Sustained Delivery of Commensal Bacteria from Pod-intravaginal Rings

Running title: Delivery of Probiotic Bacteria from Intravaginal Rings

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ABSTRACT

Topical administration of live, commensal bacteria to the vaginal tract holds significant potential as a cost-effective strategy for the treatment of sexually transmitted infections and the delivery of mucosal vaccines. Intravaginal rings (IVRs) releasing probiotics embody significant theoretical advantages over traditional, daily dosage forms, such as sustained and controlled delivery leading to improved adherence to therapy compared to frequent dosing. Conventional IVR designs, however, are not amenable to the delivery of live bacteria. We have developed a novel pod-IVR technology, where polymer-coated tablets (”pods”) of *Lactobacillus gasseri* ATCC 33323, a commensal microorganism of human origin, were embedded in silicone IVRs. The release rate of bacterial cells was controlled by the diameter of a delivery channel exposing a portion of the pod to external fluids. *In vitro* studies demonstrated that the prototype devices could release between $1.1-14 \times 10^7$ cells per day for up to 21 days in a controlled, sustained fashion with stable, burst-free release kinetics. The daily release rates were correlated with the delivery channel cross-sectional area. Bacteria in the IVR pods remained viable throughout the *in vitro* studies and formed biofilms on the surface of the devices. This proof-of-principle study represents the first demonstration of prolonged, sustained release of bacteria from an intravaginal device and warrants further investigation as a non-chemotherapeutic for restoration and maintenance of a normal urogenital flora.
INTRODUCTION

The burden of sexually transmitted infections (STIs) among women, particularly in resource-poor regions, highlights the urgent need for female-controlled, cost-effective approaches to prevention and treatment (1). Strategies involving the administration of commensal bacteria to the vaginal tract are emerging as a promising platform to achieve these important goals (2). Probiotics, “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (3), have been shown to promote and restore healthy vaginal microbiota in clinical trials (2-4). Both oral and intravaginal probiotic regimens for the prevention and treatment of bacterial vaginosis (BV) (5-8) and urinary tract infections (UTIs) (9, 10) have demonstrated clinical efficacy. Because probiotic lactobacilli express a number of characteristics that are antagonistic to pathogens, but complementary to host immunity their use has been proposed to improve reproductive health and pregnancy outcomes (11).

Commensal and attenuated pathogenic bacteria also are being developed as vectors for mucosal vaccines against STIs (2). *Listeria monocytogenes* is a promising candidate vaccine vector against HIV because it induces a strong cell mediated immune response and can be readily manipulated to express viral antigens. Proof-of-concept studies have been performed using recombinant *L. monocytogenes* strains that express HIV Gag in feline (12) and nonhuman primate (13) HIV models. Similarly, human vaginal isolates of *Lactobacillus jensenii* were genetically modified to secrete functional two-domain CD4 proteins, thereby inhibiting HIV-1 entry into target cells in a dose-dependent manner (14). A recombinant *L. jensenii* expressing the HIV-1 entry inhibitor cyanovirin-N demonstrated a 63% reduction in transmission of a chimeric simian/HIV (SHIV, SF162P3) following repeated vaginal challenges in macaques (15). *L. monocytogenes* expressing the H-2K(b) glycoprotein B peptide from Herpes Simplex Virus type...
4 HSV-1 triggered a robust CD8 T-cell providing protective immunity to HSV infection (16).

Intravaginally administered recombinant *Streptococcus gordonii* and *Salmonella enterica*
engineered to express antigens of human papillomavirus type 16 were evaluated in mice (17, 18)
and cynomolgus macaques (19) in an effort to develop effective topical vaccines for cervical
cancer. Recently, attenuated bacterial pathogens have been investigated as delivery vectors for
heterologous antigens that may simultaneously vaccinate against two pathogens (20).

The intravaginal administration of probiotics traditionally has been achieved using a variety
of dosage forms including tablets (21), capsules (7, 8), suppositories (9), and tampons (22).
Collectively, these approaches have led to detectable, but not optimally durable, levels of the
delivered bacteria. In women, these levels may not be sufficient to overcome existing biofilms
refractory to the administered organism. Topical delivery of commensal bacteria using
intravaginal rings (IVRs) holds significant potential for female-controlled STI prevention and
treatment. The microorganisms can be administered in a controlled manner as sustained release
formulations and adherence issues are significantly reduced compared to daily dosing.

