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Ingestible Osmotic Pill for In Vivo Sampling of Gut Microbiomes

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Technologies capable of noninvasively sampling different locations in the gut upstream of the colon enable new insights into the role of organ-specific microbiota in human health. Herein, an ingestible, biocompatible, battery-less, 3D-printed microengineered pill with an integrated osmotic sampler and microfluidic channels for in vivo sampling of the gut lumen and its microbiome upstream of the colon is discussed. The pill's sampling performance is characterized using realistic in vitro models and validated in vivo in pigs and primates. Herein, the results show that the bacterial populations recovered from the pill's microfluidic channels closely resemble the bacterial population demographics of the microenvironment to which the pill is exposed. Herein, it is believed that such lab-on-a-pill devices revolutionize the understanding of the spatial diversity of the gut microbiome and its response to medical conditions and treatments.

1. Introduction

The gut microbiota represents trillions of bacteria belonging to around 1000 species.[1] Among the various microbial communities associated with the human body, the gut microbiome is noted for its diversity, elevated concentration,[2,3] and many beneficial functions. Dysbiosis (microbial imbalance) has been associated with conditions such as inflammation, recurrent infections, and increased susceptibility to enteric pathogens.[4–8] Most studies infer the condition of the gut microbiome from the analysis of fecal DNA and fecal metabolites.[1] Because the gut environment changes as the gut content moves down the gastrointestinal (GI) tract,[9] analyses of feces are inadequate to identify abnormal conditions upstream of the distal colon. Whereas the analysis of complex bacterial populations has benefitted from new DNA sequencing techniques, our capacity to precisely and noninvasively sample different organs has not improved. Consequently, medical research is often based on easily accessible samples, like feces, in spite of the inherent limitations of the conclusions that can be drawn from the analysis of such samples.[10] For instance, samples important for understanding the interaction between enteric pathogens and the host remain out of reach, unless invasive sampling techniques are used.[11] To gain new insights into the many beneficial functions of the gut microbiota, it is essential to sample in vivo different locations in the gut, particularly organs located upstream of the colon.

Here, we describe the development and testing of an innovative noninvasive technology to sample the intestinal lumen in

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DOI: 10.1002/aisy.201900053

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vivo. Beyond the prospects and opportunities for ingestible GI sampling devices discussed in a prior article,[12] the proposed device is specifically targeted to study the gut microbiome. We created an ingestible, biocompatible, 3D-printed microengineered pill with an integrated osmotic sampler that requires no battery for its operation. Stereo-lithography (SLA)-based 3D printing was used to fabricate the miniaturized ingestible device with sophisticated microfluidic functions for spatial sampling of the gut lumen. The pill was covered with a pH-sensitive enteric coating to delay sampling until it entered the small intestine, where the coating dissolves in the higher pH environment. A magnetic holding mechanism was designed to enable the pill to sample more time from a targeted region of the gut. Natural peristaltic motion endows mobility to the pill through the GI tract without any active parts. The sampling function of the pill has been extensively validated in vitro and in vivo in pigs and primates. The magnetic hold for spatial targeting has been validated in vitro.

2. Results and Discussion

2.1. Osmotic Pill Sampler Design

The overall design of the pill is shown in **Figure 1**a. The osmotic pill sampler consists of three main parts—a top sampling head, a semipermeable membrane in the middle, and a bottom salt chamber. The head comprises four inlets, a stilling basin connected to four helical channels, all of them leading to one small chamber. The salt chamber consists of a cavity that contains dry calcium chloride salt powder, a second cavity to hold a small neodymium magnet, two tube-like reservoirs to hold fluorescent dye, and a horn-shaped exit nozzle at the bottom. The pill works on the principle of osmosis where a pressure differential is created across the semipermeable membrane, which creates a passive pumping action (see Figure 1a). This mechanism facilitates the flow of water across the membrane from the helical channels toward the salt chamber. The porosity of the membrane blocks the flow of larger particles (e.g., microorganisms), leaving them trapped in the helical channels (Figure 1a).

