an incubation period of 1h with hormones had no effect on the subsequent number of microorganisms adhering. Increased adherence was observed, however, when epithelial cells were incubated with hormones for 18h. Sugarman & Epps (1982) commented that no alterations in receptor cell morphology, total number of receptor cells or viability were noted. This, coupled with the relatively long incubation period required for a hormone effect, suggests that any modification to receptor cell structure would be secondary to the altered metabolism of the HeLa cells.

Sharma *et al.* (1987) observed the influence of a hormone preparation containing oestrogen (5mg/ml) and progesterone (50mg/ml) on exfoliated uroepithelial cells. They found that treatment for 1h with hormones gave a subsequent increase in numbers of adherent *E. coli*. In the present study, by contrast, when epithelial cells were pretreated with hormones there was decreasing *Candida* adherence after hormone pretreatment. This may have been due to non-specific inhibition.

Mammalian steroid hormones have been reported to stimulate germination of *C. albicans* in serum (at 1μM levels)(Kinsman *et al.*,1988). Many fungi use hormonal control mechanisms in the processes of their mating, growth, and development. Examples include *Coccidioides immitis* (Drutz *et al.*, 1981; Drutz & Huppert, 1983), and *Paracoccidioides brasiliensis* (Salazar *et al.*, 1988; Stover *et al.*, 1986). There may be unappreciated interactions between mammalian hormones and invading fungi. Loose and co-workers (Loose & Feldman, 1982; Loose *et al.*, 1981) established the presence of a corticosterone binding system within the cytosol of *C. albicans*. Corticosterone and progesterone were shown to bind competitively to the same site; this site was probably a protein macromolecule (Loose & Feldman, 1982). Powell *et al.* (1984) identified a second binding system in *C. albicans* and *C. glabrata*. This system involves highly specific binding of oestradiol and shows similarities to the mammalian oestrogen receptor. The presence of this binding system may permit significant interaction with physiologically unconjugated oestradiol encountered in the blood, especially during pregnancy. It is unknown whether this interaction has physiological or pathogenic significance (Powell *et al.*, 1984).

An investigation was carried out in the present study to see whether hormones would affect the adhesion properties of *C. albicans*. Yeasts were pretreated for 30 min in oestradiol or progesterone at a concentration of 1μM. With this incubation time, increased adhesion to epithelial cells was not observed. In fact, there was a slight decrease in *Candida* adherence after hormone treatment. Growth in hormone containing medium had no effect on the subsequent *Candida* adhesion to epithelial cells.

1.10 Effect of lactobacilli

This study showed that pretreatment of vaginal epithelial cells with lactobacilli could inhibit subsequent *C. albicans* adhesion. Inhibition of *Candida* adhesion to mouse, rat and human vaginal epithelial cells was observed following pretreatment with *L. acidophilus* and *L. fermentum*. Both these *Lactobacillus* species inhibited glucose-grown yeasts better than galactose-grown organisms. Galactose-grown *Candida* could adhere in greater numbers than glucose-grown yeasts, probably due to the modified yeast cell wall. It appears lactobacilli are less effective at blocking the adhesion of yeasts which have a modified cell wall.

An experiment was carried out in which mouse vaginal epithelial cells were pretreated with different concentrations of *L. acidophilus* and *L. fermentum*. Differences in the inhibition of yeast adhesion due to different pretreatment concentrations were not great. Counts were made of the number of adherent lactobacilli/vaginal epithelial cell for the different pretreatment concentrations. It was seen that for both *L. acidophilus* and *L. fermentum* the average number of adherent lactobacilli/vaginal epithelial cell was least for a pretreatment concentration of 2.5×10^8. With this concentration an average of 6.97 *L. acidophilus*/vaginal epithelial cell and 3.67 *L. fermentum*/vaginal epithelial cell were observed. Maximum numbers of lactobacilli/vaginal epithelial cell were obtained for both *L. acidophilus* and *L. fermentum* at a pretreatment concentration 1×10^9 cells/ml. At this concentration an average of 11.25 *L. acidophilus*/vaginal epithelial cell and 10.45 *L. fermentum*/vaginal epithelial cell were observed. These results are similar to those obtained by Wood *et al.* (1985) who, at a pretreatment concentration

of 10^8 lactobacilli/ml, obtained values of 3.7-6.8 lactobacilli/epithelial cell for *Lactobacillus* species isolated from yoghurt.

Three other culture collection species, *L. jensenii*, *L. casei* ssp. *rhamnosus* and *L. casei*, were tested for adherence to mouse vaginal epithelial cells; they adhered in numbers similar to *L. acidophilus* or *L. fermentum*. Lactobacilli freshly isolated from the human vagina were also tested for their ability to adhere to mouse vaginal epithelial cells. The different species adhered to different extents in the range of 5.8 bacteria/vaginal epithelial cell for *L. crispatus* 629425X to 45.9 bacteria/vaginal epithelial cell for *L. crispatus* 632697M. Four of the five fresh isolates adhered in greater numbers than the culture collection species. The numbers of lactobacilli attached/vaginal epithelial cell were comparable to those reported in a study by Sobel *et al.* (1981) and were of a similar order of magnitude to values obtained by Wood *et al.* (1985) for vaginal isolates. It is possible that culture collection strains which have been subjected to repeated subculture may be less able to adhere to epithelial cells. This effect has been observed in experiments by Westergren & Emilso (1982). Reid *et al.* (1992)b found that for some lactobacilli isolates, repeated sub-culture was accompanied by a loss of hydrophobic surface properties. Reduction in hydrophobicity has been implicated with a reduction in *Lactobacillus* adhesion to epithelial cells (Wadstrom *et al.*, 1987).

All the *Lactobacillus* species tested were able to inhibit the adhesion of *C. albicans* GDH 2346 to different extents. The isolates which adhered to epithelial cells in greatest numbers gave the greatest inhibition of yeast adhesion. It was found that the fresh vaginal isolates of lactobacilli were able to adhere to vaginal

cells in greatest numbers. Sobel *et al.* (1981) reported a mean number of 26.7 and 22.8 adherent lactobacilli/vaginal cell for the two strains they tested, these values were similar to those observed in this study for fresh vaginal isolates. With their strains, Sobel *et al.* (1981) observed a 29.3% and 40.3% inhibition of *Candida* adhesion after pretreatment of vaginal epithelial cells with 10^8 lactobacilli/ml. These values were similar to the inhibition obtained in the present study when epithelial cells were pretreated with culture collection strains of lactobacilli. The fresh vaginal isolates gave greater inhibition of *Candida* adhesion than that observed by Sobel *et al.* (1981).

Although the bacteria readily attached to the vaginal epithelial cells the entire surface of the epithelial cell was never completely occupied by lactobacilli. This finding is similar to those of Chan *et al.* (1985) who investigated the ability of lactobacilli to block attachment of *E. coli* to uroepithelial cells.

The fresh vaginal isolate *L. crispatus* 63269M was used to pretreat mouse VEC before adhesion assays with four different strains of *C. albicans*. The inhibition of adherence of *C. albicans* GDH 2346 was not as great as had been obtained in an earlier experiment (Table 25); this may be because on subculture the *Lactobacillus* isolate had lost some of its ability to adhere. The isolate inhibited adhesion of the different *C. albicans* strains to different extents. Inhibition was greatest with the yeast strains which had shown least adhesion when pretreatment with lactobacilli was omitted. Least inhibition of adhesion was observed with yeast strains which had been able to adhere in greatest numbers. It may be that in the human vagina a *C. albicans* strain which is poorly adherent and more inhibited by lactobacilli would be unable to cause

disease; this proposition is supported by the fact that *C. albicans* strain GRI 682 was isolated from a symptomless carrier.

As has been seen, different strains of lactobacilli are able to inhibit *Candida* adhesion to different extents. It is possible that in certain combinations, a *Lactobacillus* strain may inhibit a strain of *C. albicans* sufficiently well to prevent disease. It may be possible to screen many strains of lactobacilli to find one which could offer protection against *Candida* adhesion and thus infection. There is a popular belief that the use of preparations which contain lactobacilli, either orally or intravaginally, may achieve cures of pathological *Candida* infections. A recent study claimed to show daily ingestion of yoghurt containing *L. acidophilus* decreased candidal colonization and infection (Hilton *et al.*, 1992). A common folk remedy for the treatment of 'thrush' is intravaginal application of yoghurt. Moreover, there are increasing numbers of *Lactobacillus* preparations commercially available which claim to maintain a balance between 'desirable' and 'undesirable' bacteria and fungi and also to combat infection. Such preparations are often termed 'probiotics'. The *Lactobacillus* species used in these preparations are often of dairy origin and may contain strains unsuitable for human colonization. In some cases the *Lactobacillus*-containing products currently available either do not contain the *Lactobacillus* species advertised and/or contain other bacteria of questionable benefit (Hughes & Hillier, 1990). Despite these limitations, observations that lactobacilli are of clinical value have been noted by general practitioners (Will 1979; Sandler 1979). At the present time, such observations have not been backed up with microbiological evidence to establish the role of the *Lactobacillus* treatment.

1.11 Effect of pH

Human vaginal epithelial cells come from an environment which is normally acidic (pH 4.5) and so the effect of low pH on *Candia* adhesion was investigated. It was found that *Candida* adhered to human vaginal cells in greatest numbers when the adhesion assay was carried out at pH 7.2. This increase in adhesion was not significant by the Student t-Test; however, the Chi-square statistic showed a highly significant difference between the distribution of adherent *Candida* to epithelial cells at pH 7.2 and 4.5. Incresed adhesion at pH 7.2 is in agreement with previous studies which gave an optimal pH value for adherence of 6-8 (Persi *et al.*, 1985; King *et al*, 1980; Sobel *et al.*, 1981). In contrast, Samaranayake & MacFarlane (1982b) found maximal adherence of *C. albicans* to HeLa cells was at pH 3. However, it has been suggested that the effect of pH may be dependent on *C. albicans* strain and the source of mucosal cells (Mehentee & Hay, 1989).

Other authors have investigated the effect of pH on *Lactobacillus* adherence. Henriksson *et al.* (1991) found that the adhesion of *L. fermentum* strain 104-S to porcine gastric squamous epithelial cells was greatest at a pH value of 7. They found that the adhesion of strains 104-R to epithelial cells was unaffected by pH. It appears that the effect of pH on *Lactobacillus* adherence may be strain dependent. Nagy *et al.* (1992) investigated the binding of lactobacilli to fibronectin. They found nine of the 54 strains tested bound fibronectin at pH 7.2. Lowering the pH value to 4.0 increased the binding capacity of all the lactobacilli tested and binding occured with strains which had previously failed to bind fibronectin at pH 7.2. They suggested that increased binding of

lactobacilli to fibronectin at low pH may play a role in the maintenance of the ecological balance of the vagina.

In this study, inhibition of yeast adhesion by lactobacilli was greatest at pH 7.2. However, because other yeast and lactobacilli strains were not tested, no overall conclusions can be drawn as to the effect of vaginal pH on the interactions between *C. albicans* and vaginal lactobacilli.

1.12 Effect of concentrated culture supernatant

L. acidophilus and *L. fermentum* were tested to see whether they released substances into their culture medium which would inhibit *Candida* adhesion. A study by Markham *et al.* (1975) demonstrated the presence of lipoteichoic acid (LTA) in the culture fluid of *S. mutans, S. sanguis, S. salivarius* and *S. mitior*. Sherman & Savage (1986) found extracts from the culture fluids of *L. acidophilus* and *L. fermentum* contained LTA or deacylated LTA. LTA was suggested as a possible *Lactobacillus* adhesin. In a different study, Conway & Kjelleberg (1989) indicated that proteinaceous *Lactobacillus* adhesins may be found in spent culture fluids. Coconnier *et al.* (1992) also found *L. acidophilus* to release a proteinaceous adhesin-promoting factor into spent culture fluids. The studies by Sherman & Savage (1986), Conway & Kjelleberg (1989), Coconnier *et al.* (1992) and Markham *et al.* (1975) indicated it may be possible for spent culture fluid to contain adhesins - either LTA or proteins. If adhesins were released, a pretreatment of vaginal epithelial cells with spent culture fluid may block subsequent *Candida* adhesion. Chan *et al.* (1985) showed that sonicated cell-wall fragments of lactobacilli inhibited adherence of *E. coli, K. pneumoniae* and *Psuedomonas aeruginosa* to uroepithelial cells,

although to a lesser extent than did whole cells. Because cell wall fragments were less effective at blocking adherence than whole cells they suggested that steric hind rance of receptor sites is important in the inhibition of adhesion, particularly as *E. coli*, *K. pneumoniae* and *P. aeruginosa* are likely to have separate and distinct receptor sites. Chauviere *et al.* (1992) postulated that heat-killed *L. acidophilus* strain LB inhibited the attachment of diarrhoeagenic *E. coli* to human intestinal epithelial cells by steric hind rance. In the present study pretreatment of epithelial cells with concentrated spent culture supernatant had no significant effect on subsequent *Candida* adhesion.

2. Effect of lactobacilli on growth of *Candida*

2.1 Growth of *Candida* and lactobacilli over a wide pH range

Initial studies showed that the *Lactobacillus* and *Candida* strains tested were able to grow over a similar range of pH values. This suggests that they are likely to grow in the same environmental conditions and therefore interact. Lactobacilli lowered the pH value of their growth medium to pH 4.2; this would not be low enough to inhibit the growth of *Candida* which could grow at pH values down to 3.

2.2 Growth of lactobacilli and *Candida* in mixed broth cultures

In this study it was found that lactobacilli did not inhibit *Candida* when the two organisms were grown together in mixed broth cultures. Jack *et al.* (1990) showed that when *C. albicans* and *L. acidophilus* were grown in mixed culture, after an initial depression in the growth of *Candida*, the yeasts rapidly outgrew *L. acidophilus*. An initial depression in the growth of *C. albicans* was probably caused

by increased competition for nutrients. This effect may not have been seen in the present study because only a single count of yeast and bacterial numbers was taken at 24h when the stationary phase should have been reached. Young *et al.* (1956) carried out similar experiments, using oral strains of lactobacilli and *C. albicans* grown in mixed culture. They observed a decrease in yeast numbers and an increase in bacterial numbers when compared with pure cultures. This effect was seen even after 120h incubation. By contrast, the initial decrease in yeast growth observed Jack *et al.* (1990) did not continue beyond a 24h incubation period. Young *et al.* (1956) suggested that a counterbalance exists between the two organisms in the normal mouth, with yeasts providing nutritional stimulation for the lactobacilli, and the latter producing lactic acid which prevents the excessive development of *Candida*.

