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In vitro Evaluation of Small Molecule Inhibitors and Probiotic Byproducts on Growth and Viability of Vaginal Microorganisms

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Authors' contributions

This work was carried out in collaboration between all authors. Authors BL and FDS were responsible for designing this research and negotiating funding with the funding agency. All research was carried out in the laboratory of authors BL and FDS reviewed the results throughout the course of the research. Author MH handled all laboratory technical procedures and processes and contributed to the presentation of the results and statistical analysis of the data. All three authors contributed to the manuscript and agree with its contents.

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Original Research Article

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ABSTRACT

Background: Interest in avoidance of antibiotic resistance development through non-antibiotic therapeutics prompts development of topical vaginal preparations that may

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inhibit vaginal microorganisms. Prior data on boric acid, zinc salts and probiotic microorganisms suggests a potential role in female genital tract infections.
Aims: Determine antimicrobial effects of boric acid, zinc sulfate and metabolites of probiotic lactobacilli alone or in combination on *Candida albicans*, *Gardnerella vaginalis* and *Streptococcus agalactiae*.
Methodology: Absolute counts of test organisms cultivated with or without inhibitory compounds were determined by a flow cytometric method and compromise of microbial cell integrity was demonstrated by propidium iodide staining, also determined by flow cytometry. All microbial count growth experiments were conducted in triplicate and averages were reported.
Results: The three microbial species challenged with boric acid, zinc sulfate or spent medium from probiotic lactobacilli showed varying degrees of susceptibility to these inhibitors, with boric acid showing consistent activity against *C. albicans* and also showing yeast cell damage demonstrated by propidium iodide uptake. Boric acid showed greater antibacterial activity against *S. agalactiae* than against *G. vaginalis* and neither of the bacterial organisms showed propidium iodide staining. Zinc sulfate inhibition was greatest for *Candida* and least for *Gardnerella*. Probiotic *Lactobacillus* spent media was also inhibitory toward the three test organisms with *Gardnerella* being slightly less susceptible than the other two test organisms. When binary combinations were tested, the combination of boric acid and *Lactobacillus* spent medium was the most effective in vitro against all three organisms.
Conclusion: Zinc sulfate did not prove any more effective in vitro against the three test organisms than did boric acid or *Lactobacillus* spent media. The most potent binary combination against the three test microorganisms was boric acid plus spent media from probiotic Lactobacilli.

Keywords: Vaginal therapy; zinc; boric acid; probiotic; Candida albicans; Gardnerella vaginalis; Streptococcus agalactiae.

1. INTRODUCTION

The vaginal microenvironment is host to a variety of organisms which may be commensal and may likewise participate in interactions with the host that result in vaginal symptomatology. These include the yeast, *Candida albicans*, bacterial vaginosis-associated species of which *Gardnerella vaginalis* is typical, or Group B *Streptococcus* predominantly of concern as a cause of neonatal infectious complications or maternal urinary tract infection [1]. While molecular approaches to the microbiome and its interaction with the host are providing more scientifically satisfying insights into interactions between tissue and microorganisms, efforts to identify safe and effective strategies to re-balance the microbiotia continue. Antibiotic pharmaceutical drugs are primarily used for common infectious vaginal conditions, but interest remains strong in developing new therapies based on compounds that do not engender resistant strains. Small molecules with minimal host toxicity or probiotic microorganisms are frequently considered in this context. Indeed, our previous studies with boric acid as an inhibitor of *Candida albicans* [2] suggest that this could be part of a multi component vaginal microbicide. In the present study, we investigate the antibacterial and antifungal activity of four potentially probiotic lactobacilli individually and in combination with boric acid and/or zinc sulfate which has been investigated as an antiviral therapy or preventive [3-5] and possesses antibacterial activity as well [6].

2. METHODOLOGY

2.1 Bacteria

Bacterial strains obtained from American Type Culture Collection (ATCC) included *Gardnerella vaginalis* 14081 and *Streptococus agalactiae* 12386, grown initially according to instructions provided by ATCC. For growth studies *G. vaginalis* was grown in a broth consisting of the components of V-agar [7] without human blood or agar. This media which is hereafter referred to as GV medium was supplemented with inhibitory substances or not and inoculated with the *G. vaginalis* test organism and incubated in 5% CO_2 atmosphere prior to cell counting on the flow cytometer. *S. agalactiae* experiments were conducted in Todd-Hewitt broth with or without inhibitory additives in a manner similar to the *Gardnerella* experiments.