The fabrication of this pill is explained in detail in the Experimental Section. Briefly, the pill sampling head and the bottom salt chamber are 3D printed using an SLA 3D printer. A semipermeable membrane is used to construct the osmotic pump. The membrane itself is made of woven 5 μm-thick cellulose acetate fibers commonly used in reverse osmosis–based water filters (Figure 1e,f). A small neodymium magnet is placed and sealed inside the salt chamber. To facilitate locating the pill after it is excreted in the feces, two lines of a green fluorescent dye are painted and sealed in the salt chamber. The salt chamber is filled with calcium chloride salt. The top and bottom chambers are separated by the semipermeable membrane assembled and attached together using a UV curable adhesive. The fabricated osmotic pill sampler is shown in Figure 1b. Note that the proposed pill has no electrical components or moving parts. We have used a biocompatible photocurable polymer for the pill casing. Furthermore, the only chemical used in the pill is common salt. Considering the materials and chemicals used in the construction of the pill, it is safe to conclude that the pill is biocompatible.

2.2. Pill Operation Concept

2.2.1. Priming the Pill

The pill is primed by injecting approximately 200 μL water into the salt chamber through the exit nozzle using a syringe with a 36 G needle. Similarly, the helical channels are filled with water from a marked inlet, which is connected to one of the helical channels (see Figure 1a, and Supplementary video 1). By aspirating water through the marked inlet, the water flows first through the corresponding channel and then fills the chamber at the bottom of the sampling head and finally fills all the other helical channels and the stilling chamber on the top of the pill in this sequence. The head can hold approximately 120 μL of sample.

2.2.2. Sampling Strategy

After priming, the pill is ready for oral administration. To avoid sampling the stomach lumen and limit the sampling to the small and large intestine, we placed the pill in a commercially available size-0 enteric coating capsule. The enteric coating resists the acidic environment of the stomach and only dissolves in the neutral/basic environment of the intestine. The dissolution profile for the enteric coating can be controlled by an appropriate choice of polymer(s). Testing of different enteric coatings was not the focus of this study. The osmotic pill sampler moves down the GI tract primarily due to natural peristalsis. The pill, however, can be held at a specific location inside the GI tract using an external magnet. This is made possible by a small neodymium magnet embedded in the pill (Figure 1a). Immobilizing the pill enables preferential sampling of specific regions of the gut, so that more of the collected sample originates from this region. Without this magnet, the pill would sample more or less uniformly along the entire length of the GI tract. We have added two fluorescent marks on the pill to facilitate detection following excretion (Figure 1d). The details of our sampling strategy are shown in Figure 1g.

2.3. Physical Characterization of the Pill

Osmosis is a net movement of a solvent through a semipermeable membrane toward a region of high solute concentration. Once the pill is primed, the process of osmosis causes solvent (water) to flow across the membrane from the helical channels into the salt chamber (Figure 1f). The flow rate depends on the properties of this membrane (e.g., thickness, porosity, area) and the salt gradient across it. The fluid sampler consists of an osmotic pump that continuously pulls the fluid from the gut into the narrow microfluidic collection channels ($<$1 mm diameter) at a flow rate of 2–20 $\mu L\,h^{-1}$. We characterized the flow created by osmosis across the semipermeable membrane, which drives the sampling function of the pill (see Section 1a, Supporting Information). The flow rate through the membrane assembled in the pill was also measured over time (Figure S1b, Supporting Information). Initially, the flow rate was $\approx$2.5 $\mu L\,h^{-1}$ and remained approximately constant for almost 48 h. The flow rate decreased to around $\approx$0.5 $\mu L\,h^{-1}$ after 4 days.

