Sustained Delivery of Commensal Bacteria from Pod-Intravaginal Rings

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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. Probiotic-releasing intravaginal rings (IVRs) embody significant theoretical advantages over traditional daily-dosage forms, such as sustained and controlled delivery leading to improved adherence to therapy compared to that of frequent dosing. The 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* strain ATCC 33323, a commensal microorganism of human origin, are embedded in silicone IVRs. The release rate of bacterial cells is controlled by the diameter of a delivery channel that exposes a portion of the pod to external fluids. *In vitro* studies demonstrated that the prototype devices released between 1.1 × 10⁷ and 14 × 10⁷ 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 cross-sectional area of the delivery channel. Bacteria in the IVR pods remained viable throughout the *in vitro* studies and formed biofilms on the surfaces of the devices. This proof-of-principle study represents the first demonstration of a prolonged, sustained release of bacteria from an intravaginal device and warrants further investigation of this device as a nonchemotherapeutic agent for the restoration and maintenance of normal urogenital flora.

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* when 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* organism expressing the HIV-1 entry inhibitor cyanovirin-N demonstrated a 63% reduction in the transmission of a chimeric simian HIV strain (SIVHF162P3) following repeated vaginal challenges in macaques (15). *L. monocytogenes* expressing the H-2K(b) glycoprotein B peptide from herpes simplex virus 1 (HSV-1) triggered a robust CD8 T cell response providing protective immunity against 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 sustainable, levels of the delivered bacteria. In women, these levels may not be sufficient to overcome existing biofilms refractory to the administered organism. The 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 in 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 sexually contracted HIV (23) and HSV acquisition (24), as well as for the treatment of recurrent genital herpes (25). However, microbical IVR technologies based on the established matrix and reservoir designs (23, 26), in which the antimicrobial agent is dispersed and diffuses through the cross-sectional area of the delivery channel. Bacteria in the IVR pods remained viable throughout the *in vitro* studies and formed biofilms on the surfaces of the devices. This proof-of-principle study represents the first demonstration of a prolonged, sustained release of bacteria from an intravaginal device and warrants further investigation of this device as a nonchemotherapeutic agent for the restoration and maintenance of normal urogenital flora.

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IVRs throughout the study.

Controlled-release kinetics. The bacteria remained viable in the IVRs for up to 21 days in vitro (see below) and remained at 90% relative to that of the lyophilized cells stored at 4°C.

**Bacterial enumeration.** The concentrations of bacterial cells in 150-μl aliquots of release medium collected at predetermined time points were measured as a function of the optical density at 600 nm (OD600) in a 96-well format using a SpectraMax Plus absorbance microplate reader (Molecular Devices, Inc.). The OD600 reading was converted to the number of viable bacterial cells per ml of medium (N) according to the equation:

\[ \text{OD}_{600} = \text{N} \times 2.35 \times 10^{8} \times 2.38 \times \text{OD}_{600} \]

The factor 2.35 × 10^8 represents the number of viable cells ml^−1 providing an extinction of 1 absorbance unit (AU) cm^−1 at 600 nm. This value was determined experimentally and is well within the normal range for bacterial cells (30). The factor 2.38 corrects the optical path length 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 medium consisting of 1 ml sterile 1× phosphate-buffered saline (PBS; pH 7.2) and were incubated at room temperature (25 ± 2°C) with shaking. The medium was replaced every 24 h and the segments were thoroughly rinsed with 1× PBS before placing into fresh sterile release buffer.

**Bacterial viability.** The viability of the *Lactobacillus gasseri* cells in the release medium was measured every 6 days by culture. The aliquots (150 μl) were inoculated into 100 μl MRS medium and incubated for 24 h at 37°C and 130 rpm. The OD600 of the resulting culture was used as a surrogate measurement of growth.

**SEM.** IVR segments with 2.0-mm-diameter delivery channels were incubated in release medium for 16 days at 23°C and 100 rpm. The segments were rapidly frozen by immersion in liquid propane and were pre-cooled in liquid nitrogen. The frozen segments were subsequently freeze-substituted in ethane (at −80°C) followed by warming to ambient temperature and critical point drying. The resulting segments were cut lengthwise, mounted on metal specimen stubs, coated with a 10-nm-thick platinum film, and imaged using an XL30-SFEG 6 SEM (FEI Company, Hillsboro, OR) operating at 5 kV.

