



National Library  
of Canada

Bibliothèque nationale  
du Canada

Canadian Theses Service

Service des thèses canadiennes

Ottawa, Canada  
K1A 0N4

## NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

## AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

*TRICHOMONAS VAGINALIS* AND VAGINAL FLORA -  
INTERACTIONS IN A MOUSE MODEL

A Thesis Submitted to the  
School of Graduate Studies  
University of Ottawa

In Partial Fulfillment of the Requirements for the Degree of  
Master of Science  
Department of Microbiology and Immunology  
Faculty of Medicine

By

Karen C. Meysick



Karen C. Meysick, Ottawa, Canada, 1990



National Library  
of Canada

Bibliothèque nationale  
du Canada

Canadian Theses Service    Service des thèses canadiennes

Ottawa, Canada  
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-60008-X

Canada



UNIVERSITÉ D'OTTAWA  
UNIVERSITY OF OTTAWA

## ABSTRACT

The flagellated parasite, *Trichomonas vaginalis*, is one of the most commonly encountered genital pathogens. Despite its frequent incidence, pathogenic mechanisms of *T. vaginalis* are not well characterized.

The first section of this project involved the ability of *T. vaginalis* to grow in serum-free medium. By employing a cell culture system, it was demonstrated that *T. vaginalis* can exhibit growth in the absence of serum. McCoy cells utilized in the system did not appear to secrete growth factors responsible for this proliferation.

The second part of this project involved establishment of a mouse model for *T. vaginalis* infection and subsequent employment of the model in determining the effects of *T. vaginalis* on vaginal flora and pH. After the endemic flora and pH in mice were identified, other factors which may have influenced flora, were investigated. Both estrogenization and inoculation of media appeared to have no effect on individual vaginal species, and only appeared to increase the number of species found per mouse. *T. vaginalis* infection did not appear to alter vaginal pH or flora *in vivo*. *In vitro* studies of the interaction between *T. vaginalis* and *Lactobacillus acidophilus* indicated that indirect factors, such as secreted products, may mediate the alteration in flora and pH evident during infection.

## ACKNOWLEDGEMENTS

I wish to thank Dr. G. Garber, who has made this project possible and whose encouragement, guidance and optimism was greatly appreciated. Thanks also goes to my thesis committee, especially Drs. Dimock and Filion, and Dr. Wright for numerous suggestions and advice in writing this thesis.

I would also like to thank L. Lemchuk-Favel for all the hints and suggestions throughout this project, and Dr. E. Kemeny and the Microbiology staff at the Ottawa General Hospital for allowing me the use of their facilities and all their technical help.

Finally I want to thank all past and present graduate students, especially W. Lauzon, for their support and camaraderie over the past 3 years. They made the bad days a little easier to take.

A special thanks also goes to my family and Mark Wood for all their support.

TABLE OF CONTENTS

	PAGE
ABSTRACT . . . . .	i
ACKNOWLEDGEMENTS . . . . .	ii
TABLE OF CONTENTS . . . . .	iii
LIST OF FIGURES . . . . .	vi
LIST OF TABLES . . . . .	vii
LIST OF ABBREVIATIONS . . . . .	ix
INTRODUCTION	
<i>TRICHOMONAS VAGINALIS</i> . . . . .	1
VAGINAL FLORA AND pH . . . . .	11
LACTOBACILLUS . . . . .	20
RATIONAL . . . . .	26
OBJECTIVES . . . . .	28
MATERIALS AND METHODS	
STRAINS . . . . .	29
TISSUE CULTURE . . . . .	30
GROWTH ASSAYS . . . . .	31
SUBCUTANEOUS MOUSE ASSAY . . . . .	32
INTRAVAGINAL INOCULATION OF BALB/c MICE	
WITH <i>T. VAGINALIS</i> . . . . .	34
SAMPLING OF MOUSE VAGINAL FLORA AND pH . . . . .	36

<i>IN VITRO</i> COMPETITIVE ASSAYS BETWEEN	
<i>T. VAGINALIS</i> AND <i>L. ACIDOPHILUS</i> .....	41
STATISTICAL ANALYSIS .....	43
ANIMAL CARE .....	43
<b>RESULTS</b>	
GROWTH OF <i>T. VAGINALIS</i> IN SERUM-FREE MEDIUM .....	44
SUBCUTANEOUS MOUSE ASSAYS .....	47
ESTABLISHMENT OF A MOUSE MODEL FOR	
<i>T. VAGINALIS</i> INFECTIONS .....	50
FLORA AND pH IN UNTREATED MICE .....	55
ASSESSMENT OF VARIOUS FACTORS IN	
ALTERATIONS OF VAGINAL FLORA AND pH .....	55
<i>IN VITRO</i> COMPETITIVE ASSAYS BETWEEN	
<i>T. VAGINALIS</i> AND <i>L. ACIDOPHILUS</i> .....	68
<b>DISCUSSION</b>	
SERUM-FREE GROWTH OF <i>T. VAGINALIS</i> .....	73
PATHOGENICITY AS DETERMINED BY	
THE SUBCUTANEOUS MOUSE ASSAY .....	74
AN INTRAVAGINAL MOUSE MODEL FOR	
<i>T. VAGINALIS</i> INFECTION .....	77
THE VAGINAL FLORA AND pH IN BALB/c MICE .....	80
THE INFLUENCE OF VARIOUS FACTORS ON	
MURINE GENITAL FLORA AND pH .....	82

*IN VITRO* INTERACTIONS BETWEEN *T. VAGINALIS*  
AND *L. ACIDOPHILUS* . . . . . 88  
IMPORTANCE OF FUTURE STUDIES . . . . . 91  
CONCLUSIONS . . . . . 92  
LIST OF REFERENCES . . . . . 94  
APPENDIX . . . . . 104

LIST OF FIGURES

	PAGE
1. Growth of <i>T. vaginalis</i> isolate 002 compared in serum and serum-free cell culture systems . . . . .	46
2. Comparison of the frequency of isolation of vaginal organisms in single-estrogen treated, <i>T. vaginalis</i> inoculated mice during a two week sampling period . . . . .	63
3. Vaginal pH alterations in <i>T. vaginalis</i> inoculated mice . . . . .	64
4. Comparison of the frequency of isolation of vaginal organisms in weekly estrogen treated, <i>T. vaginalis</i> inoculated mice during a two week sampling period . . . . .	66
5. Growth characteristics of <i>L. acidophilus</i> and <i>T. vaginalis</i> in coincubation competitive assays . . . . .	69

LIST OF TABLES

	PAGE
1. Ranking of <i>T. vaginalis</i> Isolates to clinical presentation . . . . .	33
2. Primary isolation: media and incubation methods . . . . .	38
3. Growth of <i>T. vaginalis</i> in a McCoy cell culture system in the presence and absence of serum . . . . .	45
4. Subcutaneous mouse assay performed with clinical <i>T. vaginalis</i> isolates . . . . .	48
5. Establishment of a mouse model for <i>T. vaginalis</i> infection . . . . .	51
6. Modifications to mouse model . . . . .	53
7. Association between the stage of estrous and the susceptibility of mice to intravaginal infection with <i>T. vaginalis</i> . . . . .	54
8. Vaginal flora and pH in untreated mice . . . . .	56
9. Vaginal flora and pH in estrogenized mice . . . . .	58
10. Floral changes induced by inoculation with agar-free medium . . . . .	60
11. Floral changes induced by inoculation with agar-containing medium . . . . .	61

12. Growth characteristics of  
*Lactobacillus acidophilus* . . . . . 71

13. Growth characteristics of  
*Trichomonas vaginalis* . . . . . 72

**LIST OF ABBREVIATIONS**

BV	bacterial vaginitis
CDC	Center for Disease Control
CDF	cell-detaching factor
CNA	colistin sulfate and nalidixic acid
CPE	cytopathic effects
coag -ve Staph	coagulase negative Staphylococci
FBS	fetal bovine serum
GDS	Group D Streptococci
KD	kilodalton
La	<i>Lactobacillus acidophilus</i>
Lacto	Lactobacillus species
MRS	Lactobacilli MRS broth
NGU	nongonococcal urethritis
PBS	phosphate buffered saline
PEA	phenyl-ethyl alcohol
pi	post-inoculation
PMN	polymorphonuclear leucocyte
STD	sexually transmitted disease
Tf	<i>Tritrichomonas foetus</i>
Tsoy	tryptic soy broth
Tv	<i>Trichomonas vaginalis</i>

TYI  
um

Diamond TYI-S-33 medium  
micrometer

## INTRODUCTION

### *Trichomonas vaginalis*

#### General Background

The flagellated trophozoite protozoa, *Trichomonas vaginalis*, was first described by Donne in 1836. *Trichomonas vaginalis* ranges in size from 10-30 um, and morphologically consists of four anterior flagella, a long axostyle, which bisects the cell longitudinally and protrudes through the posterior end, an oval nucleus and an undulating membrane (Jirovec and Petru 1968, Honigberg 1978). The motion of the flagella and undulating membrane create *T. vaginalis*'s characteristic jerky, non-directional movement.

*T. vaginalis* is the only pathogenic member of the three trichomonad species (*T. hominis*, *T. tenax*, *T. vaginalis*) that parasitize man and is specific to the genito-urinary system (Brown 1972, Jirovec and Petru 1968, Honigberg 1978, Krieger 1981). Long considered a harmless inhabitant of the vagina (Jirovec and Petru 1968, Honigberg 1978), it was not until the early 1950's, when a significant increase in the incidence of sexually transmitted diseases (STDs) other than syphilis and gonorrhea were recognized, that investigators began to view *T. vaginalis* as a potentially significant urogenital pathogen. *T. vaginalis* is now considered one of the most frequently encountered STDs with an estimated 5 million women in the United States (Jarecki-Black *et al.* 1988) and 180 million people worldwide annually infected (Brown 1972).

In women, the classical presentation of the disease trichomoniasis includes a profuse and often malodorous vaginal discharge, and an erythema of the vaginal epithelium commonly termed "strawberry vagina". *T. vaginalis* has also been associated with an increased incidence of prolonged postpartum fever and endometritis (Jirovec and Petru 1968, Higier *et al.* 1983, Rein and Chapel 1975), premature rupture of membranes (Grice 1974, Pilawski and Malecha 1983) and cytological changes in cervical cell morphology (Opacki and Dyner 1983, Rein and Muller 1984). Several investigators also believe that *T. vaginalis* may act as a vector in upper genital tract infections since trichomonads are able to migrate to the level of the fallopian tubes and can carry bacteria on their outer surfaces (Keith *et al.* 1986). As well, up to 25% of women with positive cultures for *T. vaginalis* may be asymptomatic (Krieger 1981).

*T. vaginalis* infections can also occur in males and although most are asymptomatic, it has been implicated in approximately 5-15% of cases of nongonococcal urethritis (NGU) (Kuberski 1978, Wisdom and Dunlop 1965), in addition to epididymitis (Fisher and Morton 1969), prostatitis (Kuberski 1978, Kuberski 1980) and balanitis (Soendjojo and Pinda 1981). *T. vaginalis* may also be linked to sterility in males as the parasite has exhibited deleterious effects on sperm motility both through direct contact and secreted soluble products (Jarecki-Black *et al.* 1988, Paulsen *et al.* 1985, Tuttle *et al.* 1977).

Despite being one of the most common STDs, problems still persist in both the diagnosis and treatment of *T. vaginalis* infections. Diagnosis has proven difficult since the most widely employed techniques for detection, either direct

microscopic examination of fresh material in wet mounts or fixed and stained vaginal smears, are only 60-75% accurate in detecting positive cultures (Higier *et al.* 1983, Rein and Muller 1984). Cultivation has been shown as a more sensitive technique (Fouts and Kraus 1980, Krieger 1981) allowing detection of positive cultures 90% of the time in women attending STD clinics (Higier *et al.* 1983, Rein and Muller 1984), but is costly and time consuming. Diagnosis of infection in asymptomatic women and men with NGU was also considered a problem until Garber *et al.* (1987) developed a culture technique employing a McCoy cell system which could detect *T. vaginalis* growth with an inocula as low as  $< 10^2$ /mL. Treatment of *T. vaginalis* infections, for the last 20 years, has been with the nitroimidazole derivative, metronidazole. Although a generally effective therapy, recently metronidazole failures have been reported with increasing frequency (Krieger 1981). Reasons for treatment failure may be due to an apparent increase in the incidence of resistant strains of *T. vaginalis*, poor absorption of the drug from the gastro-intestinal tract or inadequate penetration of metronidazole into vaginal secretions (Davis *et al.* 1984). The lack of standard susceptibility testing is also a complicating factor. Metronidazole is teratogenic and carcinogenic in laboratory animals (Honigberg 1978) and although still the drug of choice, its long-term effects with repeated doses are unknown.

Although a common STD, the pathogenic mechanisms of *T. vaginalis* have not been well characterized. What is not clearly understood is how the clinical presentation of the disease can vary from a totally asymptomatic vaginal infection to florid vaginitis (Krieger 1981, Rein and Muller 1984). Further complicating

this issue is the fact that 1/3 of women found to be asymptomatic will develop frank vaginitis during the subsequent 6 month period (Krieger 1981). Several different methods have been employed to study the pathogenicity of *T. vaginalis* and included among these are: growth requirements, Honigberg's subcutaneous mouse assay, the cytotoxic effects seen on cell culture monolayers, intravaginal infections in animal models and the alterations of the endemic vaginal flora and pH in the establishment of infection.

#### Growth in Serum-free Medium

For many years, serum has been considered an essential ingredient in the preparation of media commonly used to sustain the axenic growth of *T. vaginalis* (Diamond 1957, Johnson and Trussell 1943, Sprince and Kupferberg 1947). Serum can be used from a variety of mammals and provides a host of nutrients including lipids, fatty acids, amino acids and trace metals. Concentrations of serum, ranging from 5 to 20%, will support the growth of *T. vaginalis* (Diamond 1986, Honigberg 1978). In conventional broth medium, however, *T. vaginalis* cannot be grown or maintained in the absence of serum regardless of the inoculum size used.

In the past serum had been replaced with such ingredients as cream and cholesterol (Samuels 1965), hen-egg homogenates (Barbarowski 1966), and milk and cholesterol (Nakabayashi and Miyata 1967, 1968). Recent investigations have now devised a serum replacement that is based upon the addition of bovine serum albumin (BSA) and cholesterol, together with either a glyceryl fatty acid

ester or a defined mixture of fatty acids (Linstead 1981). Although advances in the formation of a serum replacement medium have begun in earnest, all media commercially available in the United States still requires the addition of serum (Schmid *et al.* 1989). The development of a serum-free medium is of importance because it would allow for more defined studies of the nutritional requirements of *T. vaginalis* and would eliminate the possible interference of serum proteins to *T. vaginalis* secreted soluble products. It has already been established that the presence of serum in culture media can impede the *in vitro* evaluation of the pathogenic factors associated with *T. vaginalis*. Extracellular protease activity is only one factor which has been demonstrated to be inhibited by high serum concentrations (Garber and Lemchuk-Favel 1989b). The use of a serum-free system for *T. vaginalis* growth may permit the detection and subsequent isolation of other soluble virulence factors previously thought not to exist.

#### Honigberg's Subcutaneous Mouse Assay

Since its development in the early 1960's, Honigberg's subcutaneous mouse assay has become the "gold" standard for the determination of *T. vaginalis* pathogenicity. At the time of development, there was some doubt as to the pathogenicity of *T. vaginalis*, however, the assay was able to demonstrate that *T. vaginalis* isolates expressed an inherent physiological virulence (Honigberg 1961). The assay consists of the subcutaneous injection of viable *T. vaginalis* into the flanks of mice and the measurement of abscesses produced six days post-inoculation, the volumes of which can then be correlated to the

clinical presentation. The creation of the abscess or nodule has been found to be an orderly, cyclic progression which involves (1) the initial influx of polymorphonuclear leukocytes into the injection pocket, (2) multiplication of the organism and (3) death of the leukocytes, destruction of host tissues and lysis of the abscess wall promoting edema of the surrounding tissues. *T. vaginalis* infection can then spread and the cycle repeats (Frost and Honigberg 1962). This cyclic progression results in the formation of pure mantles of either leukocytes or *T. vaginalis* against the inner wall of the injection pocket. Honigberg was also able to show a positive correlation between the different clinical and pathological presentations of the vaginal disease and corresponding abscess size (Honigberg *et al.* 1966, Kulda *et al.* 1970). The assay also made available a means for detecting changes in pathogenicity in a given *T. vaginalis* strain (Honigberg 1961, Kuczynska *et al.* 1984). The histopathological processes and the time sequences involved have also been found to vary with the strain of *T. vaginalis* used and may relate to the strain's level of pathogenicity.

#### Cytopathic Effects on Cell Culture Monolayers

Over the years, numerous studies have been performed in order to understand the behaviour of *T. vaginalis* when grown in cell culture. It is widely accepted that *T. vaginalis* has cytotoxic effects on cell culture monolayers. This effect has been shown to be directly proportional to the inoculum size and the duration of time the parasite is incubated with the monolayer (Alderete and Pearlman 1984, Garber *et al.* 1987, Garber *et al.* 1989a, Heath 1981, Krieger

*et al.* 1985) however, the actual mechanisms involved in inducing the cytopathic effects (CPE) have been an issue of debate. Two basic schools of thought have evolved to explain the CPE. The first endorses the role of direct contact and adhesion of *T. vaginalis* to the cells as the major component in causing CPE. The second school of thought considers CPE to be produced by secreted soluble toxins since vaginitis is often diffuse whereas the organism itself may not be uniformly distributed.

The evidence for direct contact producing CPE first became apparent in the late 1950's to early 60's when Christian *et al.* (1963) reported that injuries inflicted upon HeLa cells were dependent upon the mechanical activities of *T. vaginalis* incubated with the cells. Since that time, it has been demonstrated by several different groups that upon incubation with monolayers *T. vaginalis* forms clumps or rosettes that adhere to the monolayers and cause destruction only in those focal areas of contact (Alderete and Pearlman 1984, Heath 1981, Krieger *et al.* 1985, Rasmussen *et al.* 1986). This damage to cell lines was thought to result from tensions generated by the parasites during movement (Heath 1981). Direct contact appeared to cause the peripheral cytoplasm of epithelial cells to retract and the cells to round up and detach from the monolayer. Also implicated in this cytopathogenicity was *T. vaginalis's* microfilaments as cytochalasin D, which inhibits microfilamentation, prevented cytolytic events from occurring (Krieger *et al.* 1985). Substantiating evidence involving reports of specific parasite-cell associations also supported the theory of direct contact. Specific receptor sites on cell surfaces and specific ligands on microorganisms

have already been documented for *Neisseria gonorrhoeae* and Group B Streptococci (Martinotti *et al.* 1986). Recently *T. vaginalis* has been shown to adhere to host cells via parasite surface proteins in the same receptor-ligand fashion (Alderete and Garza 1985, Alderete *et al.* 1988, Alderete and Garza 1988). The identification of four adhesion proteins first found to be involved in HeLa cell surface recognition (Alderete and Garza 1988) and later with human vaginal epithelial cells (Alderete *et al.* 1988) suggested that *T. vaginalis*'s mechanism of cell destruction was dependent on contact.

Those who supported contact-dependent CPE, did not rule out the possibility that diffusible chemical factors could be secreted by *T. vaginalis*, but rather believed the substances to be present at very low levels or inhibited by the medium (Alderete and Pearlman 1984, Heath 1981, Rasmussen *et al.* 1986). The initial hypothesis that *T. vaginalis* affected tissue culture cells by secreting soluble products was first made in 1943 by Hogue. Hogue demonstrated that by incubating cells with *T. vaginalis* filtrate, similar cytopathic effects to monolayers could be achieved. Several studies subsequently showed that CPE could be achieved without extensive invasion or contact of the cells by *T. vaginalis* (Honigberg and Ewalt 1961, Farris and Honigberg 1970, Nielsen and Nielsen 1975). Concerns to the validity of this hypothesis, however, involved the inability of other investigators to readily detect the presence of a cell-free cytotoxin (Alderete and Pearlman 1984, Krieger *et al.* 1985). It was not until some forty years later that the hypothesis was validated when Pindak *et al.* (1986) were able to successfully demonstrate a reproducible cell-detaching factor

(CDF) from cell-free filtrates. This CDF caused detachment and clumping of cells, and when co-incubated with freshly seeded cells would not allow the formation of monolayers (Pindak *et al.* 1986). Although in the initial study, few of the physical properties of CDF were examined, it was established that the toxin's production was not limited to any particular strain of *T. vaginalis* or to cultivation with any specific type of tissue culture. Further characterization of CDF by Garber *et al.* (1989a) showed it to be a 200,000 dalton, immunogenic glycoprotein that possessed trypsin-like activity. This work also revealed 3 factors that were important in the production of CDF: (1) the duration of *T. vaginalis* growth prior to preparation of the filtrate, (2) the initial inoculum size of *T. vaginalis* and (3) the pH of the filtrate at the time of harvest. CDF action was acid labile being optimal at a pH of 6.5 and inactivated at pH < 5.0. This might provide a possible reason as to why other investigators had previously failed to detect the presence of the toxin. Finally, the secretion of this product was shown to be dependent solely upon the viability of *T. vaginalis* rather than the cell line used. Aside from CDF, several other soluble products have been shown to be secreted by *T. vaginalis* including hydrolases, and more recently proteases. Intracellular proteases of *T. vaginalis* have been well documented (Coombs and North 1983, Lockwood *et al.* 1984, 1985, 1986) but few reports have considered extracellular proteases produced by *T. vaginalis*. This lack of information may have been in part due to the fact that serum, a basic ingredient in *T. vaginalis* culture media, could likely interfere or inhibit any protease activity in culture filtrates. By using a modified cell culture system (Garber *et al.* 1987), which

permits *T. vaginalis* growth in the absence of sera, Garber and Lemchuk-Favel (1989b) were able to isolate two cysteine-like extracellular proteases (60KD and 30KD) with optimal active: at an acidic pH. Though these proteases did not produce any cytopathic effects on cell culture monolayers, they may still play an integral role in the establishment of infection in the acidic environment of the vagina.

#### Animal Models for *T. vaginalis*

Another method by which to study virulence markers and the effects of different pathogenic strains of *T. vaginalis* has been through *in vivo* vaginal infection models. Although animal genital tract models have been well established for such sexually transmitted diseases as *Ureaplasma urealyticum* (Iwasaka *et al.* 1986) and *Chlamydia trachomatis* (Taylor-Robinson and Tuffrey 1987), only a handful of attempts have been made at establishing intravaginal models for *T. vaginalis*. In the mid-1950's, intravaginal infection was established in Golden Hamsters with 20-30% initial infectivity rates (Jirovec and Petru 1968, Honigberg 1978). Studies employing rats also begun in the late 1950's and demonstrated the ability to achieve up to 80-100% infectivity in rats in permanent estrous (Jirovec and Petru 1968, Honigberg 1978).