2.1.1 Probiotic organisms

Lactobacillus crispatus, *Lactobacillus jensenii*, *Lactobacillus gasseri*, and *Lactobacillus rhamnosus* were supplied by Centro Sperimentali del Latte, Zelo Buon Perisco, Italy and were propagated in MRS broth. These organisms were supplied as lyophilized powders in sealed packages with certificates of analysis indicating the number of viable organisms per gram of powder. Starter cultures were prepared by inoculating $1x10^6$ cfu from the powdered cultures and then dispensed these into 100 mL volumes of MRS broth to develop a stock of spent culture fluid. Spent cultures of the lactobacilli were prepared as described below and were used in lieu of mixing the lactobacilli with the target organisms because of the difficulty of distinguishing the lactobacilli from the *G. vaginalis, C. albicans or S. agalactiae* either by plate count or by flow cytometry.

Spent cultures to challenge the test organisms were prepared by growing each *Lactobacillus* in MRS broth for 72 hours at 37ºC. as well as a mixture of the four species inoculated into MRS broth in equal numbers, based on counts reported on the certificate of analysis. After growth of the probiotic organisms in MRS broth, the culture supernatant was obtained first by centrifugation at 3000xg for 20 minutes at 4ºC. The supernatant fluids were then passed through a 0.22uM filter and the sterile filtrates were used in the subsequent experiment. The pH of each filtrate was determined, but no additional characterization of the supernatants was performed. For challenge of the test organisms, growth medium appropriate to each test organism was inoculated and mixed with an equal volume of the spent *Lactobacillus* medium. Thus, spent medium was tested at a two-fold dilution but contained sufficient fresh medium to support growth of the test organisms. After combining spent culture filtrates with test organism culture and after overnight growth at 37ºC. absolute counts were determined by flow cytometry.

2.1.2 Clinical isolates

Candida albicans were clinical isolates recovered from a variety of body sites (skin, mucosa, lung, abscess, fingernail) including 3 strains from vaginal cultures. These were cultivated on Sabouraud's dextrose agar and grown in Sabouraud's broth with or without inhibitory substances for demonstration of antifungal effect of test materials. To minimize the number of subcultures of experimental organisms, the stock cultures were mixed with equal parts sterile glycerol and frozen at -80ºC. and grown from frozen stock for each experiment. Unless otherwise stated, test media were inoculated with test organisms and grown

overnight as a starter culture. Starter cultures were diluted 100-fold and 10uL of that dilution added to 1ml of fresh media with or without inhibitors.

2.2 Growth Studies

The inhibitory activity of the test articles was determined by counting test organisms (*Candida*, *Streptococcus* or *Gardnerella*) present after overnight culture with a range of inhibitors. Counts employed flow cytometry on the Accuri C6 cytometer (Becton-Dickenson, San Jose CA) which has a fluidics system that allows for absolute counts [8]. Absolute counts may allow for greater sensitivity in detecting differences in growth yield that is less than one log as is typical for serial-dilution plate count experiments.

For cytometer counts, the growth media with additives but no microorganisms is run on the flow cytometer. A forward scatter threshold is established for each organism so that artifact particles present in the growth media are not counted and only the microorganisms are recorded in the forward scatter channel (FSC). The cytometer is set to accumulate 10,000 events per experimental sample and the volume of sample to contain 10,000 events is used to calculate the absolute cell count. Each test organism was grown to early logarithmic phase in appropriate liquid medium and diluted to contain between $1x10^5$ and $1x10^6$ organisms per ml and then 10uL was used to inoculate 1mL experimental wells of a 48 well microtiter plate each containing dilutions of inhibitory materials (boric acid, zinc or probiotic). After inoculation, cultures were incubated overnight and then mixed by means of 5 repetitions of pipetting up and down and counted on the flow cytometer. These cultures were planktonic in nature because overnight growth furnished insufficient time for biofilm formation.

Because controls for inhibition testing were maximal growth, the conversion of data to percent inhibition normalized data from different strains as maximal absolute counts differed from strain to strain. For reference, 90% inhibition is equal to one log below control culture absolute counts and 99% inhibition is equal to two logs below control counts.

2.3 Propidium Iodide Staining

Microorganisms were tested for cell integrity by adding propidium iodide solution to cultures that had been grown in media with and without inhibitory factors. Propidium idodide reagent was prepared by making a stock solution of 100uM propidium iodide in dimethyl sulfoxide and subsequently diluting this stock 1:10 in water. Staining involved adding 0.01mL of the diluted stain to 1mL of bacterial or yeast culture. Staining was indicated by FL2 fluorescence of the organisms. A positive damage control was prepared for each test organism by exposing a culture to 65ºC. For ten minutes. The FL2 fluorescence of the non-damaged organisms was used to set quadrants whereby at least 95% of the non-damaged controls were placed in the lower two quadrants. The relative amount of damaged cells was taken to be the percent of events in the upper 2 quadrants of the FL1–FL2 scatter plot.