Intravaginal rings are being explored for the delivery of small molecule antiviral agents
(microbicides) to protect against sexual HIV (23) and HSV acquisition (24), as well as for the
treatment of recurrent genital herpes (25). Microbicide IVR technologies based on the
established matrix and reservoir designs (23, 26), where the antimicrobial agent is dispersed in
and diffuse through the ring elastomer are not, however, amenable to the delivery of live
bacteria.

Consequently, we have developed a novel pod-IVR (27) formulated with the commensal
organism *Lactobacillus gasseri* ATCC 33323, a neotype strain of human origin (28, 29).
Sustained delivery of *L. gasseri* was obtained for up to 21 days *in vitro* with controlled release kinetics. The bacteria remained viable in the IVRs throughout the study.

**MATERIALS & METHODS**

**Preparation of *L. gasseri* tablets.** *Lactobacillus gasseri* (ATCC 33323) cultures were inoculated from frozen stock into de Man, Rogosa, and Sharpe (MRS) media and incubated for 24 h at 37°C and 130 rpm. Cells were harvested via centrifugation for 30 min at 3000×g, 4°C. Pooled cell pellets (ca. 15 g) were dried by lyophilization and the resulting powder was blended with sodium carboxymethyl cellulose (CMC, 25% w/w) by gentle tumbling for 24 h. The resulting mixtures were compacted into 3.0 mm o.d. tablets in a manual pellet press (Parr Instrument Company).

**Manufacture of silicone intravaginal rings.** Human-sized polydimethylsiloxane (PDMS, silicone) pod-IVRs were prepared in a multi-step process that has been described in detail elsewhere (27). Tablets containing ca. 30 mg of lyophilized *L. gasseri* (ca. $7 \times 10^{14}$ cells) were coated with 5% w/v poly(D,L-lactide) in dichloromethane-ethyl acetate (1:1, v/v) to produce pods that were dried at room temperature for 72 h. Pods were embedded in IVRs (10 per ring) with a single, mechanically punched delivery channel for each pod. Delivery channels were 2.0, 1.5, 1.0, or 0.75 mm diameter, depending on the target release rates. IVRs were cut into single-pod segments for *in vitro* evaluation. The viability of bacteria encapsulated in the IVRs was compared to the lyophilized material by culturing and typically exceeded 90%. The viability of the IVR bacteria after 21 days of evaluation *in vitro* (*vide infra*) remained at 90% relative to lyophilized cells stored at 4°C.
Bacterial enumeration. The concentration of bacterial cells in 150 µL aliquots of release media collected at predetermined time-points was measured as a function of the absorbance at 600 nm ($OD_{600}$) in 96-well format using a SpectraMax® Plus Absorbance Microplate Reader (Molecular Devices, Inc.). The $OD_{600}$ reading was converted to number of viable bacterial cells per mL of media ($N$) according to eq. 1.

$$N = 2.35 \times 10^8 \cdot 2.38 \cdot OD_{600}$$

eq. 1

The factor $2.35 \times 10^8$ represents the number of viable cells mL$^{-1}$ providing an extinction of 1 AU cm$^{-1}$ at 600 nm that was determined experimentally and is well within the normal range for bacterial cells (30). The factor 2.38 corrects the optical pathlength to the 150 µL volume in a 96-well plate.

In vitro studies. In vitro release studies were carried out in triplicate using procedures presented elsewhere (27). Briefly, the IVR segments were placed in dissolution media consisting of sterile 1× phosphate-buffered saline (PBS, pH 7.2, 1 mL) and were incubated at room temperature (25 ± 2°C) with shaking. The media was replaced every 24 h and the segments were thoroughly rinsed with 1× PBS before placing into fresh sterile release buffer.

Bacterial viability. Viability of the $L. gasseri$ cells in the release media was measured every 6 days by culture. Aliquots (150 µL) were inoculated into 100 µL MRS media and incubated for 24 h at 37°C, 130 rpm. The $OD_{600}$ of the resulting culture was used as a surrogate measurement of growth.