The two most recent attempts at establishing animal models have employed mice and squirrel monkeys. Mice were first used for intravaginal infection by Cappuccinelli *et al.* (1974) who achieved 100% infection in two mouse strains by inoculating  $5 \times 10^5$  *T. vaginalis*/mL on two consecutive days to estrogenized

mice. Although Cappuccinelli *et al.* (1974) reported this to be a simple system for the intravaginal infection of mice, the model was not pursued further until Coombs *et al.* (1986a) described a similar procedure. Coombs *et al.* (1986a) reported that the susceptibility to infection apparently varied with the stage of estrous in mice (Bremner *et al.* 1986, Coombs *et al.* 1986a). In direct opposition to Cappuccinelli *et al.* (1974), Coombs *et al.* (1986a) found that 100 % infectivity was not the norm, and was not achievable regardless of a variety of modifications to the protocol. Both groups were, however, in agreement that pretreatment of the mice with estrogens was essential for establishing infection.

The infection of squirrel monkeys (*Saimiri sciureus*) with *T. vaginalis* has also met with some success. The infection in monkeys is similar in several respects to that of women (Gardner *et al.* 1987, Street *et al.* 1983). Infected monkeys had a vaginal discharge plus local IgG and IgA responses. As in the mouse model, hormonal status of the primates also appeared important in successfully establishing the vaginal infection.

## Vaginal Flora and pH

### Importance of Endemic Vaginal Flora and pH

Since the acceptance of *T. vaginalis* as a true genital pathogen, various aspects relating to its pathogenicity have been studied intensively but little work has been done in elucidating the interactions between the parasite and the endemic vaginal flora and pH. To fully comprehend the interactions which occur

during establishment and maintenance of the infection, a knowledge of the existing normal vaginal flora and pH must be established.

### Normal Vaginal Flora and pH

There are many reasons to study the normal flora of asymptomatic hosts. It is well known that endemic flora may be a source of organisms involved in intrinsic infections after surgical procedures. As well the interactions of normal flora with mucosal surface pathogens may be of importance.

The first definitive study on vaginal flora in women was published by Döderlein in 1892. Döderlein described the prominence of facultative gram-positive rods simply called "Döderlein bacillus" although now referred to as *Lactobacillus* species. It was accepted, at that time, that the vaginal flora of a normal healthy woman was comprised of Döderlein bacillus, and any departures from this homogeneity were reflective of abnormal vaginal flora and an unhealthy state within the vagina. Over time, advances in sampling and culturing techniques refuted this concept of homogeneity in the vagina. Döderlein bacillus was actually found to consist of a heterogeneous group of *Lactobacillus* species and other microorganisms, in addition to *Lactobacilli*, were considered normal inhabitants of the female genital tract.

Numerous qualitative studies of vaginal flora have been done since the late 1800's. Although culturing techniques and identification of organisms have differed between studies, an overall composition of bacterial flora in the vagina can be made. The predominant facultative gram-positive rods are *Lactobacillus*

species and diphtheroids, mainly from the genus *Corynebacterium*. Lactobacilli dominate in the vagina and are found in approximately 54-96% of women cultured (Bartlett and Polk 1984, Hill *et al.* 1984, Larsen and Galask 1980, 1982, Ohashi *et al.* 1981, Paavonen 1983, Sautter and Brown 1980). The facultative gram-positive cocci most frequently isolated include *Staphylococcus* species and *Streptococcus* species, in particular *Staphylococcus epidermidis*, Group B *Streptococci* and Group D *Streptococci*, including enterococci (Hill *et al.* 1984, Larsen and Galask 1980). *Staphylococcus aureus*, which causes toxic shock syndrome, is an infrequent inhabitant of vaginal flora. Gram-negative rods occur at a low incidence with *Escherichia coli* most frequently isolated from the genital tract in 15-30% of women (Bartlett and Polk 1984). Qualitative studies have also demonstrated that the majority of women (70-90%) are colonized by one or more anaerobic species. In fact, anaerobes outnumber aerobic organisms by a factor of ten (Larsen and Galask 1980, 1982). The most frequent anaerobic organisms isolated include the gram-positive cocci (*Peptococcus asaccharolyticus* and *Peptostreptococcus anaerobius*), Lactobacilli species, Bifidobacterium, Eubacterium and various *Bacteroides* species which includes a low prevalence of *B. fragilis* (Bartlett and Polk 1984, Larsen and Galask 1980, 1982).

Few quantitative studies of vaginal flora have been performed. Quantitation may be important, however, if specific concentrations of a given bacteria are required in providing a protective mechanism. The concentration of vaginal bacteria can range widely from  $10^5$  to  $10^{11}$  organisms/gram of secretion (Bartlett and Polk 1984, Sautter and Brown 1980). Approximately  $10^8$

aerobic organisms/gram and a mean concentration of  $10^9$  anaerobic organisms/gram are found in the vagina (Bartlett and Polk 1984, Larsen and Galask 1980). From quantitative studies two important facts emerge: (1) that anaerobic flora outnumber aerobes and (2) *Lactobacillus* species are found in a mean concentration of  $10^8$  organisms/gram making them the numerically dominant aerobic species recovered.

The above description provides a general bacterial composition of the female vagina, however, there is substantial variability in the flora between individuals. The female genital flora is a dynamic ecosystem. Thus there are multiple possibilities to explain why entire populations have different flora, or why subpopulations can exist within groups. Variables which could cause heterogeneity among groups include such factors as age, sexual activity and practices, hygiene, method of contraception, nutrition, douching, menstrual activity and hormonal fluctuations. As well the sampling and culturing techniques used in various studies may alter the frequency of isolation of certain bacterial types. The methods used to collect the sample, delays in transport to the laboratory, processing of the culture, and analysis and interpretation of results are all important factors.

Of the variables which can influence the vaginal flora, only the hormonal status of the individual and changes during menstruation have been scrutinized. It has long been the popular theory that control of the vaginal flora was dependent upon the host's production of estrogen (Hill *et al.* 1984, Larsen and Galask 1980, Paavonen 1983). Estrogenic stimulation causes a thickening of the

vaginal epithelium and concurrently an increase in glycogen deposits in the tissue. This high level of glycogen could be metabolized to lactic acid by *Lactobacillus* species to produce an acidic environment which would restrict the remaining flora to acid-tolerating species. Due to the normally low vaginal pH of 4.5 and the high rates of isolation of *Lactobacilli*, this was viewed as a cause and effect relationship. The hypothesis is also reinforced by the fact that *Lactobacilli* are most prevalent during the female reproductive years, particularly during pregnancy when estrogen levels are high, and are low before puberty and after menopause (Larsen and Galask 1980, 1982). Recently this theory has been challenged on several points including: (1) that if the predominance of *Lactobacilli* is the result of the selection of acid-tolerant organisms, the acid could not have been initially produced by *Lactobacilli*, (2) the cervix is less acidic than the vagina although the two areas are similar in terms of flora and (3) many *Lactobacillus* species have recently been shown not to ferment glycogen (Larsen and Galask 1980, Wilks and Tabaqchali 1987). Fluctuations in vaginal flora during the estrous cycle is another area currently under debate (Bartlett and Polk 1984, Larsen and Galask 1980, 1982, Mehta 1982, Wilks and Tabaqchali 1987). Proponents of floral fluctuations during the hormonal cycle believe that this is a general effect without the involvement of specific organisms (Bartlett and Polk 1984, Larsen and Galask 1980, 1982). An overlooked possibility may be the controlling influence of a nutritional substrate which could be released from proliferating and exfoliating vaginal epithelium cells during the course of the estrous cycle.

### Vaginal Flora and pH During Genital Disease

Although the basic bacterial flora and pH of the female genital tract has been determined, the role of flora and pH in genital disease has not been well established. The interactions between genital pathogens and the endemic flora could provide a comprehensive understanding of the factors involved in establishment and maintenance of the urogenital pathogen in question. It may also provide insight into the inherent pathogenic potentials of the causative agents.

Aside from trichomonal infections, most studies have involved the determination of floral changes in *Candida albicans* infections, and quantitative microbiological investigations of women suffering from bacterial vaginosis (BV). In terms of vaginal yeast infections, there appears to be no significant effect on vaginal flora (Drake *et al.* 1980, Ohashi 1982, Wilks *et al.* 1984). Lactobacillus species continued to predominate during yeast infections and vaginal pH remained in the acidic range of 4.3 - 4.6 (Drake *et al.* 1980). With regard to BV, where *Gardnerella vaginalis* is a marker of infection, changes in the vaginal flora have been documented (Chattopadhyay and Teli 1984, Ohashi 1982, Piot *et al.* 1982). A normal vaginal discharge of pH 4.5 or less, in most circumstances, yields predominantly Lactobacilli, anaerobes and only very rarely *G. vaginalis*. With BV, however, vaginal discharge becomes alkaline and vaginal flora changes drastically. *G. vaginalis* and high concentrations of anaerobic gram-negative bacilli (Bacteroides species and Mobiluncus), and anaerobic gram-negative and gram-positive cocci (Veillonella, Peptococcus and

Peptostreptococcus) have been associated with BV while a significant reduction in the Lactobacillus population occurs. Several investigators now believe that a symbiotic relationship between *G. vaginalis* and these specific anaerobic bacteria are associated with BV.

Only a single study has been undertaken to observe vaginal changes in genital infections with *Chlamydia trachomatis*, *Mycoplasma hominis* and *Ureaplasma urealyticum*. Investigators have found higher frequencies of isolation of these pathogens at higher (6.0 - 7.5) vaginal pH levels (Hanna *et al.* 1985). Flora was not sampled in this study so it is not known whether this rise in vaginal pH was at all related to a decrease in the Lactobacillus population.

#### Floral and pH Changes with *T. vaginalis* Infection

The normal physiological state of the vagina, which includes many epithelial cells, a predominance of Lactobacilli, few polymorphonuclear leucocytes (PMNs), and an acidic pH of 4.5, drastically changes during trichomoniasis. The course of *T. vaginalis* infection can be differentiated into four phases involving the microbiotic state of the vagina (Jirovec and Petru, 1968, Hollander 1983, Honigberg 1978). The first phase seen a few days after infection is termed "Trichomoniasis Acuta" and consists of reproducing trichomonads in the vaginal secretions, a reduction in the numbers of epithelial cells, the presence of Lactobacillus and the onset of inflammation manifested by a considerable number of PMNs. Other bacteria do not appear to be significantly affected by this generally short phase. "Culminating Trichomoniasis" continues the trend set

in the first stage. At this time, there are numerous trichomonads with more than  $1 \times 10^5$  parasites per mL (Demes *et al.* 1988) along with an outpouring of PMNs and a rise in several different bacterial species. *Lactobacillus* species and epithelial cells have all but disappeared during this phase which may persist for weeks or months if untreated.

In "Chronic Trichomoniasis", trichomonads begin to fluctuate in number as epithelial cell populations begin to rise and fewer PMNs appear. This apparent reduction in PMNs may be due to their so-called "clustering" or "rosetting" about epithelial cells. It has also been noticed in this phase that *T. vaginalis* has a tendency to cytoadhere to loose epithelial cells and that the PMNs can then react with the parasite in a process possibly involving opsonins (Hollander 1983). During this phase, there is a significant mixture of rods and cocci, most notably *Corynebacterium*, *Streptococci* and *Staphylococci* species, but no isolation of *Lactobacillus* species.

In the final phase, "Latent Trichomoniasis", the normal numbers of *Lactobacilli* and epithelial cells are restored as levels of trichomonads and PMNs decrease. The important trend that evolves during this four phase pattern is the loss of *Lactobacilli* during the stages of prolonged infection. This trend possibly correlates with the increase in vaginal pH from 4.5 to >5.0 in women with trichomoniasis (Hanna *et al.* 1985), but the mechanisms involved in the disruption of *Lactobacilli* populations has not been explored.

### In Vitro Interactions Between *T. vaginalis* and Other Microorganisms

Previous work, involving women suffering from trichomoniasis, has indicated apparent interactions between *T. vaginalis* and other flora. These floral changes may be an integral component in establishing and maintaining infection, but further work along these lines has not been pursued. As well, little has been done to ascertain the relationship between *T. vaginalis* and other microorganisms *in vitro*. When *T. vaginalis* was grown in the presence of *Staphylococcus aureus* or *Escherichia coli*, growth of the *T. vaginalis* population was possible and several trends in growth patterns were demonstrated (Horwat 1985). In the logarithmic phase of growth, *T. vaginalis* strains coincubated with bacteria had an increased growth rate compared to axenic cultures and also an accelerated rate of extinction. The majority of *T. vaginalis* strains, when combined with cultures of *S. aureus* and *E. coli*, achieved peak concentrations more rapidly than the axenic cultures but concentrations were smaller than those of controls. The same trend in *T. vaginalis* growth was also demonstrated in co-incubations with *Candida albicans* (Kurnatowska and Horwatt 1983).

*T. vaginalis* interactions with other genital pathogens *in vitro* has also been investigated to a small extent. If *T. vaginalis* is an actively phagocytic protozoa, it could possibly enhance the pathogenicity of other genital pathogens by ingesting them and establishing an endocytobiotic relationship. This ingestion would protect the pathogen from host defences and the inhibitory effects of antibiotics, while allowing transport of the pathogen to uncolonized areas of the urogenital tract (Francioli *et al.* 1983, Street *et al.* 1984). Studies have shown

that in the case of *Neisseria gonorrhoeae* (Francioli *et al.* 1983, Street *et al.* 1984) and *Mycoplasma hominis* (Street *et al.* 1984), bacteria were ingested by *T. vaginalis* and the intracellular viable organisms killed by the action of lysosomal enzymes within 6 hours for most gonococci and 3 hours for all mycoplasmas. When *Chlamydia trachomatis* was tested, there was no evidence that the organism even persisted in mixed cultures with *T. vaginalis* (Street *et al.* 1984). Although this work disproved the suggestion of ingested organisms forming the source of persistent infections, it certainly did not rule out the possibility of microorganisms being transported within the genital tract by adherence to the external surfaces of *T. vaginalis*.

## Lactobacillus

### Vaginal Lactobacillus

Though the human vaginal flora is predominately composed of Lactobacilli, it is in no way homogeneous. The vaginal Lactobacilli represent both homo- and heterofermentative species typically in a 4:1 ratio (Giorgi *et al.* 1987). Among the species isolated from vaginal specimens, *L. acidophilus* and *L. fermentum* are considered to be the dominant species with *L. acidophilus* in approximately 88.8% of vaginal samples (Giorgi *et al.* 1987, Magliano *et al.* 1984). *L. jensenii*, *L. casei*, *L. plantarum*, *L. bevis*, *L. delbrueckii* and *L. salivarius* can also be isolated occasionally from the vagina. The recurrent problem associated with the identification of vaginal Lactobacilli is the unreliability of the classical biochemical tests to differentiate species.

Identifications now made by gas-liquid chromatography and DNA homology may improve the current taxonomy of human vaginal Lactobacilli and allow absolute identification of dominant Lactobacillus species.

#### Lactobacillus Mechanisms of Protection

Since the late 1800's, when Lactobacillus was found to predominate in the genital flora, this microorganism was considered to play an active role in the maintenance of a healthy genital tract. The protective activities of Lactobacillus and the mechanisms by which they exert this protective effect have yet to be elucidated though several possibilities are currently being investigated.

#### Protection by Secreted Products

It has long been considered that Lactobacillus contributes to the maintenance of an acidic vaginal pH by production of lactic acid and that this is its primary protective effect against pathogens. Lactic acid has been shown to be strongly germicidal, and at a pH of 3.6 can cause 100% mortality of *Escherichia coli* after one hour of incubation (Tramer 1966). However, the production of lactic acid has created a problem in studies of other possible inhibitory products since the acid can spread through media employed in inhibition studies. It became apparent in experiments involving single layer agar inhibitions between Lactobacillus and other bacteria that zones of inhibition coincided with the extent of spread of acidity, with inhibition zones generally being wider at pH 5.5 than at 6.0 (Skarin and Sylwan 1986, Tramer 1966). All

that these studies were able to identify was the positive correlation between growth-inhibition zones, the ability of *Lactobacillus* to acidify solid agar, and the susceptibility of the inhibited strains to low pH. While this work emphasized the critical pH inhibitory effect produced by *Lactobacillus* it did not permit identification of other factors that may be of equal importance in inhibition.

Aside from lactic acid production, *Lactobacilli* have demonstrated the ability to excrete other compounds with antimicrobial activity including hydrogen peroxide ( $H_2O_2$ ) and inhibitory proteins. *Lactobacillus* utilizes flavoproteins which generally convert oxygen to  $H_2O_2$ . This mechanism along with the absence of the heme protein catalase results in the formation of  $H_2O_2$  in amounts above the degradation capacity of the bacteria. This  $H_2O_2$  can inhibit or even kill other microorganisms which have low levels or lack  $H_2O_2$ -scavenging enzymes such as catalase peroxidase. The microbicidal activity of  $H_2O_2$  can actually be enhanced in the vagina by the enzyme peroxidase in the presence of halide ions which are present in genital tract secretions. The  $H_2O_2$  production could represent an additional mechanism by which *Lactobacilli* regulate the growth of other organisms in the vagina. Evidence to substantiate this claim has involved a study of the vaginal flora in normal women and those with bacterial vaginosis. Work has demonstrated the presence of  $H_2O_2$ -producing facultative *Lactobacillus* species in the vaginas of 96% of normal women as compared to 6% in women suffering from bacterial vaginosis (Eschenbach *et al.* 1989). These results indicate the possibility that absence of  $H_2O_2$ -producing *Lactobacillus* species could constitute a primary defect in endemic flora, such that women with this

aberration could be more susceptible to overgrowth of particular organisms that could result in bacterial vaginosis.

Secreted inhibitory proteins of *Lactobacillus* have also been postulated as a protective mechanism against urogenital pathogens. Over the years the names of these compounds have grown to include lactolins, acidolins, lactocidins, lactobacillins and lactocins. Regardless of the lack of coordination in categorizing these compounds, it is evident that *Lactobacilli* produce bacteriocins and compounds inhibitory to other microorganisms. Bacteriocins are antagonistic proteins or peptides that show bactericidal activity only against closely related species. Lactacin B isolated and purified from *L. acidophilus* N2 has been one of the most recent bacteriocins isolated and is active against *L. leichmannii*, *L. bulgaricus*, *L. helveticus* and *L. lactis* (Barefoot and Klaenhammer 1984).

Possibly more important in the maintenance of the endemic vaginal flora may be the inhibitory proteins secreted by *Lactobacillus* that are active against a wide range of microorganisms. First considered by Sabine (1963), substantial efforts have been made to elucidate these compounds. Investigators using different strains of *Lactobacillus* have been able to demonstrate inhibition against a variety of gram-positive and negative aerobes and facultative anaerobes including: *Pseudomonas* species, *Staphylococcus aureus*, *Escherichia coli*, *Neisseria gonorrhoeae* and *Candida albicans* (Collins and Hardt 1980, Mardh and Soltesz 1983, McGroarty and Reid 1988, Mehta *et al.* 1983a, Reid *et al.* 1987, Saigh *et al.* 1978, Sanders *et al.* 1982). No inhibition has been found

against strict anaerobes (Mardh and Soltesz 1983). Although these inhibitory activities appear to vary from strain to strain, several properties of the inhibitory substances have been demonstrated. The inhibitory agent produced by *L. casei* GR-1 was found to be heat-labile, acid-stable and active after four hours of growth in either air, CO<sub>2</sub> or under anaerobic conditions. The substance also had a molecular weight greater than 12,000 - 14,000 (McGroarty and Reid 1988, Reid *et al.* 1988). Another inhibitory protein from *L. acidophilus* AC<sub>1</sub> has also been purified and found to be a 5.4 kilodalton polypeptide sensitive to trypsin and chymotrysin treatment while resistant to pepsin. This heat-labile protein is active over a wide pH range but optimally active at a pH between 4.0 and 5.5 (Mehta *et al.* 1983a, 1983b). The differences between the inhibitory proteins mentioned and results showing immunological differences between inhibitory proteins isolated from *L. acidophilus* AC<sub>1</sub> and AR<sub>1</sub> (Mehta *et al.* 1983b) confirms the diversity of inhibitory substances produced by various strains of Lactobacillus. Each strain may, however, confer a protective activity against specific pathogens in the vagina.

#### Protection Provided by Adherence

In conjunction with the secretion of inhibitory products, another factor deemed important in providing protection to the endemic flora has been vaginal colonization and adherence. Colonization of the vaginal epithelium can be influenced by binding sites, nutritional status and a variety of other physiological factors such as pregnancy, menses, and hormonal levels. It has also been

demonstrated that both gram-positive and gram-negative aerobes and anaerobic organisms attach to the vaginal walls. The attachment of these organisms is considered to be related to specific receptors present on the mucous membrane. Gram-positive organisms such as Group B Streptococci have been shown to use lipoteichoic acids for their attachment while gram-negative bacteria use fimbriae and attach to specific sugars (Galask 1988). The adherence of *Lactobacillus* to uroepithelial cells and their ability to interfere with the colonization of pathogenic organisms has been considered to be another protective mechanism. Though strain to strain variability is encountered, investigators have demonstrated that, *in vitro*, *Lactobacillus* can block the attachment of uropathogenic bacteria to surfaces of vaginal epithelial cells (Chan *et al.* 1984, Chan *et al.* 1985, Reid *et al.* 1987, Sobel *et al.* 1982, Wood *et al.* 1985). Lipoteichoic acid has been found to be responsible for the adherence of *Lactobacilli* to uroepithelial cells, but the actual mechanism of competitive exclusion seems to be due to steric hindrance as opposed to specific blockage of receptor sites (Chan *et al.* 1985). The protection conveyed by the adherence of *Lactobacillus* and the influence the menstrual cycle has on this adherence has yet to be fully elucidated. It appears that adherence of vaginal *Lactobacillus* follows a bicyclic repetitive pattern during the menstrual cycle (Chan *et al.* 1984), with peaks at day 10 and again at days 17-18. The cyclic patterns during menses may in part be due to changes in cell viability and availability of receptor sites since bacteria have been demonstrated to adhere in particular to cells in the process of desquamating and have shown little adherence to young newly dividing cells (Sobel *et al.* 1982).

### Rational

How can *T. vaginalis*, which normally requires a pH of 6.0 - 6.5 to proliferate *in vitro*, not only survive but establish infections in the acidic environment (pH 4.5) of the female vagina. Jirovec and Petru (1968) demonstrated reduction to total loss of the Lactobacillus population as a result of infection. The fact that Lactobacillus is intimately involved in maintaining the acidic vaginal pH and providing protective activity in the vagina against other pathogens, suggests that in some way *T. vaginalis* may have a deleterious effect on Lactobacillus. The mechanism that *T. vaginalis* employs to alter the Lactobacillus population is not understood, but several hypotheses can be constructed.

