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Prevention of vaginal SHIV transmission in macaques by a live recombinant *Lactobacillus*

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Abstract

Most HIV transmission in women occurs through the cervicovaginal mucosa, which is coated by a bacterial biofilm including *Lactobacillus*. This commensal bacterium plays a role in maintaining healthy mucosa and can be genetically engineered to produce anti-viral peptides. Here, we report a 63% reduction in transmission of a chimeric simian/human immunodeficiency virus ($SHIV_{SF162P3}$) after repeated vaginal challenges of macaques treated with *Lactobacillus jensenii* expressing the HIV-1 entry inhibitor cyanovirin-N. Furthermore, peak viral loads in colonized macaques with breakthrough infection were reduced 6-fold. Colonization and prolonged anti-viral protein secretion by the genetically engineered lactobacilli did not cause any increase in proinflammatory markers. These findings lay the foundation for an accessible and durable approach to reduce heterosexual transmission of HIV in women that is coitally independent, inexpensive, and enhances the natural protective effects of the vaginal microflora.

INTRODUCTION

Halting the global HIV/AIDS epidemic requires interventions that have practical and achievable goals in the developing world setting. Topical prevention strategies designed to

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Conflict of interest: LAL, XL, YL, and PPL work for Osel Inc., a company developing this approach as a topical microbicide.

DISCLOSURE

LAL, XL, YL, and PPL work for Osel Inc., Mountain View, CA, a company developing this approach as a topical microbicide. BIOQUAL (BES) and ABL (RP) are for-profit institutions and are subawardees to Osel, Inc.

interdict infection at the mucosal surface represent one approach. Early prevention trials examined non-specific microbicides, such as detergents and polyanions (e.g., Nonoxynol-9 and cellulose sulfate), which were wholly ineffective or in some cases may have increased the risk of HIV acquisition due to the inflammatory and viral-enhancing properties of the compounds employed [1–7]. These clinical trials also revealed that coitally-dependent products, such as Carraguard, were used less frequently than women reported [8], highlighting the difficulty of achieving the level of adherence required for efficacy of such microbicides. Recent results from the CAPRISA 004 trial provided a much-needed proof of concept that a topical agent, the specific antiretroviral drug tenofovir delivered as a gel, can prevent the acquisition of HIV in women [9]. However, the efficacy of this approach was modest (39%), probably in large part because it is a coitally dependent regimen and the gel must be applied within 12 hours both before and after sexual intercourse for maximum efficacy. Concluded from the trial results was that the main goals are to improve adherence and to develop coitally independent microbicide strategies.

In many cultural settings, women need a product that can be used covertly without obtaining the permission of their sexual partner. Additionally, the cost of the HIV prevention must be affordable to the developing world. Thus, there is still a need for products that block HIV transmission, are safe and easy to use, and are coitally independent, discreet, as well as cost effective.

The majority of viral transmissions in women occur during unprotected vaginal intercourse on the mucosal surfaces of the endocervix, cervix and vagina. In macaques [10] and human vaginal explant tissues [11,12], Langerhans cells and intraepithelial CD4+ T cells are the targets of HIV-1 viral entry and initial replication [12]. Mucosal surfaces of the lower female reproductive tract are normally coated with a biofilm of commensal bacteria including lactobacilli, which are an important component of the vaginal microbiome and provide the first line of defense against invading pathogens [13]. Lactobacilli acidify the vagina by producing lactic acid [14] and may reduce the risk of acquiring HIV-1 and other sexually transmitted infections [15,16].

One practical and cost-effective approach to protect the vaginal epithelium is to colonize it with a recombinant *Lactobacillus* expressing an anti-viral protein, thus transforming the vaginal microflora into a “live” bioshield [17]. This is a novel approach to preventing the transmission of HIV, and eventually other mucosal pathogens, and thereby represents a platform approach to intervention of disease transmission that is both discreet to the user and partner. Importantly, it is also inexpensive to manufacture.

Cyanovirin-N (CV-N), a protein originally isolated from the cyanobacterium, *Nostoc elipsosporum*, was selected as a model HIV inhibitor for this approach. CV-N has broad cross-clade potency against HIV [18,19] including primary isolates [20,21]. Previous studies showed that CV-N protein, formulated in a gel, protected macaques from vaginal and rectal challenge [22,23].

We previously described a *Lactobacillus* strain, *L. jensenii* 1153–1666, that is genetically engineered to secrete an amino-terminally modified CV-N protein (P51G) [24]. CV-N protein

isolated from the culture media of this strain has potent anti-HIV activity against CCR5- and CXCR4-tropic viruses *in vitro* whereas control media from the parental strain, *L. jensenii* 1153 OSEL 175, is inactive [24]. Compared to the parental strain, *L. jensenii* 1153–1666 displays no loss of fitness, grows to the same levels and produces similar levels of lactic acid and hydrogen peroxide [24]. Although *L. jensenii* 1153–1666 persisted in the estrogenized-CD1 mouse model [24], mice do not support *Lactobacillus* colonization as they have significantly different vaginal biology from primates. Therefore, to advance this platform technology, a non-human primate model was developed.

The rhesus macaque model is commonly used for microbicide and vaccine studies, but has not previously been used for testing live microbicides; therefore we performed extensive characterization to determine whether rhesus macaques were suitable as a model. We found that Chinese and Indian-origin rhesus macaques typically harbor abundant endogenous vaginal *L. johnsonii* as their predominant species, and, like humans, generally have only one vaginal *Lactobacillus* strain at a time [25]. We examined the DNA fingerprints and biochemical profiles of *L johnsonii* strains and found that most are strong H_2O_2 producers and are similar to the human strain *L. gasseri* [25]. We further characterized changes in the vaginal microbiome during menses. Unlike humans, the endogenous *Lactobacillus* flora of the macaque varies more throughout the menstrual cycle, with the lowest levels found at or around menstruation [25,26]. Vaginal pH in macaques is also higher than in humans with the average value around 6.3 among the animals in this study. Higher vaginal pH is also found in women with bacterial vaginosis (BV), which predisposes them to increased risk of HIV infection [16]. Thus it has recently been suggested that the macaque may be a useful model for HIV protection studies because they resemble women with BV [27]. Displacement of endogenous lactobacilli followed by long-term colonization of rhesus macaques with human *L. jensenii* 1153 strain has been achieved, making this animal model useful for pre-clinical evaluation of live *Lactobacillus*-based anti-viral approaches [25].

Here, we used the macaque model to test colonization of recombinant lactobacilli, CV-N protein expression and efficacy of the recombinant *L. jensenii* 1153–1666 in a repeated vaginal SHIV challenge model, and demonstrate that colonized animals show a 63% reduction in acquisition of the virus. We also examined vaginal pH, histology and biomarkers of inflammation to assess the safety of this approach.

RESULTS

Colonization and protein expression of recombinant *Lactobacillus* in macaques

First we examined the duration of colonization and *in situ* protein expression using *L. jensenii* 1153–1666 in macaques. Vaginal inoculation of two macaques, M1 and M2, with live *L. jensenii* 1153–1666 delivered in hydroxyethylcellulose HEC on five successive days led to sustained colonization and secretion of full length CV-N that was detected in cervical vaginal lavage (CVL) as early as 24 hr post inoculation, and persisted at three and at six weeks post inoculation (Fig.1). Decreases in the CV-N levels in CVL, as seen in animal M1 at week 3, appear to be due to lower levels of *Lactobacillus* colonization during menses [25]. CV-N in CVLs collected from macaques was measured at a concentration of 83–160 ng/ml.