2.4 Inhibitory Materials

Boric acid and zinc sulfate were analytical grade chemicals and each was diluted into sterile growth medium to achieve the desired final concentration. Controls consisted of test microorganisms grown in the growth medium without added inhibitors and counts obtained from these control wells were considered to be 100% of maximal growth which allowed

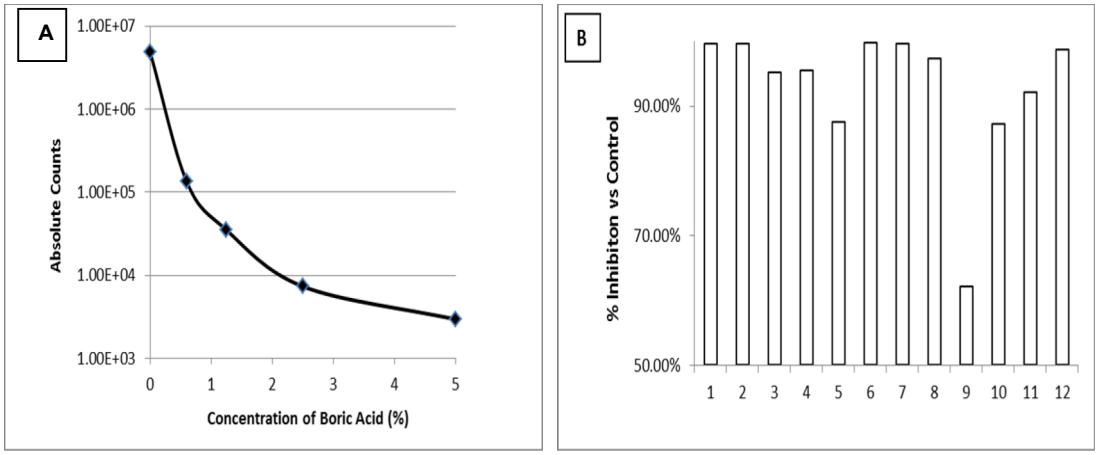
expression of restricted growth as % inhibition relative to controls. Because counts obtained were absolute counts, it was possible to determine ratios of experimental to control counts that provided meaningful data even when there was less than a ten-fold difference between experimental and control wells.

3. RESULTS AND DISCUSSION

3.1 RESULTS

3.1.1 Boric acid

Previous studies provided evidence that boric acid produces an antifungal effect on *C. albicans* and provided the basis for testing of 12 clinical isolates. Titration of the effect of boric acid in Sabouraud's broth in overnight culture is depicted in Fig. 1a which includes concentrations of boric acid up to 5% w/v (0.82M) which produces complete inhibition of growth above the *Candida* concentration in the experimental inoculum. Even at 0.625% (0.1M) greater than ten-fold inhibition compared to Sabouraud's broth control was observed. As shown in Fig. 1b all test strains were inhibited to various degrees by 0.625% boric acid. In all but 4 *Candida* strains, 0.625% boric acid reduced absolute counts by at least 95% compared to Saboruaud's growth controls. Only one strain of *Candida* showed growth greater than 15% of absolute counts of the control.



Figs. 1A and B. *C. albicans* growth (average of 12 strains) in a range of concentrations of boric acid (panel A) and the inhibition of for 12 strains of *Candida* (arbitrary strain number shown below bars) on at 0.625% boric acid concentration (panel B) as 100-[(absolute counts in boric acid/absolute counts in control)x100]. Inhibition of 90% is equal to 1 log decrease relative to growth in inhibitor-free media; absolute counts of controls varied among strains

To determine if exposure of *C. albicans* to boric acid was accompanied by loss of cellular integrity, propidium iodide staining after culture in 0.625% boric acid was performed. Staining was defined as the percent of microorganisms which had staining above the level seen among yeast grown overnight in Sabouraud's broth. All 12 of the clinical isolates of *Candida* were tested and all 12 isolates showed propidium staining greater than that observed among the controls. As shown in Fig. 2, the average percent of yeast taking up

propidium iodide in Sabouraud's cultures was 1.85% compared to an average of 21% staining among cultures exposed to boric acid. For comparison, 32% of yeast heated for 10 minutes at 65ºC. stained with propidium iodide. Staining was significantly higher for boric acid treated or heated yeast ($p<0.0001$, paired student's t test) compared to control culture.

Based on FL-2 fluorescence (flow cytometry) the percent of fluorescent cells represented the population having cell integrity compromise. As a positive control for cell damage, an aliquot of cells grown in Sabouraud's medium were exposed to elevated temperature. Error bars represent SD for data from 12 *Candida* strains and are very small due to the statistical power of counting 10,000 events.