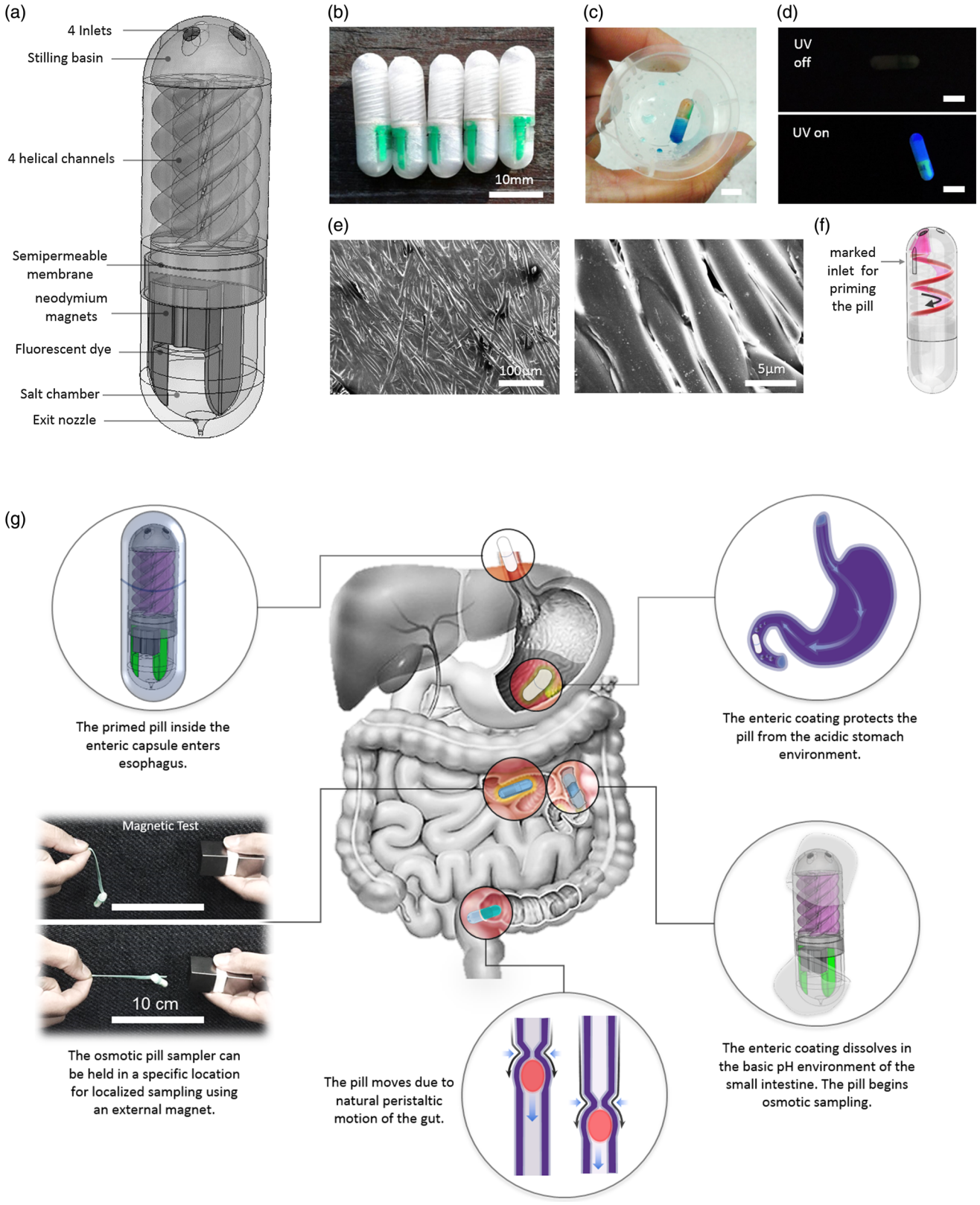

Figure 1. Osmotic pill sampler design and working principle. a) A 3D schematic of the overall design of the pill, b) fabricated osmotic pill samplers, c) proof of concept, the pill samples of a water solution containing blue food dye (the sample did not penetrate the salt chamber), d) the pill surface and the fluorescent dye in the pill are detectable under UV light, e) scanning electron microscope (SEM) images of the osmotic membrane used in the pill, f) marked inlet at the top chamber is designed for initiating the pill (priming), and g) overall working principle of the osmotic pill sampler in an enteric capsule.