It is possible that a phagocytic mechanism is generating losses in the Lactobacillus numbers. *T. vaginalis* is an actively phagocytic protozoa that has already been documented to ingest other urogenital pathogens (Francioli *et al.* 1983, Street *et al.* 1984). It is unlikely, however, that phagocytosis could result in such extensive damage to Lactobacillus numbers that it would allow a pH rise and establishment of the parasite. This is supported by the fact that many *T. vaginalis* organisms first introduced into the vagina would perish in the acidic environment before substantial numbers of Lactobacillus could be phagocytosed.

Another hypothesis could be that secreted soluble products of *T. vaginalis* play an integral role in the alterations of vaginal flora. Recently two *T. vaginalis* soluble products have been isolated, a cell-detaching factor (CDF) and two cysteine-like proteases (Garber *et al.* 1989a, Garber and Lemchuk-Favel

1989b). It is unlikely that the CDF is important in the establishment of infection in the vagina since it has been found to be active at pH >6.5 and would be rendered inactive in the acidic vagina. The secreted proteases, which are optimally active at vaginal pH, are more likely to be factors in altering vaginal flora and establishing infection. The action of the proteases may be directly against the *Lactobacillus* population, possibly disrupting the bacteria's cell wall, or may indirectly involve an alteration of the *Lactobacillus*-secreted inhibitory proteins rendering them inactive. If the proteases did act to modify the *Lactobacilli*, directly or indirectly, this could account for the reduction in the population and the subsequent rise in pH seen in trichomoniasis. The rise in pH would also allow for activation of CDF, which would cause the sloughing off of vaginal epithelial cells evident in the clinical presentation of the disease, and the proliferation of *T. vaginalis* organisms already present in the vagina. Studies of this nature would allow a better understanding of the environmental factors favouring the establishment and maintenance of *T. vaginalis* infection. Insight would also be provided into which environmental factors may effect the expression of inherent pathogenic potentials of various strains of *T. vaginalis*.

### OBJECTIVES

1. To determine if *T. vaginalis* can grow in the absence of sera.
2. To establish a mouse model for *T. vaginalis* infection.
3. To study the interactions between *T. vaginalis*, vaginal flora, and pH in the mouse model.
4. To determine the mechanism(s) by which *T. vaginalis* alters vaginal flora.

## MATERIALS AND METHODS

### Strains

#### Trichomonas species

Isolates of *T. vaginalis* (Tv) were obtained from vaginal secretions of women suffering from vaginitis. *Tritrichomonas foetus* (Tf) (ATCC 30003), a nonpathogenic trichomonad found in cattle, was purchased from the American Type Culture Collection, Rockville, Md. As previously reported (Garber *et al.* 1987), organisms were grown in glass, screw-capped tubes (16 by 125mm) in 10 mL of Diamond TYI-S-33 medium (TYI) pH 6.2 (Diamond *et al.* 1978) supplemented with 10% heat inactivated fetal bovine serum (FBS)(Gibco Laboratories, Life Technologies Inc. Grand Island, N.Y.), 100 U/mL penicillin, 100 ug/mL streptomycin (Penicillin-Streptomycin Solution Gibco Laboratories) and 100 U/mL nystatin (Gibco Laboratories). These tubes were incubated in 5% CO<sub>2</sub> at 37° C at a 45° angle. Cultures were passed every 2-3 days. Axenic cultures were mixed with an additional 10% heat inactivated FBS and 10% dimethyl sulfoxide and stored at -70° C until required.

#### Killed *T. vaginalis*

Killed *T. vaginalis* was produced by first allowing approximately  $1 \times 10^4$  *T. vaginalis*/mL in TYI with FBS and antibiotics to incubate at 37° C for 4 days. After this allotted time, the culture was centrifuged for 10 min. at 140 x g in a Sorvall GLC-1 centrifuge (serial # 1840 Sorvall, Newtown, Connecticut), the

supernatant discarded, and the pellet resuspended in TYI (pH 4.0) without FBS. This culture was incubated for 48 hrs at 37° C after which time it was moved to 4° C for an additional 48 hrs. When this incubation was complete, the culture was left overnight under ultraviolet light to ensure not only *T. vaginalis* death but also that of any possible bacterial contaminants. Dead *T. vaginalis* was identified by trypan blue exclusion under a hemacytometer.

#### Lactobacillus species

*Lactobacillus acidophilus* (La) (ATCC 4356) was purchased from the American Type Culture Collection, Rockville, Md.. Organisms were grown in either Bacto Lactobacilli MRS Broth (MRS)(Difco Laboratories Detroit, Michigan) or on MRS plates (Bacto Lactobacilli MRS Broth with an additional 1.5% Bacto Agar) (Difco Laboratories) at 37° C in 5% CO<sub>2</sub>. Cultures were passed every 2-3 days. Axenic cultures in MRS broth were mixed with an additional 10% glycerol and stored at -70° C until required.

#### Tissue Culture

McCoy cells were grown in CMGA media (Garber *et al.* 1987) in 75 cm<sup>2</sup> tissue culture flasks and incubated at 37° in 5% CO<sub>2</sub>. For growth assays, McCoy cell monolayers were washed with phosphate-buffered saline solution (PBS)(6.8 M KH<sub>2</sub>PO<sub>4</sub>, 3.7 M KCl, 73 mM NaCl, 657 mM Na<sub>2</sub>HPO<sub>4</sub> pH 7.2), trypsinized and CMGA suspensions of 1 mL containing 4 x 10<sup>5</sup> McCoy cells/mL were inoculated into glass shell vials (Garber *et al.* 1987). After 24 hours incubation, confluent

monolayers were achieved. The CMGA was aspirated off and the monolayers washed three times with PBS prior to inoculation with *T. vaginalis*.

### Growth Assays

*T. vaginalis* isolates, grown in TYI to log phase, were harvested by centrifugation at 140 x g for 10 minutes in a Sorvall GLC-1 centrifuge. The pellet was washed three times with PBS and resuspended in either a 2:1 mixture of CMGA/TYI or serum-free CMGA/TYI. Initial *T. vaginalis* suspensions were counted and serial dilutions of  $1 \times 10^3$ ,  $1 \times 10^4$  and  $1 \times 10^5$  *T. vaginalis*/mL were prepared in both media. One mL aliquots of each *T. vaginalis* dilution was added to the vials containing confluent, washed McCoy cell monolayers. The vials were incubated at 37° C and representative vials were sampled daily.

When sampled, the supernatant was aspirated from the vial and saved. The monolayer was washed twice with 1 mL PBS and the washings pooled with the supernatant. This final suspension was centrifuged at 140 x g for 10 minutes in a Sorvall GLC-1 centrifuge. The pellet was resuspended in 1 mL of PBS and viable *T. vaginalis* was assessed by counting motile *T. vaginalis* and by trypan blue exclusion using a hemacytometer.

To determine if the McCoy cell line was secreting a possible growth factor for *T. vaginalis*, serum-free cell culture media (CMGA/TYI) was overlaid on confluent McCoy cell monolayers and incubated at 37° C with 5% CO<sub>2</sub> for 4 days. This cell culture supernatant fluid was used in growth assays to determine if it could sustain *T. vaginalis* growth in the absence of a cell monolayer.

Viability of the monolayer and disruption of the cells during incubation was monitored by visual appearance of the monolayer and by trypan blue exclusion. *T. vaginalis* viability and growth was also assessed by trypan blue exclusion using a hemacytometer.

#### Subcutaneous Mouse Assay

Twelve clinical isolates of *T. vaginalis* collected from vaginal secretions of women attending STD clinics were used in this assay. Each isolate had been previously ranked according to the clinical presentation of the disease as either asymptomatic, moderate or chronic/severe (see TABLE 1). A non-pathogenic strain, *Tritrichomonas foetus* and killed *T. vaginalis* organisms were also used as controls.

Each isolate was grown to log phase in TYI and harvested by centrifugation for 10 minutes at 140 x g in a Sorvall GLC-1 centrifuge. The supernatant was discarded and the pellet was washed three times by resuspension in PBS and centrifuged for 10 min. at 140 x g. After the last wash, the pellet was resuspended in pre-warmed Bacto Fluid Thioglycollate Medium (Difco Laboratories) and adjusted to a final concentration of  $1.8 \times 10^6$  *T. vaginalis*/mL. This *T. vaginalis* inocula was loaded into 1 mL syringes and injected subcutaneously in 0.5 mL amounts into the shaved flanks of 22-24 gm female BALB/c mice (Charles River Company, Montreal). Control mice received an injection of 0.5 mL pre-warmed fluid thioglycollate medium into the flank. Six days post-inoculation, mice were sacrificed and the length, width and height

**TABLE 1**  
Ranking of *T. vaginalis* Isolates to Clinical Presentation

Isolate #	Rank	Discharge (Amount/Quality)	Erythema
387	Asymptomatic	small/white	none
386	Asymptomatic	small/white	none
364	Asymptomatic	small/white	none
396	Asymptomatic	small/white	none
335	Moderate	small/white	cervix & vulva
202	Moderate	moderate/green	vagina & vulva
263	Moderate	moderate/green	cervix
256	Moderate	small/yellow	cervix & vagina
277	Severe	large/green	vagina & vulva
330	Severe	large/green	cervix & vagina
002	Chronic	large discharge for > 1 month	
86-2	Chronic	large discharge for > 1 month	

of any abscess produced in the flank was measured with calipers and a metric ruler. The total volume of the abscess was obtained from the formula:  $L \times W \times H \times 0.5236$  (Honigberg *et al.* 1966) and expressed in  $\text{mm}^3$ . In assays where killed organisms were used the abscesses were drained after 6 days. This drained material was incubated in pre-warmed TYI for up to 10 days to determine if viable *T. vaginalis* was present.

#### Intravaginal Inoculation of BALB/c Mice with *T. vaginalis*

Two days before infection, 22-24 gm female BALB/c mice were treated subcutaneously with 0.05 mL of Delestrogen (Estradiol Valerate 10 mg/mL, Squibb Canada Inc. Montreal, PQ) (Cappuccinelli *et al.* 1974, Coombs *et al.* 1986). The mice were inoculated intravaginally with  $5 \times 10^5$  log phase *T. vaginalis*/mL on two successive days, Day 0<sub>1</sub> and Day 0<sub>2</sub>. Intravaginal inoculations were done using a Eppendorf pipet which was inserted into the vagina and the contents dispelled. Depending upon the initial concentration of *T. vaginalis*, the actual inoculated amount normally ranged from 20-40  $\mu\text{L}$ .

Six different *T. vaginalis* clinical isolates grown in TYI were used. Prior to inoculation, the organisms were pelleted by centrifugation for 10 minutes at  $140 \times g$  in a Sorvall GLC-1 centrifuge and washed three times in PBS. The pellet was finally resuspended in Diamond's TYI medium supplemented with 10% FBS and 0.32% Bacto Agar (Coombs *et al.* 1986) but void of antibiotics which may have otherwise caused changes to the normal vaginal flora. Each mouse's stage of estrus was also determined prior to inoculation by the vaginal

smear technique of Fox and Laird (1970) which employed staining vaginal smears with Methylene Blue and comparing the numbers of leucocytes and cornified epithelial cells present on the slide.

On Day 3 post-inoculation, the first in a series of vaginal washings was begun to determine the duration of infection. These washings were done with 40 uL of pre-warmed TYI supplemented only with 10% FBS. For washings, the disposable Eppendorf pipet tip was inserted into the vagina, the media ejected and aspirated several times so that the TYI became turbid. The wash material was collected and put into tubes of 10 mL pre-warmed TYI with 10% FBS, 300 U/mL penicillin, 300 ug/mL streptomycin, 300 ug/mL kanamycin (Gibco Laboratories), 300 ug/mL vancomycin/gentamicin (Gibco Laboratories) and 100 U/mL nystatin. Antibiotic levels were raised in order to overcome bacterial contamination. Tubes were incubated at 37° C and examined daily for the presence of motile *T. vaginalis* under an inverted microscope.

After the Day 3 washing, mice were maintained and sampled for the presence of motile *T. vaginalis* weekly until two consecutive negative readings occurred. Later two slight modifications were made to this inoculation procedure to determine if infectivity rates could be increased. First weekly estrogenization (Cappuccinelli *et al.* 1974) was attempted, in which case the mice received 0.05 mL Delestrogen subcutaneously every seven days over the course of the experiment. The second modification was a weekly estrogen treatment, but with an increased dose of 0.1 mL Delestrogen (Coombs *et al.* 1986).

## Sampling of Mouse Vaginal Flora and pH

### Untreated and Estrogenized Mice

In experiments, two groups of female 22-24 gm BALB/c mice were run in parallel. One group was left untreated while the second group was injected subcutaneously with 0.05 mL Delestrogen two days before vaginal sampling was done.

When sampling for pH and vaginal flora the following protocol was followed. First, the pH of each individual mouse was taken. This was carried out by attaching pH paper to the tip of a capillary tube (Fisher Microhematocrit Capillary Tubes 02-668-68 diameter 1.1-1.2 mm) which was inserted into the vagina, rotated several times, removed and read. Two types of pH paper were used, one with a range of pH 5-6.6 (Accutint Indicator Paper, Anachemia Chemicals Ltd, Montreal) and the other with a range of pH 5.3-7.0 (Accutint Indicator Paper, Anachemia Chemicals Ltd, Montreal). Averages of these two readings were used to establish the vaginal pH.

Vaginal flora was sampled by a 50 uL wash with 0.9% sterile saline using an Eppendorf pipet. The pipet tip was inserted into the vagina and the saline aspirated several times until turbid. This wash material was halved so that 25 uL was added to 2.5 mL of Bacto Tryptic Soy Broth (T-soy)(Difco Laboratories Detroit, Michigan) and the remaining 25 uL was added to 2.5 mL of reduced Bacto Fluid Thioglycollate Medium enriched for anaerobes with 1% Hemin Solution (APPENDIX) and 1% Vitamin K Solution (APPENDIX). T-soy broths were incubated aerobically at 37° C for 12-16 hrs while the enriched Thioglycollate

broths were incubated in an anaerobic chamber (Model # 1024 Forma Scientific, Mississauga, Ont.) at 37° C for 4 days.

After incubation, T-soy broths were streaked to isolate colonies on the following media: Blood Agar, Bacto MacConkey Agar CS (Difco Laboratories), Columbia CNA Agar (APPENDIX), Bacto Rogosa SL Agar (Difco Laboratories) and Chocolate Agar (APPENDIX). All plates were incubated for 24 hrs at 37° C with the exception of the Chocolate plates which were kept at 37° C but in 5% CO<sub>2</sub>. The enriched Thioglycollate broths incubated anaerobically were streaked to isolate colonies on two separate occasions, once at 48 hrs after initial sampling and a second time at 4 days after sampling. These broths were streaked onto reduced Columbia CNA Agar, reduced CDC-PEA Agar (APPENDIX) and reduced Brucella Blood Agar (APPENDIX). Plates were incubated for 48 hrs anaerobically at 37° C. Primary isolation media and incubation methods are presented in TABLE 2.

Once primary plating and incubation were complete, the plates were checked and individual colonies picked and streaked for identification. For aerobic and 5% CO<sub>2</sub> plates all individual colonies picked were streaked for purity onto Blood Agar and incubated at 37° C for 24 hrs. With anaerobic plates, secondary plating of individual colonies was made onto reduced CDC-PEA Agar which was incubated anaerobically at 37° C for 48 hrs and Chocolate Agar incubated at 37° C in 5% CO<sub>2</sub> for 24 hrs. This secondary plating allowed for the differentiation of the isolated microorganism between obligate and facultative anaerobes, or microaerophilic organisms.

TABLE 2

Primary Isolation Media and Incubation Methods

Medium	Incubation (37° C )	Purpose
Blood Agar	aerobic	general coverage
Columbia CNA Agar <sup>1</sup>	aerobic	Grm +ves
MacConkey Agar CS <sup>2</sup>	aerobic	Grm -ves
Rogosa SL Agar	aerobic	lactobacilli
Chocolate Agar	5% CO <sub>2</sub>	Grm + & -ves
Columbia CNA Agar	anaerobic	Grm +ves
CDC-PEA <sup>3</sup>	anaerobic	general anaerobes
Brucella Blood Agar	anaerobic	fastidious anaerobes

Grm +ves - Gram positive organisms

Grm -ves - Gram negative organisms

<sup>1</sup> Designed to inhibit gram negatives and swarming of Proteus species.

<sup>2</sup> Medium allows controlled swarming of Proteus species.

<sup>3</sup> Addition of phenylethyl alcohol prevents swarming of Proteus species.

After purity plating, each colony type was identified as to genus and species, where possible, by morphological characteristics, Gram stain, hemolysis reaction, catalase, oxidase (Bacto Oxidase Differentiation Disks, Difco Laboratories) and coagulase (Bacto Coagulase Plasma, Difco Laboratories). For full identification API kits: API-20s, for Streptococcus species, API-20E, for Enterobacteriaceae and other Gram negative bacteria, and API-20A, for anaerobic bacteria, were used (API Laboratory Products Ltd., St. Laurent, Quebec). The Multi-scan System (Microscan Pos Combo Panel Type 4 and Autoscan-4 with DMS, American Micro Scan, West Sacramento, Ca.) was also employed for the identification of coagulase negative Staphylococci.

#### Media Inoculated Mice

As in experiments previously described, female 22-24 gm BALB/c mice were divided into two groups. Both groups received a single subcutaneous injection of 0.05 mL Delestrogen and after two days were inoculated intravaginally with either 20 uL pre-warmed TYI with 10% FBS, and 0.32% Agar or TYI with only 10% FBS. This inoculation was repeated the following day and five days post-inoculation, the vaginal flora from both groups was sampled. For inoculated mice, three slight modifications were made to the flora sampling protocol. Rogosa SL Agar was eliminated from the primary plating since it did not appear to permit growth of any of the vaginal organisms isolated from the mouse. Since all previously isolated vaginal aerobes grew in the presence and absence of 5% CO<sub>2</sub>, Chocolate Agar was deleted from the aerobic plating. This

agar was still employed, however, in determining whether isolates grown under anaerobic conditions were obligate or facultative anaerobes. The last modification was the elimination of Columbia CNA Agar for anaerobic plating since vaginal isolates all appeared to grow adequately on the other media employed. Microorganisms present in the vaginal wash were isolated and identified by methods previously described.

#### *T. vaginalis* Inoculated Mice

Female BALB/c mice 22-24 gm were divided into two groups. The first group was given one 0.05 mL subcutaneous injection of Delestrogen two days prior to inoculation. The second group received the same initial estrogen treatment but were estrogenized weekly with 0.05 mL Delestrogen for the duration of the experiment.

Two days after initial estrogen treatment, all mice were inoculated intravaginally with  $5 \times 10^5$  log phase *T. vaginalis*/mL on two consecutive days. Only one *T. vaginalis* clinical isolate (263) was used and was inoculated as previously described. The stage of estrus of each mouse was also determined by vaginal smear prior to inoculation. Control mice were intravaginally inoculated with only media in an identical manner.

After inoculation, a sampling regimen was implemented in order to monitor infectivity, vaginal pH and flora. Infectivity was monitored on Days 3, 7, 14 and 18 post-inoculation by vaginal washings previously described. Vaginal

pH readings were taken on Days 4, 8 and 15 post-inoculation while vaginal flora was sampled on Days 5, 9 and 16 both as previously described.

### **In Vitro Competitive Assays between *T. vaginalis* and *L. acidophilus***

#### **Quantitation of *Lactobacillus acidophilus***

A one litre quantity of MRS broth was inoculated with 0.1% of a pure culture of *L. acidophilus* and incubated overnight at 37° C in 5% CO<sub>2</sub>. After incubation, *L. acidophilus* was harvested by centrifugation for 10 minutes, at 5,000 x g, 4° C, in a Beckman centrifuge (Model J2-21M with JA-10 rotor). The supernatant was discarded and the *L. acidophilus* pellet resuspended in MRS broth and centrifuged again for 25 minutes at 140 x g in a Sorvall GLC-1 centrifuge. The supernatant was discarded and the pellet suspended in MRS broth. Duplicate serial dilutions in MRS broth were made from the original sample and all samples including a MRS blank were read for absorbance at 650 nm in a spectrophotometer (Beckman DU-88). Each dilution was also used to produce 10-fold serial dilutions in PBS. These serial dilutions were incorporated into pour plates with MRS agar so that colony counts of *L. acidophilus* were possible. Poured plates were incubated for 48 hrs at 37° C. Each plate was counted for colony-forming units and a standard curve of optical density versus colony-forming units/mL plotted with the line of best fit, produced by the method of least squares.

### In Vitro Competitive Assays

A single clinical isolate of *T. vaginalis* (263) was grown to log phase in TYI supplemented with 10% FBS and antibiotics. Simultaneously, a pure culture of *L. acidophilus* was grown to log phase in MRS broth and the bacterial concentration determined spectrophotometrically at 650 nm. Both the *Lactobacillus* and the *T. vaginalis* cultures were centrifuged separately for 20 minutes at 140 x g in a Sorvall GLC-1 centrifuge, the supernatants discarded and the pellets resuspended in sterile PBS. Pellets were washed a total of three times in PBS by 15 minute centrifugations at 140 x g in a Sorvall GLC-1 centrifuge. After final wash, both cultures were suspended in PBS. *T. vaginalis* concentration was determined, at this time, by counting viable *T. vaginalis* using trypan blue exclusion. Appropriate dilutions of both organisms were made in pre-warmed TYI supplemented with only 10% FBS. Three different ratios of *T. vaginalis* to *L. acidophilus* were used along with *T. vaginalis* and *L. acidophilus* controls. Each ratio and controls were run in triplicate and two experimental runs were made. Once all experimental ratios and control tubes were set, tubes were incubated at 37° C in 5% CO<sub>2</sub>. Samplings were made to determine changes in the growth patterns of both *T. vaginalis* and *L. acidophilus* at 8 hour intervals for 48 hours.

When sampling the cultures, a one mL quantity was removed from each tube and used for *T. vaginalis* counts and pH measurements. An additional 50 uL was taken to determine *L. acidophilus* quantities. The 50 uL sample was used in 10-fold serial dilutions in sterile PBS and each dilution was incorporated

into duplicate MRS agar pour plates. The pour plates once solidified were incubated at 37° C for 48 hrs before colonies were counted. The pH of the one mL sample was determined by pH paper readings (Accutint Indicator Paper pH range 3.9-5.4 or 5.0-6.6, Anachemia Chemicals Ltd. Montreal). *T. vaginalis* counts of the sample were made by trypan blue exclusion of appropriate dilutions using a hemocytometer.

### **Statistical Analysis**

Where applicable, data was analyzed statistically with computer programs for Student's t-tests (T-ease Version 2.0, Institute for Scientific Information Software) and 2-way analysis of variance (ANOVA from EPISTAT software programmed by T. L. Gustafson).