SEM. IVR segments with 2.0 mm dia. delivery channels were incubated in release media for 16 d at 25°C, 100 rpm. The segments were rapidly frozen by immersion in liquid propane and were prepared for SEM as described previously (31, 32). Dehydration of the frozen segments was carried out by freeze-substitution in ethanol at -80°C followed by warming to ambient
temperatures and critical point drying. During this process, much of the biological material became detached from the IVR surface. The dried ring segments were cut lengthwise, mounted on metal specimen stubs, coated with a 10 nm thick platinum film, and imaged using an XL-30 SFEG 6 SEM (FEI Company, Hillsboro, OR) operating at 5 kV.

**Statistical Analysis.** Data were analyzed using GraphPad Prism (version 6.02, GraphPad Software, Inc.).

## RESULTS

**In vitro** kinetic studies demonstrate sustained, controlled release profiles for up to 21 days. **In vitro** cumulative and daily release profiles (Fig. 1, Table 1) from the *L. gasseri* IVR formulation exhibited burst-free, sustained release, as is typical for pod-IVRs delivering small molecules (27, 33, 34). The daily release rates, calculated from the linear portion of the cumulative release profile, displayed the expected (27) dependence on delivery channel cross-sectional area (Fig. 1B).

**IVR bacteria remain viable, even after 21 days.** The viability of the *L. gasseri* in the IVR formulations was maintained (90%, compared to lyophilized cells stored at 4°C), even after 21 days of incubation in release media (Fig. 2). These results were confirmed by labeling (LIVE/DEAD® BacLight® Bacterial Viability Kit, Life Technologies Corporation) the excised pod core and examination by fluorescence microscopy (data not shown). The concentration of viable cells in the release media, measured in terms of OD₆₀₀ in Fig. 2, was representative of the daily release rate at those time-points (see Fig. 1A).

**L. gasseri biofilms form on the IVR surface.** Bacterial biofilms were clearly visible on *L. gasseri* delivering IVR segments followed prolonged incubation in PBS. These structures were
delicate and readily detached from the IVR surface during handling. Two principal biofilm morphologies were observed by SEM examination of cryopreserved specimens (Fig. 3): first, open channels defined by bacteria embedded in extracellular polymeric substances (EPS, Fig. 3A), sometimes linked by dense networks of nanofibers (Fig. 3B); second, thick mats of aggregated bacteria (Fig. 3C). Bacterial mats also formed on the inner surface of the delivery channel (Fig. 3D-F).

DISCUSSION

The pod-IVR design (27) contains a number of key, unique features relevant to this study. The unmedicated structure that holds the bacterial pods can be made of any biocompatible elastomer (e.g., PDMS, ethylene-vinyl acetate copolymer, polyurethane, latex, etc.), providing flexibility in material choice. In conventional IVRs, such as matrix and reservoir designs (23), including segmented (35, 36) and tubular (37, 38) configurations, the device elastomer forms an integral part of the delivery system controlling drug diffusion, an approach that is not amenable to the delivery of live bacterial agents.

The pod-IVR platform was specifically designed for the sustained delivery of multiple agents, each with independently and precisely controlled delivery rates (27). We have demonstrated in pig-tailed macaques that the drug release rate can be modulated over a wide range (34). A key feature of the pod-IVR design is its versatility in the agents it can deliver, with drug substances spanning the range from hydrophobic and hydrophilic small molecules (24, 25, 33, 34, 39) to high molecular weight, highly water-soluble biomolecules (40). We reported on the design and 28-day pharmacokinetic evaluation in sheep of a five-drug pod-IVR as a proof-of-concept, advanced Multi-Prevention Technology (MPT) that combines three antiretroviral drugs...
from different mechanistic classes with a proven estrogen-progestogen contraceptive for HIV and unintended pregnancy prevention (39). No other IVR design has demonstrated the ability to deliver more than two agents. Pod-IVRs delivering antiviral agents have shown preliminary safety in pig-tailed macaques and women (25, 41), including culture-independent characterization of the vaginal microbiota (42). Based on the extensive in vivo track record of the pod-IVR design in rabbits (24), sheep (24, 33, 39), macaques (34, 41), and women (25, 42), no significant challenges are anticipated in transitioning the current IVR delivering commensal bacteria into in vivo studies.