2.3 Effect of lactobacilli on the growth of *C. albicans* in a sandwich plate test

Lactobacilli inhibited *Candida* growth in the sandwich plate assay when the plates were preincubated to allow bacterial growth prior to inoculation with *Candida*. Reid *et al.* (1988) had previously used a similar method to test the ability of 35 different *Lactobacillus* strains to inhibit the growth of 11 uropathogens. In that study, the strains of lactobacilli used showed great variation in inhibiting the growth of uropathogens.

The ability of lactobacilli to inhibit the growth of other microorganisms has been known for some time (Table 2 and Table 3). In this study, viable cells were not necessary to give inhibition of *Candida* growth. When lactobacilli were killed before overlay, inhibition was still seen. The use of the overlay technique

demonstrated that inhibition could not be due to nutrient depletion, as fresh nutrients were supplied in the overlay. Moreover, killed lactobacilli would be unable to provide any competition for nutrients yet inhibition of *Candida* growth was still observed. These initial experiments suggest that the lactobacilli are producing an inhibitor which can build up within the assay plate. As inhibition of yeast growth is seen in the agar plate assay and not when the organisms are grown together in a mixed liquid culture, there may be a need for the close proximity of yeasts and bacteria to provide a high localized concentration of inhibitor. The production of bacteriocins (Tagg *et al.*, 1976) and inhibitor activity (Shahani & Chandan,1979) by lactobacilli is well known.

Further investigation of *Candida* inhibition revealed that greater numbers of lactobacilli in the base of the sandwich plate gave greater yeast inhibition. Also, the longer the lactobacilli were allowed to preincubate before overlaying with *Candida*, the greater was the yeast inhibition observed. Therefore, it appears that the substance(s) produced by lactobacilli is (are) relatively stable.

2.4 Production of antimicrobial substance(s) by lactobacilli grown in MRS broth

By use of the lawn growth inhibition assay, it was shown that no activity was seen in the cell pellet or sonicated cell pellet. The activity of the antimicrobial substance was tested against a range of fungi. Concentrated culture supernatant of *L. acidophilus* and *L. fermentum* showed activity against *C. albicans*, *C. neoformans*, *A. niger* and *T. metagrophytes*. The butanol-extracted culture

supernatants of *L. acidophilus* and *L. fermentum* were also tested against a range of bacteria; antibacterial activity was seen against *S. aureus, E. coli, B. subtilis, B. megaterium,* and *P. vulgaris.* A similar spectrum of antimicrobial activity produced by various *Lactobacillus* cultures has been previously reported by DeKlerk & Coetzee (1961), Vincent *et al.* (1959), Hamdan & Mikolajcik (1974), Vanderbergh & King (1989), Talarico & Dobrogosz (1989) and Talarico *et al.* (1988). DeKlerk & Coetzee (1961) found that the supernatants of various *Lactobacillus* isolates inhibited growth of *D. pneumoniae* and enterococci; however, no activity was seen against enterobacteria and *Staphylococcus.* Vincent *et al.* (1959) observed an antimicrobial substance of *L. acidophilus* was active against *P. vulgaris, Salmonella, Pasturella, Neisseria, E. coli, Mycobacterium, Trichophyton, Microsporum* and *Corynebacterium.* Hamden & Mikolajcik (1974) reported activity against *S. aureus, Salmonella, E. coli, Streptococcus* and the viruses Vaccinia and Polio. Vanderbergh & King (1989) found that an extract of *L. casei* var. *rhamnosus* could inhibit the growth of *Penicillium oxalicum.* Talarico & Dobrogosz (1989) and Talarico *et al.* (1988) observed that the substance reuterin, formed by a culture of *L. reuteri,* was able to inhibit *Escherichia, Shigella, Salmonella, Proteus, Pseudomonas, Staphylococcus, Streptococcus, Clostridium, Bacillus, Leuconostoc, Lactobacillus* and *Saccharomyces* species, and could also prevent virus replication.

The wide spectrum of activity, which was found in this study, distinguished the antimicrobial substance from the bacteriocins which are known to be produced by lactobacilli (Barefoot & Klaenhammer, 1983; Klaenhammer, 1988; Vaughan *et al.*, 1992). Bacteriocins are characterized by a narrow range of inhibitory

action only affecting closely related species within the *Lactobacillaceae*.

When different strains of lactobacilli were assayed for their ability to inhibit *C. albicans*, it was apparent that the production of growth inhibiting substance(s) was common to those strains tested. As had been seen with the sandwich plate assays, the amount of activity in culture supernatants increased with increasing age of the *Lactobacillus* culture.

2.5 Production of antimicrobial substance(s) by *L. acidophilus* and *L. fermentum* grown on cellulose-covered agar plates

The active substance(s) produced by *L. acidophilus* and *L. fermentum* passed through a cellulose membrane indicating a molecular mass less than 10,000 Da. This distinguished the substance(s) produced in this study from the bacteriocins which other authors have investigated. These bacteriocins include Lactocin 27 produced by *L. helveticus* which was reported to have a molecular mass in excess of 12,400 Da (Upreti & Hin sdill, 1975). Joerger & Klaenhammer (1986) isolated Helveticin J, also produced by *L. helveticus*, which was found on purification to be a 37,000 Da protein. Other bacteriocins, although not fully characterized, have been shown to be retained within a dialysis membrane (DeKlerk & Cotzee, 1961; Vincent *et al.*, 1959; Daeschel *et al.*, 1986) and thus distinguishable from substance(s) found in this study.

2.6 Purification of antimicrobial substance(s) produced by *L. acidophilus* and *L. fermentum*

As the chemical composition of the antimicrobial substance(s) was unknown, a number of different procedures were carried out in an attempt to purify and concentrate antimicrobial activity. Solvent extraction was used to remove medium components from the concentrated culture supernatant. Similar extraction procedures have been employed in previous efforts to purify antimicrobial activity (DeKlerk & Coetzee, 1961; Hamdan & Mikolajcik, 1974). In this study extraction with butanol allowed greatest concentration of antimicrobial activity. As lactic acid is known to be produced in *Lactobacillus* cultures, and is known to be an inhibitor of microbial growth (Tramer,1966), samples of lactic acid were tested in a plate assay. This assay showed that not all of the activity displayed by the butanol extract was due to lactic acid.

Columns of ion-exchange resins did not bind the antimicrobial substance(s) suggesting that it was uncharged. When fractional distillation was carried out, increasing distillation of active component was seen with increasing temperature. This method allowed the separation of some media components. It also demonstrated that the antimicrobial substance(s) was heat stable. The substance characterized by Hamdan & Mikolajcik (1974) was also heat stable. All these methods of purification were of limited value. Solvent extraction of freeze-dried culture supernatant was carried out using different water-miscible solvents. Ethanol extraction gave some purification of antimicrobial activity as some media components remained undissolved and could be removed from the solvent phase. Extraction with ethanol had an advantage over butanol extraction in that the solvent control showed only a trace of background activity.

The use of ethanol in the extraction eliminated the possibility that the antimicrobial substance was identical to lactocidin, a component originally described by Vincent *et al.* (1959), since the activity of this substance is destroyed by ethanol.

Column chromatography of an ethanol extract of *L. acidophilus* culture supernatant was carried out using Sephadex LH 20. This material separates not only by gel filtration, but also by adsorption and partition chromatography. It has been designed for use with organic solvents and has wide applicability in the fractionation of small bio-molecules. Most bioactivity was eluted in a fraction which absorbed at 280nm (A_{280}); this indicated that the antimicrobial substance co-eluted with media peptide or protein components. Therefore, the pooled active fractions were subjected to thin layer chromatography using a butanol/ glacial acetic acid/ water solvent system which is known to separate peptides. This allowed the removal of more of the media components from the active fraction. As some activity was found in a non-peptide fraction extracted from the TLC plate this material was chromatographed in a chloroform/ methanol/ water solvent which is used for the separation of lipids. Further experiments showed that the antimicrobial substance(s) was not a lipid. This conclusion was reached because the lipid-containing fraction extracted from the preparative TLC plate showed no antimicrobial activity. It appeared that TLC in the second solvent system had separated more of the contaminating media components from the antimicrobial substance(s). Mass spectrometry established that the antimicrobial substance(s) had a molecular mass of 419, 433, or 447 Da. The differences in molecular mass were probably due to an increasing number of $-CH_2$ groups. Finding the molecular mass enabled the substance(s) to be distinguished from

other antimicrobial compounds previously identified as products of lactobacilli e.g. the substance produced by *L. acidophilus* which has an approximate molecular mass of 200 Da (Hamdan & Mikolajcik, 1974). The substance(s) found here is also distinct from acidophilin (Shahani *et al.*, 1972) which has a molecular mass of 284 Da; reuterin, produced by *L. reuteri*, with a molecular mass of 148 Da (Dobrogosz & Lindgren, 1988); Lactacin F, produced by *L. acidophilus* with a molecular mass of 2500 Da (Muriana & Klaenhammer, 1991); and Sakacin M, produced by *L. sake*, with an apparent molecular weight of 4640 (Sobrino *et al*, 1992). Moreover, the antimicrobial activity demonstrated in this study was clearly not due to lactic acid which has a relative molecular mass of 90.

3. *In vivo* studies

3.1 An animal model for the investigation of vaginal candidosis

In vitro experiments were used to investigate the effect of various individual factors, e.g. lactobacilli and pH, on *Candida* adhesion and growth. However, the vaginal environment is complex and many factors will simultaneously affect the pathogenesis of vaginal candidosis. For a study of pathogenesis it is highly desirable to develop a suitable vaginal infection in a laboratory animal.

The first investigator to produce vaginal candidosis in an animal was Colpe in 1894. He isolated a yeast from the vagina of a patient and inoculated the organism into the vagina of a rabbit. In 1960 Taschdjian *et al.* succeeded in infecting the vaginas of mice with *C. albicans* and, in the same year, Scholer described an experimental vaginal candidosis in rats. These experimental infections differed from the human disease as clinical signs of inflammation and purulent discharge were not seen.

Infection of the rodent vagina with *Candida* correlates strongly with the oestrous cycle, which lasts in mice for 5 days. At the oestrous phase, lasting 1-2 days, a multilayer pavement epithelium is produced with an upper layer of keratinised, anucleated cells which can be released into the lumen of the vagina. Taschdjian *et al.* (1960) reported that successful vaginal colonization by *Candida* occurred only at the onset of oestrous. Histological sections taken 24h after infection showed filamentous fungi growing in the lower layers of the stratum corneum. This infection lasted only 2-5 days until metoestrous began and the layers of pavement epithelium were shed to restore dioestrous, characterized by an epithelium of squamous cells with numerous polymorphonuclear leucocytes.

3.2 Treatment of the mouse vagina with different concentrations of *C. albicans.*

In animal experiments carried out in this study, a state of pseudooestrous was induced by a subcutaneous injection of oestradiol benzoate. Two days later, when animals were inoculated with *C. albicans*, an infection was established.

Different yeast concentrations were used to infect the mouse vagina. An initial experiment showed that a larger *Candida* inoculum gave a greater percentage infection of the mice and higher total vaginal counts. A different overall picture of the course of infection was obtained from results in Table 56. Again, different yeast concentrations were used to infect the mouse vagina but in this experiment, 5 animals were taken from each treatment group at different times after infection. On day 7, the highest total vaginal counts were obtained for animals inoculated with a *Candida*

suspension containing 10^7 yeasts/ml. Higher total vaginal counts were obtained on earlier days in the experiment, but by day 7 clearance of *Candida* from the vagina had begun. The clearance coincided with an end to the induced oestrous and an influx of polymorphonuclear leucocytes (PMNs) into the vagina with the onset of dioestrous. This suggests that the absence of PMNs is important for the establishment and maintenance of infection. Such a hypothesis is supported by experiments by Taschdjian *et al.* (1960) who inserted yeast cells into the mouse vagina during dioestrous. The fungal cells were eliminated within 12h due to phagocytosis by the numerous PMNs which were present at the dioestrous stage of the oestrous cycle. Kita *et al.* (1985) found that injecting mice with oestradiol affected the function of PMNs responsible for eliminating gonococci. It may be that the administration of oestradiol could also enable *C. albicans* to infect the vagina as a consequence of impaired leucocyte activity. Baranao *et al.* (1991) suggested female steriods affected macrophage functionality, probably by regulating surface receptors that are involved in phagocyte activity.

Yeasts were eliminated more rapidly from animals which had been infected with the higher concentrations of *C. albicans* (10^8 and 10^9 yeasts/ml). The larger numbers of *Candida* may have triggered a stronger clearing response whilst the lowest infection concentration established a low-level, more persistent colonization of the vagina.

During experiments with the mouse model, the vagina was removed and homogenised, and after appropriate dilution, a plate count was carried out. This is the most accurate quantitative method of assessing vaginal infection. Ryley & McGregor (1986) compared different methods of assessing infection. They obtained different results when they used vaginal smears on BiGGY agar as compared with

total counts using vaginal homogenates. Vaginal homogenates showed maximum infection on day 7, but smears on BiGGY agar gave an impression of a much more persistent infection. The method most suitable for quantifying a vaginal *Candida* infection in rodents will depend on the precision and sensitivity required, and the need or otherwise to keep the animals for further study. The magnitude of time and effort required to perform the various techniques may also be a consideration in the choice of the most suitable method.

When the course of infection was followed for diabetic mice after treatment of the vagina with different concentrations of *C. albicans*, the infection was seen to be much greater than for normal mice. This agrees with results obtained by Fromtling *et al.* (1987). They carried out experiments which showed a greater susceptibility of diabetic mice to mortality and degree of colonization with *C. tropicalis* compared with normal mice.

3.3 Colonization of the rat vagina with *Lactobacillus acidophilus.*

In this study experiments were also carried out using the rat model. The expense of these animals, combined with the necessity to follow the course of infection, led to the use of a vaginal swabbing method for determining numbers of microorganisms present.

The overall objective was to investigate *in vivo* interactions between lactobacilli and *C. albicans* within the rat vagina. Initially, however, the ability of *L. acidophilus* NCTC 4504 to colonize the rat vagina was determined. Lactobacilli have previously been isolated as part of the normal rat vaginal flora, although only at low frequency and not in such large numbers as are seen in the human vagina (Larsen *et al.* 1976a).