### **Animal Care**

All experimental procedures involving animals meet with approval of the University of Ottawa Animal Care Committee, protocol number MI-33.

## RESULTS

### Growth of *T. vaginalis* in Serum-Free Medium

As an initial project, the growth rate of *T. vaginalis* in a serum-free cell culture system was compared to growth in the same system but with the addition of serum. *T. vaginalis* isolate 002 (TABLE 3) was employed and growth assays were performed in triplicate. In comparing the serum and serum-free systems, evaluation of the presence or absence of growth was defined as a >50% increase in *T. vaginalis* numbers above the initial inoculum. Both TABLE 3 and FIGURE 1 summarize the growth kinetics of isolate 002 in the two types of systems tested. *T. vaginalis* reached a higher peak concentration in a serum containing cell system than in a serum-free system (Table 3). This difference was considered significant using initial inocula of log 3.0 *T. vaginalis*/mL ( $p=0.0114$  Student t-test) and log 4.0 *T. vaginalis*/mL ( $p=0.0058$  Student t-test). *T. vaginalis* isolate 002 was also shown to have a longer doubling time in the serum-free system than in the serum system for all inocula concentrations examined. Generally doubling time without serum was found to be approximately twice that found in the serum system. FIGURE 1 presents graphically the comparison of growth of *T. vaginalis* in the two cell culture systems. Growth in serum-free medium was longer in duration and the organism did not reach the high concentrations achieved in the serum containing system. In spite of the differences in growth characteristics, *T. vaginalis* isolate 002 was able to proliferate in the absence of serum using the McCoy cell system. In order to determine if growth in the

TABLE 3

Growth of *T. vaginalis* in a McCoy Cell Culture System in the Presence and Absence of Serum

Initial Conc'n (Log Tv/mL)*	Peak Con'n Attained (Log Tv/mL)		Doubling Time (hrs)	
	serum	serum-free	serum	serum-free
3.00	6.27 ± 0.31	5.13 ± 0.32	6.45	14.90
4.00	6.25 ± 0.22	5.51 ± 0.09	3.77	7.62
5.00	6.03 ± 0.17	5.57 ± 0.30	11.65	36.12

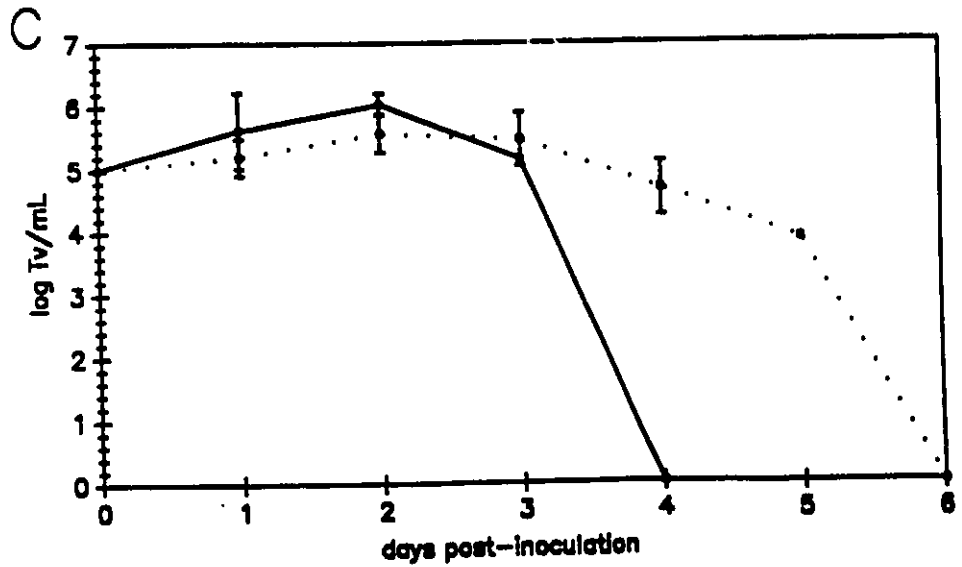
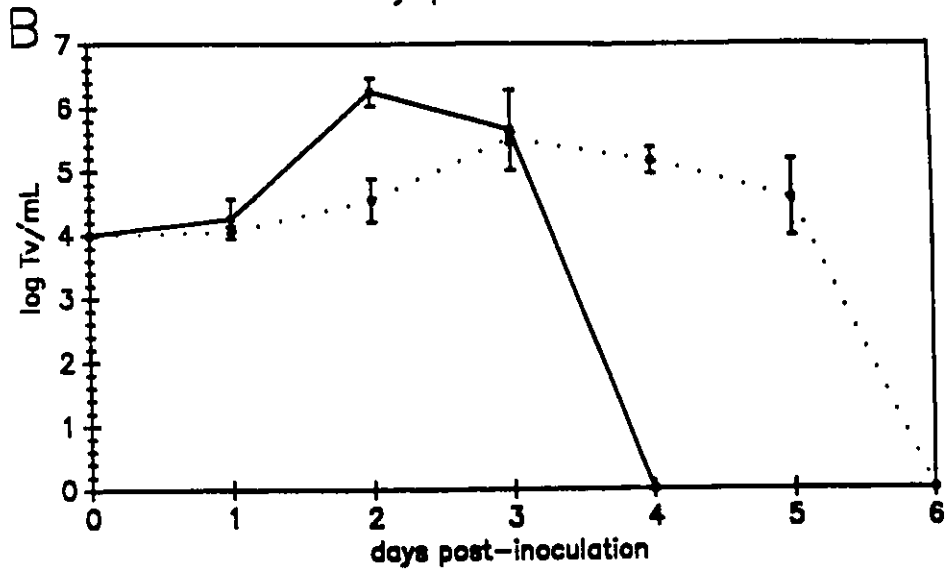
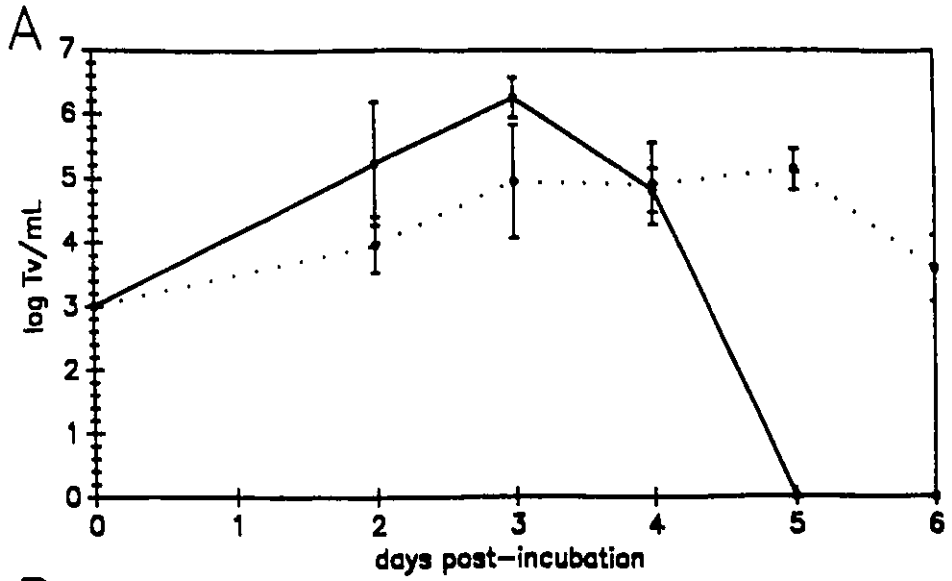
\* *T. vaginalis* isolate 002 (Tv) run in triplicate.

**FIGURE 1**

Growth of *T. vaginalis* isolate 002 compared in serum and serum-free cell culture systems. Figure 1A: initial inoculum log 3 *T. vaginalis*/mL. Figure 1B: initial inoculum log 4 *T. vaginalis*/mL. Figure 1C: initial inoculum log 5 *T. vaginalis*/mL.

————— Solid lines - growth in the presence of serum

. . . . . Dotted lines - growth in the absence of serum



absence of serum was merely a unique characteristic of isolate 002, six other *T. vaginalis* strains were tested in the growth assay system. Although there was variability between isolates, all demonstrated the ability to grow in the absence of serum.

During the growth assays the conditions of the McCoy cell monolayers were also monitored. In the absence of *T. vaginalis*, McCoy cell monolayers remained intact and viable in both serum and serum-free media for up to 10 days. Only in the presence of *T. vaginalis* growth in excess of  $5 \times 10^5$  *T. vaginalis*/mL was disruption of the monolayer and loss in cell viability evident.

McCoy cells were examined for the possible secretion of soluble growth factors for *T. vaginalis*. Using only serum-free medium, which had been overlaid on confluent monolayers for four days, *T. vaginalis* showed no proliferation regardless of the inoculum size applied. Serum-free media overlaid on McCoy cell monolayers was not observed to cause disruption of the monolayer and up to 75% of the monolayer remained confluent during the overlay incubation.

### Subcutaneous Mouse Assays

Before *T. vaginalis* (Tv) isolates were employed in a mouse model, their pathogenicity was determined by Honigberg's subcutaneous mouse assay. Each isolate, at the initial time of isolation, had been ranked by the clinical presentation of the disease as asymptomatic, moderate or severe/chronic (TABLE 1). Results of the subcutaneous assay for each isolate is presented in TABLE 4.

TABLE 4

Subcutaneous Mouse Assays Performed with Clinical *T. vaginalis* Isolates

<u>Isolate</u>	<u>Clinical Presentation</u>	<u>n</u>	<u>Mean Volume of 6 day lesions (mm<sup>3</sup>)</u>
Tv 387	asymptomatic	6	163.7 ± 47.32
Tv 386	asymptomatic	6	140.8 ± 34.97
Tv 364	asymptomatic	6	91.8 ± 28.35
Tv 396	asymptomatic	6	207.7 ± 57.81
Tv 202	moderate	6	254.2 ± 138.2
Tv 256	moderate	6	161.0 ± 51.93
Tv 263	moderate	5	244.8 ± 49.00
Tv 335	moderate	6	112.2 ± 62.94
Tv 277	severe	6	180.0 ± 85.12
Tv 330	severe	6	247.5 ± 66.72
Tv 002	chronic	6	140.5 ± 51.93
Tv 86-2	chronic	6	254.0 ± 119.8
Tf 30003	non-pathogenic	6	54.3 ± 39.14
Killed 263	killed	6	00.0
Killed 002*	killed	6	23.8 ± 13.31

Tv - *Trichomonas vaginalis*

Tf - *Tritrichomonas foetus*

\* - nodules drained to confirm presence of viable *T. vaginalis*

Each *T. vaginalis* isolate was tested on the flanks of three mice and the mean volume of lesions produced at 6 days post-inoculation (pi) is presented with standard deviation. Three additional isolates were also tested, *Tritrichomonas foetus*, (Tf 30001) a non-pathogenic trichomonad strain, and two killed strains of *T. vaginalis*. Control mice received subcutaneous injections of sterile media. At no time did media inoculated mice show signs of lesion formation at day 6 pi.

For each clinically ranked isolate, the mean volume of lesions produced was compared to lesion volumes of different rankings using 2-way analysis of variance. The average volume of lesions at day 6 pi were not significantly different between the three clinical rankings ( $p=0.2017$  2-way analysis). The non-pathogenic (Tf) and killed strains of *T. vaginalis* were similar in their lesion size, but each was found to be significantly different from the clinically ranked pathogenic *T. vaginalis* isolates ( $p=0.005$  for Tf,  $p<0.001$  for killed Tv, 2-way analysis).

Two killed *T. vaginalis* strains Tv 263 and Tv 002 were used in the subcutaneous assays (TABLE 4). Killed Tv 263 did not produce lesions in mice at day 6 or day 12 pi. Killed Tv 002 did show small lesions after 6 days although significantly smaller than those attained when the live form of the strain was employed. To control for bacterial contamination in the inoculum the assay was repeated after ultraviolet irradiation of the sample, prior to its injection. This additional step should eliminate microbial contamination. Nodules were still produced with this killed strain 6 days pi after irradiation. Drainage and

culturing of the nodule contents indicated the absence of viable, motile *T. vaginalis* which may have survived the killing process. Nodules drained after the injection of live organisms 6 days pi have been shown to contain motile parasites.

#### Establishment of a Mouse Model for *T. vaginalis* Infections

Six clinical isolates of *T. vaginalis* were employed to demonstrate intravaginal growth in BALB/c mice. As shown in TABLE 5, BALB/c mice could be infected with *T. vaginalis*, although 100% infectivity could not be achieved. Initial infectivity, recorded by the presence of *T. vaginalis* at day 3 pi, ranged from 44-89%. After infection was established, it could persist for as long as 56 days. The length of infection did not appear to correlate with the clinical pathogenicity of the isolate employed. In all strains examined, a drop in the percentage of infected mice at day 7 pi was apparent. This decline in the infectivity rate, which in some cases was as high as 72%, appeared to occur within a 4 day period. Control mice received intravaginal inoculations of media and were treated in the same manner as mice inoculated with *T. vaginalis*. Control mice showed no *T. vaginalis* or related parasites upon culturing. On two separate occasions "false negative" readings were encountered (TABLE 5). False negatives were defined as mice negative for *T. vaginalis* during the first sampling session but exhibiting *T. vaginalis* upon subsequent sampling. In isolated cases infected mice presented clinical features of trichomoniasis including swelling and erythema of the vagina. The clinical manifestations presented by the

TABLE 5

Establishment of a Mouse Model for *T. vaginalis* Infection

<u>Tv strain</u>	<u>n</u>	<u>% mice infected at</u>			<u>Maximum # of days infected</u>
		<u>Day 3</u>	<u>Day 7</u>	<u>Day 14</u>	
002	17	47(8)	18(3)	12(2)*	28
202	19	88(8) <sup>o</sup>	16(3)	16(3)*	56 <sup>a</sup>
86-2	9	44(4)	0	0	3
256	9	55(5)	11(1)	0	7
263	9	89(8)	33(3)	33(3)	49 <sup>b</sup>
330	9	55(5)	22(2)	11(1)	42

Numbers in parentheses are raw data

\* - "false-negative" (see text)

<sup>a</sup> - average maximum number of days infected was 49.

<sup>b</sup> - average maximum number of days infected was 46.

<sup>o</sup> - only 9 of 19 mice inoculated were sampled on Day 3.

mice did not appear, however, to correlate to the clinical pathogenicity of the *T. vaginalis* isolate employed.

In an attempt to improve the infection rate, two modifications to the standard protocol were applied (TABLE 6). The first modification was a weekly estrogenization of mice with 0.05 mL Delestrogen, the second was also a weekly estrogenization but with double the dose (0.1 mL) of Delestrogen. Two *T. vaginalis* isolates (202 and 263), which had shown high initial infectivity (TABLE 5), were used and the modifications applied. Neither modification produced 100% infectivity rates or substantially improved the percentage of infected mice beyond day 7 pi.

It had been previously reported that the stage of estrous was a crucial component in the successful establishment of intravaginal infections (Coombs *et al.* 1986). To test the validity of this claim, the association between the stage of estrous at the time of infection and the susceptibility of mice to *T. vaginalis* was analyzed. Applying the standard inoculation protocol, mice were examined to establish their stage of estrous prior to infection. The majority of mice (53%) were found to be in metestrous while only 36% were in estrous prior to inoculation (TABLE 7). Fifty-six percent of mice infected with *T. vaginalis* at day 3 pi were in metestrous before initial inoculation as compared to 28% in estrous. Mice infected at day 7 and beyond did not appear to be in metestrous prior to inoculation, but were instead in either estrous or proestrous stages.

**TABLE 6**  
Modifications to Mouse Model

---

<u>WEEKLY ESTROGEN TREATMENT (0.05 mL)</u>					
<u>Tv strain</u>	<u>n</u>	<u>% mice infected at</u>			<u>Maximum # of</u>
		<u>Day 3</u>	<u>Day 7</u>	<u>Day 14</u>	<u>days infected</u>
202	9	67(6)	22(2)	11(1)	35
263	8	75(6)	50(4)	25(2)	48

---

<u>WEEKLY ESTROGEN TREATMENT (0.1 mL)</u>					
<u>Tv strain</u>	<u>n</u>	<u>% mice infected at</u>			<u>Maximum # of</u>
		<u>Day 3</u>	<u>Day 7</u>	<u>Day 14</u>	<u>days infected</u>
263	8	63(5)	25(2)	0	7

---

Numbers in parentheses are raw data.

TABLE 7

Association Between the Stage of Estrous and the Susceptibility of Mice  
to Intravaginal Infection with *T. vaginalis*

Stage of Estrous	Dioestrous	Proestrous	Estrous	Metestrous
Condition at Day 0* (% mice) (n = 45)	2	9	36	53
Condition at Day 0* of mice +ve for Tv Day 3 (% mice) (n = 25)	4	12	28	56

\* stage of estrous was determined prior to inoculation.

All mice were estrogenized with a single 0.05 mL dose of Delestrogen 2 days prior to estrous determination and Tv inoculation.

### Flora and pH in Untreated Mice

To study the interactions between *T. vaginalis* and vaginal flora in the mouse model, a basic knowledge of the normal genital flora of the mouse was required. No previous work had been done in this area so initial experiments were performed to identify the normal flora and pH.

Vaginal flora and pH were examined in 25 untreated BALB/c mice (TABLE 8). In untreated mice, the most commonly isolated organisms appeared to be *Staphylococcus aureus* and Group D Streptococci. These species were followed in frequency by Lactobacillus species and enteric organisms (*Escherichia coli* and *Proteus mirabilis*). The incidence of both coagulase-negative Staphylococci and anaerobic organisms was considerably low. In total, BALB/c mice appeared to harbour  $1.56 \pm 0.651$  different bacterial species in the vagina. Vaginal pH was recorded to be near neutral at a value of  $6.47 \pm 0.317$ . The low incidence of anaerobic species was at first considered to be a reflection of the inadequacy of the sampling technique employed. To determine if this was the case, twenty mice were sampled strictly for anaerobes with all culturing and plating performed in an anaerobic chamber. This revised sampling/culturing technique did not increase the overall number of anaerobes isolated.

### Assessment of Various Factors in Alterations of Vaginal Flora and pH

#### Estrogen Treatment

Once the bacterial composition and pH of untreated BALB/c mice had been established, alterations to the flora and pH, with *T. vaginalis* infections,

**TABLE 8**  
Vaginal Flora and pH in Untreated Mice

---

<u>Microorganism</u>	<u>Frequency of Isolation</u>
<i>Staphylococcus aureus</i>	56%
<i>Streptococcus faecium</i>	32%
Lactobacillus species	16%
<i>Escherichia coli</i>	12%
<i>Proteus mirabilis</i>	12%
<i>Micrococcus luteus</i>	12%
<i>Staphylococcus xylosus</i>	8%
<i>Staphylococcus epidermidis</i>	4%
<i>Staphylococcus sciuri</i>	4%
Anaerobic <i>Streptococcus constellatus</i>	4%

n = 25

Average vaginal pH:  $6.47 \pm 0.317$  (n=45)

Average # of species/mouse:  $1.56 \pm 0.651$  (n=25)

---

were to be studied. Since several factors in the *T. vaginalis* inoculation procedure may have influenced flora, it was necessary to study the factors individually. The first such factor considered was estrogenization since it was imperative in establishing *T. vaginalis* infection in mice. BALB/c mice were estrogenized and vaginal flora determined two days later, corresponding in time to when *T. vaginalis* would be inoculated under normal circumstances. Estrogenized mice exhibited the same small homogeneous array of bacterial species that had been isolated in untreated mice (TABLE 9). There were no differences in the frequency of isolation of vaginal species between estrogenized and untreated groups. Estrogenization appeared only to produce a higher number of bacterial species isolated per mouse (2.08 compared to 1.56  $p=0.034$  Student t-test), and a lower vaginal pH (6.23 versus 6.47  $p=0.034$  Student t-test) than that determined in untreated mice.

#### Media Inoculations

The infection protocol required *T. vaginalis* to be suspended in medium at the time of intravaginal inoculation. For this reason, alterations of the vaginal flora by medium inoculation were studied. Two types of media were examined for their effect on vaginal flora. The first consisted of TYI supplemented with 10% FBS (agar-free). The second medium was identical to the first except for the addition of 0.32% agar (agar-containing). For both media experiments, 25 mice were employed in the standard infection protocol except inoculations were with medium only. Five days pi vaginal flora was sampled.

**TABLE 9**  
Vaginal Flora and pH in Estrogenized Mice

---

<u>Microorganism</u>	<u>Frequency of Isolation</u>
<i>Staphylococcus aureus</i>	76%
<i>Streptococcus faecium</i>	28%
<i>Streptococcus faecalis</i>	28%
Lactobacillus species	24%
<i>Escherichia coli</i>	24%
<i>Proteus mirabilis</i>	12%
<i>Staphylococcus xylosus</i>	12%
Anaerobic <i>Streptococcus constellatus</i>	8%
<i>Micrococcus luteus</i>	4%
Anaerobic <i>Bacteriodes fragilis</i>	4%

n = 25

Average vaginal pH: 6.23 ± 0.450 (n=25)

Average # of species/mouse: 2.08 ± 0.997 (n=25)

---

Both agar-free (TABLE 10) and agar media (TABLE 11) inoculated mice showed similar frequencies of isolation, and numbers of different bacterial species per mouse. The same homogeneous yet small bacterial populations evident in untreated mice were demonstrated in both groups of media-inoculated mice and no differences were found in comparisons of the frequency of isolation of any vaginal species with respect to untreated mice. Comparison of the numbers of bacterial species isolated per mouse, however, indicated that significantly higher numbers of species were isolated from media-inoculated mice ( $p=0.029$  agar-free,  $p=0.005$  agar-containing) in relation to untreated mice.

#### *T. vaginalis* Inoculated, Single Estrogenized Mice

To establish the effects of *T. vaginalis* on the genital flora, 25 mice were given a single estrogen treatment and inoculated with *T. vaginalis* strain 263 (TABLE 1). Mice were monitored for infectivity and alterations to vaginal pH and flora on selected days over a 2 week course of infection. Initial infectivity was 57% at day 3 pi, but dropped markedly so that by day 18 pi there were no mice positive for *T. vaginalis*.

During the experiment, mice were subdivided into 2 groups. The first group was "infected" and defined as mice positive for *T. vaginalis* upon sampling prior to the time vaginal pH and flora was determined. "Cleared" mice represented the second group and were negative for *T. vaginalis* in samples prior to the recording of vaginal pH and flora. Control mice, which received

**TABLE 10**  
**Floral Changes Induced by Inoculation with Agar-free Medium**

<u>Microorganism</u>	<u>Frequency of Isolation</u>
<i>Staphylococcus aureus</i>	72%
Group D Streptococci*	40%
<i>Proteus mirabilis</i>	36%
Lactobacillus species	32%
Coagulase Negative Staphylococci**	12%
<i>Listeria grayi</i>	4%
Anaerobic <i>Propionibacterium acnes</i>	4%
n = 25	
Average # of species/mouse: 2.08 ± 0.954 (n=25)	

\* Group D Streptococci was comprised of 40% *S. faecium* and 8% *S. faecalis*.

