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Testing of viscous anti-HIV microbicides using *Lactobacillus*

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Abstract

The development of topical microbicides for intravaginal use to prevent HIV infection requires that the drugs and formulated products be nontoxic to the endogenous vaginal *Lactobacillus*. In 30 min exposure tests we found dapivirine, tenofovir and UC781 (reverse transcriptase inhibitor anti-HIV drugs) as pure drugs or formulated as film or gel products were not deleterious to *Lactobacillus* species; however, PSC-RANTES (a synthetic CCR5 antagonist) killed 2 strains of *Lactobacillus jensenii*. To demonstrate the toxicity of formulated products a new assay was developed for use with viscous and non-viscous samples that we have termed the *Lactobacillus* toxicity test. We found that the vortex mixing of vaginal *Lactobacillus* species can lead to reductions in bacterial viability. *Lactobacillus* can survive brief, about 2 sec, but viability declines with increased vortex mixing. The addition of heat inactivated serum or bovine serum albumin, but not glycerol, prevented the decrease in bacterial viability. *Bacillus atrophaeus* spores also demonstrated loss of viability upon extended mixing. We observed that many of the excipients used in film formulation and the films themselves also afford protection from the killing during vortex mixing. This method is of relevance for toxicity for cidal activities of viscous products.

Keywords

HIV; Microbicide; *Lactobacillus*; Vortex mixing; bacterial quantitation

1. Introduction

Different solid or viscous formulations are under development for microbicide delivery including vaginal rings, gels and films. These products should optimally prevent infection by HIV without disturbing the normal vaginal microflora since this flora is a key component of the innate immune environment which can reduce the HIV risk(Martin et al., 1999). We

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have focused on films and gel formulations; both of which rely on similar or the same viscosity enhancing agents such as species of polyvinyl alcohols and methylcelluloses.

The minimum cidal concentration (MCC) assay detects a decrease in microbial viability after a 30 min exposure of 10^4 colony forming units (CFUs)/mL or greater (Moncla and Hillier, 2005, Moncla et al., 2008). The test was useful in our search for materials that would kill HIV and bacterial sexually transmitted pathogens but lacked the sensitivity to determine *Lactobacillus* killing of less than 4 logs. Minimum inhibition concentration tests (MIC) with microbicides do not provide relevant information because they only determine inhibition. The MCC assay may suggest that inhibition is occurring but more complex methods are required to differentiate killing from inhibition (Moncla and Hillier, 2005, Moncla et al., 2008). However, the MCC assay is difficult to use with drugs formulated for vaginal use because they contain viscous excipients. In order to test these products for cidal activity against *Lactobacillus*, the products must be diluted before testing, resulting in drug and excipient concentrations that are far below that expected during product use. Therefore, the assay may underestimate deleterious effects, undermine toxicity testing against *Lactobacillus* or misrepresent the efficacy of the drug. We therefore developed a new assay to assess the effects of drugs, excipients and formulated products that closely mimic concentrations anticipated during use.

In the studies reported here two different classes of anti-HIV drugs (reverse transcriptase inhibitors, RTIs and antimicrobial peptides) were screened for toxicity (non-deleterious effects) against *Lactobacillus* species. A new assay for toxicity against Lactobacillus was developed and we found vortex mixing killed *Lactobacillus* species; however, most of the films, gels, excipients and formulations were not detrimental to *Lactobacillus* species.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Reference strains of *Lactobacillus* were obtained from the American Type Culture Collection, ATCC (Manassas, VA 20109, USA). Field isolates were obtained from human vaginal samples. *Lactobacillus* species were identified using colony morphology, Gram-stain reaction and catalase production and were confirmed using DNA-DNA hybridization to DNA from reference strains (Antonio et al., 1999). Organisms were stored frozen at −70° C in litmus milk until needed. Stock cultures were revived by plating onto blood agar plates (Columbia blood agar base, PML Microbiologicals, Wilsonville, OR 97070-9234, USA). Cultures were incubated at 35° C in air enriched to 6 % CO_2 overnight or until good growth was observed. *Bacillus atrophaeus* spore suspensions at a concentration of 2.6×10^7/mL were obtained from STERIS Corporation (Mentor, OH 44060, USA).