The most common organisms within the rat vagina are α- and non-haemolytic streptococci, *Pasteurella pneumotropica* (an organism not isolated from the human vagina) and diphtheroid bacilli (in numbers comparable to those found in the human vagina). There is also a high frequency of isolation of Gram-negative rods, notably *Proteus* species. Frequencies of isolation of *S. epidermidis* similar to those found in humans are observed (Larsen *et al.*, 1976a). In the study by Larsen *et al.* (1976a) more than half the rats had 5 or 6 species comprising their vaginal flora and all rats had at least 3 species. There were thus fundamental differences between the normal flora of the animal model and the human subject. This may be due to a fundamental difference in the microenvironments of the two hosts. The high percentage of rats carrying *Proteus* species may be due to the association of rats with faecal material in their cages. The relatively high vaginal pH values in rats observed by Larsen *et al.* (1976a) were confirmed in this study. These values may be related to the low frequency of lactobacilli.

Not only is *Candida* infection associated with the oestrous cycle but so also is bacterial colonization. Larsen *et al.* (1976b, 1977b) found rats having normal oestrous cycles showed cyclic variation in the number of bacteria present. With cornification of the vaginal epithelium which occurs 3 days post oestradiol treatment, bacterial colonization was clearly seen (Larsen *et al.*, 1977a). Bacterial forms appeared to associate with the vaginal epithelium predominantly at intercellular borders (Larsen *et al.*, 1978) and frequently formed microcolonies although some bacteria were seen singly (Larsen *et al.*, 1977a). Exfoliated cells were colonized by a variety of bacterial forms; some of the bacteria were in the process of division which was a significant finding since it

suggests that these organisms were multiplying *in situ* and not simply transients. Larsen *et al.* (1977a) suggested a bacterial growth-promoting substrate may be present in the oestrogen-stimulated vaginal epithelium that is released from the exfoliating cells.

The vaginas of ovarectomised oestradiol-treated rats were infected with lactobacilli ($5x10^8$ cells) and on days 1, 2, 3 and 7 *Lactobacillus* colonization was assessed. On MRS agar, four different colony types were seen, a number similar to that observed by Larsen *et al.* (1976a). Lowering the pH value of the medium did not eliminate the non-*Lactobacillus* colonies.

Total numbers of bacteria in the vagina rose on day 1. From the swab samples taken, it was apparent that there was a similar increase in bacterial numbers associated with the vaginal cells, thus indicating colonization. Distinguishing the lactobacilli by colony morphology gave a different picture of colonization, with maximal numbers at day 2/3. This disproportionate increase in numbers of lactobacilli, compared with the increase in total numbers of bacteria, indicates that lactobacilli may have replaced other members of the vaginal flora. The *Lactobacillus* inoculum initially introduced into the vagina was relatively large. However, with no change in the environment to maintain the increased *Lactobacillus* population, it would appear that prolonged colonization cannot occur and the normal flora is re-established by day 7.

An attempt was made to prolong this transient colonization of the rat vagina with lactobacilli by giving repeated inoculations (five in all). The colonization that resulted maintained *Lactobacillus* numbers but could not establish a permenantly elevated

Lactobacillus population. The higher vaginal pH in rodents may be an important factor in the inability of lactobacilli to colonize.

Nevertheless, lactobacilli could exist in the rat vagina and so the influence of such a population on *Candida* adhesion was investigated.

3.4 Effect of colonization by lactobacilli on subsequent *Candida* infection in the rat vagina

A complex relationship exists between members of the microbial flora of the human vagina. Bacteria and yeasts can coexist within the vagina as members of the normal flora (Ryley, 1986). The classic concept was that vaginal lactobacilli protected the vaginal epithelium from various infective agents. However, Goplerud *et al.* (1976) failed to substantiate this idea and even suggested that when *Lactobacillus* colonization is greatest, yeast colonization can also be at a maximum. Oestrogen-treated rats possess a more abundant flora than untreated rats (Scholer, 1960). However, it is also known that an oestrogen pretreatment (which would increase bacterial numbers) is necessary for the establishment of a *Candida* infection of the rodent vagina (Taschdjian *et al.*, 1960; Scholer, 1960).

A model of chronic vaginal candidosis in rats was devised by Sobel *et al.* (1985). The initial procedure was similar to that used with mice except that rats had to be ovarectomised before administering oestradiol valerate; without ovarectomy no permanent oestrous is obtained, probably due to a surplus production of progesterone. Sobel *et al.* (1985) examined histologically the vaginal mucosa of rats and found that within 48h of inoculating with oestrogen, the vaginal mucosa was profoundly altered.

The vaginal mucosa in rats is normally composed of non-cornified columnar cells which undergo cytoproliferation and transformation to a stratified squamous epithelium with extensive keratinization after administration of oestadiol. The role of keratinization in human vaginal candidosis is not certain as human vaginal epithelium is not keratinized and does not possess a stratum corneum.

Scholer (1960) found a correlation between the production of vaginal candidosis and the oestrous cycle in rats. When a state of long-lasting oestrous was induced by hormone treatment of ovarectomised rats, a persistent vaginal candidosis was regularly produced within 24h using an inoculum of 10^5-10^6 *C. albicans* cells. The maximal intensity of the infection was reached after 24h and remained more or less unchanged over a period of 14 days. Thereafter, the mycosis slowly diminished, and 40 days after the infection all animals were free of the fungus. Scholer (1960) noted that the decrease of infection was accompanied by the appearance of leukocyctes and was obviously due to phagocytosis.

Scholer (1960) noted that very few signs of disease were evident in the rats in the course of a long term infection. By microscopic examination of vaginal smears, abundant fungal hyphae were observed 24h after inoculation. They then become progressively longer and perhaps more numerous. From day 10 of infection they diminished in number, whereas more blastospores appeared in the vaginal smears. Scholer (1960) found that the fungus penetrated the entire horny layer; however, it was unable to invade the vital nucleated cells of the deeper layers of the epithelium. An intracanalicular spread of the fungus to the cervices and the uterine horns occurred, in most animals after a delay of some days.

During experiments in this study, establishment of a *Lactobacillus* population in the rat vagina provided no significant protection against infection with *C. albicans*. This was a result similar to that reported by Larsen & Galask (1984) who found that high bacterial counts in the rat vagina did not prevent yeast colonization. They suggested that the physiological conditions promoting *Lactobacillus* colonization may also enhance yeast growth. In this study, the observation that inoculation of the rat vagina with a *Lactobacillus* population had no protective effect against subsequent *Candida* infection may reflect inadequecies of the rat model and does not necessarily preclude a role for *Lactobacillus-Candida* interactions in the pathogenesis of human infections. The pretreatment strain of *Lactobacillus*, which was of human origin, had been seen in earlier experiments to be unable to give prolonged colonization of the rat vagina. Differences between the numbers and type of epithelial cell receptor, the normal flora and average pH of the rat vagina compared to the human vagina may have made it impossible for this strain of *Lactobacillus* to establish and inhibit subsequent *Candida* infection within the rat vagina. It may have been appropriate to use a rat vaginal isolate of lactobacilli to investigate *Lactobacillus-Candida* interactions in the rat model. A rat vaginal isolate may have been able to give prolonged *Lactobacillus* colonization within the rat vagina and thus inhibit subsequent *Candida* infection.

3.5 Effect of a butanol extract of *Lactobacillus* culture supernatant on *Candida* infection of the rat vagina

As a butanol extract of *Lactobacillus acidophilus* culture supernatant had shown an ability to inhibit *Candida* growth *in vitro*, its effect on *Candida* infection of the rat vagina was investigated. Treating the rat vagina with the culture extract had no protective effect against *Candida* infection. A possible explanation for this is that the pH within the rat vagina inactivated the antimicrobial component. The pH value of the butanol extract was 4.5; at neutral pH the extract was seen to lose its inhibitory action in the plate bioassay. Shahani *et al.* (1972) similarly found that the antimicrobial substance acidophilin was unstable to heat and storage under neutral pH conditions. The rat vagina was found to have a neutral pH value; it is thus possible that the antimicrobial substance was inactivated by the pH conditions. However, within the human vagina, which has a pH value of 4.5, inactivation may not occur and therefore a role for the antimicrobial substance(s) produced by lactobacilli in the pathogenesis of human infection cannot be ruled out.

The results of animal experiments showed a large standard error. This is not unusual for animal work as the number of replicates is usually limited and individual animals can differ widely in their response to treatments. This is another factor which limits the usefulness of the rat model for studying interactions of vaginal lactobacilli and *C. albicans*.

To further investigate the *in vivo* interactions between *C. albicans* and vaginal lactobacilli, it may be necessary to consider the use of an alternative animal model. This is because of fundamental differences, in vaginal pH and normal flora, between the

rodent model and the human subject. Like humans, the monkey is known to have an acidic vaginal pH (Ryley, 1986) and also to have glycogen present in the vagina (Cruickshank & Sharman, 1934a). This would make the monkey a suitable candidate for the development of an amimal model for *Candida* vaginitis. However, expense and difficulties in animal handling may make a monkey model unsuitable.

Because of the limitations of the animal model, further information on the *in vivo* interactions between *C. albicans* and vaginal lactobacilli may come from clinical observations. There are increasing numbers of clinical studies investigating the use of lactobacilli as therapy for vaginitis. It may be possible by careful *in vitro* screening to select *Lactobacillus* strains with specific properties, e.g. ability to adhere to vaginal epithelial cells and also produce antimicrobial substance(s), to be tested *in vivo.* With better techniques to identify the artificially administered lactobacilli, the length of colonization, if any, and numbers of bacteria within the vagina may be able to be established and related to *Candida* colonization. Only by further study will the interactions between *C. albicans* and vaginal lactobacilli be fully elucidated.

REFERENCES

AMBARTSUMIAN, K.F., SARKISIAN, B., ELOIAN, D.V., ANANIAN, A.S., ERZINKIAN, L.A., CHARIAN, L.M., AKOPIAN, L.G. (1989) The effect of the acidic milk mixture "Narine" on intestinal microflora in patients with functional and inflammatory bowel disease. Klin. Med.(Mosk.) **67**: 72-74

ANDERSON, M.L. ODDS, F.C. (1985) Adherence of *Candida albicans* to Vaginal Epithelia: Significance of Morphological Form and Effect of Ketoconazole. Mycosen **28**: 531-540

ANDERSSON, R. (1986) Inhibition of *Staphylococcus aureus* and spheroplasts of Gram-negative bacteria by an antagonistic compound produced by a strain of *Lactobacillus plantarum*. Int. J. Food Microbiol. **3**: 149-160

AUGER, P., JOLY, J. (1980) Microbial Flora Associated with *Candida albicans* Vulvovaginitis. Obstet. Gynecol. **55**: 397-401

AYRE, W.B. (1951) The Glycogen-Estrogen Relationship in the Vaginal Tract. J. Clin. Endocrinol. **11**: 103-110

BAR, W., EUTENEUER, B., SCHUSTER, S. (1987) Bacteremia caused by *Lactobacillus plantarum* in endocarditis lenta. Immun. Infeckt. **15**: 173-174

BARANAO, R.I., TENENBAUM, A., RUMI, L.S. (1991) Effects of sexual steriod hormones on the functionality of murine peritoneal macrophages. Steriods **56**: 481-485

BAREFOOT, S.F., KLAENHAMMER, T.R. (1983) Detection and activity of Lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. **45**: 1808-1815

BARRET-BEE, K., HAYES, Y., WILSON, R.G., RYLEY, J.F. (1985) A comparison of phospholipase activity, cellular adherence and pathogenicity of yeasts. J.Gen. Microbiol. **131**: 1217-1221

BARROW, P.A., BROOKER, B.E., FULLER, R., NEWPORT, M.J. (1980) The attachment of bacteria to the gastric epithelium of the pigs and its importance in the microecology of the intestine. J. Appl. Bacteriol. **48**: 147-154

BARTELT, M.A., DUNCAN, J.L. (1978) Adherence of group A streptococci to human epithelial cells. Infect. Immun. **20**: 200-208

BERG, A.D., HEIDRICH, F.E., FIHN, S.D., BERGMAN, J.J., WOOD, R.W., STAMM, W.E., HOLMES, K.K. (1984) Establishing the cause of genitourinary symptoms in women in a family practice. J. Am. Med. Assoc. **251**: 620-625

BEACHEY, E.H. (1981) Bacterial Adherence: Adhesin-Receptor Interactions Mediating the Attachment of Bacteria to Mucosal Surfaces. J. Infect. Dis. **143**: 325-345

BERKLEY, R.C.W., LYNCH, J.M., MELLING, J., RUTTER, P.R., VINCENT, B. (Eds.) (1980) Microbial adhesion to surfaces. John Wiley & Sons Ltd., Chichester

BIBEL, D.J., ALY, R., LAHTI, L., SHINEFIELD, H.R., MAIBACH, H.I. (1987) Microbial adherence to vulvar epithelial cells. J. Med. Microbiol. **23**: 75-82

BOSE, S.K., GOSWAMI, P.C. (1986) Enhancement of adherence and growth of *Chlamydia trachomatis* by estrogen treatment of HeLa cells. Infect. Immun. **53**: 646-650

BOTTA, G.A. (1981) Possible role of hormones in the observed changes in adhesion of several microorganisms to epithelial cells from different body sites. FEMS Microbiol. Lett. **11**: 69-72

BOTTA, G.A. (1979) Hormonal and type-dependant adhesion of group B streptococci to human vaginal cells. Infect. Immun. **25**: 1084-1086

BROOKER, B.E., FULLER, R. (1975) Adhesion of lactobacilli to the chicken crop epithelium. J. Ultrastruct. Res. **52**: 21-31

BROWN, D. (1986) Therapeutic alternatives and new treatment modalities in vulvovaginal candidasis. J. Reprod. Med. **31**: 653-654

BRUCE, A.W., REID, G. (1988) Intravaginal instillation of lactobacilli for prevention of recurrent urinary tract infections. Can. J. Microbiol. **34**: 339-343

BURD, R.S., RAYMOND, C.S., DUNN, D.L. (1992) Endotoxin promotes synergistic lethality during concurrent *Escherichia coli* and *Candida albicans* infection. J. Surg. Res. **52**: 537-542

BUTLER, B.C., BEAKLEY, J.W. (1960) Bacterial Flora in Vaginitis. Am. J. Obstet. Gynec. **79**: 432-440

BYBEE, J.D., ROGERS, D.E. (1964) The phagocytic activity of polymorphonuclear leukocytes obtained from patients with diabetes mellitus. J. Lab. Clin. Med. **64**: 1-13

CALDERONE, R.A., LEHRER, N., SEGAL, E. (1984) Adherence of *Candida albicans* to buccal and vaginal epithelial cells: ultrastructural observations. Can. J. Microbiol. **30**: 1001-1007

CARLSON, E.(1982) Synergistic effect of *Candida albicans* and *Staphylococcus aureus* on mouse mortality. Infect. Immun. **38**: 921-924