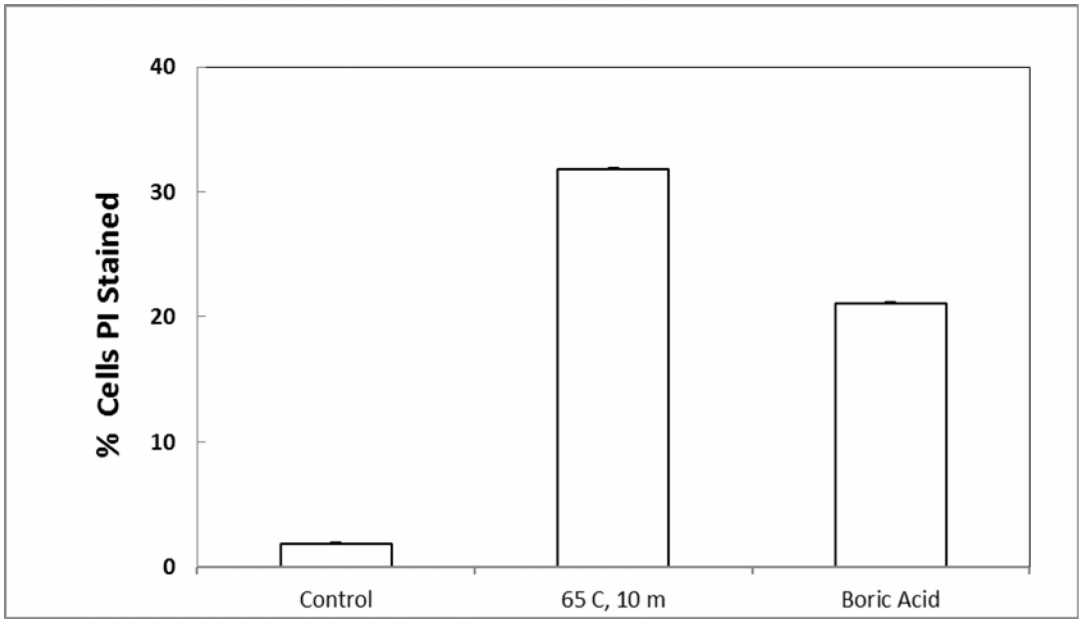


Fig. 2. *C. albicans* treated with boric acid or heat were stained with propidum iodide

Prior information on bacterial response to boric acid is limited, consequently, both *G. vaginalis* and *S. agalactiae* were each challenged with boric acid at 0.625%. The relative inhibition for each of these organisms is provided in Fig. 3 compared to the absolute counts obtained in growth controls. While *S. agalactiae* was more susceptible to boric acid, with nearly 2 logs difference in counts compared to the growth control, *G. vaginalis* showed less than a 1 log decrease in counts compared to control. At the conclusion of the incubation, neither *G. vaginalis* nor *Streptococcus agalactiae* showed detectable levels of propidium iodide staining suggesting bacterial growth inhibition occurred without loss of cell integrity.

3.1.2 Zinc sulfate

Having established the level of antibacterial and antifungal effects of boric acid we determined the activity of zinc sulfate in relationship to the three vaginal microorganisms. This experiment was guided by the work of Bourne et al. [3] who used 100 and 200mM concentrations in mouse experiments designed to inhibit in vivo vaginal viral infection. Accordingly, we determined the counts of *C. albicans*, *G. vaginalis* and *S. agalactiae* with and without 100mM zinc sulfate after overnight cultivation at 37ºC. Absolute counts

furnished by data from the FSC channel of the cytometer were evaluated by dividing the counts in the presence of zinc by the counts in the corresponding control medium which allowed us to establish the percent inhibition as was done for yeast experiments. Table 1 summarizes the results of test.