\*\* Coagulase Negative Staphylococci included *S. xylosus*, *S. cohnii*, and a rare biotype that was not identified.

TABLE 11

Floral Changes Induced by Inoculation with Agar-containing Medium

<u>Microorganism</u>	<u>Frequency of Isolation</u>
<i>Staphylococcus aureus</i>	76%
Group D Streptococci*	64%
Lactobacillus species	24%
<i>Proteus mirabilis</i>	20%
<i>Escherichia coli</i>	12%
Other Enteric species*	8%
Coagulase Negative Staphylococci**	8%
<i>Listeria grayi</i>	4%
Anaerobic <i>Propionibacterium acnes</i>	4%

n = 25

Average # of species/mouse: 2.2  $\pm$  0.866 (n=25)

\* Group D Streptococci was composed of *S. faecium* 40%, and *S. faecalis* 24%.

\* Other Enteric species included *Escherichia hermannii* and *Enterobacter cloacae*.

\*\* Coagulase Negative Staphylococci included: *S. xylosus* and *S. epidermidis*.

estrogen-treatment and inoculations with *T. vaginalis*-free media, were also included.

Flora, in regard to the frequency of isolation of each vaginal species, were similar in control, infected and cleared groups of mice both on individual days and between sampling times (FIGURE 2). The only significant difference encountered was in Lactobacillus frequencies on days 5 and 9 in cleared mice. In this case, the high frequency of Lactobacillus apparent at day 5 (62%) dropped to only 14% by day 9 ( $p=0.01$  Student t-test). Vaginal pH in control, infected and cleared mice ranged from  $6.28 \pm 0.202$  to 6.8 (FIGURE 3A). In all three of the sampling groups, vaginal pH was found to be comparable on separate sampling days and between days.

The spectrum of bacterial species, identified in this experiment, were similar to those previously demonstrated in untreated mice. Regardless of the study group analyzed, the incidence of *S. aureus*, Group D Streptococci, enteric bacilli, coagulase negative Staphylococci and anaerobic species was comparable to that in untreated mice. Only the frequency of Lactobacillus in all three of the study groups, particularly at day 5, was significantly higher ( $p=0.03$ ) than that established in untreated mice. The average number of vaginal species isolated per mouse ranged from 1 to 2.1 in the three study groups and was found, in general, to be slightly higher than that demonstrated in untreated mice. Vaginal pH in the experimental mice was not found to differ significantly from that of untreated mice.

## FIGURE 2

Comparison of the frequency of isolation of vaginal organisms in single-estrogen treated *T. vaginalis* inoculated mice during a two week sampling period.

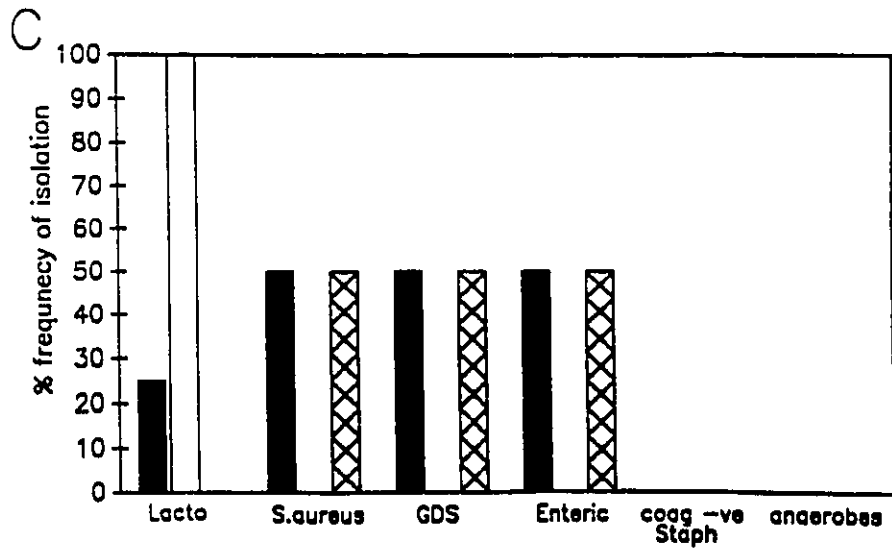
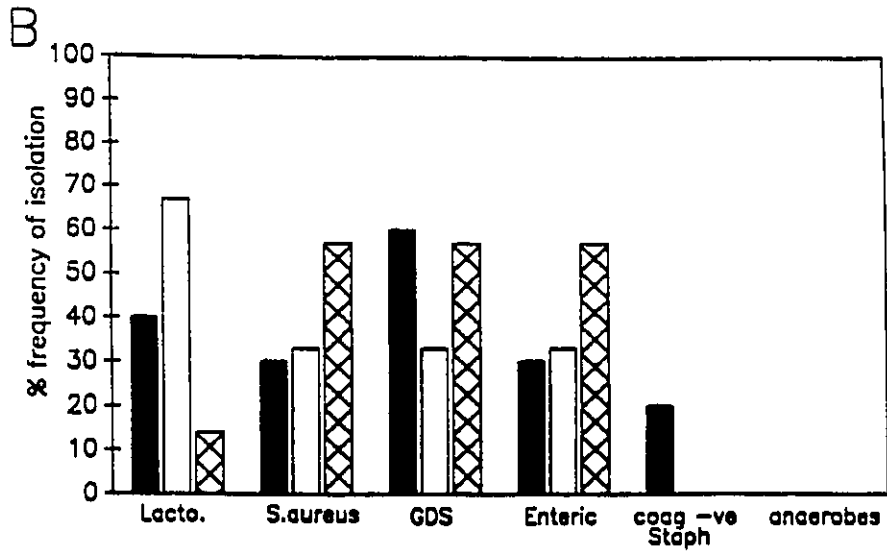
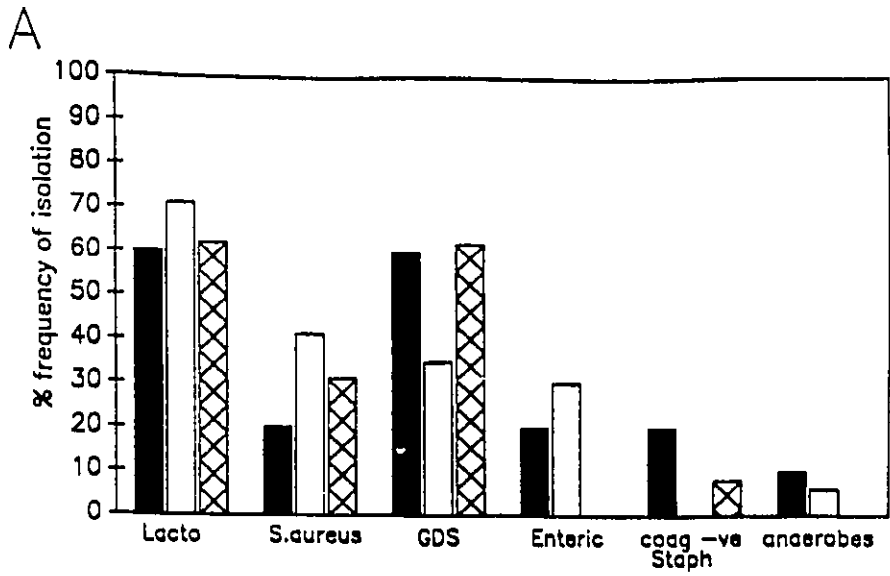
### Legend:

A - day 5 post-inoculation	■	Control
B - day 9 post-inoculation	□	Infected
C - day 16 post-inoculation	☒	Cleared

Lacto. - Lactobacillus species  
 S.aureus - *Staphylococcus aureus*  
 GDS - Group D Streptococci  
 Enteric - *Proteus mirabilis* & *Escherichia coli*  
 coag-ve Staph - Coagulase negative Staphylococci  
 anaerobes - Anaerobic species

### Sample Sizes:

<p>In A: control n= 10          infected n= 17          cleared n= 13</p>	<p>In B: control n= 10          infected n= 3          cleared n= 14</p>
<p>In C: control n= 4          infected n= 1          cleared n= 2</p>	



### FIGURE 3

Vaginal pH alterations in *T. vaginalis* inoculated mice. Figure 3A represents mice treated with a single dose of estrogen and 3B, mice that were estrogenized weekly. Solid bars - control mice inoculated with medium. Open bars - *T. vaginalis* infected mice. Hatched bars - mice which have cleared the infection

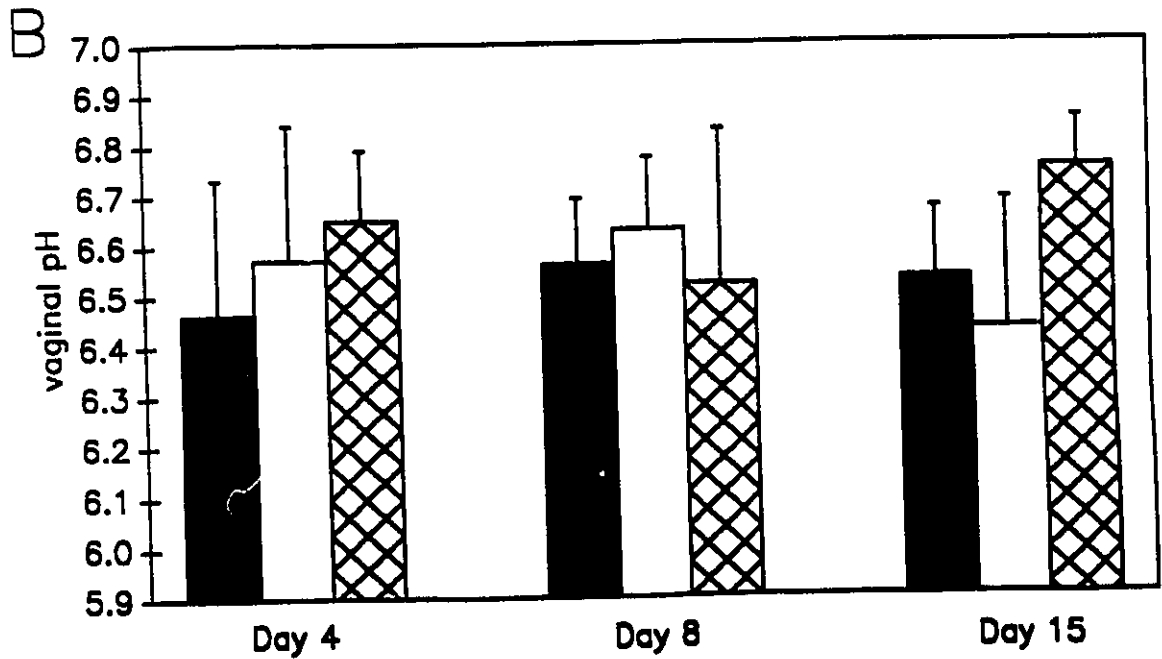
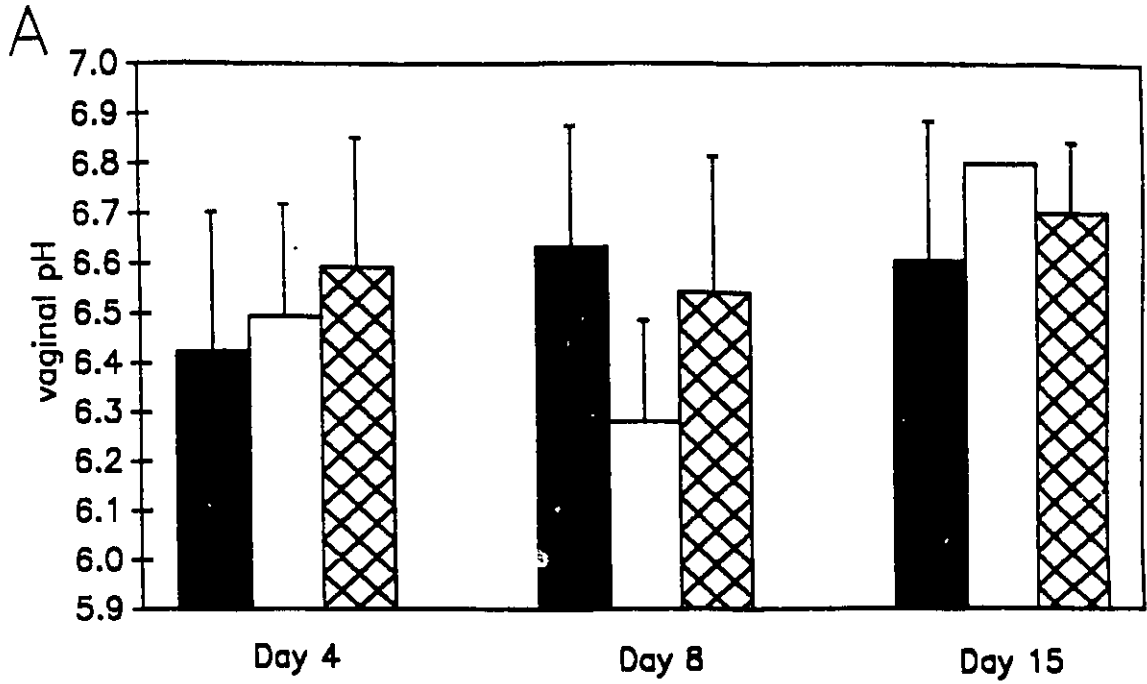
#### Sample Sizes:

##### Figure 3A

Day 4- control n= 10	Day 8- control n= 10
infected n= 17	infected n= 3
cleared n= 13	cleared n= 14
Day 15- control n= 4	
infected n= 1	
cleared n= 2	

##### Figure 3B

Day 4- control n= 9	Day 8- control n= 9
infected n= 25	infected n= 12
cleared n= 8	cleared n= 13
Day 15- control n= 9	
infected n= 8	
cleared n= 4	



*T. vaginalis* Inoculated, Weekly Estrogenized Mice

In the initial study of floral alterations during *T. vaginalis* infection, the small population sizes may have not been representative of the effects seen in the general population. For this reason a similar experiment was conducted employing *T. vaginalis* inoculated, weekly estrogenized mice. Mice, in this study, were estrogenized weekly in the hope that permanent estrous with a lower vaginal pH could be achieved. A low vaginal pH would permit detection of any changes to the pH by *T. vaginalis*. In this experiment initial infectivity was recorded at 76% and allowed larger sample sizes in the infected and cleared groups studied.

As had been the case in all previous experiments, the vaginal flora in control, infected and cleared were again found to harbour the same homogeneous population of vaginal species (FIGURE 4). At both single samplings and between sampling days, the frequency of isolation of vaginal species was similar in control, infected and cleared mice with three exceptions noted. One situation involved the low incidence of *Staphylococcus aureus* in cleared mice at day 9 pi ( $p=0.04$  Student t-test). The second incidence of significance was the high frequency of enteric bacilli in cleared mice at day 16 pi ( $p=0.03$  Student t-test). The third significant situation, and most interesting, involved the decline in the frequency of *Lactobacillus* species in cleared mice from days 5 to 16 ( $p=0.0136$  Student t-test). In this study, the number of vaginal species per mouse ranged from 1.6 to 2.3 and was similar in all three of the study groups regardless of sampling times. Vaginal pH in control, infected and

#### FIGURE 4

Comparison of the frequency of isolation of vaginal organisms in weekly estrogen treated *T. vaginalis* inoculated mice during a two week sampling period.

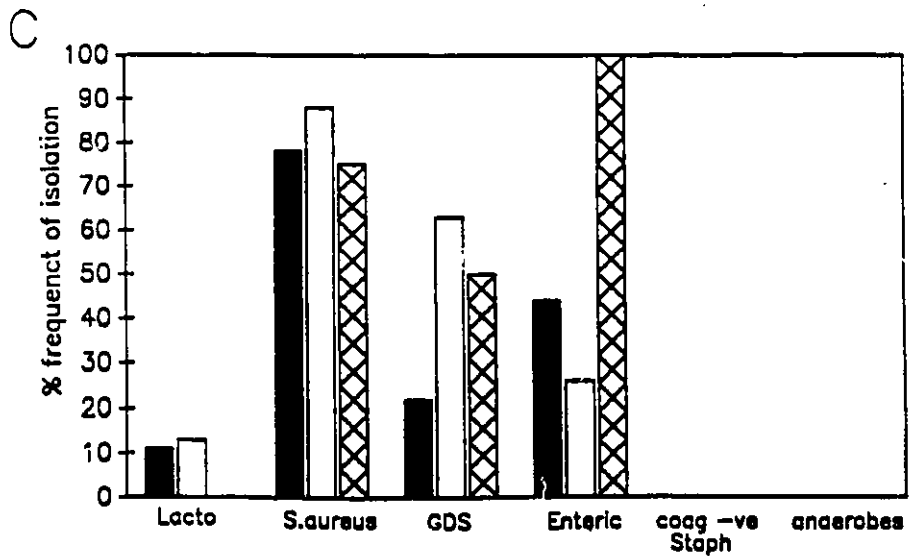
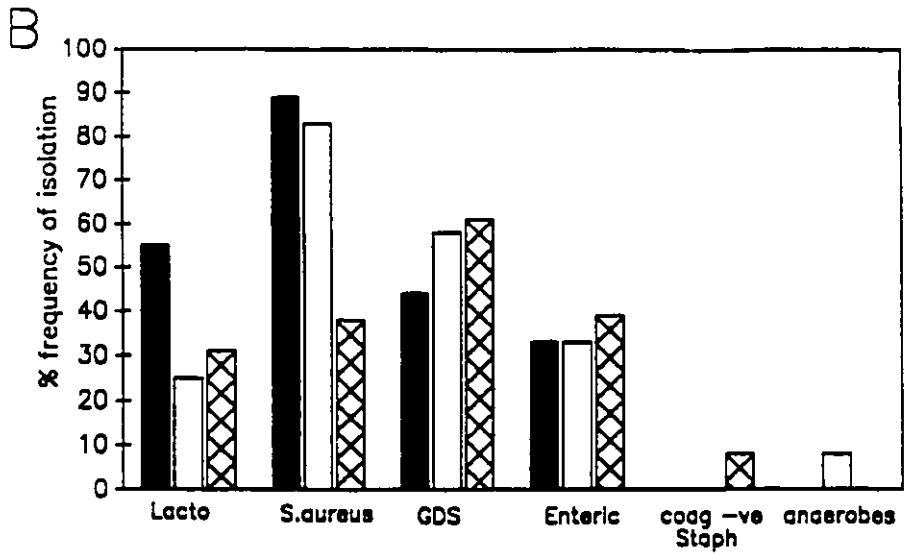
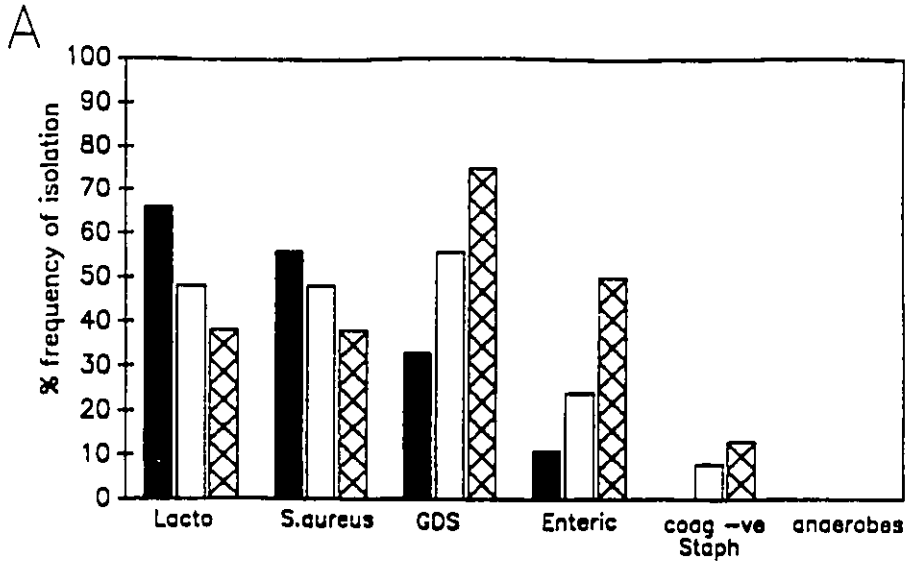
#### Legend:

- |                             |                                     |          |
|-----------------------------|-------------------------------------|----------|
| A - day 5 post-inoculation  | <input checked="" type="checkbox"/> | Control  |
| B - day 9 post-inoculation  | <input type="checkbox"/>            | Infected |
| C - day 16 post-inoculation | <input checked="" type="checkbox"/> | Cleared  |

Lacto - Lactobacillus species  
 S.aureus - *Staphylococcus aureus*  
 GDS - Group D Streptococci  
 Enteric - *Proteus mirabilis* & *Escherichia coli*  
 coag-ve Staph - Coagulase negative Staphylococci  
 anaerobes - Anaerobic species

#### Sampling Sizes:

In A: control n= 9	In B: control n= 9
infected n= 25	infected n= 12
cleared n= 8	cleared n= 13
In C: control n= 9	
infected n= 8	
cleared n= 4	



cleared mice was found to range from  $6.43 \pm 0.258$  to  $6.75 \pm 0.10$  (FIGURE 3B) and except for significantly higher readings in cleared mice at day 15 ( $p=0.0383$  compared to infected,  $p=0.0191$  compared to control Student t-tests), the pH was similar between study groups on separate samplings and between sampling days.

The frequency of isolation of the various vaginal species in this experiment was similar to that recorded in untreated mice with the exception of *Lactobacillus* species. This experiment again exhibited a particularly high incidence of *Lactobacillus* species at day 5 in both control, infected and cleared mice ( $p=0.0212$  Student t-test) as compared to that in untreated mice. The numbers of vaginal species isolated per mouse were also higher in this group of mice than in untreated mice. Vaginal pH in the control, infected and cleared mice did, however, remain similar to that recorded for untreated mice.

Both single and weekly estrogenized *T. vaginalis* inoculated mice and their controls were generally found to be similar in regards to the incidence of vaginal species isolated, the number of vaginal species found per mouse and the vaginal pH. One common situation encountered in both *T. vaginalis* inoculation experiments occurred at day 4, when the vaginal pH of cleared ( $p=0.0107$  single estrogen,  $p=0.0143$  weekly estrogen Student t-test) and infected mice ( $p=0.039$  single estrogen,  $p=0.002$  weekly estrogen Student t-test) was significantly higher than that demonstrated in estrogenized mice (TABLE 9). Vaginal pH of control mice was, however, similar to the recorded pH of estrogenized mice.