CARLSON, E.(1983a) Enhancement by *Candida albicans* of *Staphylococcus aureus*, *Serratia marcescens*, and *Streptococcus faecalis* in the Establishment of Infection in Mice. Infect. Immun. **39**: 193-197

CARLSON, E. (1983b) Effect of Strain of *Staphylococcus aureus* on Synergism with *Candida albicans* Resulting in Mouse Mortality and Morbidity. Infect. Immun. **42**: 285-292

CARLSTEDT-DUKE, B., ALM, L., HOEVERSTAD, T., MIDTVEDT, A.C., NORIN, K.E., STEINBAKK, M., MIDTVEDT, T. (1987) Influence of clindamycin, administered together with or without lactobacilli, upon intestinal ecology in rats. FEMS Microbiol. Ecol. **45**: 251-259

CATTERAL, R.D. (1971) Influence of gestogenic contraceptive pills on candidiasis. Br. J. Vener. Dis. **47**: 45-47

CENTENO, A., DAVIS, C.P., COHEN, M.S. WARREN, M.M. (1983) Modulation of *Candida albicans* Attachment to Human Epithelial Cells by Bacteria and Carbohydrates. Infect. Immun. **39**: 1354-1360

CHAN, R.C.Y., REID, G., IRVIN, R.T., BRUCE, A.W., COSTERTON, J.W. (1985) Competitive Exclusion of Uropathogens from Human Uroepithelial Cells by *Lactobacillus* Whole Cells and Cell Wall Fragments. Infect. Immun. **47**: 84-89

CHAN, R.C., BRUCE, A.W., REID, G. (1984) Adherence of cervical, vaginal and distal urethral normal microbial flora to human uroepithelial cells and the inhibition of adherence of gram-negative uropathogens by competitive exclusion. J. Urol. **131**: 596-601

CHAUVIERE, G., COCONNIER, M.H., KERNEIS, S., DARFEUILLE-MICHAUD, A., JOLY, B., SERVIN, A.L. (1992) Competitive exclusion of diarrheagenic *Escherichia coli* (ETEC) from human enterocyte-like Caco-2 cells by heat killed *Lactobacillus*. FEMS. Microbiol. Lett. **91**: 213-217

CHEN, K.C.S., FORSYTH, P.S., BUCHANAN, T.M., HOLMES, K.K. (1979) Amine content of vaginal fluid from untreated and treated patients with non-specific vaginitis. J. Clin. Invest. **63**: 828-835

COCONNIER, M.H., KLAENHAMMER, T.R., KERNEIS, S., BERNET, M.F., SERVIN, A.L. (1992) Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell lines in culture. Appl. Environ. Microbiol. **58**: 2034-2039

COLLINS, E.B., HARDT, P. (1980) Inhibition of *Candida albicans* by *Lactobacillus acidophilus*. J. Dairy Sci. **63**: 830-832

COLLINS-LECH, C., KALBFLEISCH, J.H., FRANSON, T.R., SOHNLE, P.G. (1984) Inhibition by Sugars of *Candida albicans* Adherence to Human Buccal Mucosal Cells and Corneocytes *in vitro*. Infect. Immun. **46**: 831-834

COLPE, J. (1894) Hefezellan als krankheitserreger im weiblichen genitalcanal. Arch. Gynaekol. **47**: 635-645

CONWAY, P.L., ADAMS, R.F. (1989) Role of Erythrosine in the Inhibition of *Lactobacillus fermentum* Strain 737 to Mouse Stomach Tissue. J. Gen. Microbiol. **135**: 1167-1173

CONWAY, P.L., KJELLEBERG, S. (1989) Protein-mediated Adhesion of *Lactobacillus fermentum* Strain 737 to Mouse Stomach Squamous Epithelium. J. Gen. Microbiol **135**: 1175-1186

CORBISHLEY, C.M. (1977) Microbial flora of the vagina and cervix. J. Clin. Path. **30**: 745-748

COSTERTON, J.W., MARRIE, T.J., CHENG, K.J. (1985) Phenomena of bacterial adhesion. In Bacterial adhesion, mechanisms and physiological significance ed. Savage, D.C., Fletcher, M. pp.3-43. New York: Plenum Press

COX, F. (1986) *Candida albicans* adherence in newborn infants. J. Med. Vet. Mycol. **24**: 121-125

CRITCHLEY, I.A., DOUGLAS, L.J. (1987a) Isolation and partial characterization of an adhesin from Candida albicans. J. Gen. Microbiol. **133**: 629-636

CRITCHLEY, I.A., DOUGLAS, L.J. (1987b) Role of glycosides as epithelial cell receptors for Candida albicans. J. Gen. Microbiol. **133:** 637-643

CRUICKSHANK, R., SHARMAN, A. (1934a) The Biology of the Vagina in the Human Subject I. Glycogen in the vaginal epithelium and its relation to ovarian activity. J. Obstet. Gynaecol. Br. Emp. **41:** 190-207

CRUICKSHANK, R., SHARMAN, A. (1934b) The Biology of the Vagina in the Human Subject II. The bacterial flora and secretion of the vagina at various age-periods and their relation to glycogen in the vaginal epithelium. J. Obstet. Gynaecol. Br. Emp. **41:** 208-225.

DAESCHEL, M.A., McKENNEY, M.C., McDONALD, L.C. (1986) Characterization of a bacteriocin from *Lactobacillus plantarum*. ASM Bacteriol. Proc. abstr. P -13, p.277

DARWAZEH, A.M., MacFARLANE, T.W., McCUISH, A., LAMEY, P.J. (1991) Mixed salivary glucose levels and candidal carraige in patients with diabetes mellitus. J. Oral. Pathol. Med. **20:** 280-283

DE KLERK, H.C., COETZEE, J.N.(1961) Antibiosis among lactobacilli. Nature **192:** 340-341

DE KLERK, H.C., SMIT, J.A. (1967) Properties of *Lactobacillus fermenti* bacteriocin. J. Gen. Microbiol. **48:** 309-316

DE MAN, J.C., ROGOSA, M., SHARPE, M.E. (1960) A Medium For The Cultivation Of Lactobacilli. J. Appl. Bact. **23:** 130-135

DE SIMONE, C., TZANTZOGLOU, S., BALDINELLI, L., DI FABIO, S., BIANCHI-SALVADORI, B., JIRILLO, E., VESELY, R. (1988) Enhancement of host resistance against *Salmonella typhimurium* infection by diet supplemented with yoghurt. Immunopharmacol. Immunotoxicol. **10**: 399-415

DICKGIESSER, U., WEISS, N., FRITSCHE, D. (1984) *Lactobacillus gasseri* as the cause of septic urinary infection. Infection **12**: 14-16

DOBROGOSZ, W.J., LINDGREN, S.E. (1988) Antibiotic reuterin. International Patent, World Intellectual Property Organisation. International publication number WO 88/08452

DO CARMO-SOUSA, L. (1969) Distribution of yeasts in nature. In: Rose AH & Harrison JS (eds). The Yeasts(volume 1) pp. 79-105. Academic Press, London

DOUGLAS, L.J. (1987) Adhesion of *Candida* species to Epithelial Surfaces. CRC Crit. Rev. Microbiol. **15**: 27-43

DOUGLAS, L.J. (1985) Adhesion of pathogenic *Candida* species to host surfaces. Microbiol. Sci. **2**: 243-247

DOUGLAS, L.J., HOUSTON, J.G., McCOURTIE, J. (1981) Adherence of *Candida albicans* to human buccal epithelial cells after growth on different carbon sources. FEMS Microbiol. Lett. **12**: 241-243

DOUGLAS, L.J., McCOURTIE, J. (1983) Effect of tunicamycin treatment on the adherence of *Candida albicans* to human buccal epithelial cells. FEMS Microbiol. Lett. **16**: 199-202

DOUGLAS, L.J., McCOURTIE, J. (1981) Adherence of *Candida albicans* to denture acrylic as affected by changes in cell-surface composition. In: Stewart GG & Russell I. (eds) Current Developments in Yeast Research. pp. 375-380. Pergamon Press, Toronto

DRUTZ, D.J., HUPPERT, M. (1983) Coccidioidomycosis: factors affecting the host-parasite interaction. J.Infect. Dis. **147**: 372-390

DRUTZ, D.J., HUPPERT, M., SUN, H.S., McGUIRE, W.L. (1981) Human sex hormones stimulate the growth and maturation of *Coccidioides immitis*. Infect.Immun. **32**: 897-907

DUBOS, R., SCHAEDLER, R.W., COSTELLO, R.,HOET, P. (1965) Indigenous, normal and autochthonous flora of the gastrointestinal tract. J. Exp. Med. **122**: 67-76

EDMAN, J., SOBEL, J.D., TAYLOR, M.L. (1986) Zinc status in women with recurrent vulvovaginal candidiasis. Am. J. Obstet. Gynecol. **155**: 1082-1085

EKLUND, T., JARMUND, T. (1983) Microculture model studies on the effect of various gas atmospheres on microbial growth at different temperatures. J. Appl. Bacteriol. **55**: 119-125

ELEGBE, I.A., BOTU, M. (1982) A preliminary study on dressing patterns and incidence of candidiasis. Am. J. Public Health **72**: 176-177

ELEGBE, I.A., ELEGBE, I. (1983) Quantitative relationships of *Candida albicans* infections and dressing patterns in Nigerian women. Am. J. Public Health **73**: 450-452

ESCHENBACH, D.A., DAVICK, P.R., WILLIAMS, B.L., KLEBANOFF, S.J., YOUNG-SMITH, K., CRITCHLOW, C.M., HOLMES, K.K. (1989) Prevalence of Hydrogen Peroxide-Producing *Lactobacillus* Species in Normal Women and Women with Bacterial Vaginosis. J. Clin. Microbiol. **27**: 251-256

FLEURY, F.J. (1986) A comparative study of butoconazole vs. clotrimazole. J. Reprod. Med. **31** (suppl.) : 664

FREDRICSSON, B., ENGLUND, K., NORD, C.E., WEINTRAUB, L. (1992) Could bacterial vaginosis be due to the competitive suppression of lactobacilli by aerobic microorganisms? Gynecol. Obstet. Invest. **33**: 119-123

FREDRICSSON, B., ENGLUND, K., WEINTRAUB, L., OLUND, A., NORD, C.E. (1989) Bacterial vaginosis is not a simple ecological disorder. Gynecol. Obstet. Invest. **28** : 156-160

FROMTLING, R.A., ABRUZZO, G.K., GILTINAN, D.M. (1987) *Candida tropicalis* Infection in Normal, Diabetic, and Neutropenic Mice. J. Clin. Microbiol. **25**: 1416-1420

FULLER, R. (1973) Ecological Studies on the Lactobacillus Flora Associated with the Crop Epithelium of the Fowl. J. Appl. Bact. **36**: 131-139

FULLER, R., TURVEY, A. (1971) Bacteria Associated with the Intestinal Wall of the Fowl (*Gallus domesticus*). J. Appl. Bact. **34**: 617-622

FURR, P.M., TAYLOR-ROBINSON, D. (1989) Oestradiol-induced Infection of the Genital Tract of Female Mice by *Mycoplasma hominis*. J. Gen. Microbiol. **135**: 2734-2749

GARVIE, E.I., COLE, C.B., FULLER, R., HEWITT, D. (1984) The effect of yoghurt on some components of the gut microflora and on the metabolism of lactose in the rat. J. Appl. Bacteriol. **56**: 237-245

GHANNOUM, M.A., BURNS, G.R. ELTEEN, K.A., RADWAN, S.S. (1986) Experimental Evidence for the Role of Lipids in Adherence of *Candida* spp. to Human Buccal Epithelial Cells. Infect. Immun. **54**: 189-193

GILLESPIE, H.L., BINMON, W.B., SLATER, V. (1960) Incidence of *Candida* in the vagina during pregnancy. Study using the Pagano-Levin culture medium. Obstet. Gynecol. **16:** 185-188

GOPLERUD, C.P., OHM, M.J., GALASK, R.P. (1976) Aerobic and anaerobic flora of the cervix during pregnancy and the puerperium. Am.J. Obstet. Gynecol. **126:** 858-868

GORBACH, S.L., MENDA, K.B., THADEPALLI, H., KEITH, L.(1973) Anaerobic microflora of the cervix in healthy women. Am. J. Obstet. Gynecol. **117:** 1053-1055

GREGOIRE, A.T., KANDIL, O., LEDGER, W.J. (1971) The glycogen content of human vaginal epithelial tissue. Fertil. Steril. **22:** 64-68

GREGOIRE, A.T., RAMSEY, H., ADAMS, A. (1967) The Effect of Various Doses of Oestradiol 17-β on Glycogen Deposition in the Rat Uterus, Cervix and Vagina. J. Reprod. Fert. **14:** 231-234

GUILLOT, N. (1958) Production by *Lactobacillus acidophilus* of a substance active against *Candida albicans*. Ann. Inst. Pasteur. **95:** 194-207

HAAN, E., BROWN, G., BANKIER, A., MITCHELL, D., HUNT, S., BLAKEY, J., BARNES, G. (1985) Severe illness caused by the products of bacterial metabolism in a child with a short gut. Eur. J. Pediatr. **144:** 63-65

HALLEN, A., JARSTRAND, C., PAHLSON, C. (1992) Treatment of bacterial vaginosis with lactobacilli. Sex. Transm. Dis. **19:** 146-148

HAM, A.W. (1969) Histology. 6th Edition. J.B. Lippincott company. Philidelphia & Toronto

HAMDAN, I.Y., MIKOLAJCIK, E.M. (1974) Acidolin: an antobiotic produced by *Lactobacillus acidophilus*. J. Antibiot. **27:** 631-636

HAMILTON, J.R., OVERALL, J.C., GLASGOW, L.A. (1976) Synergistic effect on mortality in mice with murine cytomegalovirus and *Pseudomonas aeruginosa*, *Staphylococcus aureus* or *Candida albicans* infections. Infect. Immun. **14**: 982-989

HENRIKSSON, A., SZEWZYK, R., CONWAY, P.L. (1991) Characteristics of the adhesive determinants of *Lactobacillus fermentum* 104. App. Environ. Microbiol. **57**: 499-502

HENZL, M. (1986) Vulvovaginal candidiasis: Historical perspectives and current trends. J. Reprod. Med. **31**: 641-644

HERTHELIUS, M., GORBACH, S.L., MOLLBY, R., NORD, C.E., PETTERSSON, L., WINBERG, J. (1989) Elimination of vaginal colonization with *Escherichia coli* by administration of indigenous flora. Infect. Immun. **57**: 2447-2451