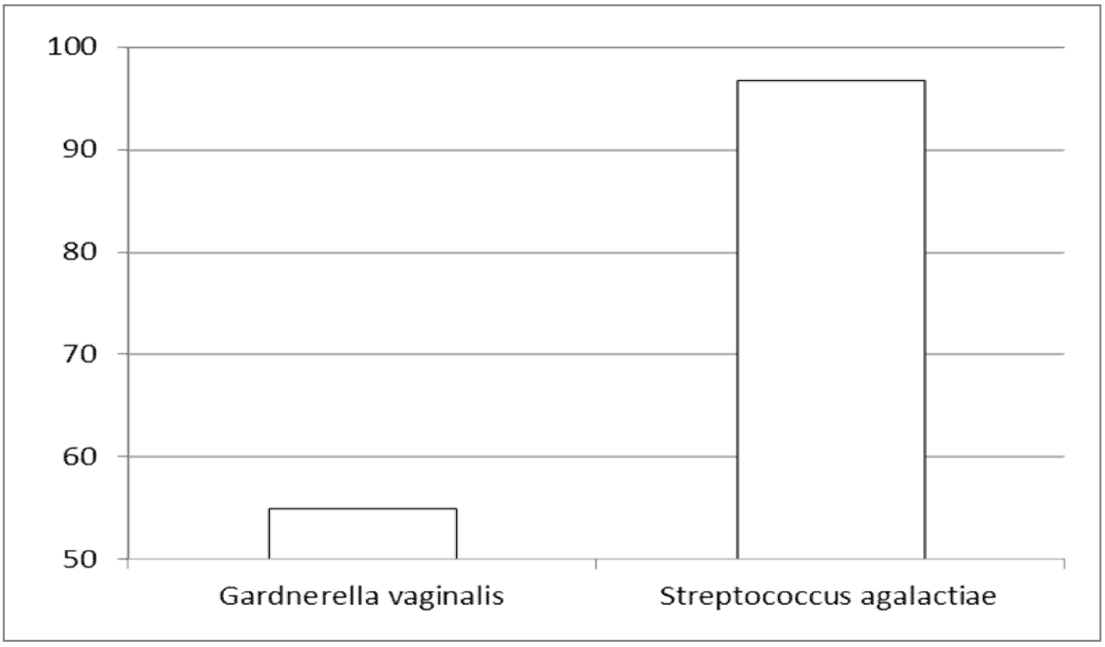


Fig. 3. Growth inhibition of *Gardnerella vaginalis* exposed to 0.625% boric acid and *Streptococcus agalactiae* growth inhibition after overnight cultivation compared to medium without inhibitor. Inhibition of 90% is equal to 1 log decrease relative to growth in inhibitor-free media

Table 1. Growth yield of microorganisms in 0.1M $ZnSO_4$ versus controls

Organism	Absolute counts/mL		% Inhibition*
	Control	$ZnSO_4$	
C. albicans	$1.00X10^7$	$1.35X10^5$	98.65
G. vaginalis	$1.87X10^5$	$5.70X10^4$	69.52
S. agalactiae	$2.00X10^6$	$4.00X10^5$	82.00

**100–[(Absolute counts in Zn/Absolute counts in control)x100]*

The results demonstrated that some inhibitory activity was obtained with this concentration of zinc sulfate, but in relative terms the fungal isolate seemed more sensitive to the antimicrobial effect of zinc compared to the bacterial isolates.

3.1.3 Probiotics

Probiotics represent a third potential approach to restoring or maintaining vaginal health. Available in our laboratory are four *Lactobacillus* strains which could individually or in combination, be part of a vaginal probiotic product. As described in detail in the Methods Section, we used spent culture medium to challenge the test organisms. All filtered, spent

media from *Lactobacillus* cultures showed similar acidic reaction as follows: *L. jensenii*, pH 4.38; *L. crispatus*, pH 4.33, *L. rhamnosus*, pH 4.36; *L. gasseri*, pH 4.34 and the mixture or organisms, pH 4.36. Prior to inoculation, the MRS broth in which the lactobacilli were grown had a pH of 5.44 and when boric acid at a final concentration of 5% was added a small decrement in pH was observed (pH 5.27) and MRS with 0.625% boric acid (final concentration was prepared, the pH measured 5.36. Fig. 4 depicts the results of this study which showed consistent inhibitory activity by spent media from lactobacilli against *C. albicans*. Except for the mixture, each of the 4 probiotic strains of *Lactobacillus* furnished between 98.5 and 99.5% inhibition (average for 12 strains of yeast) or approximately 2 logs less than growth in media without added spent lactobacillus medium. The mixture provided a smaller average degree of inhibition at just above 97%.

Also presented in Fig. 4 is the propidium iodide staining accompanying the exposure of *C. albicans* to spent media from various species of *Lactobacillus*. There was no obvious relationship between the level of cell integrity compromise and the absolute counts of yeast grown in various *Lactobacillus* spent cultures. However, because between 12 and 50% of *Candida* exposed to *Lactobacillus* spent culture fluid showed positive PI uptake, it was apparent that *Lactobacillus* spent medium represented more than just an unsatisfactory growth medium, but had a definite biological effect on cell integrity of *Candida*.