2.2. Lactobacillus toxicity testing (LTT) and MCC testing

Saline and ACES buffer [(N-2-acetamido)-2- aminoethanesulfonic acid] (pH 7.0) (Sigma-Aldrich, St. Louis, MO 63178, USA) were prepared in house. The isotonic strength of each lot of buffer was determined using a Vapro Pressure Osmometer 5520 (Westcor Inc., Logan UT, 84321 USA) and adjusted to 200–300 mosm/kg with sodium chloride prior to use. Heat inactivated fetal bovine serum (FBS) was obtained from Cellgro, (Mediatech Herndon, VA 20109, USA) bovine serum albumin (BSA) was obtained from Sigma-Aldrich (St. Louis, MO 63178, USA). Serum, BSA and glycerol were added to buffer at a final concentration of 7.5 %, 3.75% and 3.75% (vol/vol) respectively and filter sterilized through a 0.45 μ filter and stored at 4° C until used; buffers were prepared fresh weekly.

Bacterial suspensions were prepared in saline or ACES buffer with or without additions as indicated in the figures to a density of 2 McFarland units, approximate concentration of 2.0

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$\times 10^8$ bacteria/mL. Sample materials were weighed into the bottom of a 50 mL conical centrifuge tube (about 0.25 to 0.5 g) and equivalent volume of buffer added and mixed. Then an equal volume of bacterial suspensions were added, mixed for 2 sec, and sampled for colony forming units (CFUs). The samples were then mixed on a vortex mixer set to speed of 10 (the fastest available speed) for 30 sec, unless otherwise indicated. Alternatively, the mixtures were mixed by hand using a gentle swirling motion. The starting concentrations of bacteria in the inoculum were determined by plate count of the initial suspension of bacteria at a density of 2 McFarland units. Experiments where the CFUs, as determined from the starting solution and the CFUs from the 2 sec sample did not agree were rejected. The number of CFUs for each datum was taken from the dilution plate that contained between 50 and 300 colonies. All reported values represent the average of triplicate samples. If the number of colonies on the individual sample plates did not agree within 20% they were rejected. MCCs and MICs (agar dilution) assays were performed exactly as previously described (Moncla and Hillier, 2005, Moncla et al., 2008). When drug availability limited the number of tests we could perform the drug was tested at the concentration anticipated during use or the highest obtainable concentration. Octylglycerol (OG), a well characterized microbicide was used as a control. The OG was kindly provided by Charles Isaacs (New York State Institute of Basic Research in Developmental Disabilities, Staten Island, NY 10314 USA). Unless stated otherwise each experiment was repeated on at least 2 separate occasions.

2.3. Excipients, Drugs, and Substances tested

The viscosity increasing excipients evaluated in these studies were methylcellulose (4000 centipoise) and polyvinyl alcohol (PVA) [30 to 70 kilo-daltons (kDA) and 9 to 10 kDa], Methy-β-cyclodextrin MβCD (approximate formula weight 1320) and hydroxypropyl- β-cyclodextrin, HPβCD (approximate FW 1380) were from Sigma Aldrich (St. Louis, MO 63178, USA). Carbopol 974P was obtained from Noveon, Inc., (Cleveland, OH 44141, USA), and hydroxyethycellulose (HEC) 250HX was from Hercules Incorporated (Wilmington, DE 19801). PSC-RANTES (a synthetic peptide CCR5 antagonist) was synthesized by the Peptide Synthesis Facility at the University of Pittsburgh (Fontenot et al., 1991), and the molecular mass confirmed by mass spectrometry. The non-nucleoside reverse transcriptase inhibitors (NNRTIs) UC781 drug and tenofovir drug and gel product (1% drug) were provided by CONRAD (the Contraceptive Research and Development a Division of the Department of Obstetrics and Gynecology of Eastern Virginia Medical School, 1911 North Fort Myer Drive, Suite 900, Arlington, Virginia 22209) (Schwartz et al., 2006). A gel containing 0.01% UC781 was prepared in a carbomer/methylcellulose base formulated as previously described in animal studies (Patton et al., 2006, Patton et al., 2007). The hydroxyethyl cellulose (HEC) universal placebo was prepared as previously described (Tien et al., 2005). A proprietary formulation of dapivirine (TMC120) gel was provided by the International Partnership for Microbicides (Silver Spring, MD 20910, USA). Octylglycerol, dapivirine and UC781 containing films were formulated using a polyvinyl alcohol (PVA) polymer base.