Histology and immunohistochemistry were performed on vaginal biopsies taken from control macaques or macaques colonized with *L. jensenii* 1153–1666. Colonization by the recombinant *Lactobacillus* did not lead to observable inflammation or disruption of squamous epithelial cell integrity as indicated by examination of vaginal histology sections from *L. jensenii* 1153–1666 colonized macaques compared to controls (Fig. 2a). To detect CV-N *in situ*, biopsies were incubated with control serum or anti-CV-N serum. The CV-N specific serum revealed co-localization of CV-N protein with *Lactobacillus* bacteria on the vaginal mucosa and in the vaginal lumen (Fig. 2b).

Examination of mucosal markers of inflammation, vaginal pH and immunogenicity

To determine the effects of the recombinant *Lactobacillus* on the vaginal environment, we performed sequential measurements of proinflammatory cytokines and pH in CVL of animals in the absence of detectable *Lactobacillus*, in the presence of spontaneously occurring endogenous *L. johnsonii*, and in the presence of introduced recombinant *L. jensenii* 1153–1666. The cytokine measurements focused on four pro-inflammatory mediator cytokines that are often used as biomarkers of inflammation and indicators of mucosal barrier function: IL-1β, IL-1ra, IL-6, and IL-8 [28,29]. As shown in Fig. 3 a–d, there was a high variation from animal to animal in the measured values of these cytokines, but no consistent or statistically significant differences as a function of bacterial colonization status. The average vaginal pH value of the macaques used in this study, as determined by seven sequential measurements, was 6.3 ± 0.78 (mean ± SEM). Comparison of uncolonized animals to those harboring endogenous *L. johnsonii* or introduced *L. jensenii* 1153–666 did not reveal any significant variations (Fig. 3e). Additionally, macaques that were repeatedly exposed to *L. jensenii* 1153–1666 had no detectable IgG, IgA or IgM response to either CV-N or killed *Lactobacillus* measured in CVL whereas macaques immunized via the intramuscular route with 100 μg/ml purified CV-N delivered in adjuvant exhibited a robust mucosal and systemic immune response to CV-N (data not shown).

Inhibition of HIV-1 infection in an EpiVaginal tissue model

The MatTek EpiVaginal™ Tissue Model, was used to assess the activity of the *L. jensenii* 1153 (parental strain) vs. *L. jensenii* 1153–1666 (producing CV-N). Infection was monitored by p24 release at day 3, 5 and 8-post infection. It was shown that co-cultivation of the tissue model with the *L. jensenii* 1153 parental strain reduced infection by 23%, compared to a control with no added bacteria ($p=0.1$), whereas co-cultivation with CV-N-producing *L. jensenii* 1153–1666 inhibited infection by 72% ($p<0.008$) (Fig. 4).

SHIV challenge protocol and results

To determine the efficacy of the *L. jensenii* 1153–1666 live prevention strategy in the macaque model, we designed a repetitive vaginal challenge protocol using the well-characterized CCR5-tropic virus $SHIV_{SF162P3}$. The repeated challenge model was chosen because it more closely resembles human infection than does a single challenge using either a very high viral dose or pretreatment with DepoProvera®. The study protocol is shown in Fig. 5a. Twelve menstrually cycling adult female macaques were given vaginal azithromycin suppositories to reduce endogenous *Lactobacillus* flora. They were then

colonized with *L. jensenii* 1153–1666 for five days initially, and again at 48 and 24 hrs prior to each of six weekly challenges. Each challenge was performed a minimum of 24 hr after the bacterial inoculation, a period sufficient for CV-N protein accumulation to occur (Fig 1a) as well as to demonstrate the potential of coital independence. The challenge contained 300 median tissue culture infectious doses ($TCID_{50}$) of $SHIV_{SF162P3}$; this amount of virus was previously determined to infect approximately 1 of 3 animals after each challenge. We confirmed the presence and quantity of *L. jensenii* 1153–1666 by culture and the expression of CV-N by analysis of recovered *L. jensenii* 1153–1666 (Table 1).

The results of the challenge study are shown as a Kaplan-Meier analysis (Fig. 5b). Twelve control macaques were infected at a rate of 35.4% following each vaginal $SHIV_{SF162P3}$ challenge; 31 challenges were required to infect 11 controls (11/31 = 35.4%), with one control macaque remaining uninfected following six challenges. By contrast, 12 macaques treated with *L. jensenii* 1153–1666 (LB-CVN) were infected significantly less frequently: 61 challenges were required to infect eight LB-CVN treated macaques (8/61 = 13.1%), with four macaques remaining uninfected following six challenges. Thus, this live prevention strategy reduced the infection rate by 62.9% ($p = 0.0039$).

The data were further analyzed by separately considering the three sub-groups of the 12 control animals that received no exogenous *Lactobacillus*: those that received no treatment of any type (n=4), HEC only (n=4) or azithromycin + HEC (n=4). Fig. 5c shows that each of these subgroups has a greater infection rate than the experimental animals, and this difference was significant for the non-treatment group ($p = 0.008$) and the HEC only group ($p = 0.002$).

Unexpectedly, we also observed that the peak SHIV RNA plasma viral levels in macaques with breakthrough infection were 6-fold lower in experimental animals that had been colonized with *L. jensenii* 1153–1666 compared to infected non-colonized control animals (Fig. 6). Despite variability in the peak viral loads, this difference was significant ($p = 0.029$).

The serological response to SHIV was evaluated by testing all plasma samples for seroconversion and quantifying antibody titers to p27, a simian immunodeficiency virus (SIV) core antigen. All macaques with detectable plasma viral load also generated antibodies to SIV specific antigens with a prominent p27 protein band in Western blots and positive titers to p27 by ELISA, whereas all protected animals had no detectable SIV-specific antibodies by either Western blot or ELISA (data not shown).

In addition, we measured TRIM5α alleles for 20/24 macaques used in the study, including all 12 Lactobacillus inoculated animals and 8 control animals (Supplemental Table 1). We found no correlation between TRIM5α resistant or sensitive phenotype and rate of infection or viral load.

DISCUSSION

This study examined a live recombinant *Lactobacillus* producing an HIV-entry inhibitor on the cervical/vaginal mucosa as a means to prevent transmission of $SHIV_{SF162P3}$ to

macaques. Genetically engineered lactobacilli, which are capable of forming a colonizing biofilm on the cervical/vaginal mucosa, can provide a continuous delivery system for anti-viral proteins. Furthermore, they provide beneficial protective effects of a normal vaginal microbiome, which helps to control a variety of vaginal infections through multiple mechanisms including H_2O_2 and lactic acid production [13–16].

Given the multifactorial nature of this approach, the present study was designed to evaluate the combined, natural and engineered antiviral properties of L. *jensenii* 1153–1666 rather than to focus only on anti-viral protein expression. Control animals were not experimentally colonized with wild-type lactobacilli, but. two control subgroups did receive either HEC placebo gel, which was used as a carrier for the *L. jensenii* 1153–1666, or HEC plus azithromycin, an antibiotic that was administered to reduce endogenous flora. There was a statistically significant difference in protection between *L. jensenii* 1153–1666 colonized animals and the control group as whole and between *L. jensenii* 1153–1666 colonized animals and the control non-treated or HEC only subgroups. However, the number of animals in the individual subgroups was too small for a definitive sub-group comparison.

Now that efficacy of *L. jensenii* 1153–1666 has been established, further experiments are required to differentiate the effects of the host organism as compared to the produced protein. *Lactobacillus* is associated with good vaginal health, and may have some degree of indigenous protective activity against a variety of pathogens including HIV. Initial *in vitro* experiments using HIV infection of a human vaginal tissue model showed that co-cultivation with the *L. jensenii* 1153 parental strain reduced infection by 23%, (but did not reach statistical significance $p=0.10$) compared to a control with no added bacteria, whereas co-cultivation with CV-N-producing *L. jensenii* 1153–1666 inhibited infection by 72% ($p<0.008$). By contrast, in a MAGI cell attachment assay, total conditioned media from the parental strain showed no activity compared to strong inhibition by material from CV-N-producing cells, suggesting that whatever inhibition is due to the innate activity of *Lactobacillus* occurs through a mechanism distinct from the fusion inhibiting activity of CV-N (data not shown).