The design of the exit nozzle in the salt chamber is important because it facilitates the discharge of water being accumulated in the salt chamber as a result of osmotic action. Numerical simulations were carried out to study flow through the discharge hole for two different hole diameters, 100 μm and 50 μm (see Figure S2, Supporting Information). The maximum fluid velocity for the 50 μm hole was 0.6 mm s^{-1}, which was 4.28 times higher than that for the hole of 100 μm size. A higher continuous flow rate at the discharge point has two advantages: first, it reduces the diffusion from the environment into the salt chamber, essentially acting as a one-way valve, and second, a high flow rate reduces the chance of blockage of the discharge hole by solid debris present in the gut.

2.4. In Vitro Sampling

The gut microbiome comprises prokaryotic and eukaryotic cells that may have no active motility or may be able to swim propelled by flagella or other mechanisms. We validated whether the osmotic pump can sample microorganisms regardless of their motion by testing the pill in vitro with nonmotile solid particulates as well as with highly motile bacteria. The first environment included polystyrene microparticles of 10 μm diameter in a highly viscous liquid (160 mPa s^{-1}) to mimic the gut environment. These particles exhibit negligible diffusion, especially in the highly viscous solution of 50% w/v polyethylene glycol (PEG) hydrogel. Because these particles are neutrally buoyant in this solution, the effect of gravity on their motion is insignificant. The second environment included highly motile bacteria, wild-type *Bacillus subtilis*, in an aqueous solution. These bacteria perform a run-and-tumble motion,[13] meaning that they swim in a certain direction for a short time (≈1 s) and then change their swimming direction at random. This results in a highly diffusive transport of the bacteria over a long time with effective diffusion coefficients comparable to small gas molecules in water.

2.5. In Vitro Sampling of Nonmotile Microparticles

We tested the pill in highly viscous environments similar to the GI tract by using solutions of 50% w/v of PEG, with a molecular mass of 10 000, in two different aqueous buffers of pH 4 and pH 8. Different pH conditions help model the fact that pH varies along the GI tract. The measured viscosity of the solution was 160 ± 5 mPa s^{-1} at 25 °C (USS-DVT4 Rotary Viscometer Viscosity Meter, U.S. Solid Inc., Cleveland, OH, USA). Next, polystyrene particles of 10 μm diameter were added to these solutions to mimic partially digested food particles in the GI tract. These microparticles are large enough so that Brownian motion can be ignored. Therefore, any microparticles captured by the pill would have entered the helical channels mainly due to osmotic flow. Initially, pills were primed with deionized water without PEG. Then, a group of three primed pills were placed in each of the pH 4 and pH 8 solutions described earlier. At different time points, a pill was taken out of the solution, and the sample was extracted from the pill using a pipette. On average 120 ± 6 μL of the sample was recovered from each pill. The extracted sample was weighed and imaged using an optical microscope and finally dried on a hotplate at 40 °C and weighed again. The samples were weighed before and after drying to evaluate the amount of hydrogel aspirated by the pill. The number of microparticles acquired by the pill at two different time steps was also quantified (see Experimental Section for details). To exclude the effect of background fluid motion on the sampling capability of the pill, the tests were performed in static conditions with no agitation. To justify exclusion of the uptake in the pill by diffusion, we calculate the diffusion coefficient, D, for microparticles in the viscous medium through the Stokes–Einstein relation

$$D = \frac{k_b T}{3\pi \eta d} \tag{1}$$

where k_b is Boltzmann's constant, T is the absolute temperature, d is the diameter of the particle and η is the dynamic viscosity of the medium. The diffusion coefficient of a 10 μm-diameter spherical particle at 37 °C in a fluid with viscosity of 160 mPa s^{-1} is $D = 2.8 \times 10^{-4} \mu m^2\ s^{-1}$. To diffuse one particle diameter under such conditions would require a time $t \approx d^2/2D = 2.1$ days. Thus, on time scales relevant to our experiments, diffusion is ineffective in generating any appreciable uptake of particles into the pill.