3. Theory/Calculations

Vaginal products and their components should optimally have no cidal activity against *Lactobacillus* species, a key component of the vaginal ecosystem. *L. crispatus* and *L. jensenii* are used as representative species since they are the most common vaginal *Lactobacillus* (Antonio et al., 1999, Gustafsson et al., 2011). If formulated products or their components cause a reduction in the number of viable bacteria, equal to or greater than 1 Log $_{10}$, of two different strains of any of our representative species, the substance being tested is considered to potentially have a negative effect on the *Lactobacillus* if used

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clinically. Many of the materials are difficult to test because they are so viscous they must be diluted to low concentration. We reasoned that if we could mix high concentrations of bacteria with the tested material and allow them to interact for 30 minutes prior to diluting; we would be exposing the organisms to the materials under conditions similar to their *in vivo* use.

4. Results

4.1. Effects of drugs on Bacteria

The Minimum Cidal Concentration test (MCC) format was used to expose the organisms to drug and formulations (Table 1). The reverse transcriptase inhibitors (RTIs) tenofovir, dapivirine (TMC120), and UC781 in both the formulated (film or gel) or unformulated states; were not toxic to *Lactobacillus*. Tenofovir at a concentration of 1 mg/mL and the gel, diluted 1:10 for use, were not toxic. However, these concentrations did not represent the concentration anticipated during use. PSC-RANTES killed 20% of the *L. jensenii* isolates tested and inhibition of an unknown quantum was observed in 4 strains. However, these data, in the MCC format, did not address the question as to whether or not inhibition of these strains reaches the requisite 1 log decrease.

4.2. Effects of Excipients on bacterial viability

To define the LTT and make it objective we averaged all the data in Table 2 and set the cutoff as 2 standard deviations of the mean. Therefore, all results ≥ 1 $\log_{10}$ decrease in CFUs is a failed test; if the material has a failed test with two different isolates it fails the LTT test and is not used in our development scheme. Toxicity testing of different grades of PVAs as candidates for film preparation was concurrent with film development; therefore, testing was halted on any excipient that was rejected during formulation. The results of studies of effects on the viability of *Lactobacillus* species for the most promising varieties of PVAs, films and gels are presented in Table 2. The PVAs dissolved in buffer resulted in slight increases in the number of CFUs observed that ranged from 0.004 to 0.702 $\log_{10}$. However, the placebo and active films were observed to reduce the CFUs for some species and these reductions appear to be strain specific. Using saline in place of ACES buffer did not alter the results (data not shown and Fig. 1). Values less than one $\log_{10}$ could represent pipetting or indeterminate errors. Table 2 demonstrates the difference in killing observed between the strains of different species, the *L. jensenii* are more sensitive compared to *L. crispatus*. The *L. jensenii*, 3 of 5 strains, had viability losses when exposed to the gel formulations that were greater than 1 $\log_{10}$ and therefore failed the LTT. The 30 day tenofovir films also demonstrated viability losses with *L. jensenii > 1* Log and also failed the LTT. However, these decreases in viability are much less than would be observed with a truly toxic compound such as OG, see figure 1, where killing is 7 $\log_{10}$ in 30 min.

4.3 Formulated products

From an extensive screening process several viscosity increasing excipients were selected for use in preparing film and gel based preparation of the NNRTIs, Tables 2 and 3. The effects on viability were very much dependent on the species and strains used for the test. *L. jensenii* consistently demonstrated reductions in viability approaching 1 $\log_{10}$ Table 2. The calculated mean of all the data presented in Table 2 is 0.050 and the standard deviation (SD) is 0.477. Values greater than a −2 times the SD indicated killing has occurred; therefore, anything more than a one log reduction is a failure. Both of the gels and the thirty day tenofovir film failed the LTT because there were two isolates that failed for each test material (Table 2). Table 3 demonstrated the sensitivity of *L jensenii* LPB28Ab to the testing; this strain approaches $\log_{10}$ values of −1 for four different formulations. It also

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demonstrates that the gels and films may be reformulated to prevent the killing of specific strains of *Lactobacillus*.