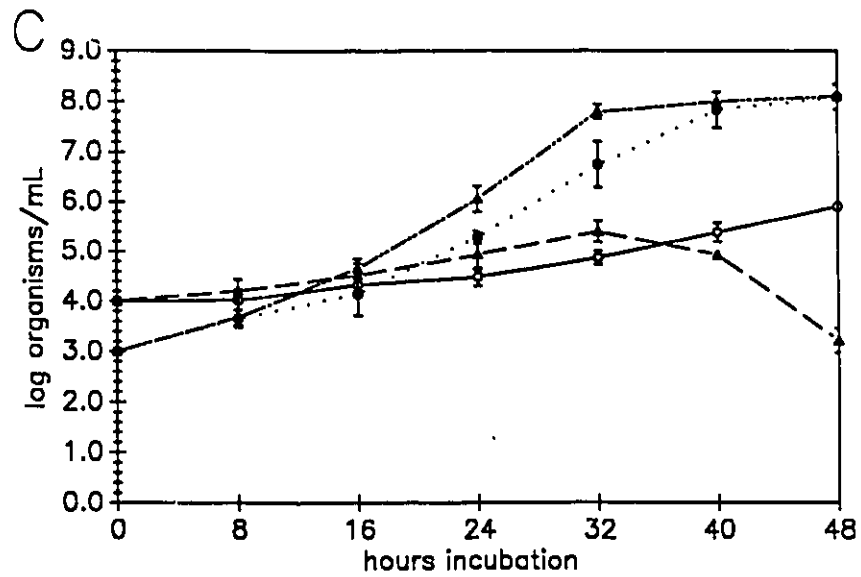
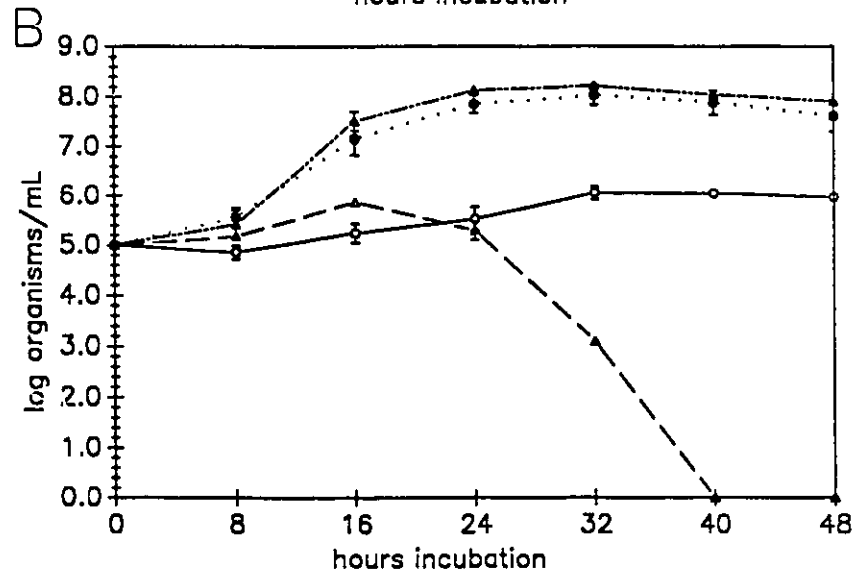
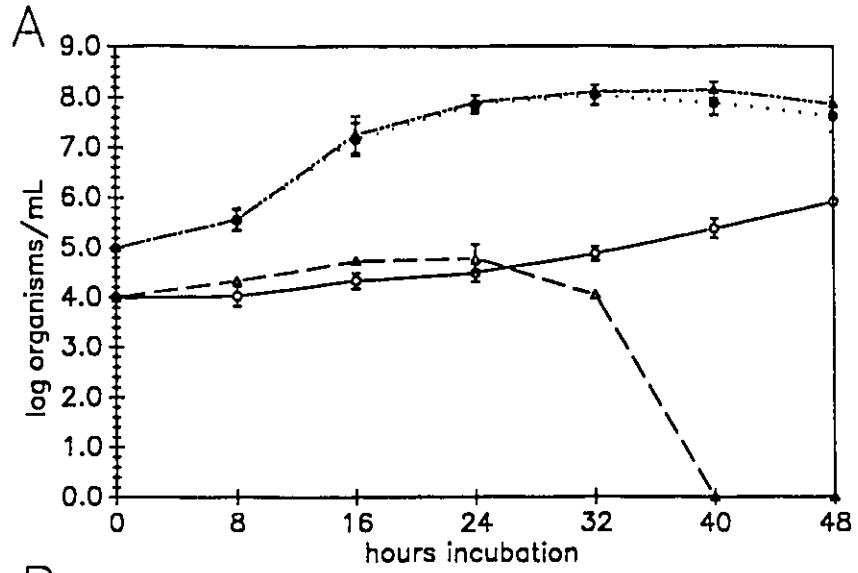
*In Vitro* Competitive Assays Between *T. vaginalis* and  
*L. acidophilus*

Analysis of the *in vitro* growth of *T. vaginalis* (Tv) isolate 263 (TABLE 1) in the presence of *L. acidophilus* (La) was conducted in order to establish the interactions between these two organisms and possibly relate this to the situation found in the vagina. Three separate ratios of *T. vaginalis* to *L. acidophilus* were used: 1 Tv:1 La, 1 Tv:10 La and 10 Tv:1 La along with appropriate Tv and La controls. Each assay was run twice in triplicate.

Growth of *L. acidophilus* in experimental ratios resulted in several significant trends (FIGURE 5). *L. acidophilus*, when coincubated with *T. vaginalis* of equal or higher concentrations, demonstrated a rapid growth rate, compared to controls (FIGURE 5B,C). The 1 Tv:1 La ratio, at optimal concentrations for both species, exhibited increased *L. acidophilus* growth between 16 and 32 hours ( $p=0.0048$  @ 16 hrs,  $p=0.003$  @ 24 hrs,  $p=0.063$  @ 32 hrs Student t-test) (FIGURE 5B) as did the 10 Tv:1 La ratio at the same time intervals ( $p=0.029$  @ 16 hrs,  $p<0.001$  @ 24 hrs,  $p<0.001$  @ 32 hrs Student t-test) (FIGURE 5C). Only when a 10-fold lower concentration of *T. vaginalis* was coincubated with *L. acidophilus* was there no significant change in the growth of the bacteria with respect to that of the control.

The pH of the media was also monitored during the assays. The original pH of the media (6.6) decreased to approximately 5 when a concentration of log 6 La/mL was achieved and dropped to a pH of 4.2 when log 7 La/mL was attained. The media at a pH of 4.2 was the maximum drop achieved. Growth





kinetics and pH values for *L. acidophilus* controls and ratios are presented in TABLE 12. No differences were found in the peak concentrations of *L. acidophilus* or in the pH, at the time of this peak, in controls or ratios. Only the time of peak concentration varied slightly between the experimental ratios and controls.

*T. vaginalis* growth showed far more deviations with respect to controls than did *L. acidophilus* (FIGURE 5, TABLE 13). Regardless of *L. acidophilus* concentration, a general pattern in *T. vaginalis* growth was demonstrated in coincubations. In all ratios, *T. vaginalis* exhibited a rapid growth rate with lower peak concentrations of organisms. In both 1 Tv:1 La and 1 Tv:10 La ratios, growth of *T. vaginalis* was significantly higher than controls in the first 16 hours of the assay (1 Tv:1 La :  $p < 0.001$  @ 8 hrs,  $p < 0.001$  @ 16 hrs, 1 Tv:10 La :  $p = 0.007$  @ 8 hrs,  $p < 0.001$  @ 16 hrs Student t-test) (FIGURE 5A,B). With a lower initial inoculum of *L. acidophilus* (10 Tv:1 La) increased growth of *T. vaginalis* occurred between 24 and 32 hours ( $p = 0.011$  @ 24 hrs,  $p = 0.001$  @ 32 hrs Student t-test) (FIGURE 5C).

For every ratio studied, the peak concentrations of *T. vaginalis* occurred earlier in time, but were significantly below that achieved with controls (1 TV:1 LA :  $p = 0.007$ , 1 Tv:10 La :  $p < 0.001$ , 10 Tv:1 La :  $p < 0.001$  Student t-test) (TABLE 13). The peak concentrations, in the ratios, occurred prior to a drop in the pH below 4.5, after which time *T. vaginalis* death was apparent.

TABLE 12

Growth Characteristics of *Lactobacillus acidophilus*

<u>Ratio</u>	<u>Peak Conc'n achieved (log cfu/mL)</u>	<u>Time of Peak Conc'n (hr)</u>	<u>pH at Peak Conc'n</u>
control 1	8.03 ± 0.197	32	4.2
1Tv:10La	8.14 ± 0.164	40	4.2
1Tv:1La	8.22 ± 0.095	32	4.2
control 2	8.08 ± 0.243	48	4.2
10Tv:1La	8.10 ± 0.252	48	4.2

control 1 - initial inoculum  $1 \times 10^5$  La/mL

control 2 - initial inoculum  $1 \times 10^3$  La/mL

TABLE 13  
Growth Characteristics of *Trichomonas vaginalis*

<u>Ratio</u>	<u>Peak Conc'n achieved (log Tv/mL)</u>	<u>Time of Peak Conc'n (hr)</u>	<u>pH at Peak Conc'n</u>
control 1	6.06 ± 0.135	32	5.8
1Tv:1La	5.87 ± 0.121	16	5.1
control 2	5.91 ± 0.103	48	6.1
10Tv:1La	5.40 ± 0.213	32	4.6
1Tv:10La	4.73 ± 0.112	16	5.1

control 1 - initial inoculum  $1 \times 10^5$  Tv/mL  
control 2 - initial inoculum  $1 \times 10^4$  Tv/mL

## DISCUSSION

### Serum-Free Growth of *T. Vaginalis*

Since serum proteins can interfere with enzyme studies and may also bind directly to *T. vaginalis* (Bromke 1986, Peterson and Alderete 1982, 1983), the establishment of a serum-free medium would be advantageous. Serum replacement medium for *T. vaginalis* has been devised and consists of bovine serum albumin and cholesterol, together with a defined mixture of fatty acids (Linstead 1981). The addition of cholesterol satisfies *T. vaginalis*'s obligate requirement for a sterol (Linstead 1981) while the 3 fatty acids: palmitate, oleate, and stearate are a basic requirement for *T. vaginalis* growth.

This study demonstrated that serum could be omitted from medium used to grow *T. vaginalis*, if a McCoy cell culture system (Garber *et al.* 1987) was employed. In the serum-free system, the growth of *T. vaginalis* did not achieve the peak concentrations evident in serum containing medium. Further, serum-free growth was slower than that found in the normal cell system. The exhibited trends did, however, corroborate results from previous studies on the establishment of a totally defined medium. In serum-replaced systems, *T. vaginalis* has demonstrated slower growth and final cell yields of only log 5 to 5.5 *T. vaginalis*/mL (Linstead 1979, 1981). Although strain to strain variability was evident, six other isolates exhibited the same trend.

Garber *et al.* (1987) have demonstrated that without the presence of eucaryotic cells in the serum-containing cell culture system, medium alone could

not support the growth of *T. vaginalis*. Experiments in this study have also shown that the supernatant from cell culture medium, incubated for up to 4 days over confluent McCoy cell monolayers, would not permit *T. vaginalis* proliferation. The physical presence of eucaryotic cells appears to be necessary for the growth of *T. vaginalis*. The possibility of a specific growth factor secreted by the cells, however, was not apparent when tested. Peterson and Alderete (1984) have demonstrated that lipoproteins are capable of supporting parasite multiplication and that plasma deficient in lipoproteins fails to support *T. vaginalis* growth. Since eucaryotic cell membranes consist of a lipid bilayer with intrinsic and extrinsic proteins, this may account for *T. vaginalis* growth in the serum-free cell culture system. If the membrane components of the cell are capable of supporting *T. vaginalis* growth, the quantity and variation of proteins found at the mucosal surfaces, where infection occurs, could be a variable not yet considered in the pathogenicity of *T. vaginalis*.

#### Pathogenicity as Determined by the Subcutaneous Mouse Assay

Prior to inoculation in the mouse model, *T. vaginalis* isolates were employed in subcutaneous assays to determine pathogenicity. The clinical presentation of each isolate had been recorded at the initial time of isolation but no record was made of the number of serial passages prior to storage at -70° C. Since *T. vaginalis* strains frozen immediately after isolation do not lose pathogenicity (Kulda *et al.* 1970), while strains serially transferred *in vitro* for long periods do become attenuated (Kuczynska *et al.* 1984), it was important to

establish if the pathogenicity of each isolate still correlated to its clinical presentation.

Upon analysis of the assay data several points were considered of interest. Deviations in the nodule size for mice injected with the same *T. vaginalis* isolate were attributed to the tendency of lesions produced by certain strains to open and weep prior to measurement. Inoculations into flanks may have also permitted mice to bite at the lesion causing drainage before measurement. The most prominent result of the assay was that *T. vaginalis* isolates, when grouped in terms of clinical presentation, and compared by the lesion volumes produced were found not to be significantly different. These results appeared to be in direct opposition to those of previous investigators (Honigberg 1961, Honigberg *et al.* 1966, Kulda *et al.* 1970) who had shown a direct correlation between the subcutaneous assay and vaginal/cervical disease. In early studies, which involved relatively few isolates, the volume of subcutaneous lesions could be related to both cervical epithelial abnormalities and manifestations of vaginal disease. Recent work (Honigberg *et al.* 1984) employing a larger array of isolates has demonstrated that only the degree of cervical epithelial cytopathologic changes could be statistically related to the mean volume of subcutaneous lesions. No relationship was evident between lesion volume measured in the mouse assay and parameters which included discharge, redness, ulceration and inflammation of the vagina and cervix. These recent findings support the presented data. No correlation was found between the clinical presentation and the lesion size produced by *T. vaginalis* isolates employed, however, the clinical ranking of the

isolates was defined by vaginal discharge, inflammation and erythema. No record of changes in cervical cytopathology was made at the initial time of isolation of each *T. vaginalis* isolate so it was not possible to test for a correlation between this parameter and the lesion volume.

Injection of both non-pathogenic trichomonads and killed *T. vaginalis* strains produced lesions that were significantly smaller than those generated by live, pathogenic *T. vaginalis*. This small lesion formation was considered to be a normal immune response elicited by the mouse to a foreign antigen. In this regard, the subcutaneous mouse assay did appear to be efficient at differentiating between non-pathogenic and pathogenic strains of *T. vaginalis*. Killed *T. vaginalis* strains were of interest since one strain showed no lesion formation when assayed, while the second strain tested generated a lesion significantly smaller than that of its live *T. vaginalis* counterpart. The lesion created by killed isolate 002 showed no viable *T. vaginalis* when drained indicating efficient killing of the parasite prior to inoculation. Bacterial contamination was also eliminated as a potential cause of the lesion. A possible explanation for variability in nodule formation in the killed strains tested may involve the antigenic heterogeneity of *T. vaginalis*. The presence of common and unique antigens associated with *T. vaginalis* has been documented in at least fifteen clinical isolates. Immunological response to different *T. vaginalis* antigens appears to vary between individuals (Garber *et al.* 1986). This may be the case in inoculated mice if a unique antigen of one isolate is generating an immune response which can cause the subsequent lesion.

The subcutaneous assay has been considered to be the most effective method in determining the inherent pathogenicity of *T. vaginalis* in relation to its clinical presentation. The assay is, however, expensive in both time and money. This fact, coupled with the recent reports of correlations only to cervical epithelial abnormalities, and the presence of unique *T. vaginalis* antigens, raises questions concerning the efficacy of the assay. Elucidation of the pathogenicity of *T. vaginalis* may require more sensitive and specific markers of virulence.

#### An Intravaginal Mouse Model for *T. vaginalis* Infection

Two *T. vaginalis* intravaginal animal models presently being studied are squirrel monkeys and mice. Infection in squirrel monkeys is similar to trichomoniasis in women (Street *et al.* 1983), however, squirrel monkeys are both costly and technically difficult to handle. Mice, on the other hand, are relatively inexpensive, extremely easy to manipulate, and do not carry indigenous vaginal trichomonad species as has been documented in squirrel monkeys (Gardner *et al.* 1987).

Six clinical *T. vaginalis* isolates tested in the mouse model were able to produce infection in BALB/c mice. Although 100% infectivity for over 40 days (Cappuccinelli *et al.* 1974) was not achieved, infectivity ranged from 44-89% and was evident up to a maximum of 56 days. The results reported in this study corroborated those of Coombs *et al.* (1986a). Infection rates in both studies appeared to differ between *T. vaginalis* isolates and individual response variations of the mice to *T. vaginalis* inoculations was apparent. The presence

of false negatives was also reported in both studies. False negatives may have been a reflection of the limitations in the culture system used to demonstrate infection. *T. vaginalis* growth in the culture system currently employed requires a minimum inocula of between log 3.5 - 4 *T. vaginalis*/mL to detect growth (Garber *et al.* 1987).

Regardless of the isolate used, *T. vaginalis* infected mice showed substantial decreases in the number infected by day 7 pi. This drop in infectivity had not been reported in previous work with the mouse model. Attempts were made to erradicate this loss in infectivity at day 7 pi by employing weekly estrogenization of the mice with varying dosages of estrogen. Weekly estrogenization had been part of the protocol of Cappuccinelli *et al.* (1974) which claimed to yield 100% infectivity for up to 6 weeks. Increased estrogen dosage had been employed by Coombs *et al.* (1986a). In the experiments presented here, modifications did not substantially increase the number of infected mice at day 7.

Previously it was reported that estrogen treatment was critical in establishing extended *T. vaginalis* infections (Cappuccinelli *et al.* 1974, Coombs *et al.* 1986a). Estrogenization in vaginal animal models was believed to enhance vaginal cell glycogen levels and therefore potentiate infection by creating a nutritionally-rich environment. In 1986 Coombs *et al.*, upon further investigation with mice, found an association between the susceptibility of mice to *T. vaginalis* and the stage of estrous at the time of inoculation. It appeared, from this work, that mice were most susceptible to infection at early proestrous. The estrous

cycle in mice occurs every 4 1/4 days and consists of proestrous, estrous, metestrous and diestrous phases. During the murine estrous cycle variations can occur in the morphology of the vaginal epithelium, the composition and amount of vaginal secretions, the level of neutrophils and the normal flora (Corbeil et al. 1985). During proestrous/estrous phases, uterine and cervical epithelial cells are covered with microvilli overlaid with mucus-like secretions. Little normal bacterial flora is present anywhere in the tract. At metestrous, neutrophils begin to migrate through the epithelium and the bacterial flora becomes extensive. Finally in diestrous, newly regenerated epithelium demonstrates a high percentage of neutrophils but a lack of normal bacterial flora.

In experimental mice, infection with *T. vaginalis* is possible in each phase of the sexual cycle (Coombs *et al.* 1986a, Kazanowska *et al.* 1983) but susceptibility appears to be greatest during proestrous/estrous when few neutrophils or bacteria are present in the vagina. Most mice in the present study were recorded to be in metestrous (56%) prior to inoculation rather than proestrous/estrous (45%). A lack of susceptibility to infection during metestrous may be due to the extensive normal flora and neutrophils present, at that time, which can provide a measure of protection against invading *T. vaginalis*. The reduction in the number of infected mice at day 7 pi may be a result of initial inoculations occurring while mice were in metestrous. *T. vaginalis* inoculated during metestrous may survive neutrophil and bacterial competition for the first 3 days, given the large initial inoculum concentrations, but possibly could not survive to day 7 after encountering abundant neutrophils in both metestrous and

diestrous. Mice infected for over two weeks were generally found to be in proestrous/estrous at the time of inoculation. Inoculations during these phases of low bacterial and neutrophil populations may have allowed enough time for *T. vaginalis* colonization and adaptation prior to the influx of bacteria and neutrophils at metestrous. Equally important in susceptibility to infection may be the concentration of lactoferrin, an iron-binding protein present in the vagina. In women, lactoferrin, in vaginal mucus, is under hormonal control with highest concentrations just after menses and low amounts detected prior to menses (Cohen *et al.* 1987). If a cyclic presentation of lactoferrin occurs in the mouse, it may also increase susceptibility to *T. vaginalis* since the parasite requires iron for its normal metabolism.

It appears that although estrogen treatment is required for successful infectivity by *T. vaginalis*, its administration does not guarantee progression into the proestrous/estrous phases. Despite identical procedures in administration, mice appear to have individual responses to estrogen treatment. The varied susceptibility of mice to *T. vaginalis* does not negate the usefulness of the model in studying trichomoniasis, but does make clear the inadequacies which must be considered prior to utilization of the model.

#### The Vaginal Flora and pH in BALB/c Mice

Employing the mouse model to study trichomoniasis required an understanding of the murine vaginal flora. Only one previous study, that demonstrated relative amounts of vaginal flora in the mouse during the estrous

cycle, has been reported (Corbeil *et al.* 1985). This study, lacking in culturing techniques, merely quantitated the vaginal bacterial flora of the mouse without identification of the bacteria. Identification of the vaginal flora and pH in mice was therefore considered important.

The vaginal flora of mice was found to consist primarily of *Staphylococcus aureus* and Group D Streptococci. Enteric bacilli and *Lactobacillus* species were moderate in their frequency of isolation while both coagulase negative Staphylococci and anaerobic organisms were infrequently isolated. In relation to the genital flora apparent in women, aside from the relatively high incidence of alpha-hemolytic (Group D) Streptococci, murine and human genital tracts exhibited a wide divergence. The high frequency of *Staphylococcus aureus* in the mouse coupled with a low incidence of coagulase negative Staphylococci was in direct opposition to the situation documented in women (Hill *et al.* 1984, Larsen and Galask 1980). The incidence of enteric bacilli, particularly *Proteus mirabilis*, was also higher in mice compared to that reported in the human vagina (Bartlett and Polk 1984) but may have been attributed to fecal origin. Another difference between murine and human genital flora was the occurrence of anaerobic organisms. Mice had an extremely low incidence of anaerobic species while in the human vagina anaerobes frequently outnumber aerobic species in incidence (Larsen and Galask 1980,1982). Perhaps the most striking difference in vaginal flora between mice and women involved *Lactobacillus* species. Although dominant in a woman's vagina, *Lactobacillus* was harboured by only a small percentage of mice. The differences in flora may have been

mediated by a variation in vaginal pH. Women exhibit an acidic vaginal pH while that of mice has been recorded near neutral. Although variation in vaginal flora and pH was considerable between humans and mice, studies involving other laboratory animals (Koiter *et al.* 1977, Larsen *et al.* 1976, Larsen *et al.* 1977, Skangalis *et al.* 1979) were found to be no better in their comparisons to the human vagina. It appears that only humans harbour a high incidence of vaginal *Lactobacillus* and a corresponding acidic vaginal pH.