An essential criterion for a successful microbicide is that it not cause inflammation, which is well known to increase susceptibility to HIV infection [16,30–32]. In our study, we found no evidence for *L. jensenii* 1153–1666-induced inflammation either by microscopic examination of vaginal biopsy samples or by measurement of multiple pro-inflammatory mediator cytokines. In addition, CV-N did not appear to be immunogenic.

In many women, lactobacilli are the dominant flora and contribute to the average pH value of around 4 through lactic acid production [14]. The pH of the vagina may also influence HIV transmission, with low pH values acting in a protective fashion. But it’s important to note that HIV-1 is introduced in semen, thus vaginal pH can transiently fluctuate to neutral or even basic pH for several hours post coitus [33]. CV-N protein is active in a range from pH 4–8.2 [17].

In this model, we show that macaques have a more dynamic and complex flora, which is more similar to women with BV. Thus, the macaque study may underestimate the potential

efficacy of recombinant lactobacilli in humans, in whom the bacteria will play a more dominant role. Importantly, a Phase 2a study has shown that 61–78% of women with BV can be re-colonized with an introduced *L. crispatus* strain [34].

CV-N protein was used in this study because it has broad activity against a wide range of HIV-1 isolates, it is stable in the vaginal environment, and it is potent at achievable concentrations. Huskins *et al.* [35] have questioned the safety of CV-N as a topical microbicide because they observed mitogenic activity and enhanced expression of T cell activation in peripheral blood mononuclear cells exposed to certain preparations of the protein. However, the clinical relevance of the Huskins study is unclear since it is not known whether the proteins used in their analysis were monomeric (P51G) [36] or dimeric (P51) or whether they were contaminated with endotoxin. By contrast, a study by Buffa *et al*. found low levels of T-cell proliferation only during prolonged exposure to extremely high CV-N concentrations (360,000 nM) with no tissue toxicity [20]. We observed that expression of the low 1–2 μg/ml (92–183 nM) levels of monomeric CV-N (P51G) produced by *L. jensenii* 1153–1666 did not enhance expression of typical markers associated with mucosal barrier function or inflammation. While additional biomarkers evaluation is needed to determine any toxicity that may be seen with long-term use, these preliminary studies suggest that this low level is safe.

With the recent characterization of the early HIV-1 infection events and findings that a majority of HIV transmissions involve a single founder virus [37], there has been a re-examination of SHIV and SIV models in an attempt to mimic the physiologic dosage seen in natural infection. This has led to the use of lower doses with repeated challenges, requiring more prolonged studies but being more physiologically relevant than high dose models. For example, low-dose rectal inoculations of macaques with SIV were shown to more closely recapitulate human mucosal infection by HIV-1 [38] in terms of the number of viral variants that were transmitted. Repeated challenge approaches also increase the statistical power achieved [26,39]. Perhaps most importantly, lower challenge doses may be responsive to clinically achievable doses of microbicides; protection against high doses typically requires extraordinarily high and potentially toxic doses of the tested compounds [22,23,40–42]. For example, when tested in a cynomolgus macaque model, 0.5–2% CV-N, equivalent to 5–20 mg/ml (454 μM-1.8 mM) was required to inhibit 83% of infections in a very stringent vaginal challenge of 5000 $TCID_{50}$ on epithelium that was thinned with medroxyprogesterone [22]. Such early studies lead to the belief that vast excesses of microbicide were needed to provide efficacy and led to concerns about toxicity of the products used at such high levels.

These factors led us to the selection of a repeated low-dose vaginal challenge model for testing *L. jensenii* 1153–1666. The $SHIV_{SF162P3}$ stock was diluted to a level that infects untreated macaques at approximately 30% per challenge. This infection rate is still higher than that for women infected heterosexually, thus providing a stringent test of protection, yet more closely mimics natural infection than use of single high dose.

The reduction in viral load in macaques receiving *L. jensenii* 1153–1666 was an unexpected finding. It has been hypothesized that small founder populations of virus undergo local

expansion prior to systemic dissemination [43]. It is possible that these initial foci of infection are still accessible to the mucosally produced CV-N, providing some level of viral control after the initial infection event. Certain virus strains, such as SIV_{E660}, are restricted by TRIM5α alleles. We found no correlation between susceptibility related to TRIM alleles. Restrictive MHC alleles, such as MamuA01, are rare in Chinese-origin macaques, but more common in Indian-origin macaques [44,45].

In this study, macaques were repeatedly colonized with *L. jensenii* 1153–1666 at least 24 hrs prior to each viral challenge. However, because this *Lactobacillus* strain was shown to colonize and secrete CV-N for at least six weeks following administration, it likely has the capacity to provide considerably more durable protection with relatively infrequent re-colonization. The ability of the recombinant *Lactobacillus* to continually colonize also suggests that our introduction of a single gene into the bacterial chromosome did not have a deleterious effect on its growth and survival *in vivo*. Importantly, because healthy women have 10-fold higher levels of *Lactobacillus* colonization in the cervical and vaginal mucosa than do female rhesus macaques, this approach may actually lead to even greater protection in humans than observed in this model.

The live *L. jensenii* 1153–1666 reduced the rate of $SHIV_{SF162P3}$ acquisition by 63% in the macaque model. Statistical modeling predicts that a reduction in the rate of HIV acquisition by 50% using either a partially effective vaccine or microbicide could have a significant impact on the global HIV epidemic [46,47]. Our data suggest that this approach to HIV prevention is effective and safe. It is also highly flexible since the modular nature of the *Lactobacillus* expression system readily allows the expression of other HIV inhibitor proteins, such as the CCR5 inhibitor C1C5 RANTES [48], or even the production of multiple inhibitors. Furthermore, because lactobacilli are inexpensive to ferment and manufacture, this approach is eminently suitable for large-scale manufacturing for delivery to women in the resource-limited settings of the developing world.

METHODS

Study animals, procedures, and pH analysis

Twenty-four normal cycling female Chinese-origin rhesus macaques (*Macaca mulatta*), ages 3.5–7 yrs were used in this study. Animals were housed either at BIOQUAL, Inc. in Rockville, MD, or at Advanced Biosciences Laboratories (ABL), Kensington, MD. Nonhuman primate housing, care, and treatments were performed in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), the Animal Welfare Act as amended, the Public Health Service Policy on Humane Care and Use of Laboratory Animals, 2002 and the NIH guidelines for Research Involving Recombinant DNA Molecules. In all studies animals were sedated with 10 mg/kg ketamine and 1 mg/kg acepromazine when required. Vaginal pH was monitored using a portable HI 99181 Skin pH Meter Hanna Instrument® (Woonsocket, RI). We monitored menses by inserting a clean saline dampened cotton-tipped applicator into the monkey's vagina. The swab was then removed and a recording from 0 to 3 was made (0=no blood, 1=slight, 2=moderate, 3=heavy bleeding). The monkeys were trained for this procedure by giving food as a reward. Cervicovaginal lavages (CVLs) were taken by

instilling 2 mL of PBS spiked with complete, EDTA-free protease Inhibitor (Roche Applied Science, Indianapolis, IN) into the vagina, flushing it back and forth four times, and then collecting the fluids on ice. Samples were clarified by centrifugation at 820×g at +4°C and snap frozen on dry ice, prior to storage at −80°C until analysis. Samples were collected in all phases of the menstrual cycle.