To differentiate the underlying cause, the effects of vortex mixing were studied using *L. crispatus* and two strains of *L. jensenii*. Fig. 2 demonstrates the killing occurs over a brief time, 30 sec. The ATCC type strain of *L. crispatus* (ATCC 33197) demonstrated only a slight decrease in the colony forming units after 30 seconds of vortex mixing while *L. jensenii* strains were more sensitive to killing (Fig. 2). Killing was found to be time dependent. The addition of 7.5% heat inactivated horse serum (Fig. 1), 3.75% BSA but not 3.75% glycerol prevented *L. jensenii* killing during vortex mixing (data not shown). *Bacillus atrophaeus* was also tested by mixing for up to 5 minutes. This resulted in a reduction in viability > 6 logs, the limit of detection in our system. *B. atrophaeus* was not destroyed when 7.5% serum was present during the mixing (Fig. 1). Loss of viability over that observed in controls was never observed when bacterial or spore suspensions were mixed by hand or when vortex mixing was performed in a round bottom 50 mL centrifuge tube (data not shown). Using phase contrast microscopy we were unable to discern any differences in the chain lengths or clumping of organisms after mixing by either method.

4.4. Underlying mechanism

The reductions in CFUs were only observed in the films and not with the unformulated PVA used suggesting: the polymers were protective; manipulation of the bacteria was causing the decrease in viability observed or the films were toxic.

5. Discussion

The reverse transcriptase inhibitors (RTIs) used in this study did not demonstrate toxicity against any of bacteria used in our study. This was an expected result since reverse transcriptases have not been reported in these organisms. The killing and inhibition of *Lactobacillus* by peptide anti-retroviral PSC-RANTES was surprising. *Lactobacillus* lack any known targets for these compounds. Killing was inconsistent among species and strains. For example, two isolates of *L. jensenii* were killed by PSC-RANTES while four isolates were inhibited. The *L. vaginalis* and *L. gasseri* strains were not affected. Intra-species variations in S-layer proteins could explain these differences (Antikainen et al., 2002, Claus et al., 2005).

Women in different parts of the world appear to have the same dominant species of vaginal *Lactobacillus* species: *L. crispatus, L. jensenii* and *L. gasseri* (Antonio et al., 1999, Anukam et al., 2006, Gustafsson et al., 2011, Pavlova et al., 2002, Vasquez et al., 2002). Therefore, our findings are relevant to locations outside of North America.

Most other studies have used gel formulated microbicides (Patton et al., 2006, Patton et al., 2007, Schwartz et al., 2006) and we encountered sampling difficulties with these formulations. In our studies we observed killing by gel formulations. As we explored other delivery vehicles such as films it became obvious the current methods (MCC) require sample dilution to concentrations far less than encounter during use, for example we diluted tenofovir gels 1:10 before assaying. Thus an alternative method for toxicity testing was needed that would approximate *in vivo* concentrations of drugs or excipients.

Killing during vortex mixing was observed for 3 of 4 strains of *L. crispatus* and all 4 strains of *L. jensenii* examined; suggesting the effect may be widespread among the *Lactobacillus*. Importantly, the organisms can be protected by the addition of BSA or serum to the diluent. *B. atrophaeus* spores were studied because we reasoned they are more resistant to killing by mechanical forces, and would not show an increase in CFUs due to chain cleavage as could

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be with *Lactobacillus*. As with the *Lactobacillus*, *B. atrophaeus* spores were killed by vortex mixing that did not occur in the presence of added protein or in a round bottom tubes. This suggests that conical tubes disrupt the smooth mixing process and introduce deleterious mechanical stresses that lead to cellular damage that is not limited to species of *Lactobacillus*.

The use of the deleterious effects of vortex mixing have been noted in microbiology (Gerhardt, 1981, Holmquist and Kjelleberg, 1993, Ranhand, 1974, Sadhu et al., 1989, Seung Won et al., 2008). Sadhu et al., 1989 used extensive vortex mixing to examine vaginal morphotypes but did not report the effects on flora viability. Vortexing *Lactobacillus* with glass beads disrupts *Lactobacillus* chain lengths to varying degrees depending on the species (Ranhand, 1974). Strains of *S. pyogenes* exhibited 100% increase in CFUs after 30 sec but with increased mixing viability decreased (Ranhand, 1974). This demonstrates that mechanical action was disrupting groups of streptococci resulting in higher colony counts. We did not observe this to any significant degree.

Seung et al. noted a lower recovery of *Bacillus* from the ventilation filters if the samples were mixed using a vortex mixer than if they were mixed by hand. Our data suggests they were observing loss of viability rather than a decrease in recovery of organisms from the filter materials(Seung Won Kim, 2008).