Although major deviations exist between the genital flora of women and that of laboratory animals, the mouse still appears to be the most cost efficient and simple model in which to study genital tract disease *in vivo*. The major limitations of this model appear only to lie in the techniques employed to determine vaginal flora and pH. Vaginal washing, to establish bacterial flora, may be inaccurate if care is not taken in avoiding fecal contamination, or if adherent bacterial species cannot be removed from the vaginal epithelial walls during the wash. Measurement of vaginal pH may also be imprecise if, after urination, residual liquid remains present in or around the vaginal area prior to sampling.

### The Influence of Various Factors on Murine Genital Flora and pH

#### Estrogen Treatment

Estrogenization has been shown to be a critical factor in achieving *T. vaginalis* infectivity in inoculated mice. It was not known, however, if estrogen treatment effects vaginal flora and if this permits enhanced infectivity.

Comparisons of vaginal flora from both estrogenized and untreated mice revealed the same populations of vaginal flora and no differences in the frequency of isolation of any vaginal species. Estrogenization appeared only to lower the vaginal pH and increase the number of bacterial species present in the mouse vagina. The lower vaginal pH may have been a result of a slight rise in the *Lactobacillus* population linked to increased glycogen deposits in the vaginal epithelium as a result of estrogen treatment. Lower pH may have also been related to the estrous cycle, since reports of low pH in rats has been made during proestrous/estrous (Koiter *et al.* 1977). Higher numbers of vaginal species isolated per mouse may also relate to increased vaginal glycogen levels and subsequent higher nutrient levels. Increased bacterial counts and species numbers have been documented for both mice and rats in estrous (Corbeil *et al.* 1985, Koiter *et al.* 1977). Estradiol administration, which induces estrous in rats, has also been shown to elevate bacterial counts (Larsen *et al.* 1977). The effects of estrogen on the microbial flora is believed to be indirect and possibly a result of alterations in vaginal cytology, mucus secretions and vascularity of the vagina. In the mouse model, estrogenization did not appear to produce any significant changes in the vaginal flora which might have permitted enhanced *T. vaginalis* infectivity.

#### Media Inoculation

*T. vaginalis* was suspended in medium at the time of intravaginal inoculation. Since floral alterations produced by inoculation could have been a

consequence of either the parasite or the medium, inoculation of medium was tested separately to determine its effects on vaginal flora. Experiments employed two types of media, agar-free (Cappuccinelli *et al.* 1974) and agar containing (Coombs *et al.* 1986). Both agar and agar-free medium inoculated mice demonstrated similar bacterial species with no differences found in terms of the incidence of bacterial species or the number of vaginal species isolated per mouse. This result implied that either media could be employed in the inoculation procedure without the possibility of the agar, used in one, directly effecting the vaginal flora.

Both types of media inoculated mice also revealed similar frequencies in the isolation of vaginal species to that recorded in untreated mice. In comparison, only the number of bacterial species isolated per mouse was considerably higher in media inoculated than in untreated mice. This result is interesting considering the fact that media inoculated mice had been estrogenized 9 days prior to vaginal sampling. In this length of time, mice should have returned to a normal estrous cycle and resembled untreated mice in terms of the number of vaginal species isolated. The high findings in this experiment may imply that medium inoculation enhanced bacterial growth and possibly allowed isolation of bacterial species previously too small in numbers to detect. Additional nutrients in the medium may have permitted this increased growth of small populations. Although medium inoculations did not appear to alter vaginal flora, its effect on vaginal pH was not determined.

### *T. vaginalis* Inoculations

After establishing the influence of estrogenization and media inoculation on vaginal flora, the direct effects produced by *T. vaginalis* were investigated over a 2 week course of infection. The initial study employed a single estrogen treatment of the mice prior to inoculation. Mice presented a low level of initial infectivity and coupled with a subsequent drop at day 7 posed a problem since large sample sizes of experimental groups could not be maintained. Trends in the general populations may have therefore not been evident from the small sample sizes employed.

The frequency of isolation of different vaginal species was similar in control, infected and cleared groups of mice except for one incident detected in cleared mice between days 5 and 9. The frequency of Lactobacillus species dropped significantly during this time period and may have implied that in combating *T. vaginalis* infection the Lactobacillus population was for some reason depleted. *T. vaginalis* did not appear to affect either the number of bacterial species isolated per mouse or the vaginal pH of the mice.

The significance of any results produced in this study became questionable, however, when comparisons were made to previously studied mice. Vaginal pH of mice employed in this investigation closely resembled those of untreated mice. Although pH may have been lowered directly after estrogenization, it was not possible to determine if the subsequent rise was due to the return of the normal estrous cycle, the effects of media or the infection with *T. vaginalis*. The number of bacterial species isolated per mouse was also

higher in control, infected and cleared mice as opposed to untreated mice. Since media inoculations could mediate such a change, the ability to discern if the increase reported in the study was caused by medium or by the presence of the parasite was not possible. Perhaps the most unexpected result was the high frequency of *Lactobacillus* found in all experimental mice at day 5. Whereas the high frequency of *Lactobacillus* in infected and cleared mice may have implied some possible interaction between parasite and flora, the equally high *Lactobacillus* levels in control mice cast doubts as to the validity of any conclusions drawn. Control mice should have been similar to media inoculated mice in their frequency of isolation of *Lactobacillus* since both underwent identical procedures.

With both an unexplained higher incidence of *Lactobacillus* and small sample sizes studied, it was considered beneficial to conduct a second experiment. In this study, weekly estrogenization was employed in an attempt to maintain a relatively low vaginal pH. Initial infectivity in this experiment was higher than that of the first so analysis of larger sample sizes was possible. Despite larger populations, however, the recorded results were similar to those of the first *T. vaginalis* infection experiment. The frequency of isolation of vaginal species was similar for control, infected and cleared groups with three exceptions. Two of these situations, one involving *Staphylococcus aureus* and the other enteric bacilli, may have been a reflection of bacterial adhesion related to sexual cycling or fecal contamination. The third incident was the significant decline in *Lactobacillus* frequencies from days 5 to 16 in cleared mice. This

result coincided with that reported in the previous experiment and may have again implied that in clearing *T. vaginalis* infection, the Lactobacillus population was adversely affected by an unknown mechanism.

In relation to untreated mice, the results of *T. vaginalis* inoculations were similar. Weekly estrogenization, even in control groups, did not appear to lower the vaginal pH and it remained similar to that recorded in untreated mice. Whether the high vaginal pH was caused by the medium or infection with the parasite could not be determined. Interesting to note, however, was that in both *T. vaginalis* inoculation experiments, control mice at day 4 demonstrated a vaginal pH similar to that of estrogenized mice while cleared and infected mice exhibited a significantly higher pH (single estrogen  $p=0.039$  infected and  $p=0.01$  cleared, weekly estrogen  $p=0.002$  infected and  $p=0.014$  cleared Student t-test). This may imply that at least initial inoculation of *T. vaginalis* could increase vaginal pH to levels higher than what might be achieved simply by medium inoculation. Numbers of vaginal species isolated per mouse continued to remain high but this effect may have been solely influenced by the inoculation of medium. If *T. vaginalis* infection did increase the number of bacterial species isolated per mouse, it could not be ascertained in these studies. Finally, there was the aberrantly high incidence of Lactobacillus recorded in the *T. vaginalis* infection experiments. Control mice in these studies were treated in an identical manner to mice inoculated with medium. One possible explanation of this result may have involved the relatively small numbers of mice employed in *T. vaginalis* infection experiments. If, as in women, both substantial variations occur between

individuals, and different subpopulations exist in sampling groups, this may partially account for some of the discrepancy in the results.

In lieu of this major deviation, all that could be concluded definitively was that *T. vaginalis* infection did not appear to significantly alter vaginal flora. Until determination of the influence of media inoculation on vaginal pH, the effects the parasite may have on vaginal pH can not be elucidated. Contrary to the situation encountered in women, *T. vaginalis* did not appear to have an effect on vaginal flora in the mouse model. This situation apparent in mice may be related to the divergence in genital flora and pH between the two species. If the mouse flora could be manipulated to parallel the situation found in women, this may allow the model to become an even greater *in vivo* tool in ascertaining the interactions between the parasite and the vaginal flora.

#### *In Vitro* Interactions Between *T. vaginalis* and *L. acidophilus*

In conjunction with *in vivo* experiments monitoring alterations in flora during *T. vaginalis* infection, *in vitro* interactions between *T. vaginalis* and Lactobacillus were also examined. In the healthy vagina, Lactobacillus predominates, but during *T. vaginalis* infections a reduction to total loss in this bacterial species has been demonstrated (Jirovec and Petru 1968). The mechanism employed by *T. vaginalis* to mediate this reduction has, however, yet to be defined. Two theories have been proposed in attempts to define this mechanism of interaction. The first involves the phagocytosis of Lactobacilli in the vagina by invading *T. vaginalis*. The second possibility involves indirect

effects on the *Lactobacillus* population mediated by the action of *T. vaginalis* secreted products. *In vitro* competitive assays were employed to evaluate the action of *T. vaginalis* isolate 263 on *Lactobacillus acidophilus*, a predominant vaginal *Lactobacillus* strain which has been reported to secrete inhibitory substances against bacteria (Giorgi *et al.* 1987, Magliano *et al.* 1984, Mehta *et al.* 1983a, 1983b).

*L. acidophilus* incubated with equal or greater concentrations of *T. vaginalis* grew at a significantly higher rate than controls. Coincubation with *T. vaginalis* concentrations initially lower than *L. acidophilus* showed no changes in the growth rate compared to controls. All *L. acidophilus* concentrations, whether control or ratios, achieved peak concentrations of log 8 *L. acidophilus*/mL and decreased pH to 4.2 by the end of the assay. Interesting was the ability of *L. acidophilus* to exhibit increased proliferation rates with equivalent or higher concentrations of *T. vaginalis*. In previous coincubation studies between *T. vaginalis* and *Escherichia coli* or *Staphylococcus aureus* no reports were made of changes in bacterial growth (Horwatt 1985). *T. vaginalis* appeared to induce a pH independent, high proliferation rate of *L. acidophilus*. *T. vaginalis* may provide additional nutrient sources that allow increased proliferation of *L. acidophilus* in certain phases of its growth cycle. This can only be speculation, however, until experiments employing *T. vaginalis* spent medium are used to grow *L. acidophilus*. If a secreted nutritional source is the reason for enhanced *L. acidophilus* growth, it should be demonstrated by this work.

*T. vaginalis* growth was also significantly different when coincubated with *L. acidophilus*. Regardless of the initial inoculum size, *T. vaginalis*, in experimental ratio studies, had a higher initial growth rate and subsequently achieved early peak concentrations. The peak concentrations attained were, however, significantly less than those produced by controls. The pH of the media was considered to play an integral role in this unique growth pattern of *T. vaginalis*. In ratios, a drop in the media's pH to 4.5 appeared to correspond to *T. vaginalis* cell death. Control *T. vaginalis* media did not drop to a pH of under 5 throughout the assay. The general trend of rapid early growth, and lower peak concentrations when *T. vaginalis* was coincubated with *L. acidophilus*, was similar to reports of *T. vaginalis* growth in coincubations with *E. coli*, *S. aureus* and *Candida albicans* (Horwatt 1985, Kurnatowska 1983). Explanations as to why this trend occurs may include two separate components. First, the increased proliferation rate of *T. vaginalis* when coincubated with *L. acidophilus* may involve phagocytosis of the bacteria as an additional nutrient source (Francioli *et al.* 1983, Street *et al.* 1984). Phagocytosis of *L. acidophilus* by *T. vaginalis* may occur during the early portions of the assay, but may not be reflected in *L. acidophilus* growth due to its rapid doubling time as compared to that of *T. vaginalis*. The second component involves *T. vaginalis* pH-dependent growth. *L. acidophilus* production of lactic acid in the experimental ratios may have accounted for the lower peak concentrations of *T. vaginalis* and rapid death at pH 4.5. To verify if *T. vaginalis* growth is strictly pH-dependent or is actually mediated by the direct presence of the bacteria, *L. acidophilus*

spent media at various pH values could be employed in growing *T. vaginalis*. Likewise, the assay could be repeated controlling for pH throughout the incubation period. If pH is implicated in changing *T. vaginalis* growth patterns, experiments involving sandwich plate inhibition assays (McGroarty and Reid 1988, Reid *et al.* 1988) with solid *T. vaginalis* media (Philip *et al.* 1987) may have to be devised to determine other possible interactions between the two microorganisms.

Results of the *in vitro* competitive assays appear to be directly opposed to the situation apparent in the vagina during *T. vaginalis* infection. *In vitro*, *L. acidophilus* remained unaffected or actually exhibited enhanced growth in the presence of *T. vaginalis*. It was the parasite that showed adverse effects when incubated with the bacteria. This could imply that *in vivo* the direct physical presence of *T. vaginalis* may not be the factor involved in the decline of vaginal Lactobacilli. The recent isolation and characterization of both a cell-detaching factor and proteases secreted by *T. vaginalis* (Garber *et al.* 1989a, Garber and Lemchuk-Favel 1989b) may be one of several indirect factors affecting the Lactobacillus population.

### Importance of Further Studies

Although *T. vaginalis* is one of the most commonly encountered genital pathogens, its pathogenic mechanisms are far from well characterized. Being considered a "nuisance" infection which could be easily treated with imidazole drugs may have accounted for lack of interest in *T. vaginalis*. Only upon recent

associations with other genital tract diseases and increasing numbers of resistant strains has the pathogenicity of *T. vaginalis* begun to be explored. The establishment of a mouse model, and initial studies of the murine genital flora will hopefully act as base for future studies involving *T. vaginalis* effects on vaginal flora. The interaction between *T. vaginalis* and flora may be a critical element both in the establishment and maintenance of infection. The eventual elucidation of mechanisms involved in altering vaginal flora, in particular Lactobacilli, may not only provide a greater understanding of the pathogenesis of *T. vaginalis* but may also be of clinical relevance in the development of vaccines (Gombosova *et al.* 1986, Karkut 1984, Muller and Salzer 1983, Ngumbi and Nyakeri 1984, Ruttgers and Lorenz 1982).

### Conclusions

In summary, several of the research objectives outlined in the project were fulfilled. It was determined that by employing a cell culture system, *T. vaginalis* could proliferate in the absence of serum. Although growth characteristics were slightly different than in an identical system containing serum, *T. vaginalis* proliferation was evident. McCoy cells utilized in this system did not appear to secrete soluble growth factors for *T. vaginalis*. More likely, the lipoproteins of the eucaryotic cell membrane may be responsible for this proliferation.

With this work, a mouse model for *T. vaginalis* infections was also established. Although mice tended to show individual responses to both estrogen treatment and *T. vaginalis* inoculation, infectivity could be achieved for as long

as 56 days. In order to begin to study the interactions between *T. vaginalis*, vaginal flora and pH in the mouse model, the endemic flora and pH of mice was identified. Other factors, which may have affected vaginal flora or pH during the *T. vaginalis* inoculation procedure, were also investigated. It was found that both estrogenization and media-inoculation produced no apparent effects on individual vaginal species and were only found to increase the number of species isolated per mouse. In the mouse, *T. vaginalis* infection did not appear to alter vaginal flora or pH. *In vitro* studies involving the interaction between *T. vaginalis* and *Lactobacillus acidophilus* also indicated that the parasite had no deleterious effects on the bacteria. *L. acidophilus*, however, did significantly alter *T. vaginalis* growth patterns though this may have been a pH-dependent effect caused by the production of lactic acid from the bacteria.

Although this work has not fully elucidated the mechanism by which *T. vaginalis* alters vaginal flora, it seems to indicate the possibility of indirect factors influencing flora. The secreted products of *T. vaginalis* may be a crucial element of this mechanism.

LIST OF REFERENCES

- ALDERETE, J.F. and E. PEARLMAN. 1984. *Trichomonas vaginalis* cytotoxicity to cell culture monolayers. Br. J. Vener. Dis. 60:99-105.
- ALDERETE, J.F. and G.E. GARZA. 1985. Specific nature of *Trichomonas vaginalis* parasitism of host cell surfaces. Infect. Immun. 50(3):701-708.
- ALDERETE, J.F., P. DEMES, A. GOMBOSOVA, M. VALENT, M. FABUSOVA, A. JANOSKA, J. STEFANOVIC and R. ARROYO. 1988a. Specific parasitism of purified vaginaepithelial cells by *Trichomonas vaginalis*. Infect. Immun. 56(10):2558-2562.
- ALDERETE, J.F. and G.E. GARZA. 1988b. Identification and properties of *Trichomonas vaginalis* proteins involved in cytoadherence. Infect. Immun. 56(1):28-33.
- BARBAROWSKI, H. 1966. *Trichomonas vaginalis* Donne culture on a medium without serum. An attempt to replace serum with hen-egg homogenate. Wiad. Parazytol. 12:469-470.
- BAREFOOT, S.F. and T.R. KLAENHAMMER. 1984. Purification and characterization of the *Lactobacillus acidophilus* bacteriocin Lactacin B. Antimicrob. Agents Chemother. 26(3):328-334.
- BARTLETT, J.G. and B.F. POLK. 1984. Bacterial flora of the vagina: quantitative study. Reviews of Infect. Dis. 6(S1):S67-S72.
- BREMNER, A.F., G.H. COOMBS and M.J. NORTH. 1986. Studies on trichomonad pathogenicity: The effect of *Trichomonas vaginalis* on mammalian cells and mice. Acta Universitatis Carolinae-Biologica 30:381-386.
- BROMKE, B.J. 1986. A serum-free, lipid-supplemented medium for the growth of *Trichomonas vaginalis*. J. Microbiol. Methods 6:55-59.
- BROWN, M.T. 1972. Trichomoniasis. Practitioner 209:639-644.
- CAPPUCCINELLI, P., C. LATTES, I. CAGLIANI and A.N. PONZI. 1974. Features of intravaginal *Trichomonas vaginalis* infection in the mouse and the effect of oestrogen treatment and immunodepression. G. Batteriol. Virol. Immunol. (Microbiol.) 61:31-40.
- CHAN, R.C.Y., A.W. BRUCE and G. REID. 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.

- CHAN, R.C.Y., G. REID, R.T. IRVIN, A.W. BRUCE and J.W. COSTERTON. 1985. Competitive exclusion of uropathogens from human uroepithelial cells by *Lactobacillus* whole cells and cell wall fragments. *Infect. Immun.* 47(1):84-89.
- CHATTOPADHYAY, B. and J.C. TELI. 1984. Isolation of *Gardnerella vaginalis* from routine genito-urinary tract specimens. *J. Infect.* 8:157-162.
- CHRISTIAN, R.T., N.F. MILLER, P.P. LUDOVICH and G.M. RILEY. 1963. A study of *Trichomonas vaginalis* in human cell culture. *Am. J. Obstet. Gynecol.* 85(7):947-954.
- COHEN, M.S., B.E. BRITIGAN, M. FRENCH and K. BEAN. 1987. Preliminary observations on lactoferrin secretion in human vaginal mucus: Variation during the menstrual cycle, evidence of hormonal regulation, and the implications for infection with *Neisseria gonorrhoeae*. *Am. J. Obstet. Gynecol.* 157(5):1122-1125.
- COLLINS, E.B. and P. HARDT. 1980. Inhibition of *Candida albicans* by *Lactobacillus acidophilus*. *J. Dairy Science* 63(5):830-832.
- COOMBS, G.H. and M.J. NORTH. 1983. An analysis of the proteinases of *Trichomonas vaginalis* by polyacrylamide gel electrophoresis. *Parasitol.* 86:1-6.
- COOMBS, G.H., A.F. BREMNER, D.J. MARKHAM, V.S. LATTER, M.A. WALTERS and M.J. NORTH. 1986. Intravaginal growth of *Trichomonas vaginalis* in mice. *Acta Universitatis Carolinae-Biologica* 30:387-392.
- CORBEIL, L.B., A. CHATTERJEE, L. FORESMAN and J.A. WESTFALL. 1985. Ultrastructure of cyclic changes in the murine uterus, cervix and vagina. *Tissue and Cell* 17(1):53-68.
- DAVIS, B., D.D. GLOVER and B. LARSEN. 1984. Analysis of metronidazole penetration into vaginal fluid by reversed-phase high-performance liquid chromatography. *Am. J. Obstet. Gynecol.* 149(7):802-803.
- DEMES, P., A. GOMBOSOVA, M. VALENT, H. FABUSOVA and A. JANOSKA. 1988. Fewer *Trichomonas vaginalis* organisms in vaginas of infected women during menstruation. *Genitourin. Med.* 64:22-24.
- DIAMOND, L.S. 1957. The establishment of various trichomonads of animals and man in axenic cultures. *J. Parasitol.* 43:488-490.
- DIAMOND, L.S., D.R. HARLOW, and C.C. CUNNICK. 1978. A new medium for the axenic cultivation of *Entamoeba histolytica* and other Entamoeba. *Trans. R. Soc. Trop. Med. Hyg.* 72:431-432.

- DIAMOND, L.S. 1986. In vitro cultivation of the *Trichomonadidae*: A state of the art review. *Acta Universitatis Carolinae - Biologica* 30:221-228.
- DRAKE, S.M., B.A. EVANS and A. GERKEN. 1980. Vaginal pH and microflora related to yeast infections and treatment. *Br. J. Vener. Dis.* 56:107-110.
- ESCHENBACH, D.A., P.R. DAVICK, B.L. WILLIAMS, S.J. KLEBANOFF, K. YOUNG-SMITH, C.M. CRITCHLOW, K.K. HOLMES. 1989. Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. *J. Clin. Microbiol.* 27(2):251-256.
- FARRIS, V.K. and B.M. HONIGBERG. 1970. Behaviour and pathogenicity of *Trichomonas vaginalis* Donne in chick liver cell cultures. *J. Parasitol.* 56(5):849-882.
- FISHER, I. and R.S. MORTON. 1969. Epididymitis due to *Trichomonas vaginalis*. *Br. J. Vener. Dis.* 45:252-253.
- FOUTS, A.C. AND S.J. KRAUS. 1980. *Trichomonas vaginalis*: Reevaluation of its clinical presentation and laboratory diagnosis. *J. Infect. Dis.* 141:137-143.
- FOX, R.R. and C.W. LAIRD. 1970. Sexual cycles. In *Reproduction and Breeding Techniques for Laboratory Animals*. Edited by E.S.E. Hafez, Lea and Febiger, Philadelphia pp 107-122.
- FRANCIOLI, P., H. SHIO, R.B. ROBERTS and M. MULLER. 1983. Phagocytosis and killing of *Neisseria gonorrhoeae* by *Trichomonas vaginalis*. *J. Infect. Dis.* 147(1):87-94.
- FROST, J.K. and B.M. HONIGBERG. 1962. Comparative pathogenicity of *Trichomonas vaginalis* and *Trichomonas gallinae* to mice. II. Histopathology of subcutaneous lesions. *J. Parasitol.* 48(6):898-918.
- GALASK, R.P. 1988. Vaginal colonization by bacteria and yeast. *Am. J. Obstet. Gynecol.* 158(4):993-995.
- GARBER, G.E., E.M. PROCTOR and W.R. BOWIE. 1986. Immunogenic proteins of *Trichomonas vaginalis* as demonstrated by the immunoblot technique. *Infect. Immun.* 51(1):250-253.
- GARBER, G.E., L. SIBAU, R. MA, E.M. PROCTOR, C.E. SHAW and W.R. BOWIE. 1987. Cell culture compared with broth for detection for *Trichomonas vaginalis*. *J. Clin. Microbiol.* 25(7):1275-1279.
- GARBER, G.E., L.T. LEMCHUK-FAVEL, and W.R. BOWIE. 1989a. Isolation of a cell-detaching factor of *Trichomonas vaginalis*. *J. Clin. Microbiol.* 27(7):1548-1553.