Genetic modifications of *Lactobacillus*

Lactobacillus jensenii 1153–1666 used in the study was created at Osel, Inc. and is described in Liu *et al.* [24]. Briefly, the nucleotide sequence of the CV-N gene was recoded for optimal *Lactobacillus* codon usage. In addition, proline at amino acid 51 was changed to glycine (P51G) by site-directed mutagenesis to stabilize CV-N in a properly folded monomeric form. A 4-amino-acid peptide containing, alanine, proline, valine, and threonine (APVT), corresponding to the amino terminus of the mature collagen-binding S-layer protein A (CbsA) protein of *Lactobacillus crispatus*, was inserted downstream of the CbsA signal sequence to resemble the native signal peptidase cleavage site [24]. An expression cassette containing an *L. jensenii* promoter for the ribosomal protein subunit (P_{rpsU}) directing expression of APVT-CV-N (P51G) was integrated by homologous recombination in a single copy into the *pox1* site of the genome of *L. jensenii* 1153. The resolved strain, *L. jensenii* 1153–1666, was used in the macaque studies described in this paper. *L. jensenii* 1153–1666 when cultured to ~10^8 CFU, secretes modified CV-N at concentrations of approximately 1.5–2 μg/ml [24]. APVT-CV-N has similar anti-viral activity against CCR5 and CXCR4 viruses as the original CV-N protein [24].

Lactobacillus growth conditions, colonization and sampling

Lactobacilli were cultured using Mann Rogosa Sharp (MRS) broth or agar purchased from Difco (Becton Dickinson, Franklin Lakes, NJ). A colonization model using the Chinese rhesus macaque for testing a live *Lactobacillus*-based microbicide was established prior to this study [25]. Lactobacilli were delivered to the macaques in a 3 ml volume with equal volumes of MRS and 2.7% hydroxyethylcellulose (HEC) (Hercules Incorporated, Hopewell, VA). The vaginal microflora of each animal was sampled using the Port-A-Cul™ swab/tube collection system (Becton Dickinson, Cockeysville, MD, USA). Vaginal swabs were taken two days prior to each challenge and plated to MRS agar (Difco, BD) and PRAS Brucella plates (Anaerobe Systems, Morgan Hill, CA). Lactobacilli were identified using Gram stain, and API Microorganism Identification Strips (Biomerieux, Inc, St. Louis, MO). *Lactobacillus sp.* identity was confirmed by DNA extraction and 16S ribosomal DNA (rDNA) sequencing and BLASTn DNA sequence analysis.

Detection of CV-N in CVL

Macaques were colonized with live *L. jensenii* 1153–1666 in HEC without the use of antibiotic to determine the extent and duration of colonization. CVLs were performed to detect secreted CV-N protein collected at 24 hr, 3 and 6 weeks post *Lactobacillus* inoculation. To detect CV-N, CVL samples were incubated with a rabbit pAb against CV-N (Cocalico Biologicals, Inc., Reamstown, PA). The mixture was then coupled to the pre-washed and pre-equilibrated protein-A sepharose beads (Sigma-Aldrich, St. Louis, MO) at

4°C for four hours under agitation. The beads were washed with lysis buffer, and the immunoprecipitated proteins were separated on 4–12% SDS-PAGE gel and transferred to polyvinylidene fluoride membranes (PVDF) using an IBlot system (Invitrogen, Carlsbad, CA). CV-N present in the CVL was visualized with a goat anti-CVN pAb (Cocalico Biologicals) and WesternBreeze® Chromogenic kit (Invitrogen). Control CV-N used in Western blots did not contain the N-terminal APVT sequence and had a slightly faster mobility on SDS-PAGE gels.

Vaginal biopsies and *in situ* detection of CV-N

Vaginal pinch biopsies were collected from *L. jensenii* 1153–1666 colonized macaques and either formalin fixed and stained with hematoxylin and eosin or flash frozen for immunohistochemistry (IHC). Samples for IHC were microsectioned and reacted with goat anti-CV-N antibody diluted 1:4000 in PBS containing 1% bovine serum albumin (BSA). Secondary antibody was anti-goat conjugated with immunoperoxidase diluted 1:500 in PBS-BSA. DAB (3,3'-diaminobenzidine) substrate (Sigma) was used to visualize the CV-N in tissue. All IHC procedures were performed by Histoserv Inc., Germantown, MD. The sections were visualized using light microscopy on a Leica SP5 Confocal Microscope (Leica Microsystems, Mannheim, Germany). Slides were viewed and photographed using either a 10×-low power objective or 63×NA1.4 oil immersion objective.

Cytokine and immunoglobulin measurements

The levels of IL-1β, IL-1ra, IL-6, and IL-8 in CVLs were determined from 16 of the macaques used in the challenge experiment, with a multiplexed fluorescent microsphere immunoassay using the Luminex 100 system (Luminex Corporation, Austin, TX) using Milliplex™ MAP Non-human primate cytokine kit (Millipore). Data were collected and analyzed with Bioplex Manager v3.0 software (Bio-Rad, Hercules, CA) using a 5-parameter fitting algorithm. To account for possible differences between tests, samples were analyzed in overlapping batches testing all the time points from the individual animals at the same time. CV-N specific and *L. jensenii* specific IgG, IgA and IgM from macaque CVL were measured by ELISA. NUNC Maxisorp (Thermo Scientific, Rochester, NY) plates were coated with CV-N protein or *L. jensenii* 1153 whole bacteria, blocked with 1% casein (Thermo Scientific) and 10% heat inactivated goat serum. CVL and serum samples were tested for the presence of antibodies to CV-N and *Lactobacillus* using goat anti-monkey IgA, IgG and IgM (Rockland Immunochemicals Inc., Gilbertsville, PA) and visualized with p-Nitrophenyl Phosphate, Disodium Salt (Sigma) substrate at 405 nM. Control macaques were immunized with CV-N delivered in Titermax® Gold Adjuvant (Sigma). Immunized macaques reached serum titers against CV-N of up to 1:36,000 following immunization and antibodies to CV-N were detected in CVLs collected after the second immunization.

SHIV stock

For expansion of $SHIV_{SF162P3}$, a naïve Indian rhesus macaque, which was shown to be free from SRV, STLV, B and foamy viruses, was infected intravenously with 1 ml of cell-free $SHIV_{SF162P3}$ stock. The infected macaque was monitored for plasma viremia and when the RNA load reached ~10^8 copies/ml, PBMCs were harvested, depleted of CD8+ T cells and

activated with PHA for 72 hours. Activated CD4+ T- cell-enriched PBMCs were then cocultured with PHA-activated PBMCs pooled from three macaques. Culture media was replenished as needed with fresh medium every 2–3 days and fresh PHA-activated rhesus PBMCs were added after five days. The culture was monitored for the presence of SIV Gag p27 protein by antigen capture ELISA kit (ABL) and when the SIV p27 level reached >50 ng/ml, cell free supernatant was harvested, filtered, aliquoted into 1.0 ml aliquots, and stored in the vapor phase of liquid nitrogen.

MatTek culture

The MatTek EpiVaginal™ Tissue Model VLC-100-FT (MatTek Corporation, Ashland, MA) was used to assess the effect of *L. jensenii* 1153–1666 vs. *L. jensenii* 1153 (parental strain) on HIV-1 replication. Epivaginal tissue VLC-100 FT, which includes vaginal-ectocervical epithelial cells, fibroblast-containing lamina propria and dendritic cells, was infected with HIV-1_{SF162} at a p24 concentration of 3 ng/ml in 100 μl, in the presence of 10^7 CFU *L. jensenii* 1153–1666 or 10^7 CFU *L. jensenii* 1153 (parental strain). Two assays were performed with duplicate tissue replicates for each sample. Samples of tissue culture media were harvested for p24 analysis at day 1, 3, 5 and 8-post infection. HIV-1 p24 concentrations were determined using ELISA (PerkinElmer, Inc., Waltham, MA).