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**RESULTS**

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

**IVR bacteria remained viable, even after 21 days.** The viability of the *L. gasseri* bacteria in the IVR formulations was maintained in vitro for up to 21 days, as demonstrated by the OD600 measurements (Table 1). The OD600 measurements were used as a surrogate measurement of growth.

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

<table>
<thead>
<tr>
<th>No. of viable cells</th>
<th>2.0</th>
<th>1.5</th>
<th>1.0</th>
<th>0.75</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^7 viable cells per day</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 ± standard deviation (n = 3).*

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**MATERIALS AND METHODS**

**Preparation of *L. gasseri* tablets.** *L. gasseri* (ATCC 33323), a neotype strain of human origin (28, 29), was obtained for up to 21 days in vitro with controlled-release kinetics. The bacteria remained viable in the IVRs throughout the study.

**Manufacture of silicone intravaginal rings.** Human-sized polydimethylsiloxane (PDMS; silicone) pod-IVRs were prepared in a multistep process that has been described in detail elsewhere (27). Tablets containing ca. 30 mg of lyophilized *L. gasseri* (ca. 7 × 10^14 cells) were coated with 5% (wt/vol) poly[(R)-l-lactide] in dichloromethane-ethyl acetate (1:1 [vol/vol]) to produce pods that were dried at room temperature for 72 h. The pods were embedded in IVRs (10 per ring) with a single mechanically punched delivery channel for each pod. The delivery channels were 2.0, 1.5, 1.0, or 0.75 mm in diameter, depending on the target release rate. The IVRs were cut into single-pod segments for in vitro evaluation. The viability of the bacteria encapsulated in the IVRs was compared to that of the lyophilized material by culturing and typically exceeded 90%. The viability of the IVR bacteria after 21 days of evaluation in vitro (see below) remained at 90% relative to that of the lyophilized cells stored at 4°C.

no text
tion of the cryopreserved specimens (Fig. 3): (i) open channels and readily detached from the IVR surface during handling. Two ing prolonged incubation in PBS. These structures were delicate were clearly visible on L. gasseri nanofibers rate delivery IVRs, such as matrix and reservoir designs (23), including seg- latex), providing flexibility in material choice. In conventional (Fig. 3C). Bacterial mats also formed on the inner surface of the (90%, compared to lyophilized cells stored at 4°C), even after 21 days of incubation in release medium (Fig. 2). These results were confirmed by labeling (with the LIVE/DEAD BacLight bacterial viability kit; Life Technologies Corporation) the excised pod core and examining by fluorescence microscopy (data not shown). The concentration of viable cells in the release medium, measured in terms of the OD_{600} (Fig. 2), was representative of the daily release rate at those time points (Fig. 1A).

L. gasseri biofilms form on the IVR surface. Bacterial biofilms were clearly visible on L. gasseri-delivering IVR segments follow- ing 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 the cryopreserved specimens (Fig. 3): (i) open channels defined by bacteria embedded in extracellular polymeric sub- stands (EPS) (Fig. 3A), sometimes linked by dense networks of nanofibers (Fig. 3B), and (ii) thick mats of aggregated bacteria (Fig. 3C). Bacterial mats also formed on the inner surface of the delivery channel (Fig. 3D to F).

DISCUSSION

The pod-IVR design (27) contains a number of key unique fea- tures 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, or latex), providing flexibility in material choice. In conventional IVRs, such as matrix and reservoir designs (23), including seg- mented (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 sus- tained 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 mole- cules (24, 25, 33, 34, 39) to high-molecular-weight highly water- soluble biomolecules (M. Gunawardana, M. M. Baum, A. M. Malone, T. J. Smith, and J. A. Moss, submitted for publication). Moss et al. reported (39) the design and 28-day pharmacokinetic evaluation in sheep of a five-drug pod-IVR as a proof-of-concept advanced multipurpose prevention technology (MPT) that com- bines three antiretroviral drugs from different mechanistic classes with a proven estrogen-progestogen contraceptive for HIV and unintended-pregnancy prevention. 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, 40), including culture-independent characterisation of the vaginal microbiota (41). Based on the extensive in vivo track record of the pod-IVR design in rabbits (24), sheep (24, 33, 39), macaques (34, 40), and women (25, 41), no significant challenges are anticipated in translating the current IVR delivering commensal bacteria to 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 the 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 Lactobacil- lus fermentum RC-14 pioneered by Reid and colleagues (42), and probiotic bacteria in tandem with complementary drugs, such as estriol (43–45), a metabolic product of estradiol, and vitamin B complex (46), is possible using the pod-IVR platform.