Figure 2 shows the number of particles recovered from the helical channels for both acidic (pH 4) and basic (pH 8) environments. The number of particles increases over time (Figure 2a), which validates that the osmotic pill can indeed sample nonmotile microparticles in a highly viscous environment. We also evaluated the amount of PEG aspirated into the pill's helical channels over time. Initially, the channels contain only pure water used for priming. Over time, the PEG from the surrounding environment is sampled by the pill along with microparticles, causing the concentration of the PEG in the pill to increase. Figure 2b shows a quantitative comparison of the hydrogel concentration in the pill over time in acidic (pH 4) and basic (pH 8) conditions. The hydrogel concentration increases almost linearly with time. However, in basic conditions, a higher sampling rate was noted, as was a higher hydrogel concentration inside the pill, exceeding concentration in the surrounding environment. We believe this is because the pH protonates and deprotonates the functional groups of the membrane itself and of the molecules in solution. This effect will change the effective charge on the membrane and alter the size of the pores, which will impact the membrane's nanofiltration properties and affect the flux of water.[14,15] The zeta potential (ζ) of a cellulose acetate membrane is negative for pH 3 or higher and becomes more negative as the pH increases.[16] Therefore, the membrane is more negatively charged at pH 8 ($\zeta \approx -35$ mV) compared with pH 4 ($\zeta \approx -10$ mV), which can result in bigger pore sizes and higher flux at pH 8. We repeated these experiments for a total of 12 pills, six under acidic (pH 4) and the rest at basic (pH 8) conditions. The same 50% PEG w/v solution and black 10 μm polystyrene beads were used as discussed earlier. The results shown in Figure 2 validate the sampling capability of the pill in both acidic and basic conditions. The number of particles is larger under basic conditions as compared to acidic conditions for the reasons explained earlier and is approximately equal to the control after 24 h of operation. Here, the control is the liquid containing the particles in which pills are immersed.