SHIV challenge study

In the SHIV challenge study, azithromycin suppositories were administered vaginally to 12 macaques to clear their *Lactobacillus* flora. After a 1-week rest, the 12 macaques in the experimental arm were colonized with *L. jensenii* 1153–1666 in approximately 3 ml of a 1:1 ratio of MRS broth and HEC daily 5 times in the first week and 2 times each following week with SHIV challenge 24–48 h later. Control animals (n=12) received the following, azithromycin treatment (as described above) followed by 2.7% HEC (as described) (n=4), 2.7 % HEC (as described) (n=4) or no treatment (n=4).

SHIV challenges were performed 24–48 hours after *Lactobacillus* or HEC inoculation. One ml of RPMI containing 300 $TCID_{50}$ $SHIV_{SF162P3}$ in RMPI was intravaginally inoculated using a 3cc syringe in an atraumatic manner. Animals were challenged up to 6 times or until infection was established by a positive plasma viral load.

Viral load determination and seroconversion

Quantitative SHIV RT-PCR was performed as described previously [49]. Protection was defined as absence of plasma viremia (RNA viral load) at weekly time points from 7 to 70 days after challenge. Infection was defined by consecutive positive viral RNA detections >50 copies SHIV RNA/ml. Plasma samples obtained from all macaques were tested for seroconversion and antibody titers to p27, a simian immunodeficiency virus (SIV) core antigen, using an ELISA (performed at ABL). In addition, all plasma samples were tested by Western Blot Assay (ZeptoMetrix Corporation, Buffalo, NY) to detect antibodies to SIV antigens using a modified procedure recommended by the manufacturer.

Trim5α allele testing

TaqMan RT-PCR was used to amplify Trim5α alleles from PBMCs in 15 macaques (BIOQUAL, Inc). In five macaques genomic sequence was determined from blood (Harvard NEPRC, Southborough, MA). Four macaques were not tested due to insufficient numbers of PBMCs available.

Statistical analysis

Paired Student's T tests were used to analyze the vaginal pH data. The cytokine data and HIV-1 inhibition in MatTek cultures were analyzed with nonparametric Wilcoxon matched-pair signed-rank test using Prism Version 5 (GraphPad Software, La Jolla, CA). The proportion of uninfected vs infected animals was analyzed using Kaplan-Meier survival analysis, and peak viral loads compared using the Student's T test, in JMP Version 8 (SAS, Cary, NC). All the hypothesis tests were two-tailed, and a p value of ≤ 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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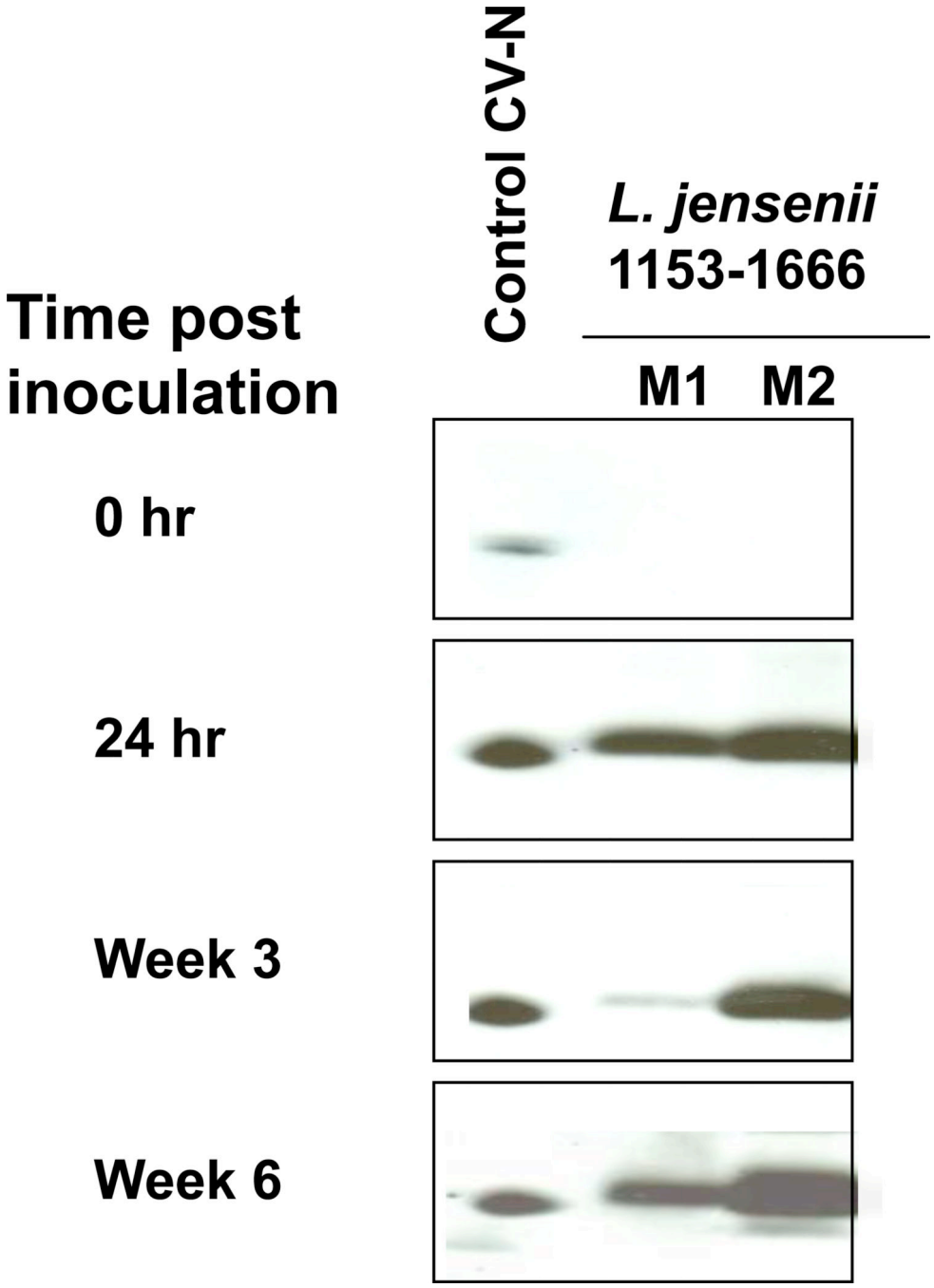


Fig. 1.

Expression and detection of CV-N *in situ* in the macaque vagina. *L. jensenii* 1153–1666 was used to colonize macaques for a 5-day period. Following this dosing regimen, cervicovaginal lavages (CVLs) were taken at 24 hr, 3 weeks and 6 weeks. Samples of each CVL were immunoprecipitated and then visualized by Western blot. Expression of CV-N was constitutive for up to 6 weeks post inoculation. At week 3, monkey 1 (M1) had a very low but detectable CV-N protein band, likely caused by reduced levels of CV-N-producing lactobacilli due to menstruation. *L. jensenii* 1153–1666, measured in CVL, grew on vaginal

epithelial cells *in vivo* at concentrations up to 7×10^6 CFU and yielded CV-N at 83–160 ng/ml.