HILLIER, S.L., KROHN, M.A., KLEBANOFF, S.J., ESCHENBACH, D.A. (1992) The relationship of hydrogen peroxide-producing lactobacilli to bacterial vaginosis and genital microflora in pregnant women. Obstet. Gynecol. **79**: 369-373

HILTON, E., ISENBERG, H.D., ALPERSTIEN, P., FRANCE, K., BORENSTEIN, M.T. (1992) Ingestion of yoghurt containing *Lactobacillus acidophilus* a prophylaxis for candidal vaginitis. Ann. Intern. Med. **116**: 353-357

HOOD, S.K., ZOTTOLA, E.A. (1987) Electron microscopic study of the adherence properties of *Lactobacillus acidophilus*. J. Food Sci. **52**: 791-792

HOROWITZ, B.J., EDELSTEIN, S.W., LIPPMAN, L. (1987) Sexual Transmission of *Candida*. Obstet. Gynecol. **69**: 883-886

HOROWITZ, B.J., EDELSTEIN, S.W., LIPPMAN, L. (1984) Sugar chromatography studies in recurrent *Candida* vulvo-vaginitis. J. Reprod. Med. **29**: 441-443

HUGHES, V.L., HILLIER, S.L. (1990) Microbiologic characteristics of *Lactobacillus* products used for colonization of the vagina. Obstet. Gynecol. **75:** 244-248

ISENBERG, H.D., PISANO, M.A., CARITO, S.L., BERKMAN, J.I. (1960) Factors Leading to Overt Monilial Disease. 1. Preliminary Studies of the Ecological Relationship Between *Candida albicans* and Intestinal Bacteria. Antib. Chemo. **10:** 353-363

ISOLAURI, E., JUNTUNEN, M., RAUTANEN, T., SILLANAUKEE, P., KOIVULA, T. (1991) A human *Lactobacillus* strain (*Lactobacillus casei* sp strain GG) promotes recovery from acute diarrhea in children. Pediatrics **88:** 90-97

JACK, M., WOOD, J.B., BERRY, D.R. (1990) Evidence for the involvement of thiocyanate in the inhibition of *Candida albicans* by *Lactobacillus acidophilus*. Microbios **62:** 37-46

JACOBSON, J.B. (1986) Current thinking on the pathogenesis of vulvovaginal candidiasis. J. Reprod. Med. (suppl.) **31:** 645-646

JOERGER, M.C., KLAENHAMMER, T.R. (1986) Characterisation and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. J. Bacteriol. **167:** 439-446

JONSSON, E. (1986) Persistence of *Lactobacillus* strain in the gut of suckling piglets and its influence on performance and health. Swed. J. Agric. Res. **16:** 43-47

JONSSON, E., OLSSON, I. (1985) The effect on preformance, health and faecal microflora of feeding *Lactobacillus* strains to neonatal calves. Swed. J. Agric. Res. **15:** 71-76

KALO-KLEIN, A., WITKIN, S.S. (1989) *Candida albicans*: cellular immune system interactions during different stages of the menstrual cycle. Am. J. Obstet. Gynecol. **161:** 1132-1136

KANDLER, O., WEISS, N. (1984) In : Krieg, N.R., Holt, J.G. Bergy's Manual for Systemic Bacteriology p 1208-1234

KANDHARI, K.C., PASRICHA, J.S., DUGGAL, S.K., PANDHI, R.K. (1969) Investigation into the causation of increased susceptibility of diabetics to cutaneous infections. Indian J. Med. Res. **57:** 1295-1302

KEARNS, M.J., DAVIES, P., SMITH, H. (1983) Variablilty of the Adherence of *Candida albicans* Strains to Human Buccal Epithelial Cells: Inconsistency of Differences Between Strains Related to Virulence. Sabouaudia **21:** 93-98

KENNEDY, M.J., VOLZ, P.A. (1985) Ecology of *Candida albicans* Gut Colonisation: Inhibition of *Candida* Adhesion, Colonization, and Dissemination from the Gastrointestinal Tract by Bacterial Antagonism. Infect. Immun. **49:** 654-663

KIMURA, L.H., PEARSALL, N.N. (1978) Adherence of *Candida albicans* to Human Buccal Epithelial Cells. Infect. Immun. **21:** 64-68

KIMURA, L.H., PEARSALL, N.N. (1980) Relationship Between Germination of *Candida albicans* and Increased Adherence to Human Buccal Epithelial Cells. Infect. Immun. **28:** 464-468

KING, R.D., LEE, J.C., MORRIS, A.L. (1980) Adherence of *Candida albicans* and other *Candida* Species to Mucosal Epithelial Cells. Infect. Immun. **27:** 667-674

KINSMAN, O.S., PITBLADO, K., COULSON,C.J. (1988) Effect of Mammalian Steroid Hormones and Luteinizing Hormone on the Germination of *Candida albicans* and Implications for Vaginal Candidosis. Mycoses **31:** 617-626

KITA, E., TAKAHASH, S., YASUI, K., KASHIBA, S.(1985) Effect of estrogen (17-Beta-estradiol) on the susceptability of mice to disseminated gonococcal infection. Infect. Immun. **49:** 238-243

KLAENHAMMER, T.R. (1988) Bacteriocins of lactic acid bacteria. Biochemie **70**: 337-349

KLAENHAMMER, T.R. (1982) Microbial consideration in selection and preparation of *Lactobacillus* strains for use in dietary adjuncts. J. Dairy Sci. **65**: 1339-1349

KLEBANOFF, S.J., COOMBS, R.W. (1991) Viricidal effect of *Lactobacillus acidophilus* on human immunodeficiency virus type 1: possible heterosexual transmission. J. Exp. Med. **174**: 289-292

KLEBANOFF, S.J., HILLIER, S.L., ESCHENBACH, D.A., WALTERSDORPH, A.M. (1991) Control of the microbial flora of the vagina by H_2O_2-generating lactobacilli. J. Infect. Dis. **164**: 94-100

KLOTZ, S.A. (1992) Fungal adherence to the vascular compartment: a critical step in the pathogenesis of disseminated candidasis. Clin. Infect. Dis. **14**: 340-347

KLOTZ, S.A., DRITZ, D.J., ZAJIC, J.E. (1985) Factors governing adherence of Candida species to plastic surfaces. Infect. Immun. **50**: 97-101

KLOTZ, S.A., DRUTZ, D.J., HARRISON, J.L., HUPPERT, M. (1983) Adherence and penetration of vascular endothelium by *Candida* yeasts. Infect. Immun. **42**: 374-384

KNIGHT, L., FLETCHER, J. (1971) Growth of *Candida albicans* in Saliva: Stimulation by Glucose Associated with Antibiotics, Corticosteroids, and Diabetes Mellitus. J. Infect. Dis. **123**: 371-377

KODAMA, R. (1952) Studies on lactic acid bacteria. II. Lactolin, a new antibiotic produced by lactic acid bacteria. J. Antibiot. **5**: 72

KOSER, S.A., HODGES, E., TRIBBY, I., STUEDELL, J.T. (1960) Growth of Lactobacilli in association with *Candida albicans*. J. Infect. Dis. **106**: 60-68

KREGER-VAN RIJ, N.J.W. (1984) The Yeasts: a taxonomic study. 3rd edition. Elsevier Science Publishers BV, Amsterdam

KVASNIKOV, E.I., SHISHLEVSKAYA, T.N., KOVALENKO, N.K. (1983) Antagonistic activity of lactic-acid bacteria with respect to agents of intestinal disease in poultry. Mikrobiol. Zh. **45**: 27-32

KWON-CHUNG, K.J., LEHMAN, D., GOOD, C., MAGEE, P.T. (1985) Genetic evidence for role of extracellular proteinase in virulence of *Candida albicans*. Infect. Immun. **49**: 571-575

LANG, W.R., FRITZ, M.A., MENDUKE, H. (1962) The bacteriological diagnosis of tricomonal, candidal and combined infections. Obstet. Gynecol. **20**: 788-791

LARSEN, B., GALASK, R.P. (1984) Influence of estrogen and normal flora on vaginal candidiasis in the rat. J.Repro. Med. **29**: 863-868

LARSEN, B., MARKOVETZ, A.J., GALASK, R.P. (1976a) The Bacterial Flora of the Female Rat Genital Tract (39261). Proc. Soc. Exp. Biol. Med. **151**: 571-574

LARSEN, B., MARKOVETZ, A.J., GALASK, R.P. (1976b) Quantitative Alterations in the Genital Microflora of Female Rats in Relation to the Estrous Cycle. J. Infect. Dis. **134**: 486-489

LARSEN, B., MARKOVETZ, A.J., GALASK, R.P. (1977a) Spatial relationship of the vaginal microflora to the vaginal epithelium of female rats: Scanning Electron Microscopy. Appl. Environ. Microbiol. **34**: 80-87

LARSEN, B., MARKOVETZ, A.J., GALASK, R.P. (1977b) Relationship of Vaginal Cytology to Alterations of the Vaginal Microflora of Rats During the Estrous Cycle. Appl. Environ. Microbiol. **33**: 556-562

LARSEN, B., MARKOVETZ, A.J., GALASK, R.P. (1978) Spatial relationship of the Genital Microflora to the Vaginal Epithelium of Female Rats: Transmission Electron Microscopy. Appl. Environ. Microbiol. **35**: 444-450

LEE, J.C., KING, R.D. (1983) Characterization of *Candida albicans* Adherence to Human Vaginal Epithelial Cells *In Vitro*. Infect. Immun. **41**: 1024-1030

LEHRER, N., SEGAL, E., BARR-NEA, L. (1983) *In Vitro* and *In Vivo* Adherence of *Candida albicans* to Mucosal Surfaces. Ann. Microbiol. (Inst. Pasteur) **134** B: 293-306

LEVISON, M.E., TRESTMAN, I., QUACH, R., SLADOWSKI, C., FLORO, C.N. (1979) Quantitative bacteriology of the vaginal flora in vaginitis. Am.J. Obstet. Gynecol. **133**: 139-144

LIDBECK, A., GUSTAFSSON, J.A., NORD, C.E. (1987) Impact of *Lactobacillus acidophilus* supplements on the human oropharyngeal and intestinal microflora. Scand. J. Infect. Dis. **19**: 531-537

LILJEMARK, W.F., GIBBONS, R.J. (1973) Suppression of *Candida albicans* by human oral streptococci in gonotobiotic mice. Infect. Immun. **8**: 846-849

LONDON, J (1976) The ecology and taxonomic status of the lactobacilli. Ann. Rev. Microbiol. **30**: 279-301

LOOSE, D.S., FELDMAN, D. (1982) Characterization of a unique corticosterone-binding protein in *Candida albicans*. J. Biol. Chem. **257**: 4925-4930

LOOSE, D.S., SCHURMAN, D.J., FELDMAN, D. (1981) A corticosteriod binding protein and endogenous ligand in *Candida albicans* indicating a possible steriod-receptor system. Nature. **293**: 477-479

LUCKEY, T.D., ONDERDONK, A.B., FINKELSTEIN, R.A., SAVAGE, D.C., WYATT, R.G. (1983) Exogenous lactobacilli fed to man - their fate and ability to prevent diarrheal disease. Intestinal Microecology., Prog. Food Nutr. Sci. **7**: 3-4

MAIKE, M., NOMOTO, K., YOKOKURA, T., YOSHIKAI, Y., MUTAI, M., NOMOTO, K. (1985) Protective Effect of *Lactobacillus casei* on *Pseudomonas aeruginosa* Infection in Mice. Infect. Immun. **48**: 480-485

MAISCH, P.A., CALDERONE, R.A. (1980) Adherence of *Candida albicans* to a fibrin-platelet matrix formed *in vitro*. Infect. Immun. **27**: 650-656

MAKRIDES, H.C., MACFARLANE, T.W. (1982) Effect of commensal bacteria on the adherence of *Candida albicans* to epithelial cells *in vitro*. Microbios Lett. **21**: 55-61

MANZELLA, J.P., HAROOTUNIAN, R. (1982) Lactobacillaemia of renal origin: A case report. J. Urol. **128**: 110-111

MARDH, P.A., WESTROM L. (1976) Adherence of Bateria to Vaginal Epithelial Cells. Infect. Immun. **13**: 661-666

MARKHAM, J.L., KNOX, K.W., WICKEN, A.J., HEWETT, M.J. (1975) Formation of Extracellular Lipoteichoic Acid by Oral Streptococci and Lactobacilli. Infect. Immun. **12**: 378-386

MARSHALL, K.C., STOUT, R., MITCHELL, R. (1971) Mechanisms of the initial events in the sorption of marine bacteria to surfaces. J. Gen. Microbiol. **68**: 337-348

MARRIE, T.J., COSTERTON, J.W. (1981) The ultrastructure of *Candida albicans* infections. Can.J. Microbiol. **27:** 1156-1164

MARTINOTTI, M.G., MARTINETTO, P., SAVOIA, D. (1986) Adherence of *Trichomonas vaginalis* to cell culture monolayers. Eur. J. Clin. Microbiol. 5: 320-323

MASUDA, K., KAWATA, T. (1983) Distribution and chemical characterization of regular arrays in the cell walls of strains of the genus *Lactobacillus*. FEMS Microbiol. Lett. **20:** 145-150

McCORMICK, E.L., SAVAGE, D.C. (1983) Characterisation of *Lactobacillus* sp strain 100-37 from the murine gastrointestinal tract - ecology, plasmid contents, and antagonistic activity toward *Clostridium ramosum* H1. Appl. Environ. Microbiol. **46:** 1103-1112

McCOURTIE J., DOUGLAS, L.J., (1985) Extracellular polymer of *Candida albicans* : isolation, analysis and role in adhesion. J. Gen. Microbiol. **131:** 495-503

McCOURTIE J., DOUGLAS, L.J., (1984) Relationship between cell surface composition, adherence and virulence of *Candida albicans*. Infect. Immun. **46:** 6-12

McCOURTIE J., DOUGLAS, L.J., (1981) Relationship between cell surface composition of *Candida albicans* and adherence to acrylic after growth on different carbon sources. Infect. Immun. **32:** 1234-1241

McGROARTY, J.A., SOBOH, F., BRUCE, A.W., REID, G. (1990) The Spermicial Compound Nonoxynol-9 Increases Adhesion of *Candida* Species to Human Epthelial Cells *In Vitro*. Infect. Immun. **58:** 2005-2007

McGROARTY, J.A, TOMECZEK, L., POND, D.G., REID, G., BRUCE, A.W. (1992) Hydrogen peroxide production by *Lactobacillus* species: correlation with susceptibility to the spermicidal compond Nonoxynol-9. J. Infect. Dis. **165:** 1142-1144