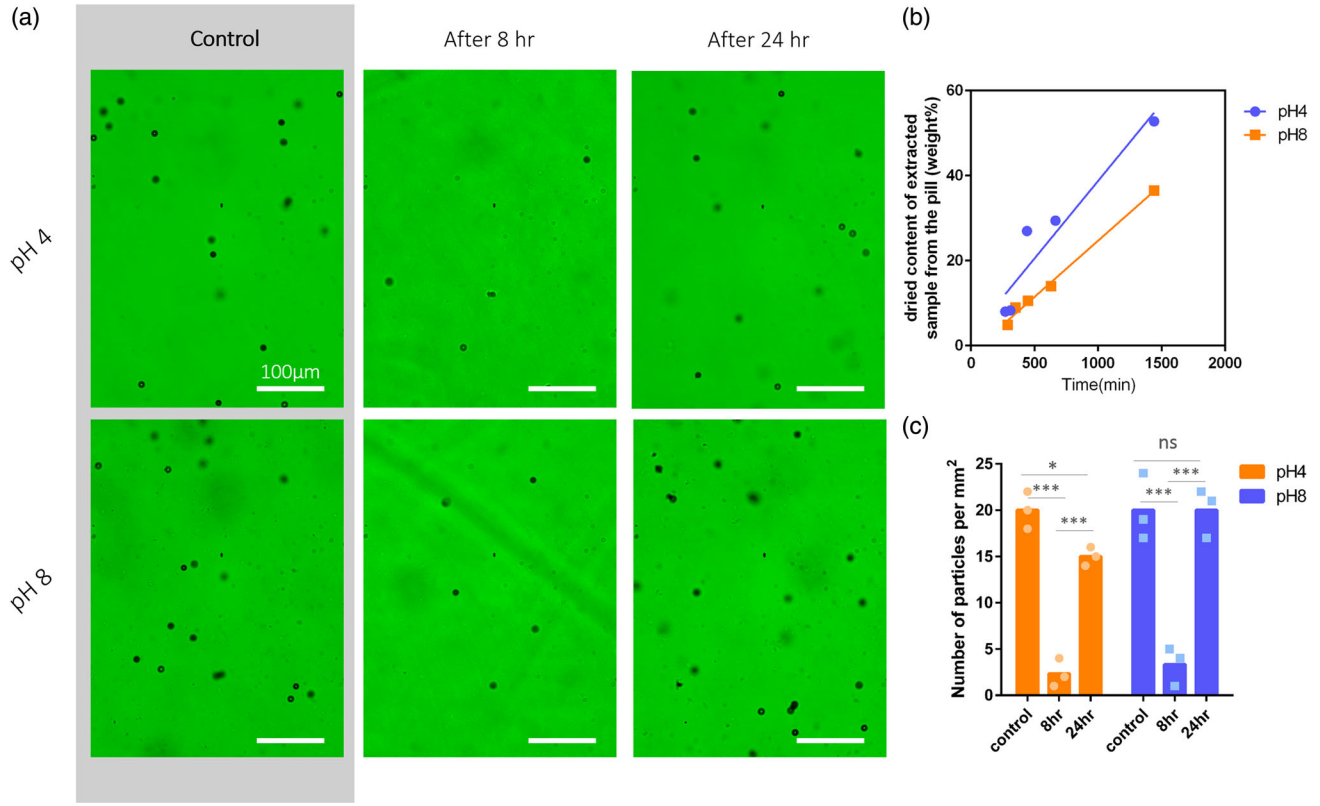

Figure 2. Osmotic sampling by the pill of solid particles in acidic and basic high-viscosity environments (50% PEG hydrogel). a) microparticles (polystyrene beads) captured by the pill after 8 and 24 h in solutions of different pH. Control shows particle suspension in which pills were immersed. b) weight percentage of the dried sample extracted from the pill at different time points. c) number of particles captured by the pill in two different pH conditions and compared with control (control is considered the environment outside of the pill).

2.6. In Vitro Sampling of Motile Bacteria

In the second in vitro test, we aimed to evaluate the sampling performance of the osmotic pills with motile bacteria. Wild-type *B. subtilis* was chosen as the test bacterium, which is ubiquitous in the GI tract of ruminants and humans. We used a fluorescent strain to facilitate imaging. *B. subtilis* is a multiflagellated bacterium that swims with an average speed of 52 $\mu m\,s^{-1}$ at room temperature (25 °C) when the flagella rotate in synchrony to form a bundle.[17] Fluctuations in flagellar rotation can cause the flagella to unbundle and generate a random reorientation of the cells. Subsequently, the bundle reforms resulting in the canonical run-and-tumble motion of the bacteria, which is characterized by an effective diffusion coefficient[18]

$$D \approx U^2\tau \tag{2}$$

where U is the swimming speed of the cells and τ is the persistence time of cell orientation, which is approximately ≈1 s for *B. subtilis*.[19] Therefore, the effective diffusion coefficient is $2700\,\mu m^2\,s^{-1}$, which is 10^7 larger than the polystyrene beads used in the previous experiment.

Figure 3 shows the motile bacteria sampled by the pill. Nine osmotic pills were placed in separate test tubes containing bacterial suspension, and three pills were removed at two time points. Their content was examined under a fluorescent microscope and compared with a sample of bacterial suspension (see Experimental Section for details). The concentration of bacteria in the pill was almost equal to the concentration in the environment at 1.5 h but continued to increase thereafter. This outcome was in fact expected as these bacteria are very motile and their diffusion factor is orders of magnitude higher than passive particles of the same size. Significantly, after 3 h, the bacteria in the pill were almost three times more concentrated than in the surrounding environment, likely the effect of continuous osmotic sampling. The osmotic membrane is water permeable, which in essence is filtering the bacteria and accumulating them in the collection chamber. Moreover, the helical geometry of the channels reduces the probability that motile bacteria can leave the collection chambers. The slight increase of the cell concentration in the surrounding medium outside the pill (control) as shown in Figure 3b is consistent with slow bacterial multiplication at room temperature. Moreover, the randomness of the position where the cells were taken from can contribute to this effect because the cell distribution inside the control volume is not completely uniform. This variation is negligible compared with a threefold increase in the concentration of bacteria inside the pill after 3 h. The in vitro test was undertaken for 24 h. As expected, the bacteria captured by the pill remained motile.