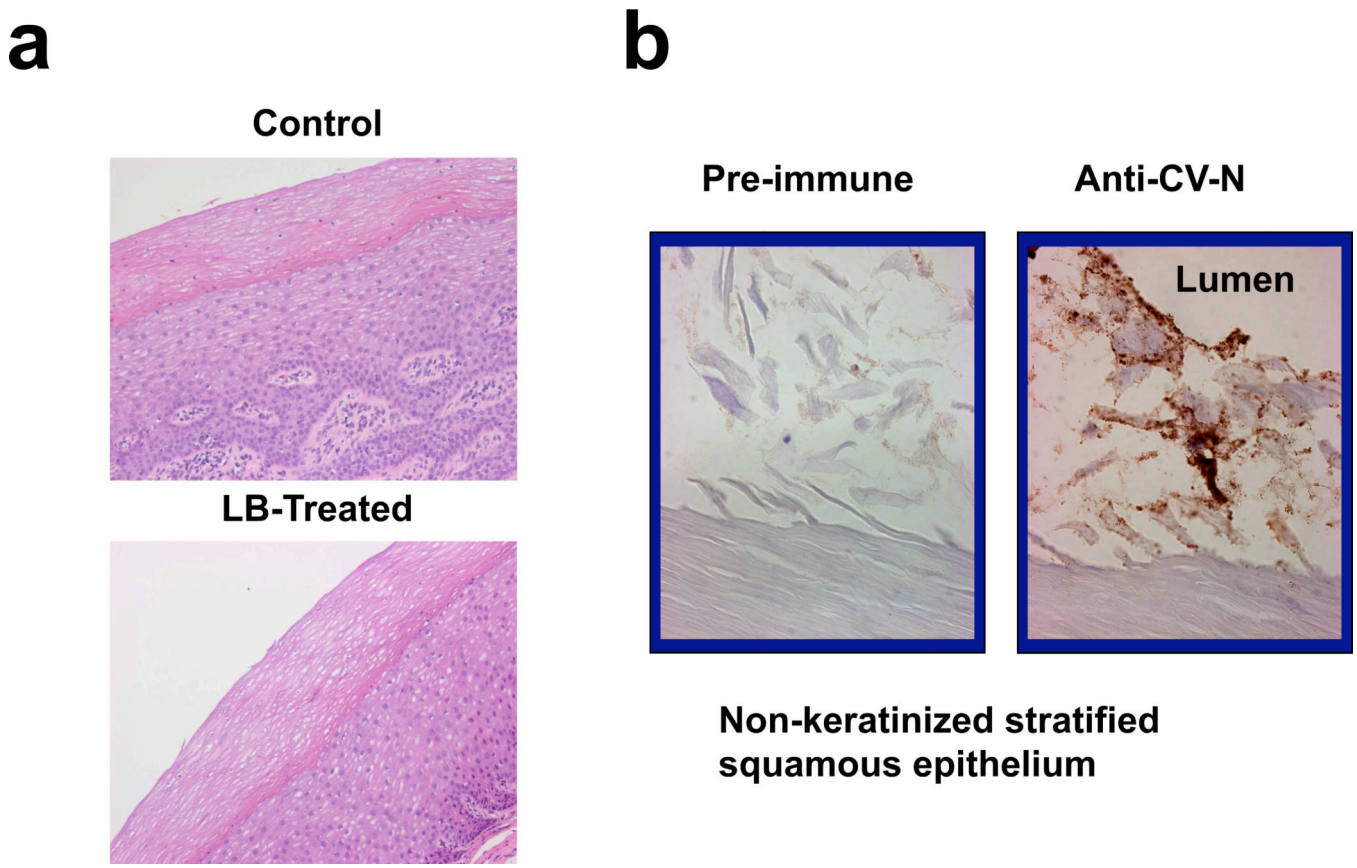


Fig. 2.

a) Histology of vaginal biopsies. A biopsy from a macaque colonized with *L. jensenii* 1153–1666 for one month (bottom) and one from a control macaque (top) were compared. Formalin-fixed sections were stained with hematoxylin and eosin and examined microscopically. There was no evidence of loss of epithelial cell integrity and no leukocytic infiltration noted. Two representative biopsies are shown at 20× magnification. b) Immunohistochemical analysis of CV-N in a vaginal biopsy. The biopsy on the left is reacted with pre-immune serum and shows no staining. In the biopsy on the right, the CV-N staining co-localized with the *Lactobacillus* bacteria on the vaginal mucosal surface and in the vaginal lumen.

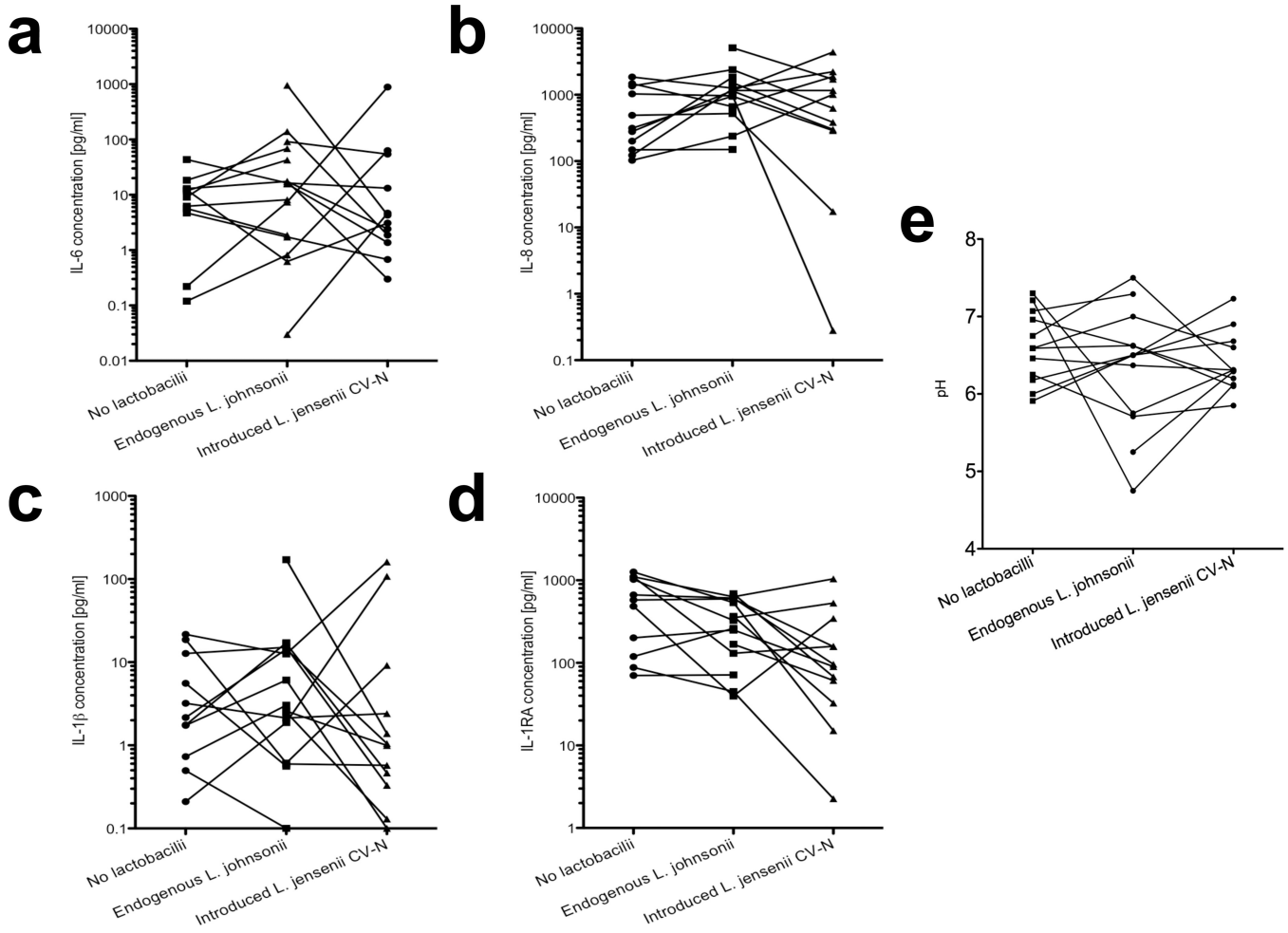


Fig. 3.