MEHENTEE, J.F., HAY, R.J. (1989) *In vitro* Adherence of *Candida albicans* Strains to Murine Gastrointestinal Mucosal Cells and Explants and the Role of Environmental pH. J. Gen. Microbiol. **135:** 2181-2188

MILES, M.R., OLSEN, L., RODGERS, A. (1977) Recurrent vaginal candidiasis. Importance of an intestinal reservoir. J. Am. Med. Assoc. **238:** 1836-1837

MILNE, J.D., WARNOCK, D.W. (1979) Effect of simulataneous oral and vaginal treatment on the rate of cure and relapse in vaginal candidosis. Br. J. Vener. Dis. **55:** 362-365

MIMS, C.A. (1987) The pathogenisis of infectious disease (3rd ed). Academic Press, London

MOHLER, R.W., BROWN, C.P. (1933) Doderleins Bacillus in the treatment of Vaginitis. Am. J. Obstet. Gynecol. **25:** 718-723

MONTES, L.F., WILBORN, W.H. (1968) Ultrastructural features of host-parasite relationship in oral candidiasis. J. Bacteriol. **96:** 1349-1356

MOORS, M.A., JONES, S.M., KLYCZEK, K.K., ROGERS, T.J., BUCKLEY, H.R., BLANK, K.J. (1990) Effect of Friend Leukemia Virus infection on susceptability to *Candida albicans*. Infect. Immun. **58:** 1796-1801

MORTON, R.S., RASHID, S. (1977) Candidal vaginitis: natural history, predisposing factors and prevention. Proc. Roy. Soc. Med. **70** (suppl.4) : 3-6

MORTVEDT, C.I., NISSEN-MEYER, J., SLETTEN, K., NES, I.F. (1991) Purification and amino acid sequence of lactocin S, a bacteriocin produced by *Lactobacillus sake* L45. Appl. Environ. Microbiol. **57**: 1829-1834

MURIANA, P.M., KLAENHAMMER, T.R. (1987) Conjugal transfer of plasmid-encoded determinants for bacteriocin production and immunity in *Lactobacillus acidophilus* 88. Appl. Environ. Microbiol. **53**: 553-560

MURIANA, P.M., KLAENHAMMER, T.R. (1991) Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. Appl. Environ. Microbiol. **57**: 144-121

NADER DE MACIAS, M.E., APELLA, M.C. ROMERO, N.C., GONZALEZ, S.N., OLIVER, G. (1992) Inhibition of *Shigella sonnei* by *Lactobacillus casei* and *Lactobacillus acidophilus*. J. Appl. Bacteriol. **73**: 404-411

NAGESHA, C.N., ANANTHAKRISHNA, N.C. (1970) Clinical and laboratory study of monilial vaginitis Am.J. Obstet. Gynecol. **107**: 1267-1268

NAGY, E., FROMAN, G., MARDH, P.A. (1992) Fibronectin binding of *Lactobacillus* species isolated from women with and without bacterial vaginosis. J. Med. Microbiol. **37**: 38-42

O'CONNER, M.I., SOBEL, J.D. (1986) Epidemiology of recurrent vulvovaginal candidiasis: identification and strain differentiation of *C. albicans*. J. Infect. Dis. **154**: 358-363

ODDS, F.C. (1988) Candida and candidosis. 2nd ed. London: Bailliere Tindall

ODDS, F.C. (1987) *Candida* infections: an overview. CRC Crit. Rev. Microbiol. **15**: 1-5

ODDS, F.C. (1979) Candida and candidosis. 1st ed. Leicester University Press

ODDS, F.C., WEBSTER, C.E., MAYURANATHAN, P., SIMMONS,P.D. (1988) *Candida* concentrations in the vagina and their association with signs and symptoms of vaginal candidosis. J. Med. Vet. Mycol. **26**: 277-283

ODDS, F.C., EVANS, E.G.V., TAYLOR, M.A.R., WALES, J.K. (1987) Prevalance of pathogenic yeasts and humoral antibodies to *Candida* in diabetic patients. J. Clin. Pathol. **31**: 840-844

OHM, M.J., GALASK, R.P. (1976) The effect of antibiotic prophylaxis on patients undergoing total abdominal hysterectomy. II. Alterations of microbial flora. Am. J. Obstet. Gynecol. **125**: 448-454

OHM, M.J., GALASK, R.P. (1975) The effect of antibiotic prophylaxis on patients undergoing vaginal operations. II. Alterations of microbial flora. Am. J. Obstet. Gynecol. **123**: 597-604

ONDERDONK, A.B., POLK, P.F., MOON, N.E., GOREN, D., BARTLETT, J.G.(1977) Methods for quantitive vaginal flora studies. Am. J. Obstet. Gynecol. **128**: 777-781

PAINE, J.R. (1958) The inhibitory actions of bacteria on *Candida* growth. Antib. Chemo. **8**: 273-281

PEDERSEN, G.T. (1969) Yeast flora in mother and child. A mycological-clinical study of women followed up during pregnancy, the puerperium and 5-12 months after delivery and of their children on the 7th day of life and at the age of 5-12 months. Nord. Med. **81**: 207-220.

PEDERSEN, G.T. (1964) Yeasts isolated from the throat, rectum and vagina in 60 women examined during pregnancy and to 1 year after labour. Acta. Obstet. Gynecol. Scand. **42** (suppl.6): 47-61

PERL, J.I., MILLES, G., SHIMOZATO, Y., (1959) Vaginal fluid subsequent to panhysterectomy. Am. J. Obst. Gynecol. **78**: 285-289

PERSI, A.M., BURNHAM J.C., DUHRING, J.L., (1985) Effects of Carbon Dioxide and pH on Adhesion of *Candida albicans* to Vaginal Epithelial Cells. Infect. Immun. **50**: 82-90

PINOTTI, J.A., DE-ARAUJO, L.T., LAVANDERAS, L.P. (1981) Use of *Lactobacillus acidophilus* in the treatment of leukorrheas. Rev. Bras. Med. **38**: 299-304

POTVLIEGE, C., VAN-HUYNEGEM, L., HANSEN, W. (1988) *Lactobacillus plantarum* septicemia. Clin. Microbiol. Newsl. **10**: 38-39

POWELL, B.L., FREY, C.L., DRUTZ, D.J. (1984) Identification of a 17 β-estradiol binding protein in *Candida albicans* and *Candida* (*Torulopsis*) *glabrata*. Exp. Mycol. **8**: 304-313

PRAJAPTI, J.B., DAVE, J.M. (1984) Nutritional and therapeutic properties of a new acidophilus preparation. Indian Dairyman **36**: 377

PRETI, G., HUGGINS, G.R. (1975) Cyclical changes in volatile acidic metabolites of human vaginal secretions and their relation to ovulation. J. Chem. Ecol. **1**: 361-376

PUROHIT, B.C., JOSHI, K.R., RAMDEO, I.N., BHARADWAJ, T.P. (1977) The formation of germ tubes by *Candida albicans*, when grown with *Staphylococcus pyogenes, Escherichia coli, Klebsiella pneumoniae, Lactobacillus acidophilus* and *Proteus vulgaris*. Mycopathologia **62**: 187-189

RAFSKY, H.A., RAFSKY, J.C. (1955) Clinical and bacteriological studies of a new *Lactobacillus acidophilus* concentrate in functional gastrointestinal disturbances. Am. J. Gastroenterol. **24:** 87-92

RAY, T.L., DIGRE, K.B., PAYNE, C.D. (1984) Adherence of *Candida* Species to Human Epidermal Corneocytes and Buccal Mucosal Cells: Correlation with Cutaneous Pathogenicity. J. Invest. Dermatol. **83:** 37-41

REID, G., McGROARTY, J.A., ANGOTTI, R., COOK, R.L. (1988) *Lactobacillus* inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. Can. J. Microbiol. **34:** 344-351

REID, G., BRUCE, A.W., TAYLOR, M. (1992) Influence of three-day antimicrobial therapy and lactobacillus vaginal suppositories on recurrence of urinary tract infections. Clin. Ther. **14:** 11-16

REID, G., CUPERUS, P.L., BRUCE, A.W., VAN DER MEI, H.C., TOMECZEK, L., KHOURY, A.H. (1992) Comparison of contact angles and adhesion to hexadecane of urogenital, dairy and poultry lactobacilli: effect of serial culture passages. Appl. Environ. Microbiol. **58:** 1549-1553

RHOADS, J.D., WRIGHT, D.C., REDFIELD, R.R., BURKE, D.S. (1987) Chronic vaginal candidiasis in woman with Human Immunodeficiency Virus infection. JAMA. **257:** 3105-3107

RICHARDSON, M.D., SMITH, H. (1981) Resistance of virulent and attenuated strains of *Candida albicans* to intracellular killing by human and mouse phagocytes. J.Infect. Dis. **144:** 557-564

RIORDAN, T., MacAULAY, M.E., JAMES, J.M., LEVENTHALL, P.A., MORRIS, E.M. NEAL, B.R., ROWLAND, J., EVANS, B.M. (1990) A prospective study of genital infections in a family-planning clinic 1. Microbiological findings and their association with vaginal symptoms. Epidemiol. Infect. **104**: 47-53

ROGOSA, M., MITCHELL, J.A., WISEMAN, R.F. (1951) A selective medium for the isolation and enumeration of oral and faecal lactobacilli. J. Bact. **62**: 132

ROMERO-PIFFIGUER, M.D., VUCOVICH, P.R., RIERA, C.M. (1985) Secretory IgA and secretory component in women affected by recidivant vaginal candidiasis. Mycopathologia **91**: 165-170

ROSENSTEIN, J.J., GRADY, D., HAMILTON-MILLER, J.M.T., BRUMFITT, W. (1985) Relationship between adhesion of *Escherichia coli* to uroepithelial cells and the pathogenisis of urinary infection: problems in methodology and analysis. J. Med. Microbiol. **20**: 335-344

RYLEY, J.F. (1986) Pathogenicity of *Candida albicans* with particular reference to the vagina. J. Med. Vet. Mycol. **24**: 5-22

RYLEY, J.F., McGREGOR, S. (1986) Quantification of vaginal *Candida albicans* infections in rodents. J. Med. Vet. Mycol. **24**: 455-460

SAIGH, J.H., SANDERS, C.C., SANDERS, W.E. (1978) Inhibition of *Neisseria gonorrhoeae* by Aerobic and Facultatively Anaerobic Components of the Endocervical Flora: Evidence for a protective effect against infection. Infect. Immun. **19**: 704-710

SALAZAR, M.E., RESTREPO, A., STEVENS, D.A. (1988) Inhibition by estrogens of conidium-to-yeast conversion in the fungus *Paracoccidioides brasiliensis*. Infect. Immun. **56**: 711-713

SAMARANAYAKE, L.P., MacFARLANE, T.W. (1982a) The Effect of Dietary Carbohydrates on the *in vitro* Adhesion of *Candida albicans* to Epithelial Cells. J. Med. Microbiol. **15:** 511-517

SAMARANAYAKE, K.P., MacFARLANE, T.W. (1982b) Factors affecting the *in-vitro* adherence of the fungal oral pathogen *Candida albicans* to epithelial cells of human origin. Archs. oral Biol. **27:** 869-873

SAMARANAYAKE, L.P., MacFARLANE, T.W. (1981) The adhesion of the yeast *Candida albicans* to epihelial cells of human origin *in vitro.* Arch. Oral. Biol. **26:** 815-820

SANDIN, R.L., ROGERS, A.L., BENEKE, E.S., FRENANDEZ, M.I. (1987a) Influence of mucosal cell origin on the *in vitro* adherence of *Candida albicans*: Are mucosal cells from different sources equivalent? Mycopathologia **98:** 111-119

SANDIN, R.L., ROGERS, A.L., FERNANDEZ, M.I., BENEKE, E.S. (1987b) Variations in affinity to *Candida albicans in vitro* among human buccal epithelial cells. J. Med. Microbiol. **24:** 151-155

SANDIN, R.L., ROGERS, A.L., PATTERSON, R.J., BENEKE, E.S. (1982) Evidence for Mannose-Mediated Adherence of *Candida albicans* to Human Buccal Cells *in vitro*. Infect. Immun. **35:** 79-85

SANDLER, B. (1979) *Lactobacillus* for vulvovaginitis. Lancet **2:** 791-792

SAUTTER, R.L., BROWN, W.J. (1980) Sequential Vaginal Cultures from Normal Young Women. J. Clin. Microbiol. **11:** 479-484

SAVAGE, D.C. (1969) Microbial Interference Between Indigenous Yeast and Lactobacilli in the Rodent Stomach. J. Bacteriol. **98:** 1278-1283

SAVAGE, D.C. (1972) Associations and physiological interactions of indigenous microorganisms and gastrointestinal epithelia. Am. J. Clin. Nutr. **25:** 1372-1379

SAVAGE, D.C. (1992) Growth phase, cellular hydrophobicity, and adhesion *in vitro* of lactobacilli colonizing the keratinizing gastric epithelium in the mouse. Appl. Environ. Microbiol. **58:** 1992-1995

SCHILLINGER, U., LUCKE, F.K. (1989) Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl. Environ. Microbiol. **55:** 1901-1906.