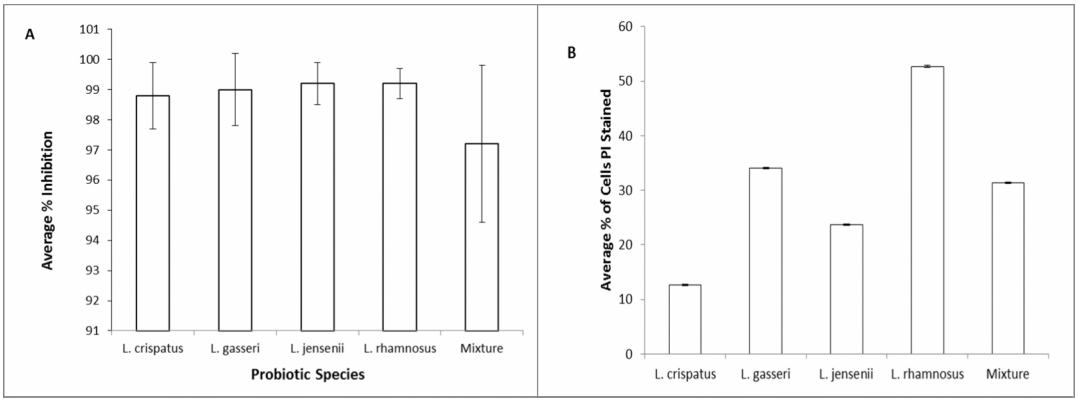


Fig. 4. Growth inhibition of *C. albicans* (average of 12 strains) by cell-free spent cultures of probiotic lactobacilli (panel A). Inhibition of 90% and 99% are equal to 1 log and 2 log decrease relative to growth in inhibitor-free media respectively; absolute counts of controls varied among strains. Propidium iodide staining (panel B) for the *C. albicans* strains shown in panel A

Antibacterial effects were exerted on *G. vaginalis* and *S. agalactiae* by spent culture fluids from *Lactobacillus* cultures as illustrated by Table 2. The magnitude of effect was determined by comparing growth in terms of decrease in cell counts compared to growth controls and expressed as percent inhibition as for *C. albicans*. Each of the *Lactobacillus* species exerted a similar degree of inhibition which was modest, in that less than 90% inhibition was noted. In terms of actual bacterial counts represented just under a one log reduction in counts compared to controls. Likewise, cellular damage was virtually absent.

In contrast to *G. vaginalis*, *S. agalactiae* was substantially more susceptible to the inhibitory effects of *Lactobacillus* spent culture medium. The magnitude of inhibition was at least 98.7% which represented nearly a 2 log decrease in bacterial counts compared to controls and *L. crispatus*, inhibition approached 3 logs. Also of note, despite significant growth inhibition, cellular integrity compromise as identified by propidium iodide staining was nil.

3.1.4 Combinations

Because it is likely that topical antimicrobials may be developed in the future as combination products, we concluded this study by evaluating combinations of the antimicrobial materials to determine if synergistic antimicrobial activity or antagonistic activity might result from cultivation of test organisms in binary combinations of antimicrobial factors. Combinations with boric acid used a 0.625% concentration of boric acid with either an equal volume of the spent culture medium from the *Lactobacillus* mixture or a 0.1M solution of $ZnSO_4$. Fig. 5 summarizes the performance of binary combinations of inhibitors with respect to the three vaginal microorganisms. The results indicated that the *Candida* and *Streptococcus* were both inhibited in combinations of boric acid and zinc or cell-free spent *Lactobacillus* culture to a degree similar to boric acid alone. This suggested the combinations were not antagonistic. The same organisms were similarly inhibited with a combination of zinc and *Lactobacillus* spent medium, also indicating that antagonism was unlikely with this combination. *Gardnerella,* as suggested by results with single antimicrobials, was least susceptible to combinations, however when boric acid and *Lactobacillus* spent medium were combined, the inhibition observed approached the magnitude of inhibition of the other two test organisms.

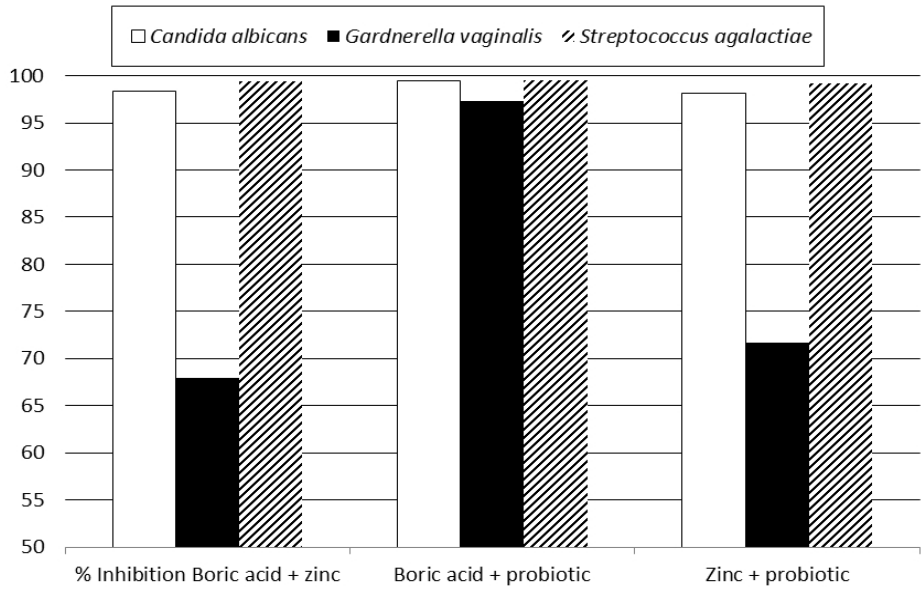


Fig. 5. Percent inhibition of three test organisms by binary combinations of the three antimicrobial agents. Inhibition of 90% and 99% are equal to 1 log and 2 log decrease relative to growth in inhibitor-free media, respectively