Measurements of biomarkers of inflammation in CVL and pH. The levels of IL-6 (a), IL-8 (b), IL-1β (c), and IL-1ra (d), were determined in CVLs of 16 macaques with no lactobacilli, or colonized with either endogenous strains of *L. johnsonii* or with introduced *L. jensenii* 1153–1666. Comparisons were made within the same macaque. e) Vaginal pH values were determined from 12 macaques at multiple time points using a portable Skin pH Meter. Comparisons were made for the same macaque with no lactobacilli or colonized either with endogenous strains of *L. johnsonii* or with *L. jensenii* 1153–1666.

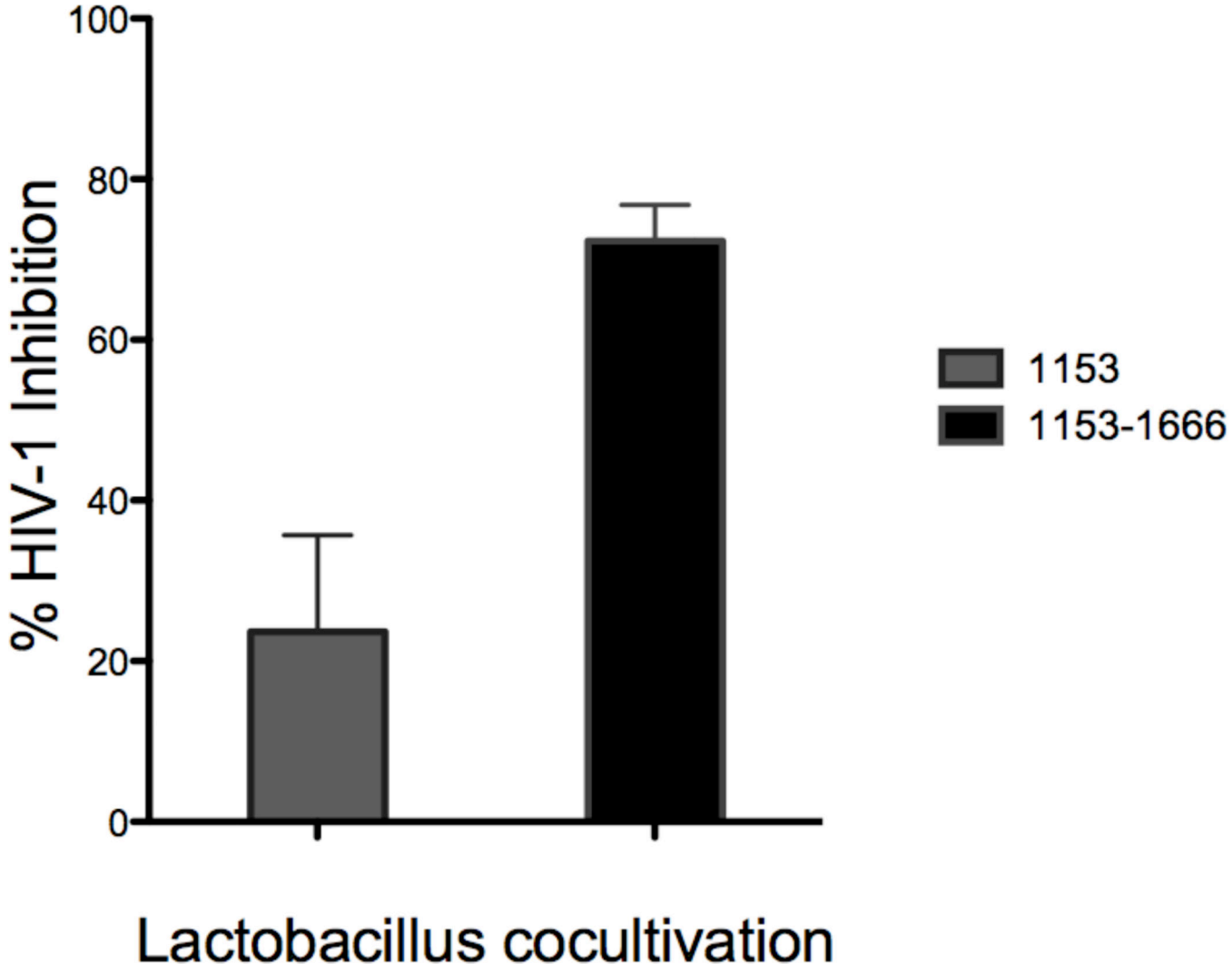
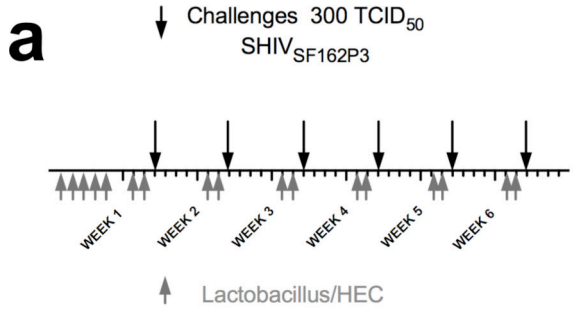


Fig. 4.

Inhibition of HIV-1 infection in a MatTek EpiVaginal tissue model. Initial *in vitro* experiments were performed with HIV-1_{SF162} infection of the MatTek EpiVaginal™ Tissue Model VLC-100 FT. We show that co-cultivation of the tissue model with the *L. jensenii* 1153 parental strain reduced infection by 23%±12.1 (mean ± SEM), compared to a control with no added bacteria, whereas co-cultivation with CV-N-producing *L. jensenii* 1153–1666 inhibited infection by 72% ±4.5 (mean ± SEM), (Fig. 4). Supernatants were harvested for p24 analysis and HIV-1 infection was determined by p24 ELISA (PerkinElmer, Inc., Waltham, MA).



Macaque	Azithromycin	L. jensenii 1153-1666	2.7% HEC	No treatment
Experimental	12	12	12	
Control	4		4	
Control			4	
Control				4

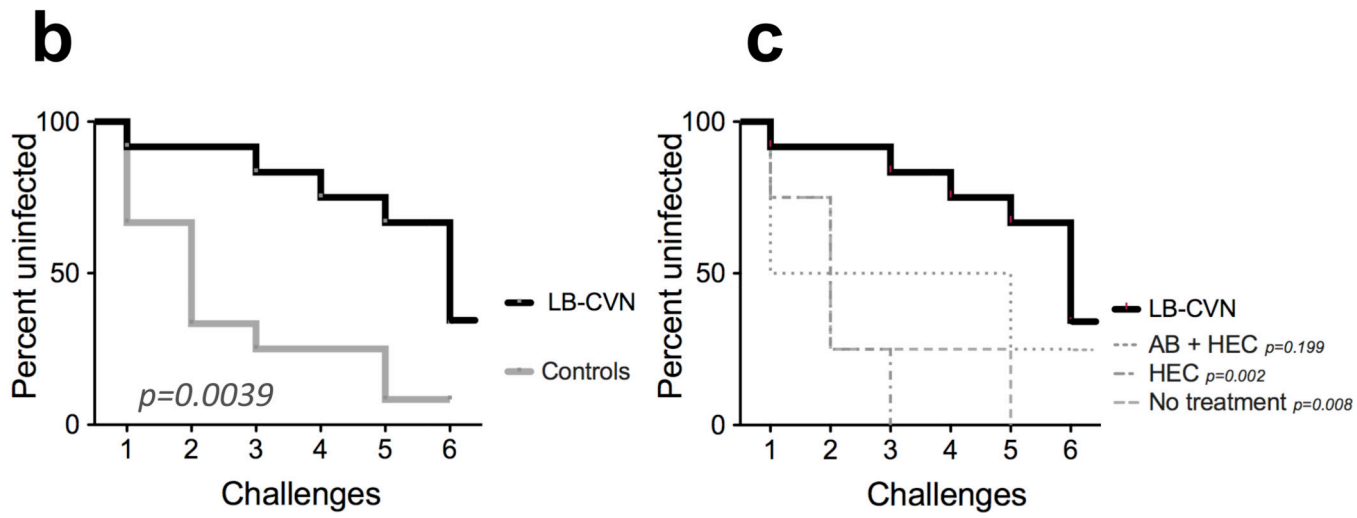


Fig. 5.