SCHMID, L., BRUNE, K. (1974) Assessment of phagocytic and antimicrobial activity of human granulocytes. Infect. Immun. **10:** 1120-1126

SCHOLER, H.L. (1960) Experimentelle vaginal-cadidiasis der ratte. Pathol. Microbiol. **23:** 62-68

SEELIG, M.S. (1966) Mechanisms by Which Antibiotics Increase the Incidence and Severity of Candidasis and Alter the Immunnological Defenses. Bacteriol. Rev. **30:** 442-458

SEGAL, E. (1987) Pathogenesis of human mycoses: role of adhesion to host surfaces. Microbiol. Sci. **4:** 344-347

SEGAL, E., SOROKA, A., SCHECHTER, A. (1984) Correlative relationship between adherence of *Candida albicans* to human vaginal epithelial cells *in vitro* and Candidal Vaginitis. Sabouraudia: J. Med. Vet. Mycol. **22:** 191-200

SEGAL, E., LEHRER, N., OFEK, I. (1982) Adherence of *Candida albicans* to human vaginal epithelial cells : inhibition by amino sugars. Exp. Cell. Biol. **50:** 13-17

SHAHANI, K.M., CHANDAN, R.C. (1979) Nutritional and healthful aspects of cultured and culture-containing dairy foods. J. Dairy Sci. **62**: 1685-1694

SHAHANI, K.M., JAYANTKUMAR, R.V., CHANDAN, R.C. (1972) Antibiotic acidophilin and process of preparing the same. US State Patent 3,689,640

SHARMA, S. MADHUR, B.S., SINGH, R., SHARMA, B.K.(1987) Effect of contraceptives on the adhesion of *Escherichia coli* to uroepithelial cells. J. Infect. Dis. **156**: 490-494

SHERMAN, L.A., SAVAGE, D.C. (1986) Lipoteichoic Acids in *Lactobacillus* Strains That Colonize the Mouse Gastric Epithelium. Appl. Environ. Microbiol. **52**: 302-304

SHERMAN, M.E., ALBRECHT, M., DeGIROLAMI, P.C., KIRKLEY, S.A., WOLF, B., ELIOPOULOS, G.M., ROHRER, R.J., MONACO, A.P. (1987) *Lactobacillus*: An unusual case of splenic abscess and sepsis in an immunocompromised host. Am. J. Clin. Pathol. **88**: 659-662

SHIBL, A.M. (1985) Effect of Antibiotics on Adherence of Microorganisms to Epithelial Cell Surfaces. Rev. Infect. Dis. **7**: 51-65

SIEGEL, J. (1986) Clinical allergies and vulvovaginitis. J. Repro. Med. **31** (suppl.) : 647

SILVA, N., JACOBUS, N.V., DENEKE, C., GORBACH, S.L. (1987) Antimicrobial substance from a human *Lactobacillus* strain. Antimicrob. Agents Chemother. **31**: 1231-1233

SJOBERG, I., HAKANSSON, S., HOLM, S.E. (1990) Accumulation of penicillin in vaginal fluid. Obstet. Gynecol. **75**: 18-21

SJOBERG, I., GRAHN, E., HAKANSSON, S., HOLM, S.E. (1992) Influence of phenoxymethylpenicillin on the vaginal ecosystem. Gynecol. Obstet. Invest. **33**: 42-46

SKARIN, A., SYLWAN, J.(1986) Vaginal lactobacilli inhibiting growth of *Gardnerella vaginalis*, *Mobiluncus* and other bacterial species cultured from vaginal content of women with bacterial vaginosis. Acta. Pathol. Microbiol. Immunol. Scand. Sect. B Microbiol. **94**: 399-403

SKERL, K.G., CALDERONE, R.A., SEGAL, E., SREEVALSAN, T., SCHELD, W.M. (1984) *In vitro* binding of *Candida albicans* yeast cells to human fibronectin. Can. J. Microbiol. **30**: 221-227

SOBEL, J.D. (1985a) Epidemiology and pathogenesis of recurrent vulvovaginal candidiasis. Am. J. Obstet. Gynecol. **152**: 924-935

SOBEL, J.D. (1985b) Management of recurrent vulvovaginal candidiasis with intermittent ketoconazole prophylaxis. Obstet. Gynecol. **65**: 435-440

SOBEL, J.D. (1984) Vulvovaginal candidiasis - what we do and do not know. Ann. Intern. Med. **101**: 390-392

SOBEL, J.D., MULLER, G., McCORMICK, J.F. (1985) Experimental chronic vaginal candidosis in rats. Sabouraudia: J. Med. Vet. Mycol. **23**: 199-206

SOBEL, J.D., MYERS, P., LEVISON, M.E., KAYE, D. (1982) Comparison of Bacterial and Fungal Adherence to Vaginal Exfoliated Epithelial Cells and Human Vaginal Epithelial Tissue Culture Cells. Infect. Immun. **35**: 697-701

SOBEL, J.D., MYERS, P.G., KAYE, D., LEVISON, M.E. (1981) Adherence of *Candida albicans* to Human Vaginal and Buccal Epithelial Cells. J. Infect. Dis. **143**: 76-83

SOBRINO, O.J., RODRIGUEZ, J.M., MOREIRA, W.L., CINTAS, L.M., FERNANDEZ, M.F., SANZ, B. (1992) Sakacin M, a bacteriocin-like substance from *Lactobacillus sake* 148. Int. J. Food. Microbiol. **16**: 215-225

SPINILLO, A., CARRATTA, L., PIZZOLI, G., LOMBARDI, G., CAVANNA, C., MICHELONE, G., GUASCHINO, S. (1992) Recurrent vaginal candidiasis. Results of a cohort study of sexual transmission and intestinal reservoir. J. Reprod. Med. **37**: 343-347

STAMEY, T.A., KAUFMAN, M.F (1975) Studies of interoital colonization in women with recurrent urinary infections II. A comparison of growth in normal vaginal fluid of common versus uncommon sero groups of *Escherichia coli*. J. Urol. **114**: 264-267

STEFFEN, R., HEUSSER, R., DUPONT, H.L. (1986) Prevention of travellers' diarrhoea by nonantibiotic drugs. Rev. Infect. Dis. **8** (suppl.2): 151-159

STEWART-TULL, D.E.S., (1964) Evidence that vaginal lactobacilli do not ferment glycogen. Am. J. Obstet. Gynecol. **88**: 676-679

STOVER, E.P., SCHAR, G., CLEMONS, K.V., STEVENS, D.A., FELDMAN, D. (1986) Estradiol-binding proteins from mycelial and yeast-form cultures of *Paracoccidioides brasiliensis*. Infect. Immun. **51**: 199-203

SUEGARA, N., MOROTOMI, M.,WATANABE, T., KAWAI, Y., MUTAI, M. (1975) Behaviour of microflora in the rat stomach *in vitro*. Infect. Immun. **12**: 173-179

SUGARMAN, B., EPPS, L.R. (1982) Effect of estrogens on bacterial adherence to HeLa cells. Infect. Immun. **35**: 633-638

TAGG, J.R., DAJANI, A.S., WANNAMAKER, L.W. (1976) Bacteriocins of Gram-positive bacteria. Bacteriol. Rev. **40**: 722-756

TALARICO, T.L., CASAS, I.A., CHUNG, T.C., DOBROGOSZ, W.J. (1988) Production and isolation of reuterin: a growth inhibitor produced by *Lactobacillus reuteri*. Antimicrob. Agents Chemother. **32**: 1854-1858

TALARICO, T.L., DOBROGOSZ, W.J.(1989) Chemical characterization of an antimicrobial substance produced by *Lactobacillus reuteri*. Antimicrob. Agents Chemother. **33**: 674-679

TANNOCK, G.W. (1988) The normal microflora: new concepts in health promotion. Microbiol. Sci. **5**: 4-8

TASCHDJIAN, C.L., REISS, F., KOZINN, P.J. (1960) Experimental Vaginal Candidiasis in Mice; Its Implications for Superficial Candidiasis in Humans. J. Invest. Dermatol. **34**: 89-94

TRAMER, J. (1966) Inhibitory Effect of *Lactobacillus acidophilus*. Nature **211**: 204-205

TAYLOR, R.S., WOODS, J.B., GUAPO, M. (1986) Correlation of vaginal hormonal cytograms with cervical mucus symptoms as observed by women using the ovulation method of natural family planning. J. Reprod. Med. **31**: 167-72

TOOLEY, P.J.H. (1985) Patient and doctor preferences in the treatment of vaginal candidosis. Practitioner **229**: 655-660

TRUMBORE, D.J., SOBEL J.D. (1986) Recurrent Vulvovaginal Candidiasis: Vaginal Epithelial Cells Susceptibility to *Candida albicans* Adherence. Obstet. Gynecol. **67**: 810-812

UPRETI, G.C., HINSDILL, R.D. (1975) Production and mode of action of lactocin 27: bacteriocin from a homofermentative *Lactobacillus*. Antimicrob. Agents Chemother. **7**: 139-145

VANDENBERGH, P.A., KING, S.W. (1989) Process for producing novel yeast and mould inhibiting products. European Patent Office. Publication number 0 302 300

Van LOOSDRECHT, M.C.M., LYKLEMA, J., NORDE, W., ZEHNDER, A.J.B. (1990) Influence of Interfaces on Microbial Activity. Microbiol. Rev. **54**: 75-87

VAUGHAN, E.E., DALY, C., FITZGERALD, G.F. (1992) Identification and characterization of helveticin V-1829, a bacteriocin produced by *Lactobacillus helveticus* 1829. J. Appl. Bacteriol. **73**: 299-308

VINCENT, J.G., VEOMETT, R.C., RILEY, R.F. (1959) Antibacterial Activity Associated with *Lactobacillus acidophilus*. J. Bacteriol. **78**: 477-484

VOLK, W.A., WHEELER, M.F. (Eds.)(1988) Basic Microbiology 6th edition. Harper and Row

VUDHICHAMNONG, K., WALKER, D.M., RYLEY, H.C. (1982) The Effect of Secretory Immunoglobulin A on the *in vitro* Adherence of the Yeast *Candida albicans* to Human Oral Epithelial Cells. Arch. Oral Biol. **27**: 617-621

WADSTROM, T., ANDERSSON, K, SYDOW, M., AXELSSON, L., LINDGREN, S., GULLMAR, B. (1987) Surface properties of lactobacilli isolated from the small intestine of pigs. J. Appl. Bacteriol. **62**: 513-520

WEINSTEIN, L., HOWARD, J.H. (1939) The effect of estrogenic hormone on the H-ion concentration and the bacterial content of the human vagina, with special reference to the Doderlein Bacillus. Am. J. Obstet. Gynecol. **37**: 698-703

WESTERGREN, G., EMILSO, C.G. (1982) Colonization and cariogenic potential in hamsters of the bacterium *Streptococcus sanguis* isolated from human dental plaque. Arch. Oral Biol. **27**: 817-822

WEST, C.A., WARNER, P.J. (1988) Plantacin B, a bacteriocin produced by *Lactobacillus plantarum* NCDO 1193. FEMS Microbiol. Lett. **49**: 163-165

WHEATER, D.M., HIRSCH, A., MATTICK, A.T.R. (1952) Possible Identity of "Lactobacillin" with Hydrogen Peroxide produced by Lactobacilli. Nature **170**: 623-624

WILKS,M., THIN, R.N., TABAQCHALI, S. (1984) Quantitative Bacteriology of the Vaginal Flora in Genital Disease. J. Med. Microbiol. **18**: 217-231

WILKS, M., TABAQCHALI, S. (1987) Quantitative bacteriology of the vaginal flora during the menstrual cycle. J. Med. Microbiol. **24**: 241-245

WILL, T.E. (1979) *Lactobacillus* overgrowth for treatment of moniliary vulvovaginitis. Lancet **2**, September 1: 482

WINNER,H.I., HURLEY, R. (1964) Candida albicans. Churchill, London

WITKIN, S.S., JEREMIAS, J. LEDGER, W.J. (1989) Vaginal eosinophils and IgE antibodies to *Canadida albicans* in women with recurrent vaginitis. J. Med. Vet. Mycol. **27**: 57-58

WITKIN, S.S., YU, I.R., LEDGER, W.J. (1983) Inhibition of *Candida albicans*-induced lymphocyte proliferation by lymphocytes and sera from women with recurrent vaginitis. Am. J. Obstet. Gynecol. **147**: 809-811

WOOD, J.R., SWEET, R.L., CATENA, A., HADLEY, W.K., ROBBIE, M. (1985) *In vitro* adherence of *Lactobacillus* species to vaginal epithelial cells. Am. J. Obstet. Gynecol. **153**: 740-743

WYLIE,J.G., HENDERSON,A. (1969) Identity and glycogen-fermenting ability of lactobacilli isolated from the vagina of pregnant women. J. Med. Microbiol. **2**: 363-366

YOUNG, G., KRASNER, R.I., YUDKOFSKY, P.L. (1956) Interactions of oral strains of *Candida albicans* and lactobacilli. J. Bacteriol. **72**: 525-529

APPENDICES

Appendix 1.

Distribution of six strains of *Candida albicans* on mouse vaginal epithelial cells by number of adhering yeasts per cell

Strain	Carbon Source[a]	Distribution of adherent yeasts on vaginal epethelial cells[b]					Chi-squared statistic
		0	1-15	6-15	16+	Total	
GDH 2346	Glucose	552	224	98	26	900	
	Galactose	330	270	150	108	900	121.47
GDH 2023	Glucose	595	209	63	33	900	
	Galactose	299	243	175	183	900	257.429
NCPF 3153	Glucose	589	187	86	38	900	
	Galactose	385	276	133	106	900	102.031
'Outbreak' Strain	Glucose	535	224	105	36	900	
	Galactose	443	268	166	73	900	38.878
GRI 681	Glucose	606	163	25	6	800	
	Galactose	673	197	27	3	900	1.975 (NS[c])
GRI 682	Glucose	715	146	33	6	900	
	Galactose	616	193	70	21	900	35.505

[a] Yeasts were grown to stationary phase in YNB medium containing either 50mM gluscose or 500mM galactose.

[b] Vaginal epithelial cells varied in their ability to bind *C. albicans*. They were segregated into arbitary sub-groups: those cells with 0 attached yeasts, those with 1-5 attached yeasts/cell, those with 6-15 attached yeasts/cell, and those with 16+ attached yeasts/cell. The total number of vaginal cells counted was 800 or 900 as specified.

[c] NS, not significant

Appendix 2.

Distribution of six strains of *Candida albicans* on vaginal epithelial cells from diabetic mice by number of adhering yeasts per cell

Strain	Carbon Source[a]	Distribution of adherent yeasts on vaginal epithelial cells[b]					Chi-squared statistic
		0	1-15	6-15	16+	Total	
GDH 2346	Glucose	483	224	66	27	800	
	Galactose	256	272	146	126	800	168.621
GDH 2023	Glucose	363	242	128	67	800	
	Galactose	238	216	165	181	800	85.225
MRL 3153	Glucose	426	181	75	18	700	
	Galactose	288	244	169	99	800	122.207
'Outbreak' Strain	Glucose	474	202	80	44	800	
	Galactose	336	190	101	73	700	26.937
GRI 681	Glucose	542	207	43	8	800	
	Galactose	432	697	69	34	800	300.151
GRI 682	Glucose	556	189	45	10	800	
	Galactose	413	546	110	49	800	247.542

[a] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[b] Vaginal epithelial cells varied in their ability to bind *C. albicans*. They were segregated into arbitiary sub-groups: those cells with 0 attached yeasts, those with 1-5 attached yeasts/cell, those with 6-15 attached yeasts/cell and those with 16+ attached yeasts/cell. The total number of vagianl cells counted was 700 or 800 as specified.

Appendix 3.