Most of the microbicides developed and tested have been gel based. In our studies only the films tested proved safe. The gel formulations always yielded greater losses in viability than film preparation, Tables 2 and 3. Setting the cutoff for passing the LTT to > two standard deviations gave us a clear and reasonable point for passing the LTT.

A cautionary note should be taken concerning the presentation of bacterial killing; for example, Simpson et al. and Baranger et al. both used percent reduction in bacterial viability in their reports of “antimicrobial” agents elafin and pre-elafin (Baranger et al., 2008, Simpson et al., 1999). Such reports tend to over emphasis small reductions in bacterial viability. In Figure 2 where the data are presented as percent of original viable bacteria the differences appear to be quite large. However, the differences are less than 1 Log_{10} and are a reminder that in reporting large numbers, such as the number of bacteria in a culture the difference in the viability or killing should use Log_{10}.

Taken together the data suggest that the use of a vortex mixer be limited in duration and for mixing only. Dispersal, or recovery of organisms from the environment or other materials should use other methods or include protective agents such as BSA, heat inactivated serum or gelatin (Gerhardt, 1981). These results demonstrate that round bottom tubes should always be used and that there is a need for careful selection and testing of diluents when sampling and dispersing *Lactobacillus* species.

Lactobacillus toxicity testing has become an integral part of microbicide development and we have developed accurate and sensitive tools and have monitored the effects by some candidates on *Lactobacillus* species viability. Currently, we feel loss of viability of < 1 log to be unimportant; however, greater loss of viability or the observation of inhibition indicates the flora should be carefully monitored during trials. Other compounds such as nonoxynol-9 that demonstrate extensive and high rates of killing should probably not enter clinical trials. The significance of the test should become known as the data from current drug trials are revealed.

In summary the non-nucleoside reverse transcriptase inhibitors studied did not exhibit toxic or inhibitory activities towards our test panel of *Lactobacillus* species while the protein based anti-HIV drug PSC-RANTES did. Analysis of formulated products requires a

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significant dilution before the products could be manipulated for testing. In many cases, the resulting drug concentrations after dilution are far below the concentration anticipated in use making it difficult to determine whether the data are meaningful. By starting at very high bacterial concentrations we could circumvent these problems; but introduced an artifact of vortex mixing. However, we have always allowed for a drop in viability less than 1 Log_{10} before we were concerned about damage to the *Lactobacillus* in the microflora. We observed protection from killing by some of the polymer excipients, films and in some cases the gel. Comparing *L. jensenii* to *L. crispatus, L. jensenii* was more sensitive to killing by vortex mixing and most sensitive to killing by gel formulations. This would suggest closer monitoring of the species is needed in future clinical trials. Selecting the proper test for *Lactobacillus* toxicity depends on the drug, its formulation and whether or not the drug inhibits or kills *Lactobacillus*. Our algorithm is to test pure materials with the MCC assay at the intended *in vivo* concentration. If killing is observed the MCC is defined and if inhibition is observed the MIC is determined. Formulated materials are tested using the *Lactobacillus* toxicity test and are considered safe if the loss of viability is less than one Log.

6. Conclusions

Round bottom tubes should be used when bacteria are mixed with a vortex mixer. The reverse transcriptase inhibitors did not demonstrate toxicity against any of our study bacteria while some peptide antimicrobials were active against some of our test species. Methylcellulose and the various molecular species polyvinyl alcohol all passed the LTT. The *Lactobacillus* toxicity test performs better than the test currently used in the field of microbicide development.

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Abbreviations

PSC-RANTES	synthetic peptide CCR5 antagonist
MCC	minimum cidal concentration
CFUs	colony forming units
MIC agar dilution	the minimum inhibitory concentration test
NNRTIs	non-nucleoside reverse transcriptase inhibitors
LL-37 and LSA-5	cathelicidin derived antimicrobial peptides
RC 101	an anti-HIV peptide microbicides
ATCC	American Type Culture Collection
STDs	sexually transmitted diseases
UC781	a thiocarboxanilide nonnucleoside reverse transcriptase inhibitor of human immunodeficiency virus (HIV-1)
TMC120	TMC120-R147681 (dapivirine) is a reverse transcriptase inhibitor
CONRAD	the Contraceptive Research and Development a Division of the Department of Obstetrics and Gynecology of Eastern Virginia Medical School, 1911 North Fort Myer Drive, Suite 900, Arlington, Virginia 22209

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ACES	buffer [(N-2-acetamido)-2- aminoethanesulfonic acid]

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Research highlights

- The standard Lactobacillus toxicity test for anti-HIV microbicides is presented.
- Vortex mixers may kill large numbers of organisms and result in false values.
- Non-nucleoside reverse transcriptase inhibitors should continue development.
- Polyvinyl alcohol based films are safe and not deleterious to Lactobacillus.
- Film based anti-HIV microbicides should continue development.