L. jensenii 1153–1666 protected rhesus macaques against repeated vaginal challenge by $SHIV_{SF162P3.}$ (a) Study protocol. Adult female rhesus macaques (n=12) were treated with the antibiotic azithromycin vaginally one week prior to the study. Animals were inoculated with *L. jensenii* 1153–1666 in 2.7% HEC for a 5-day period, and then again for 2 days prior to each challenge. A total of 12 control macaques were used. Control macaques received the following; azithromycin treatment (as described above) followed by 2.7% HEC (as described) (n=4), 2.7 % HEC (as described) (n=4) or no treatment (n=4). Macaques were challenged once weekly up to six challenges. (b) Kaplan-Meier analysis of time to detectable $SHIV_{SF162P3}$ infection. On the left, all controls were combined; the difference in protection was highly significant (p = 0.0039). (c) On the right, each subgroup within the controls was separately analyzed; the difference was significant for the non-treatment subgroup (p = 0.008) and the HEC only subgroup (p = 0.002).

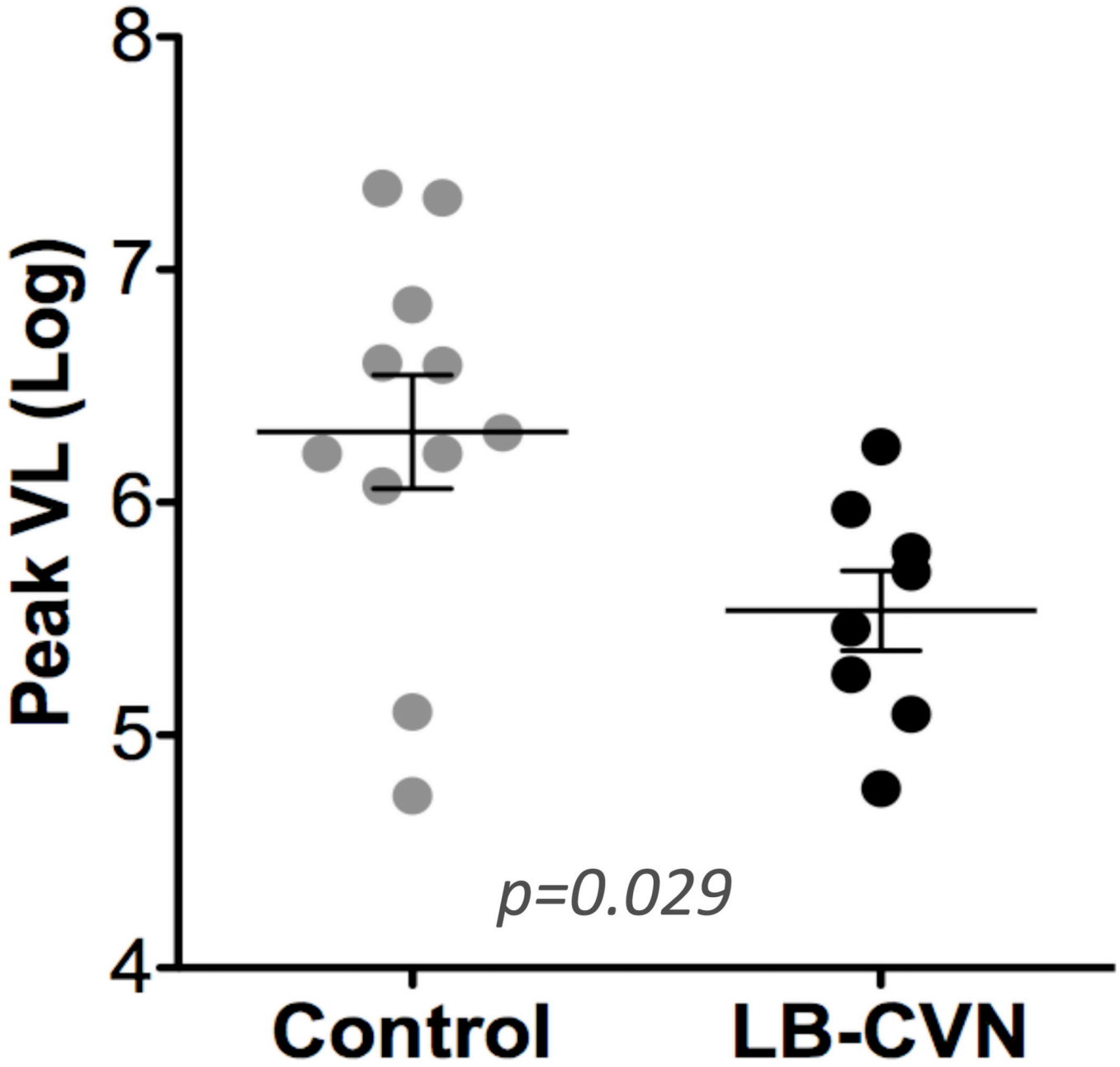


Fig. 6.

Comparison of peak viral loads in *L. jensenii* 1153–1666 colonized (LB-CVN) and control animals with breakthrough infection. Average $\log_{10}$ peak plasma viral levels were 5.5 ± 0.17 (mean ± SEM) for animals colonized with *L. jensenii* 1153–1666 vs 6.3 ± 0.24 for control animals ($p = 0.029$).

Table 1

L. jensenii 1153–1666 and CV-N protein detection in *L. jensenii* 1153–1666-colonized macaques during the course of the challenge study.

Animals	Challenge 1		Challenge 2		Challenge 3		Challenge 4		Challenge 5		Challenge 6	
	L j	CV-N	*L j*	CV-N	*L j*	CV-N	*L j*	CV-N	*L j*	CV-N	*L j*	CV-N
M4698	5	+	6	+	6	+	4	+	6	+	5	+
M4699	6	+	5	+	4	+	0	ND	6	+	6	+
M4700	0	ND	0	ND	5	+	5	+	6	+	5	+
M4701	5	+	5	+	4	+	6	+	6	+	0	ND
M4704	5	+	0	ND	0	ND	6	+	6	+	5	+
M4705	5	+	4	+	6	+	0	ND	4	−	0	ND
M4706	5	+	0	ND	6	+	5	+	6	+	4	+
M4707	5	+	5	+	5	+	5	+	4	+	5	+
M4708	5	+	5	+	0	ND	6	+	6	+	5	+
M4709	5	+	5	+	5	+	6	+	6	+	0	ND
M4711	5	+	5	+	6	+	6	+	6	+	5	+
M4713	5	+	5	+	4	+	5	+	6	+	5	+

L j = *L. jensenii* 1153–1666, 6 =10^6 colony forming units CFU/swab, 5 =10^5 CFU/swab, 4 =10^4 CFU/swab, 0 =no lactobacillus detected, ND = not determined