Human-sized pod-IVRs can accommodate 10 polymer-coated bacterial tablets, containing up to 200 mg of material each, totaling 2 g per IVR. Each pod can theoretically deliver a different agent at an independently controlled release rate determined by the polymer membrane encapsulating the tablet and by the number and cross-sectional diameter of the delivery channels in the ring, as shown in Fig. 1B. We have demonstrated simultaneous delivery of multiple agents at controlled rates from pod-IVRs in vivo (24, 39). The sustained delivery of multiple probiotics, such as the combination of Lactobacillus rhamnosus GR-1 and L. fermentum RC-14 pioneered by Reid and colleagues (43), and probiotic bacteria in tandem with complementary drugs such as oestriol (44-46), a metabolic product of estradiol, and vitamin-B complex (47) is possible using the pod-IVR platform.

Unlike oral probiotic dosage regimens (43), the intravaginal probiotic dose required to impact the vaginal microbiota has not been determined clinically; thus, target probiotic delivery rates for sustained release intravaginal products have not been established. Table 2 summarizes the intravaginal probiotic doses used in a range of clinical studies. Orally administered formulations are not included due to the uncertainly of the vaginal dose received. Based on these
data, a 28-day pod-IVR would need to deliver between $0.6 \times 10^4$ and $20 \times 10^8$ viable organisms per day, a range that is well within the capabilities of the pod-IVR platform discussed here as supported by Fig. 1. Note that Fig. 1B shows daily release rates from a single pod. A tenfold increase in release rate can be achieved simply by using 10 pods per IVR, and can be increased further by using multiple delivery channels for each pod (27). Increasing the pod size from 30 mg, as described here, to 200 mg would provide sufficient bacterial loading to last for 28 days.

The two *L. gasseri* biofilm architectures observed here show morphological similarities with the phenotypes that developed *in vivo* on pod-IVRs delivering antiviral agents in pig-tailed macaques (41) and women (25). In both cases, *Lactobacillus* spp. were well-represented in the hosts’ vaginal microbiota (41, 42). The complete genome of *L. gasseri* ATCC 33323 has been sequenced (29) and, interestingly, was found to encode 14 putative mucus-binding proteins, the highest number among lactobacilli sequenced to date. In addition, the sequence data were suggestive of a putative exopolysaccharide gene cassette contributing to features of the cell surface structure (29). These molecular findings are in agreement with our experimental observations regarding IVR surface colonization and biofilm formation by *L. gasseri* ATCC 33323. The open architecture (Fig. 3A)—designated as Phenotype II (41)—contained interwoven networks of uniform fibers (Fig. 3B) reminiscent of structures observed in monospecies *Pseudomonas* spp. laboratory cultures (32). These so-called nanowires have been observed as a consistent feature of bacterial biofilms (32, 48, 49). In our *in vivo* studies, the bacterial biofilms developed on epithelial cell monolayers covering the IVR surface (25, 41). Here, the biofilms easily became detached from the IVR surface during handling, possibly explaining why an epithelial cell monolayer was required to support *in vivo* surface adhesion of the bacterial EPS.

We observed no evidence that the biofilms affected the *in vitro* release rate of *L. gasseri*. 
Conclusion. The delivery of *Lactobacillus gasseri* from pod-IVRs in an *in vitro* model exhibited controlled release of viable cells over 21-days. The proof-of-principle study demonstrates that the modular pod-IVR platform holds promise for the sustained release of beneficial bacteria to the vaginal tract and warrants further investigation as a non-chemotherapeutic for restoration and maintenance of a normal urogenital flora. Future *in vivo* evaluation of the devices will be critical in advancing them through the development pipeline.

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REFERENCES


TABLE 1. Daily *Lactobacillus gasseri* release rates as a function of IVR configuration.

<table>
<thead>
<tr>
<th>IVR delivery channel diameter (mm)</th>
<th>2.0</th>
<th>1.5</th>
<th>1.0</th>
<th>0.75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily release ratea (10^7 viable cells d^{-1})</td>
<td>14 ± 2.2</td>
<td>4.0 ± 0.15</td>
<td>2.0 ± 0.63</td>
<td>1.1 ± 0.26</td>
</tr>
</tbody>
</table>

*a*mean ± SD, n = 3.
TABLE 2. Summary (5) of clinical trials involving intravaginal administration of probiotic formulations.