- GARBER, G.E. and L.T. LEMCHUK-FAVEL. 1989b. Characterization and purification of extracellular proteases of *Trichomonas vaginalis*. Can. J. Microbiol. 35(10):903-909.
- GARDNER, W.A., D.E. CULBERSON, J.M. SCIMECA, A.G. BRADY, F.F. PINDAK and C.R. ABEE. 1987. Experimental genital trichomoniasis in the squirrel monkey (*Saimiri sciureus*). Genitourin. Med. 63:188-191.
- GIORGI, A., S. TORRIANI, F. DELLAGLIO, G. BO, E. STOLA and L. BERNUZZI. 1987. Identification of vaginal Lactobacilli from asymptomatic women. Microbiologica 10:377-384.
- GOMBOSOVA, A., P. DEMES and M. VALENT. 1986. Immunotherapeutic effect of the Lactobacillus vaccine, SolcoTrichovac, in trichomoniasis is not mediated by antibodies cross reacting with *Trichomonas vaginalis*. Genitourin. Med. 62:107-110.
- GRICE, A.C. 1974. Vaginal infection causing spontaneous rupture of the membranes and premature delivery. Aust. N.Z. J. Obstet. Gynecol. 14:156-158.
- HANNA, N.F., D. TAYLOR-ROBINSON, M. KALIODIKI-KARAMANOLI, J.R.W. HARRIS and I.R. McFADYEN. 1985. The relation between vaginal pH and the microbiological status in vaginitis. Br. J. Obstet. Gynecol. 92:1267-1271.
- HEATH, J.P. 1981. Behaviour and pathogenicity of *Trichomonas vaginalis* in epithelial cell cultures. Br. J. Vener. Dis. 57:106-117.
- HIGIER, J., J. TUROWSKI, B. SKARZYNSKI and B. BLONSKA. 1983. Effects of *Trichomonas vaginalis* infection and mixed infections on healing of perineal and vaginal wounds in puerperium. Wiad. Parazytol. 29:71-75.
- HILL, G.B., D.A. ESCHENBACH and K.K. HOLMES. 1984. Bacteriology of the vagina. Scand. J. Urol. Nephrol. 86:23-39.
- HOGUE, M.J. 1943. The effect of *Trichomonas vaginalis* on tissue-culture cells. Am. J. Hyg. 37:142-152.
- HOLLANDER, D.H. 1983. The pathology of human *Trichomonas vaginalis* vaginitis. Wiad. Parazytol. 29:57-62.
- HONIGBERG, B.M., M.C. LIVINGSTON and J.K. FROST. 1966. Pathogenicity of fresh isolates of *Trichomonas vaginalis*: "The Mouse Assay" versus clinical and pathologic findings. Acta. Cytol. 10:353-361.
- HONIGBERG, B.M. 1978. Trichomonads of importance in human medicine. In Parasitic Protozoa Vol. 2. Edited by J.P. Kreier. Academic Press, New York. pp 275-454.

- HONIGBERG, B.M., P.K. GUPTA, M.R. SPENCE, J.K. FROST, K. KUCZYNSKA, L. CHOROMANSKI, and A. WARTON. 1984. Pathogenicity of *Trichomonas vaginalis*: Cytopathologic and histopathologic changes of the cervical epithelium. *Obstet Gynecol.* 64(2):179-184.
- HORWATT, E. 1985. The analysis of growth curves of *Trichomonas vaginalis* in the presence of *Staphylococcus aureus* and *Escherichia coli*. *Wiad. Parazytol.* 31:123.
- IWASAKA, T., T. WADA and H. SUGIMORI. 1986. Enhancement of colonization of *Ureaplasma urealyticum* in the mouse genital tract by estrogen treatment. *Am. J. Obstet. Gynecol.* 155(5):1124-1127.
- JARECKI-BLACK, J.C., W.B. LUSHBAUGH, L. GOLOSOV and A.B. GLASSMAN. 1988. *Trichomonas vaginalis*: Preliminary characterization of a sperm motility inhibiting factor. *Ann. Clin. Lab. Science* 18(6):484-489.
- JIROVEC, O. and M. PETRU. 1968. *Trichomonas vaginalis* and trichomoniasis. *Adv. Parasitol.* 6:117-188.
- JOHNSON, G. and R.E. TRUSSELL. 1943. Experimental basis for the chemotherapy of *Trichomonas vaginalis* infestations. *Proc. Soc. Exp. Biol. Med.* 54:2245-2249.
- KARKUT, G. 1984. Effects of Lactobacillus vaccine on female genital infections. *Geburtshilfe Und Frauenheilkunde* 44(5):311-314.
- KEITH, L.G., J. FRIBERG, R. BAILEY and G.S. BERGER. 1986. The possible role of *Trichomonas vaginalis* as a "vector" for the spread of other pathogens. *Int. J. Fertility* 31(4):272-277.
- KRIEGER, J.N. 1981. Urologic aspects of trichomoniasis. *Invest. Urol.* 18:411-417.
- KRIEGER, J.N., J.I. RAVDIN and M.F. REIN. 1985. Contact-dependent cytopathogenic mechanisms of *Trichomonas vaginalis*. *Infect. Immun.* 50(3):778-786.
- KOITER, T.R. M.P. HAZENBERG and T. VAN DER SCHOOT. 1977. Regulation of the bacterial microflora of the vagina in cyclic female rats. *J. Exp. Zool.* 202:121-128.
- KUBERSKI, T. 1978. Evaluation of the indirect hemagglutination technique for the study of *Trichomonas vaginalis* infections, particularly in men. *Sex. Transm. Dis.* 5:97-102.
- KUBERSKI, T. 1980. *Trichomonas vaginalis* associated with nongonococcal urethritis and prostatic. *Sex. Transm. Dis.* 7:135-136.

- KUCZYNSKA, K., L. CHOROMANSKI and B.M. HONIGBERG. 1984. Comparison of virulence of clones of two *Trichomonas vaginalis* strains by the subcutaneous mouse assay. *Z. Parasitenkd.* 70:141-146.
- KULDA, J., B.M. HONIGBERG, J.K. FROST and D.H. HOLLANDER. 1970. Pathogenicity of *Trichomonas vaginalis*. *Am. J. Obstet. Gynecol.* 108(6):908-918.
- KURNATOWSKA, A. and E. HORWATT. 1983. Quantitative study of *Trichomonas vaginalis* population in vitro with *Candida albicans*. *Wiad. Parazytol.* 29:13-18.
- KUZANOWSKA, W., K. KUCZYNSKA and R. SKRZYPIEC. 1983. Pathology of *Trichomonas vaginalis* infection in experimental animals. *Wiad. Parazytol.* 29:63-65.
- LARSEN, B., A.J. MARKOVETZ and R.P. GALASK. 1976. The bacterial flora of the female rat genital tract. *Proc. Soc. Exp. Biol. Med.* 151:571-574.
- LARSEN, B., A.J. MARKOVETZ and R.P. GALASK. 1977. Role of estrogen in controlling the genital microflora of female rats. *Applied Environ. Microbiol.* 34(5):534-540.
- LARSEN, B. and R.P. GALASK. 1980. Vaginal microbial flora: Practical and theoretic relevance. *Obstet. Gynecol.* 55(5):100S-113S.
- LARSEN, B. and R.P. GALASK. 1982. Vaginal microbial flora: Composition and influences of host physiology. *Ann. Internal Med.* 96:926-930.
- LINSTEAD, D. 1979. Evolution of a serum-free media for the cultivation of the protozoan *Trichomonas vaginalis*. *Parasitol.* 79:xlii.
- LINSTEAD, D. 1981a. New defined and semi-defined media for cultivation of the flagellate *Trichomonas vaginalis*. *Parasitol.* 83:125-137.
- LINSTEAD, D. 1981b. Further studies on the cultivation of *Trichomonas vaginalis* in a defined medium. *Parasitol.* 81:R18-19.
- LOCKWOOD, B.C., M.J. NORTH and G.H. COOMBS. 1984. *Trichomonas vaginalis*, *Tritrichomonas foetus* and *Trichomitus batrachorum*: Comparative proteolytic activity. *Exp. Parasitol.* 58:245-253.
- LOCKWOOD, B.C., M.J. NORTH and G.H. COOMBS. 1985. Purification and characterization of proteinases of parasitic protozoan *Trichomonas vaginalis*. *Biochem. Soc. Trans.* 13:336.

- LOCKWOOD, B.C., M.J. NORTH and G.H. COOMBS. 1986. Proteolysis in Trichomonads. *Acta Universitatis Carolinae-Biologica* 30:313-318.
- MAGLIANO, E.M., P. CLERICI, M.L. BESTETTI. 1984. Isolation, assay and biotyping of lactobacillary flora of the vagina. *Bollettino Dell Istituto Sieroterapico Milanese* 63(4):331-337.
- MARDH, P-A. and L.V. SOLTESZ. 1983. In vitro interactions between Lactobacilli and other microorganisms occurring in the vaginal flora. *Scand. J. Infect. Dis. Suppl.* 40:47-51.
- MARTINOTTI, M.G., P. MARTINETTO and D. SAVOIA. 1986. Adherence of *Trichomonas vaginalis* to cell culture monolayers. *Eur. J. Clin. Microbiol.* 5(3):320-323.
- McGROARTY, J.A. and G. REID. 1988. Detection of a Lactobacillus substance that inhibits *Escherichia coli*. *Can. J. Microbiol.* 34:974-978.
- MEHTA, A.M., K.A. PATEL and P.J. DAVE. 1983a. Isolation and purification of an inhibitory protein from *Lactobacillus acidophilus* AC<sub>1</sub>. *Microbios* 37:37-43.
- MEHTA, A.M., K.A. PATEL and P.J. DAVE. 1983b. Purification and properties of the inhibitory protein isolated from *Lactobacillus acidophilus* AC<sub>1</sub>. *Microbios* 38:73-81.
- MEHTA, P.V. 1982. Vaginal flora. A dynamic ecosystem. *J. Reprod. Med.* 27(8):455-458.
- MULLER, G. and H. SALZER. 1983. Long-term experience of therapy and prophylaxis of unspecific vaginal infections with a Lactobacillus vaccine. *Wiener Klinische Wochenschrift* 95(11):371-374.
- NAKABAYASHI, T. and A. MIYATA. 1967. On the isolation of *Trichomonas vaginalis* with milk media. *Trop. Med.* 9:79-88.
- NAKABAYASHI, T. and A. MIYATA. 1968. Examination of a milk medium in the cultivation of *Trichomonas vaginalis*. *Trop. Med.* 10:39-49.
- NGUMBI, P.M. and L.N. NYAKERI. 1984. Clinical experience with SolcoTrichovac in the treatment of vaginal trichomoniasis. *East African Medical J.* 61(5):372-375.
- NIELSEN, M.H. and R. NIELSEN. 1975. Electron microscopy of *Trichomonas vaginalis* Donne: Interaction with vaginal epithelium in human trichomoniasis. *Acta. Path. Microbiol. Scand. Sect. B.* 83:305-320.

- OHASHI, H., N. KOSAKAI and T. YAMADA. 1981. Clinical and bacteriological study on microbial flora in the vagina:(4) The influences of Lactobacilli to Group B Streptococcus (GBS) and other flora and the variations of vaginal microbial flora by age. *Kansenshogaku Zasshi- J. Jap. Assoc. Infect. Dis.* 55(5):373-380.
- OHASHI, H. 1982. Clinical and bacteriological study on microbial flora in the vagina: (5) Variations of vaginal microbial flora according to pregnancy and vaginitis. *Kansenshogaku Zasshi- J. Jap. Assoc. Infect. Dis.* 56(6):647-654.
- OPACKI, W. and E. DYNER. 1983. Difficulties in cytohormonal evaluation of vaginal smears in trichomonadosis. *Wiad. Parazytol.* 29:79-80.
- PAAVONEN, J. 1983. Physiology and ecology of the vagina. *Scand. J. Infect. Dis.* S40:31-35.
- PAULSON, J.P., S. LETO and P. ASMAR. 1985. *Trichomonas vaginalis* in human reproduction. *In* *Infection in Reproductive Health* Vol. 1. Edited by L.G. Keith, G.S. Berger and D.A. Edelman. MTP Press, Hingham, MA. pp137-142.
- PETERSON, K.M. and J.F. ALDERETE. 1982. Host plasma proteins on the surface of pathogenic *Trichomonas vaginalis*. *Infect. Immun.* 37:755-762.
- PETERSON, K.M. and J.F. ALDERETE. 1983. Acquisition of  $\alpha_1$ -antitrypsin by a pathogenic strain of *Trichomonas vaginalis*. *Infect. Immun.* 40:640-646.
- PETERSON, K.M. and J.F. ALDERETE. 1984. Selective acquisition of plasma proteins by *Trichomonas vaginalis* and human lipoproteins as a growth requirement for this species. *Molecular Biochem. Parasitol.* 12:37-48.
- PILAWSKI, Z. and J. MALECHA. 1983. Trichomonadosis and the pregnancy: Therapeutic problems. *Wiad. Parazytol.* 29:187-190.
- PINDAK, F.F., W.A. GARDNER, Jr., and M. MORA de PINDAK. 1986. Growth and cytopathogenicity of *Trichomonas vaginalis* in tissue cultures. *J. Clin. Microbiol.* 23(4):672-678.
- PIOT, P., E. VAN DYCK, P. GODTS and J. VANDERHEYDEN. 1982. The vaginal microbial flora in non-specific vaginitis. *Eur. J. Clin. Microbiol.* 1(5):301-306.
- RASMUSSEN, S.E., M.H. NIELSEN, I LIND and J.M. RHODES. 1986. Morphological studies of the cytotoxicity of *Trichomonas vaginalis* to normal human vaginal epithelial cells in vitro. *Genitourin. Med.* 62:240-246.
- REID, G., R.L. COOK and A.W. BRUCE. 1987. Examination of strains of Lactobacilli for properties that may influence bacterial interference in the urinary tract. *J. Urol.* 138:330-335.

- REID, G., J.A. MCGROARTY, R. ANGOTTI and R.L. COOK. 1988. Lactobacillus inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. *Can. J. Microbiol.* 34:344-351.
- REIN, M.F. and T.A. CHAPEL. 1975. Trichomoniasis, candidiasis, and the minor venereal diseases. *Clin. Obstet. Gynecol.* 18:73-88.
- REIN, M.F. and M. MULLER. 1984. *Trichomonas vaginalis*. In *Sexually Transmitted Diseases*. Edited by K.K. Holmes, P-A. Mardh, P.F. Sparling and P.J. Wiesner. McGraw-Hill, New York. pp525-536.
- RUTTIGERS, H. and U. LORENZ. 1982. Clinical experience using SolcoTrichovac in the treatment of Trichomonad infections in women. *Geburtshilfe Und Frauenheilkunde* 42(10):736-738.
- SABINE, D.B. 1963. An antibiotic-like effect of *Lactobacillus acidophilus*. *Nature* 199:811.
- SAIGH, J.H., C.C. SANDERS and W.E. SANDERS, Jr. 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(2):704-710.
- SAMUELS, R. 1965. Growth of axenic trichomonads in a serum-free medium. In *Progress in Protozoology, 2<sup>nd</sup> International Conference of Protozoology*. Excerpta Medica Foundation. International Congress Series, no. 91, pp200.
- SANDERS, C.C., W.E. SANDERS and J.E. FAGNANT. 1982. Toxic shock syndrome: An ecologic imbalance within the genital microflora of women. *Am. J. Obstet. Gynecol.* 142(8):977-982.
- SAUTTER, R.L. and W.J. BROWN. 1980. Sequential vaginal cultures from normal young women. *J. Clin. Microbiol.* 11(5):479-484.
- SCHMID, G.P., L.C. MATHENY, A.A. ZAIDI, and S.J. KRAUS. 1989. Evaluation of six media for the growth of *Trichomonas vaginalis* from vaginal secretions. *J. Clin. Microbiol.* 27(6):1230-1233.
- SKANGALIS, M., C.E. SWENSON, C.J. MAHONEY and W.M. O'LEARY. 1979. The normal microbial flora of the baboon vagina. *J. Med. Primatol.* 8:289-297.
- SKARIN, A. and J. SYLWAN. 1986. Vaginal Lactobacilli inhibiting growth of *Gardnerella vaginalis*, Mobiluncus and other bacterial species cultured from vaginal content of women with bacterial vaginosis. *Path. Microbiol. Immunol. Scand. Sect B* 94:399-403.

- SOBEL, J.D., P. MYERS, M.E. LEVISON and D. KAYE. 1982. Comparison of bacterial and fungal adherence to vaginal explanted epithelial cells and human vaginal epithelial tissue culture cells. *Infect. Immun.* 35(2):697-701.
- SOENDJOJO, A. and S. PINDA. 1981. *Trichomonas vaginalis* infection of the median raphe of the penis. *Sex. Transm. Dis.* 8:255-257.
- SPRINCE, H. and A.B. KUPFERBERG. 1947. The nutrition of protozoa. I. A simplified medium for the investigation of unknown factors in blood serum essential for the sustained growth of *Trichomonas vaginalis*. *J. Bacteriol.* 53:435-439.
- STREET, D.A., D. TAYLOR-ROBINSON and C.M. HETHERINGTON. 1983. Infection of female squirrel monkeys (*Saimiri sciureus*) with *Trichomonas vaginalis* as a model of trichomoniasis in women. *Br. J. Vener. Dis.* 59:249-254.
- STREET, D.A., C. WELLS, D. TAYLOR-ROBINSON and J.P. ACKERS. 1984. Interaction between *Trichomonas vaginalis* and other pathogenic microorganisms of the human genital tract. *Br. J. Vener. Dis.* 60:31-38.
- TAYLOR-ROBINSON, D. and M. TUFFREY. 1987. Comparison of detection procedures for *Chlamydia trachomatis*, including enzyme immunoassays, in a mouse model of genital infection. *J. Med. Microbiol.* 24:169-173.
- TRAMER, J. 1966. Inhibitory effect of *Lactobacillus acidophilus*. *Nature* 211:204-205.
- TUTTLE, J.P., Jr., T.W. HOLBROOK and F.C. DERRICK. 1977. Interference of human spermatozoal motility by *Trichomonas vaginalis*. *J. Urol.* 118:1024-1025.
- WILKS, M., R.N. THIN and S. TABAQCHALI. 1984. Quantitative bacteriology of the vaginal flora in genital disease. *J. Med. Microbiol.* 18:217-231.
- WILKS, M. and S. TABAQCHALI. 1987. Quantitative bacteriology of the vaginal flora during the menstrual cycle. *J. Med. Microbiol.* 24:241-245.
- WISDOM, A.R. and E.M.C. DUNLOP. 1965. Trichomoniasis: Study of the disease and its treatment. I The disease and its treatment in women. *Br. J. Vener. Dis.* 41:90-96.
- WOOD, J.R., R.L. SWEET, A. CATENA, W.K. HADLEY and M. ROBBIE. 1985. In vitro adherence of *Lactobacillus* species to vaginal epithelial cells. *Am. J. Obstet. Gynecol.* 153(7):740-743.

**APPENDIX****Hemin Solution:**

1.0 g Hemin (Bovine Hemin Type 1 Crystalline, Sigma Chemical Company, St. Louis, Mo) dissolved in 20 ml of 1N NaOH. Volume brought up to 100 mL with distilled H<sub>2</sub>O (dH<sub>2</sub>O). Autoclaved @ 121° c for 15 min. Stock solution 0.01 g/mL.

**Vitamin K<sub>1</sub> Solution:**

0.2 g Vitamin K<sub>1</sub> (Sigma Chemical Company, St. Louis, Mo) added to 20 mL of absolute alcohol. Stock solution 0.01 g/mL.

**Enriched Thioglycollate Medium:**

Bacto Fluid Thioglycollate Medium .....	29.8 g
1% Hemin Solution .....	0.5 mL
1% Vitamin K <sub>1</sub> Solution .....	0.1 mL
dH <sub>2</sub> O .....	1 L

**Blood Agar:**

Bacto TSA Blood Agar Base (Difco Laboratories, Detroit, Michigan) supplemented with 5% defibrinated sheep blood (Qualicum Scientific Ltd., Nepean, Ont).

**Columbia CNA Agar:**

Bacto Columbia CNA Agar (Difco Laboratories, Detroit, Michigan) supplemented with 5% defibrinated sheep blood.

**Chocolate Agar:**

**Hemoglobin Solution** - 5 g Bacto Hemoglobin (Difco Laboratories, Detroit, Michigan) dissolved in 250 mL dH<sub>2</sub>O and autoclaved.

**Medium** - 18 g Bacto GC Medium Base (Difco Laboratories, Detroit, Michigan) dissolved in 250 mL dH<sub>2</sub>O and autoclaved.

The sterile Hemoglobin Solution and Medium were cooled to 50-60° C. 5 mL of Bacto Supplement A (Difco Laboratories, Detroit, Michigan) was then aseptically added to the sterile Medium, and then the sterile Hemoglobin Solution added to the Medium containing the Supplement A. Gentle agitation would then produce a uniform medium which could be dispensed into petri plates.

**CDC-PEA Agar:**

(Center for Disease Control Anaerobic Blood Agar with Phenylethyl Alcohol)

TSA Blood Agar Base .....	40 g
Bacto Agar .....	5 g
Bacto Yeast Extract .....	5 g
*Hemin .....	0.005 g
*L-Cystine (Sigma, St. Louis, Mo) .....	0.4 g
Vitamin K <sub>1</sub> Stock Solution .....	1 mL
dH <sub>2</sub> O .....	1 L

\* Dissolved in 5 mL of 1 N NaOH before being adding to other ingredients

Ingredients were dissolved and the media adjusted to pH 7.5. 2.5 mL of 2-Phenylethanol (BDH Chemicals Ltd, Poole, England) was then added to prevent swarming of Proteus species. The Media was autoclaved, cooled to 50° C, and 5% defibrinated sheep blood was added.

**Brucella Blood Agar**

Bacto Brucella Agar (Difco Laboratories, Detroit, Michigan) was supplemented before autoclaving with 5 ug/mL of the Hemin Solution and after autoclaving with 1 mL Vitamin K<sub>1</sub> Stock Solution and 5% defibrinated sheep blood.