Table 2. Inhibitory effects *of Lactobacillus* spent media on vaginal bacteria as percent reduction in bacterial counts compared to appropriate growth medium. Propidium iodide staining was performed on the same cultures indicated the percent of bacterial cells showing compromised cell integrity

Source of spent culture	*Gardnerella vaginalis*		*Streptococcus agalactiae*	
	% Inhibition	% PI Stained	% Inhibition	% PI Stained
Control Culture*	0	0	0	0
L. crispatus	83.24%	4.0%	98.89%	0
L. gasseri	89.65%	10.0%	99.32%	0
L. jensenii	86.41%	6.50%	98.97%	0
L. rhamnosus	86.19%	9.2%	99.58%	0.30%
Mixture	81.23%	5.1%	98.70%	0.33%

**Control Culture consisted of organisms grown in the absence of inhibitor and were expected to show no cell damage*

3.2 DISCUSSION

Over the past two decades much interest, research and development efforts have been directed at vaginal microbicide discovery, spurred in large part by a desire to develop prophylactic measures for coitally-associated HIV infection. In parallel with this kind of research, interest in novel products for prevention or treatment of such common vaginal conditions as bacterial vaginosis, trichominiasis and candidosis has continued, because despite antibiotic therapies, these present ongoing clinical challenges to physicians. Small molecules other than antibiotics in addition to probiotics have been repeatedly considered in preventive and therapeutic roles.

A PubMed search on “vaginal infection and probiotics” yielded 94 matches at the time of this writing. Many of the papers were reviews or opinion papers, but despite this, a fraction contained original data on both use of oral as well as intravaginal topical probiotic preparations with various probiotic species and a broad array of clinical outcomes intended from these treatments [9,10]. Clearly interest remains strong in identifying a role for this type of treatment.

In addition, a number of non-antibiotic small molecules has attracted attention directed at sexually transmitted disease-causing agents or organisms associated with vaginitis. The present research has built on the interest of our laboratory in substances that may extend the spectrum of boric acid which we have previously investigated [2].

While the microbial flora consists of a wide diversity of species, three in particular have been associated with symptomatic vaginal conditions and have as a result been chosen for use in this study. *C. albicans* is well known as in yeast vaginitis and extensive experience in understanding pathogenesis, host response and therapy has been presented in the literature extensively [11]. Resistance to the most commonly used antifungal drugs such as fluconazole is a vexing problem, especially since overexpression of multi-drug efflux pumps may be increasingly common [12]. For many years, boric acid has been a recognized alternative intravaginal therapy for resistant yeast infection [13] and interest in this treatment has increased since our report in 2008 [2].

This study also examined *G. vaginalis* because of its known association with bacterial vaginosis and various associated risks such as preterm birth, chorioamnionitis, pelvic

inflammatory disease and HIV susceptibility in addition to its role in abnormal discharge and malodor [14]. While this organism may participate with other members of the flora in symptomatic vaginal conditions, it has proved useful in pure culture to serve as a test organism for the inhibitory substances this paper focuses on.

The third test organism, *S. agalactiae* is of primary concern as a neonatal pathogen, but also has a role in maternal urinary tract infections. While many countries have rigorous programs to detect and treat for this organism during pregnancy, concern still exists regarding the threats to neonates in underdeveloped or resource poor countries [15] which occasioned the inclusion of this organism among those challenged by boric acid, zinc sulfate and spent culture medium from four *Lactobacillus* species.

While small molecule inhibitors or other non-antimicrobial pharmaceutical therapies are theoretically attractive, the practicality of their use both in terms of activity and safety are relevant topics as well. Boric acid has been used as a 600mg capsule inserted intravaginally and assuming a 3-6ml total volume of vaginal fluid, this dose would be well above the concentrations we showed to be inhibitory toward the test organisms in this study. Moreover, the long-standing vaginal use of boric acid suggests a lack of toxicity at the high levels used clinically, but the systematic review by Iavazzo, et al. [16] has identified reports of irritation among some users of this treatment. Cell culture studies, which tend to be very sensitive and can focus on the expression of individual genes or gene products would suggest that subtle toxicities might exist [17-18] especially above 500uM.