Unlike oral probiotic dosage regimens (42), the intravaginal probiotic dose required to impact the vaginal microbiota has not been determined clinically; thus, the target probiotic delivery rates for sustained release intravaginal products have not been estab- lished. Table 2 summarizes the intravaginal probiotic doses used in a range of clinical studies. Orally administered formulations were not included due to the uncertainty of the vaginal dose re- ceived. Based on these data, a 28-day pod-IVR needs to deliver between $6 \times 10^7$ and $20 \times 10^9$ viable organisms per day, a range that is well within the capabilities of the pod-IVR platform discussed here (as supported by Fig. 1). A 10-fold increase in the release rate can be achieved simply by using 10 pods per IVR, and it 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 28 days.

The two L. gasseri biofilm architectures observed here showed morphological similarities with the phenotypes that were developed in vivo on pod-IVRs delivering antiviral agents in pig-tailed macaques (40) and women (25). In both cases, Lactobacillus spp. were well represented in the vaginal microbiota of the hosts (40, 41). 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 the lactobacilli sequenced to date. In addition, the sequence data were suggestive of a putative exopolysaccharide gene cassette contrib- uting to the features of the cell surface structure (29). These mo- lecular 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), design- nated phenotype II (40), contained interwoven networks of uni- form fibers (Fig. 3B) reminiscent of structures observed in mono- species Pseudomonas laboratory cultures (32). These so-called
nanowires have been observed to be a consistent feature of bacterial biofilms (32, 47, 48). In our in vivo studies, the bacterial biofilms developed on epithelial cell monolayers covering the IVR surface (25, 40). 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 L. gasseri from pod-IVRs in an in vitro model exhibited a controlled release of viable cells over 21 days. This 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 nonchemotherapeutic agent for the restoration and maintenance of normal urogenital flora. Future in vivo evaluations of the devices will be critical to advance them through the development pipeline.

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REFERENCES


### TABLE 2 Summary of clinical trials involving intravaginal administration of probiotic formulations

<table>
<thead>
<tr>
<th>Reference no.</th>
<th>Reason for treatment</th>
<th>Probiotic(s)</th>
<th>Dose(s)</th>
<th>Form</th>
<th>Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>49</td>
<td>BV</td>
<td>Lactobacillus acidophilus</td>
<td>$5 \times 10^8$ to $20 \times 10^9$ CFU/ml</td>
<td>5 mL fermented milk product</td>
<td>2× daily, 7 days</td>
</tr>
<tr>
<td>50</td>
<td>BV</td>
<td><em>L. acidophilus</em></td>
<td>$1 \times 10^8$ to $10 \times 10^9$ CFU/ml</td>
<td>Capsule</td>
<td>2× daily, 6 days</td>
</tr>
<tr>
<td>51</td>
<td>BV</td>
<td><em>L. acidophilus</em></td>
<td>$&gt;10^8$ CFU/ml</td>
<td>10–15 ml yogurt</td>
<td>2× daily, 7 days; repeat after 1 wk</td>
</tr>
<tr>
<td>44</td>
<td>BV</td>
<td><em>L. acidophilus</em></td>
<td>$\geq 10^8$ CFU</td>
<td>Tablet</td>
<td>1–2× daily, 6 days</td>
</tr>
<tr>
<td>45</td>
<td>Vaginitis</td>
<td><em>L. acidophilus</em></td>
<td>30 μg</td>
<td>Tablet</td>
<td>1× daily, 6 days</td>
</tr>
<tr>
<td>9</td>
<td>UTI</td>
<td>Lactobacillus crispatus GAI 98322</td>
<td>$10^8$ CFU</td>
<td>Suppository</td>
<td>Every 2 days, 1 yr</td>
</tr>
<tr>
<td>7</td>
<td>BV</td>
<td>Lactobacillus rhamnosus</td>
<td>$\geq 4 \times 10^4$ CFU</td>
<td>Capsule</td>
<td>1× wk, 6 mos</td>
</tr>
<tr>
<td>8</td>
<td>BV</td>
<td><em>L. rhamnosus</em></td>
<td>$6.8 \times 10^4$ CFU</td>
<td>Capsule</td>
<td>1× daily, 7 days on, 7 days off, 7 days on</td>
</tr>
<tr>
<td>7</td>
<td>BV</td>
<td><em>L. acidophilus</em></td>
<td>$0.4 \times 10^8$ CFU</td>
<td>Streptococcus thermophilus</td>
<td>$0.8 \times 10^8$ CFU</td>
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*See reference 5.*
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