<table>
<thead>
<tr>
<th>Study</th>
<th>Exploratory treatment</th>
<th>Probiotic</th>
<th>Dose (CFU)</th>
<th>Form</th>
<th>Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>(50)</td>
<td>BV</td>
<td><em>L. acidophilus</em></td>
<td>5-20×10⁸ mL⁻¹</td>
<td>5 mL fermented milk product</td>
<td>2× daily, 7 days</td>
</tr>
<tr>
<td>(51)</td>
<td>BV</td>
<td><em>L. acidophilus</em></td>
<td>1-10×10⁸</td>
<td>capsule</td>
<td>2× daily, 6 days</td>
</tr>
<tr>
<td>(52)</td>
<td>BV</td>
<td><em>L. acidophilus</em></td>
<td>&gt;10⁸ mL⁻¹</td>
<td>10-15 mL yoghurt</td>
<td>2× daily, 7 days; repetition after 1 week</td>
</tr>
<tr>
<td>(45)</td>
<td>BV</td>
<td><em>L. acidophilus</em></td>
<td>≥10⁷ oestriol</td>
<td>30 µg tablet</td>
<td>1-2× daily, 6 days</td>
</tr>
<tr>
<td>(46)</td>
<td>vaginitis</td>
<td><em>L. acidophilus</em></td>
<td>≥10⁷ oestriol lactose</td>
<td>30 µg 600 mg tablet</td>
<td>1× daily, 6 days</td>
</tr>
<tr>
<td>(9)</td>
<td>UTI</td>
<td><em>L. crispatus</em></td>
<td>10⁸ GAI 98322</td>
<td>suppository</td>
<td>every 2 days, 1 year</td>
</tr>
<tr>
<td>(7)</td>
<td>BV</td>
<td><em>L. rhamnosus</em></td>
<td>≥4×10⁴</td>
<td>capsule</td>
<td>1× week, 6 months</td>
</tr>
<tr>
<td>(8)</td>
<td>BV</td>
<td><em>L. rhamnosus</em></td>
<td>6.8×10⁸</td>
<td>capsule</td>
<td>1× daily, 7 days on, 7 days off, 7 days on</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>L. acidophilus</em></td>
<td>0.4×10⁸</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Streptococcus thermophilus</em></td>
<td>0.8×10⁸</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIG 1. Median in vitro release kinetics of live *L. gasseri* from single pod-IVR segments as a function of delivery channel size (*n* = 3). (A) Cumulative release: 2.0 mm dia., circles; 1.5 mm dia., squares; 1.0 mm dia., triangles; 0.75 mm dia., inverted triangles; (B) Daily release rates as a function of delivery channel cross-sectional area (SA).
FIG. 2. Viability of *L. gasseri* in IVR release media at Day 10 (circles), Day 12 (squares), and Day 21 (triangles). The OD$_{24}$ readings correspond to the absorbance at 600 nm of MRS media inoculated from the release buffer and incubated for 24 h at 37°C and 130 rpm. “CTRL”, negative control using MRS media with no inoculation.
FIG. 3. SEM images of *Lactobacillus gasseri* biofilms formed on IVR fragments *in vitro* following 16 days of incubation at 25°C, 100 rpm in PBS. (A-B) Biofilm morphologies display typical ultrastructural features of Phenotype II (41), including regular, open structures and nanowires. (D-F) Biofilms in the IVR drug delivery channel have a different morphology.
resembling a dense bacterial mat, similar to Phenotype I (41). (A) Bacteria, embedded in EPS,
assembled into open architectures including connective channels; scale bar = 2 µm. (B) Portions
of the biofilm structures contained dense nanofiber networks attached to the branching bacterial
aggregates; scale bar = 2 µm. (C) Biofilm fragment at low magnification consisted of a thick mat
of aggregated bacteria that readily detached from the IVR surface. The broken edge (arrow)
represents the fragmentation line that developed during sample processing; scale bar = 200 µm.
(D) The IVR delivery channel, at low magnification, contained a mass of material attached to the
inner surface (arrows). Traces of material on the IVR surface also were present (arrowhead);
scale bar = 10 µm. (E) The inner surface of the IVR delivery channel was covered with attached
biological material; scale bar = 20 µm. (F) The attached biofilm contained bacteria (arrows);
scale bar = 10 µm.