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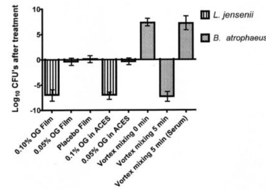

Fig. 1. Comparison of MCC method with new *Lactobacillus* Safety Test: viability of *Lactobacillus* and the effects of vortex mixing on viability

Octylglycerol was used to demonstrate that comparable results are observed using either the *Lactobacillus* Toxicity Test (bars 1, 2, and 3) or the Minimum Cidal Concentration test, (bars 4 and 5). Films were prepared so they would give a final concentration of 0.10%, 0.05% or 0.00% when dissolved in ACEs buffer for the LST, bars 1, 2 and 3 respectively. Bacteria were added to give a final concentration of approximately $2–20 \times 10^7$/mL. After 30 min at 37°C, samples were taken and the CFUs determined. Bacteria were also diluted and used in the MCC test at final OG concentrations of 0.1%, or 0.05%, bars 4 and 5 respectively. Both tests were conducted to detect as little as 50 CFUs/mL in the test solutions. In both tests *Lactobacillus* were killed (6 logs) at 0.1% but not 0.05% OG in either film formulation or a solution demonstrating the LTT test gives comparable results to the standard test (MCC). Bars 6, 7 and 8 are the results of experiments using *B. atrophaeus* spores to show the effects of vortex mixing. Spore suspensions at a concentration of 2.61×10^7/ml were prepared in ACEs buffer and mixed on a vortex mixer for 2 sec, bar 6 or 5 min bar 7. The addition of serum to the spore suspensions protected them from killing during mixing for 5 min on a vortex mixer, bar 8.

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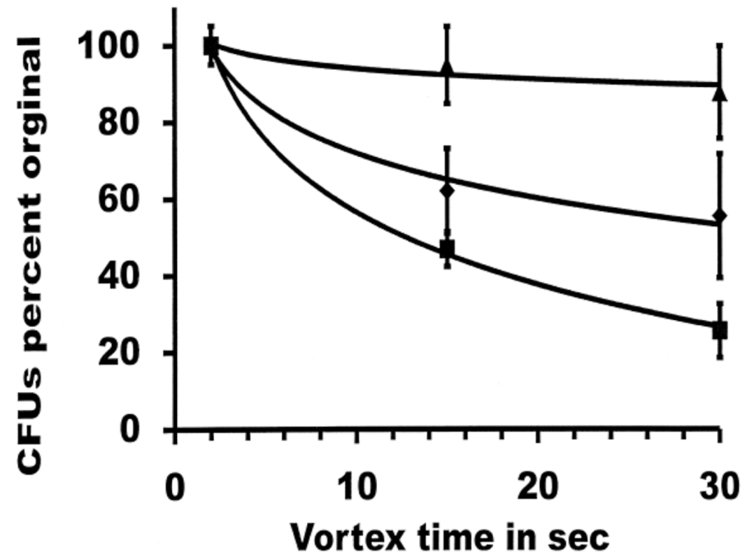

FIG. 2.
Effects of vortexing on CFUs for different *Lactobacillus* species. Strains were vortexed in ACES buffer as described in the text for the indicated seconds. For baselines (100%) samples were vortexed for 2 seconds and sampled for colony forming units. *L. crispatus* ATCC 33197 [▲], *L. jensenii* ATCC 25258 [■], *L. jensenii* LBP 90Aa [◆].

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TABLE 1

Materials tested using MCC assays[a]

Organism	Tenofovir 1mg/ml No. killed/No/tested	Tenofovir 1% gel No. killed/No/tested	UC-781 39 ng/mL No. killed/No/tested	PSC- RANTES 300 μ/mL No. killed/No/tested
Lactobacillus crispatus	0/8	0/8	0/6	0/9
L. jensenii	0/11	0/11	0/3	2/10 (4)[b]
L. vaginalis	0/10	0/10	NT[c]	0/10
L. gasseri	0/10	NT	0/9	NT
Gardnerella vaginalis	0/14	0/14	0/14	NT
Neisseria gonorrhoeae	0/8	NT	0/10	0/7 (7)

[a]All strains were tested at pH 4 and pH 7 and no differences were observed. The Minimum Cidal Concentration test (MCC) is an exposure assay that is a pass/fail tests that detects a 4 $\log_{10}$ kill in 30 min.