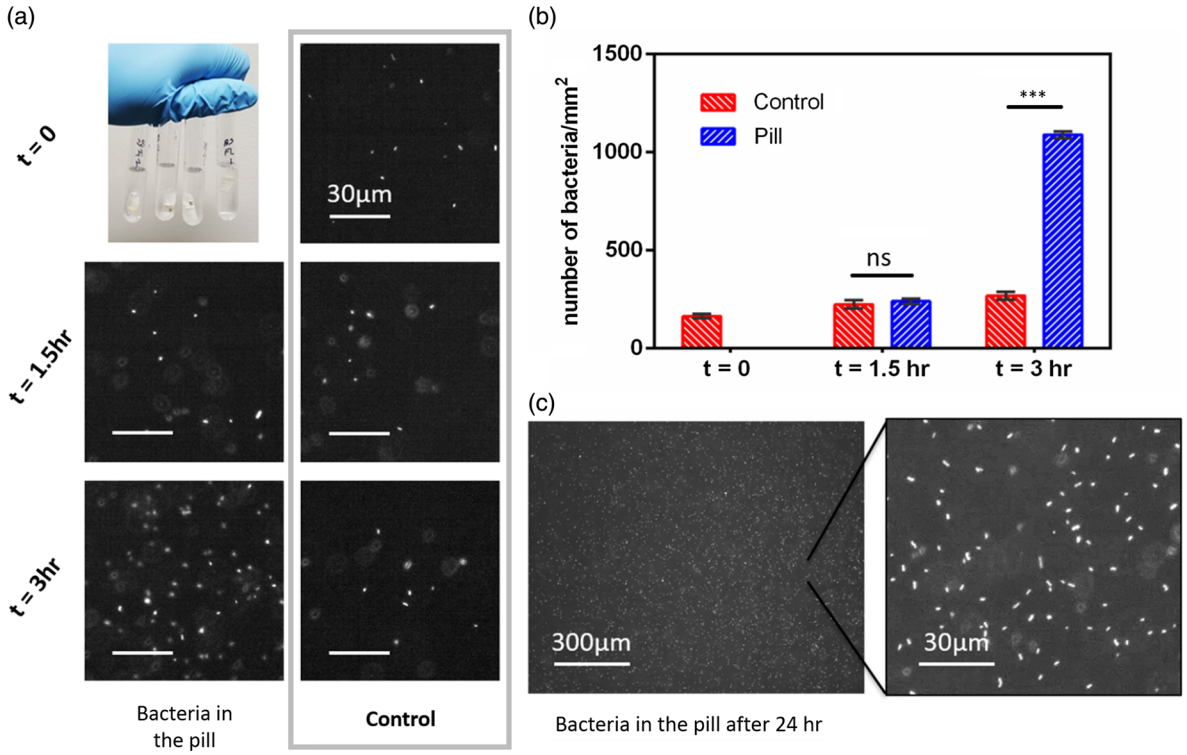

Figure 3. Osmotic sampling of motile bacteria by the pill in aqueous suspension (medium). *Bacillus subtilis* was used to model live microorganisms. a) a snapshot of bacteria concentration in the pill and surrounding environment outside of the pill (control) at different time points. b) number of bacteria sampled by the pill and outside of the pill (control). The error bars show the standard deviation of three replicate counts of the same sample. c) a snapshot of bacteria in the pill after 24 h.