The concentration of zinc sulfate was based on studies done by others and have been shown to have antiviral and antibacterial effect and lack of topical toxicity at the levels examined in this research [3-6]. Probiotic organisms have been used rather extensively for many years and most references suggest a good safety profile [19]. Of course, the actual test material we used was spent culture and not active cultures. This is an important distinction because in actual practice live probiotic organisms would probably be used and may possess greater antimicrobial activity than the metabolic products of the organism. Components of the *Lactobacillus* may have included organic acids and bacteriocins which have been characterized previously, but in vivo, it would be anticipated that such potentially antimicrobial by products would be produced continuously. Therefore, we would conclude that our in vitro studies may underestimate the antimicrobial potential of lactobacilli.

The present study shows, boric acid exerts demonstrable inhibitory activity against two species of female genital tract bacteria and clinical isolates of yeast. Metabolic factors elaborated into culture media by several lactobacilli also have activity against vaginal yeast and bacteria. Because little effort seems to have been expended on additional compounds which could enhance or diminish boric acid efficacy, we concluded our studies with examination of combinations using flow cytometry methods that were useful in identifying the antimicrobial activities of each inhibitor individually.

Considering the fact that boric acid or vaginal probiotic preparations have already been used clinically, zinc salts represent a novel inhibitor in the context of bacterial vaginosis or vulvovaginal candidiasis. Where zinc has precedence is as an antiviral [3-5] or against *S. pneumonia* [6]. This paper confirms that zinc sulfate does have antimicrobial activity, though the relative potency of zinc compared to boric acid or products of probiotic bacteria is somewhat less. It should be noted that nearly identical molarities of zinc and boric acid were compared indicating that in these experiments zinc had a lesser molar potency.

One of the important findings from this study resulted from evaluating combinations of inhibitors relative to yeast and bacterial growth. The combination that was most effective against all three organisms was *Lactobacillus* spent media and boric acid. Interestingly, a recent study combining zinc lactate and a purified component of lactobacillus (lactocin 160) was synergistic against *Gardnerella* [19]. It is possible that zinc sulfate versus zinc lactate has different intrinsic inhibitory activity which would only be revealed by a head-to-head comparison. But this study, along with others suggests that combination products could have value.

Certainly these in vitro observations need to be followed by clinical studies to determine how individual or combined actives may perform with respect to pathogenic and benign vaginal commensals. Product formulation could have a significant effect on in vivo performance and live probiotic cultures may have superior activity to a component of the spent medium. Actively growing organisms would theoretically supply inhibitory substances continuously to their environment and as Reid's work suggests [20] the interaction of lactobacilli with other microorganisms or host tissue could interfere with adherence of microorganisms associated with vaginal symptoms.

It would be advantageous to expand these studies by the use of recent clinical isolates of vaginal bacteria as laboratory strain, as used in this report may have a different susceptibility pattern to inhibitors than clinical isolates. In addition, the data presented here suggests, especially in the case of *Gardnerella* and *Streptococcus*, that cell integrity was not affected by the inhibitors tested. The basis of this difference between bacteria and yeast bears additional study as some probiotic substances have been described as membrane pore formers [21].

In evaluating potential antimicrobials in vitro, an opportunity exists to determine if compounds that might be used in combination to produce a broader spectrum of activity are readily evaluated to ensure that antagonistic effects do not occur. In addition additive and synergistic activity is possible. While we undertook a preliminary evaluation of combinations of the three antimicrobials studied in this paper, we did not find evidence of antagonism, but only one combination provided strong activity against *Gardnerella*. Combinations, like individual components did not appear to have a microbicide effect since organisms exposed to the inhibitors or their combinations did not sterilize the cultures. Despite this, inhibitors which are microbiostatic can help to restore appropriate relationships between individual beneficial and inimical species making them potentially clinically useful.

Future work with these compounds should further elucidate the mechanisms of action and mechanisms of combined action. More sophisticated methods of determining synergistic activities are available through isobole analysis and such an approach would be possible based on the methods applied in the present paper.

4. CONCLUSION

Three different candidate intravaginal antimicrobial compounds were used to challenge three microbial species relevant to lower female genital tract infections. Antimicrobial activity was demonstrated for all three microbial species through absolute counts in planktonic cultures supplemented with boric acid, zinc sulfate or spent culture fluid from lactobacilli. Only *Candida* showed altered cellular integrity in the presence of lactobacillus spent medium or boric acid. In binary combination, probiotic and boric acid furnished the broadest

antimicrobial spectrum. The utility of combination therapies will require appropriately designed and powered clinical studies.

CONSENT

No human subjects were involved in this research.

ETHICAL APPROVAL

No animal subjects were employed in the conduct of this research.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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