[b]Numbers in parenthesis are the number of strains tested demonstrating a reduction in viability without reaching a 4 $\log_{10}$ kill in 30 min.

[c]NT not tested.

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TABLE 2

Log decrease or increase in CFUs when assayed in the presence of PVAs and films.

Material tested[a]	*L. crispatus*[b]	*L. jensenii*
PVA powder-9–10 kDa (Sigma)	Pass[c] (0.010–0.370)	Pass (0.015–0.733)
PVA powder30–70 kDa (Sigma)	Pass (0.006–0.410)	Pass (0.004–0.702)
Methyl –cellulose Powder 4000 cps (Spectrum)	Pass (0.072–0.420)	Pass (0.291–0.794)
Tenofovir film T_0[d]	Pass (-0.048–−0. 414)	Pass (− 0.141–0.323)
Tenofovir film T_{30}	Pass (0.069–0.139)	Fail (−0.471–−2.120)
UC781 film	Pass (0.010–0.270)	Pass (−0.371–0.197)
UC781 gel	Pass (0.005–0.280)	Fail (0.209–1.659)
Placebo Film	Pass (0.0024–−0.159)	Pass (−0.171–0.398)
Dapivirine Film	Pass (0.042–0.130)	Pass (−0.171–0.607)
Dapivirine gel	Pass (0.025–0.280)	Fail (−0.873–1.486)

[a] Active, placebo and UC781 films use PVA (PVA 9–10 kDa). UC781 gel was formulated in a methylcellulose base.

[b] *L. crispatus* ATCC 20225, ATCC 33197 and 3 field isolates were used. *L. jensenii* ATCC 25258 and four field isolates were tested with each of the material listed. All strains were tested using the LTT with vortex mixing.

[c] Pass = the test material pasted the *Lactobacillus* Toxicity Test defined as no reduction in viability. The numbers in parenthesis are the ranges of values observed in the LTT. Data are presented as $\log_{10}$ change in CFUs, compared to the controls incubated in buffer only. The Students't-test was used for statistical analysis ($P < 0.05$). A material demonstrating a reduction in viability ≥ 1 Log_{10} for two or more of 10 isolates of *Lactobacillus* tested fails the test. All but three of the materials listed passed the LTT.

[d] Tenofovir films were tested after preparation and again after one month. T_0 samples of the films examined within 3 days of preparation, and T_{30} are the results of films stored for 30 days at 50° C. Note the films degraded by thirty days and became toxic to two strains *L. jensenii* (log reductions on the order of 1.0 to 2.1 were observed).

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TABLE 3

Gel versus Films: Effects on viability of *Lactobacillus crispatus* and *L. jensenii*

Test product	Log $_{10}$ change in CFUs[a]		
	L. crispatus ATCC 33197	*L. jensenii* LBP28Aa	*L. jensenii* ATCC 25258
PVA Film Placebo	−0.338	+0.008	−0.1686
HEC Gel Placebo	−0.062	−0.272	+0.034
MC Gel Placebo	−0.049	−0.189	+0.166
PVA Film 0.01% UC781	−0.450	−0.343	−0.095
HEC Gel 0.01% UC781	+0.119	−0.880	−0.285
MC Gel 0.01% UC781	+0.132	−1.137	+0.132
PVA Film 0.01% UC781 HPC	+0.088	−0.454	−0.105
HEC Gel 0.01% UC781 HPC	−0.105	−0.660	−0.053
MC Gel 0.01% UC781 HPC	−0.098	−0.982	+0.062
PVA Film 0.01% UC781 MC	−0.055	−0.486	−0.042
HEC Gel 0.01% UC781 MC	−0.115	−0.916	−0.215
MC Gel 0.01% UC781 MC	+0.068	−0.958	−0.402

[a]Data are present as $\log_{10}$ change in CFUs, compared to the controls incubated in buffer only. (+) Values indicate the number of CFUs increase over controls while (−) values indicate there was decrease in the CFUs, zero would represent no change. Data presented are averaged from two or in some cases three separate experiments.