Effect of growth of *C. albicans* in the presence of steroid hormone upon the distribution of adherent yeasts to mouse, rat and human vaginal epithelial cells (VEC)

Epithelial cell type	Treatment[a]	Carbon Source[b]	Distribution of adherent yeasts on VEC[c] 0	1-5	6-15	16+	Total	Chi-squared statistic
MOUSE	Oestradiol	Glucose	732	151	17	0	900	
	Progesterone	Glucose	687	200	13	0	900	
	Control	Glucose	729	146	23	2	900	17.769
	Oestradiol	Galactose	328	417	120	35	900	
	Progesterone	Galactose	372	433	67	28	900	
	Control	Galactose	378	403	88	31	900	22.550
RAT	Oestradiol	Glucose	672	115	12	1	800	
	Progesterone	Glucose	637	153	8	2	800	
	Control	Glucose	640	145	13	2	800	8.653
	Oestradiol	Galactose	354	305	101	40	800	
	Progesterone	Galactose	345	321	102	32	800	
	Control	Galactose	372	316	87	25	800	6.423
HUMAN	Oestradiol	Glucose	740	147	12	1	900	
	Progesterone	Glucose	745	141	14	0	900	
	Control	Glucose	727	166	5	2	900	8.820
	Oestradiol	Galactose	384	407	88	21	900	
	Progesterone	Galactose	406	372	106	16	900	
	Control	Galactose	472	338	74	16	900	23.07

[a] Yeasts were grown to stationary phase in medium which contained 1μM concentration of steroid hormone which had been originally solubilized in methanol. Control yeasts were grown in medium containing an equivalent concentration of methanol without the presence of steroid hormone.

[b] Yeasts were grown in medium containing either 50mM glucose or 500mM galactose as the carbon source.

[c] Vaginal epithelial cells varied in their ability to bind *C. albicans*. The total number of vaginal cells counted were segregated into arbitary sub-groups: those cells with 0 attached yeasts, those with 1-5 attached yeasts/cell, 6-15 attached yeasts/cell and 16+ attached yeasts/cell.

Appendix 4.

Effect of pretreatment of rat vaginal epithelial cells (VEC) with steroid hormones on the distribution of adherent *Candida*

Pretreatment[a]	Carbon source[b]	Distribution of adherent yeasts on VEC[c]					Chi-squared statistic
		0	1-5	6-15	16+	Total	
Oestradiol	Glucose	639	212	40	9	900	
Progesterone	Glucose	658	201	34	7	900	
Control	Glucose	620	212	53	15	900	9.512
Oestradiol	Galactose	126	265	252	257	900	
Progesterone	Galactose	98	226	275	301	900	
Control	Galactose	96	173	280	351	900	40.799

[a] Prior to adhesion assays, vaginal epithelial cells were incubated for 30 min at 37°C in PBS which contained 1μM steroid hormone which had been originally solubilized in methanol. Control cells were incubated in PBS containing an equivalent concentration of methanol without the presence of steroid hormone. After this pretreatment, vaginal cells were washed, resuspended in PBS and used in adhesion assays.

[b] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[c] Vaginal epithelial cells varied in their ability to bind *C. albicans*. They were segregated into arbitary sub-groups: those cells with 0 attached yeasts, those with 1-5 attached yeasts/cell, those with 6-15 attached yeasts/cell and those with 16+ attached yeasts/cell.

Appendix 5.

Effect of pretreatment of *Candida* with steroid hormones on the distribution of adherent yeasts to rat vaginal epithelial cells (VEC)

Pretreatment[a]	Carbon source[b]	Distribution of adherent yeasts on VEC[c]					Chi-squared statistic
		0	1-5	6-15	16+	Total	
Oestradiol	Glucose	595	229	66	10	900	
Progesterone	Glucose	586	239	63	12	900	
Control	Glucose	543	247	86	24	900	15.385
Oestradiol	Galactose	248	277	184	191	900	
Progesterone	Galactose	251	282	187	180	900	
Control	Galactose	214	247	209	230	900	15.052

[a] Prior to adhesion assays, yeasts were incubated for 30 min at 37°C in PBS which contained a 1μM concentration of steroid hormone which had been originally solubilized in methanol. Control cells were incubated in PBS containing an equivalent concentration of methanol without the steroid hormone. After this pretreatment, yeasts were washed, resuspended in PBS and used in adhesion assays.

b Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

c Vaginal epithelial cells varied in their ability to bind *C. albicans*. They were segregated into arbitary sub-groups: those cells with 0 attached yeasts, those with 1-5 attached yeasts/cell, those with 6-15 attached yeasts/cell and those with 16+ attached yeasts/cell.

Appendix 6.

Effect of *L. acidophilus* on the distribution of adherent yeasts on mouse, rat and human vaginal epithelial cells (VEC)

Epithelial cell type	Pretreatment[a]	Carbon Source[b]	Distribution of adherent yeasts on VEC[c]					Chi-squared statistic
			0	1-5	6-15	16+	Total	
Mouse	Lactobacilli	Glucose	465	116	19	0	600	12.572
	PBS	Glucose	413	155	31	1	600	
	Lactobacilli	Galactose	397	149	41	13	600	28.904
	PBS	Galactose	309	195	74	22	600	
Rat	Lactobacill	Glucose	656	133	11	0	800	18.378
	PBS	Glucose	588	188	23	1	800	
	Lactobacilli	Galactose	468	255	64	13	800	16.072
	PBS	Galactose	401	278	98	23	800	
Human	Lactobacilli	Glucose	565	255	70	10	900	11.256
	PBS	Glucose	514	287	77	22	900	
	Lactobacilli	Galactose	305	306	221	68	900	13.321
	PBS	Galactose	260	289	246	105	900	

[a] Prior to adhesion assays, vaginal epithelial cells were incubated for 30 min at 37°C in PBS or in a suspension of *L. acidophilus* NCTC 4504 (5 x 10^8 cells/ml). After this pretreatment vaginal cells were washed to remove any non-adherent lactobacilli, resuspended in PBS and used in adhesion assays.

[b] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[c] Vaginal epithelial cells varied in their ability to bind *C. albicans*. They were segregated into arbitrary sub-groups: those cells with 0 attached yeasts, those with 1-5 attached yeasts/cell, those with 6-15 attached yeasts/cell and those with 16+ attached yeasts/cell. The total number of vaginal cells counted was 600, 800 or 900 as specified.

Appendix 7.

Effect of *L. fermentum* on the distribution of adherent yeasts on mouse, rat and human vaginal epithelial cells (VEC)

Epithelial cell type	Pretreatment[a]	Carbon Source[b]	Distribution of adherent yeasts on VEC[c]					Chi-squared statistic
			0	1-15	6-15	16+	Total	
Mouse	Lactobacilli	Glucose	677	100	20	3	800	8.306
	PBS	Glucose	721	134	35	10	900	
	Lactobacilli	Galactose	499	278	94	29	900	50.163
	PBS	Galactose	363	322	157	58	900	
Rat	Lactobacilli	Glucose	748	144	5	3	900	5.847(NS)[d]
	PBS	Glucose	721	161	14	4	900	
	Lactobacilli	Galactose	470	232	156	42	900	10.822
	PBS	Galactose	412	242	181	65	900	
Human	Lactobacilli	Glucose	690	100	8	2	800	48.631
	PBS	Glucose	579	190	22	9	800	
	Lactobacilli	Galactose	424	287	143	46	900	58.889
	PBS	Galactose	272	371	170	87	900	

[a] Prior to adhesion assays, vaginal epithelial cells were incubated for 30 min at 37°C in PBS or in a suspension of *L. fermentum* ($5x10^8$ cells/ml). After this pretreatment vaginal cells were washed to remove any non-adherent lactobacilli, resuspended in PBS and used in adhesion assays.

[b] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[c] Vaginal epithelial cells varied in their ability to bind to *C. albicans*. They were segregated into arbitrary sub-groups: those with 0 yeasts attached, those with 1-5 attached yeasts/cell, those with 6-15 attached yeasts/cell and those with 16+ attached yeasts/cell. The total number of vaginal cells counted was 800 or 900 as specified.

[d] NS, not significant.

Appendix 8.

Effect of pH on the distribution of adherent *C. albicans* on mouse vaginal epithelial cells (VEC) by number of adhering yeasts per cell

pH of assay[a]	Carbon Source[b]	Distribution of adherent yeasts on VEC[c]					Chi-squared statistic
		0	1-5	6-15	16+	Total	
4.5	Glucose	789	86	15	1	900	
7.2	Glucose	618	258	21	3	900	110.80
4.5	Galactose	237	330	240	93	900	
7.2	Galactose	172	177	411	140	900	110.90

[a] Suspensions of vaginal epithelial cells and *Candida* were washed and standardized in either PBS (ph 7.2) or citrate buffer (pH 4.5) before use in the adhesion assay.

[b] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[c] Vaginal epithelial cells varied in their ability to bind *C. albicans*. They were segregated into arbitrary sub-groups: those cells with 0 attached yeasts, those with 1-5 attached yeasts/cell, those with 6-15 attached yeasts, and those with 16+ attached yeasts/cell. The total number of vaginal cells counted was 900.

Appendix 9.

Effect of *L. crispatus* 632697M on the distribution of four strains of adherent *C. albicans* on mouse vaginal epithelial cells (VEC) by number of adhering yeasts per cell

Yeast strain in assay	Carbon Source[a]	Pretreatment[b]	Distribution of adherent yeasts on VEC[c] 0	1-5	6+	Total	Chi-squared test
	Glucose	Lactobacillus	823	67	10	900	20.89
GDH 2346	Glucose	PBS	675	107	18	800	
	Galactose	Lactobacillus	643	206	51	900	38.85
	Galactose	PBS	450	273	77	800	
	Glucose	Lactobacillus	781	106	13	900	60.90
GDH 2023	Glucose	PBS	619	151	30	800	
	Galactose	Lactobacillus	569	202	129	900	72.12
	Galactose	PBS	348	236	216	800	
	Glucose	Lactobacillus	843	53	4	900	13.39
GRI 682	Glucose	PBS	710	86	4	800	
	Galactose	Lactobacillus	793	100	7	900	53.06
	Galactose	PBS	603	162	35	800	
	Glucose	Lactobacillus	741	126	33	900	5.714 (NS[d])
GRI 272	Glucose	PBS	625	146	29	800	
	Galactose	Lactobacillus	482	263	155	900	36.096
	Galactose	PBS	329	247	224	800	

[a] Yeasts were grown to stationary phase in YNB medium containing either 50mM glucose or 500mM galactose.

[b] Prior to adhesion assays, vaginal epithelial cells were incubated for 30 min at 37°C in PBS or in a suspension of *L. crispatus* (5 x 10^8 cells/ml). After this pretreatment, vaginal cells were washed to remove any non-adherent lactobacilli, resuspended in PBS and used in adhesion assays.

[c] Vaginal epithelial cells varied in their ability to bind *C. albicans*. They were segregated into arbitary sub-groups: those cells with 0 attached yeasts, those with 1-5 attached yeasts/cell, and those with 6+ attached yeasts/cell. The total number of vaginal cells counted were 800 or 900 as specified.

[d] NS, not significant.

Appendix 10.

Media

I. 50mM Glucose Yeast Nitrogen Base (50mM glucose YNB)

Glucose	9g
Difco Yeast Nitrogen Base	6.7g
Distilled water	1000ml

The medium was autoclaved at 115°C, for 10 min.

II. 500mM Galactose Yeast Nitrogen Base (500mM galactose YNB)

Galactose	90g
Difco Yeast Nitrogen Base	6.7g
Distilled water	1000ml

The medium was autoclaved at 115°C, for 10 min.

III 500mM Sucrose Yeast Nitrogen Base (500mM Sucrose YNB)

Sucrose	171g
Difco Yeast Nitrogen Base	6.7g
Distilled water	1000ml

The medium was autoclaved at 115°C, for 10 min.

IV Sabouraud Dextrose agar containing antibiotics (Penicillin and Streptomycin)

Sabouraud dextrose agar	6.7g
Distilled water	1000ml

The agar was dissolved by heating and then autoclaved at 121°C for 15 min.

Antibiotic solution:

Penicillin	2g
Streptomycin	2g
Distilled water	20ml

The solution of antibiotics was filter sterilized (Acrodisc; 0.45μm) and 1ml was added to cooled molten agar, mixed and dispensed into petri dishes. The agar was then allowed to solidify.

Appendix 11.

Buffers

I. Phosphate Buffer Saline (pH 7.2)

NaCl	16g
KCl	0.4g
Na_2HPO_4	2.3g
KH_2PO_4	0.4g
Distilled Water	2000ml

II. 0.1M citric acid - sodium citrate buffer (pH 4.5)

0.1M citric acid

$C_6H_8O_7H_2O$	21.01g
Distilled water	1000ml

0.1M sodium citrate

$Na_3.citrate.2H_2O$	29.41g
Distilled water	1000ml

495ml 0.1M citric acid was mixed with 505ml 0.1M trisodium citrate.

III. 0.1M citric acid - 0.2M disodium hydrogen phosphate buffer

0.1M citric acid

$C_6H_8O_7H_2O$	21.01g
Distilled water	1000ml

0.2M Na_2HPO_4

Na_2HPO_4	28.4g
Distilled water	1000ml

Solutions were made up and mixed to give the appropriate pH:

pH	x ml 0.1M citric acid	y ml 0.2M Na_2HPO_4
3	79.45	20.55
4	61.45	38.55
5	48.5	51.50
6	36.85	63.51
7	17.65	82.35

IV. 0.2M Tris maleate - sodium hydroxide buffer (pH 8)

0.2M tris-maleate

tris	2.4g
maleic acid	23.2g
Distilled Water	1000ml

0.2M NaOH

NaOH	8g
Distilled Water	1000ml

To obtain pH 8, 25ml of 0.2M tris-maleate was mixed with 34.5ml 0.2M NaOH and then diluted to 100ml.

V. 0.2M Sodium acetate - acetic acid buffer

0.2M sodium acetate

CH_3 COO Na $3H_2O$	27.22g
Distilled water	1000ml

0.2M acetic acid

CH_3 COO H	12.01g
Distilled water	1000ml

Solutions were made up and mixed to give the appropriate pH.

pH	x ml 0.2M sodium acetate	y ml 0.2M acetic acid
3.7	10.0	90.0
4	18.0	82.0
5	70.0	30.0

VI. 0.2M disodium hydrogen phosphate - sodium dihydrogen phosphate buffer

0.2M Na_2 HPO_4

Na_2 HPO_4	35.61g
Distilled water	1000ml

0.2M Na H_2PO_4

Na H_2PO_4	27.6g
Distilled water	1000ml

Solutions were made up and mixed to give the appropriate pH and then diluted to 100ml.

pH	x ml 0.2M Na_2 HPO_4	y ml 0.2M NaH_2PO_4
6	6.15	43.85
7	30.5	19.5
8	47.35	2.65