2.7. Pill Mobility in the Gut (Ex Vivo Study)

To study how the pill moves inside the GI tract, we conducted ex vivo experiments using intestines freshly dissected from pigs (Figure S3, Supporting Information). At a flow rate of 4 and 8 mL min^{-1}, the pills moved with an average speed of ≈10 and ≈13 cm min^{-1}, respectively. The pill, however, moves with much higher velocity (≈22 cm min^{-1}) when the flow rate is increased to 10 mL min^{-1}, demonstrating nonlinear behavior attributed to the viscoelastic nature of the intestine that expands under increased peristaltic pressure. The natural peristaltic flow rate of fluid through the proximal small intestine varies widely from an average of 2.5 mL min^{-1} in fasting subjects to as high as 20 mL min^{-1} after meals.[20–23] The ex vivo observation indicates that the pill readily moves inside the GI tract under realistic flow conditions.

2.8. In Vitro and In Vivo Studies

To further investigate the performance of the pill, three experiments were performed in vitro (see Experimental Section). In the first experiment, pills were immersed sequentially in suspensions of *Escherichia coli*, *B. subtilis*, and *Lactobacillus rhamnosus* or only in *E. coli* and *L. rhamnosus*. DNA extracted from the material collected by the pills over a 12 h immersion period was analyzed by 16S ribosomal RNA (rRNA) amplicon sequencing to quantify the relative abundance of each species and monitor the activity of the osmotic pump over time. Pill 1 was immersed for 4 h each in cultures of *E. coli*, *B. subtilis*, and *L. rhamnosus*. Pills 2 and 3 were exact replicates and were immersed for 8 h in *E. coli* culture followed by a 4 h immersion in *L. rhamnosus* culture. Because the number of rRNA operons varies by species and strain, the number of 16S sequences assigned to each species was divided by the number of operons in the genome of each strain used in the experiment. The normalized relative abundance values shown in **Table 1** are thus proportional to the number of bacteria in the sample. These data indicate that bacteria sampled in the latter part of each experiment tend to be over-represented. This conclusion is based on the observation that

Table 1. Normalized abundance of three bacterial species in samples recovered from collection channels in experiment 1.

	Experiments			Controls[b]		
Classification[a]	Pill 1	Pill 2	Pill 3	*E. coli*	*B. subtilis*	*L. rhamn*
Enterobacteriales	0.022	0.016	0.026	0.9257	0.0004	0.0000
Bacillales	0.032	0.000	0.001	0.0015	0.9827	0.0000
Lactobacillales	0.098	0.168	0.135	0.0015	0.0002	0.9606

[a]*E. coli* is classified in the order Enterobacteriales, *B. subtilis* in the order Bacillales, and *L. rhamnosus* in the order Lactobacillales; [b]DNA was extracted from a pure culture of each species.

L. rhamnosus sequences (order Lactobacillales) are more abundant than expected based on the duration of immersion in *L. rhamnosus* culture. The three rightmost columns of Table 1 show that the vast majority (>90%) of 16S sequences obtained from pure cultures of each bacterium are correctly classified.

A second in vitro experiment was conducted to confirm that the uptake of sample by the pill requires the action of the osmotic pump. Standard pills with calcium chloride in the salt chamber as shown in Figure 1a and control pills lacking salt were immersed in parallel for 12 h in the fecal slurry prepared by homogenizing a volume of approximately 5 mL of cat or mouse feces into 500 mL of distilled water. Bacterial DNA extracted from each pill's collection channels was quantified using quantitative real-time polymerase chain reaction (PCR) as described in the Experimental Section. To confirm that the action of the osmotic pump is necessary for sampling, this analysis showed that after a 12 h immersion in fecal slurry, the concentration of bacterial DNA in the no-salt pills was from 2.4- to 59-fold more dilute than in the regular pills.

The ability of the pill to sample gut luminal content was tested in a weaned pig and in four adult rhesus macaques (*Macaca mulata*). A principal coordinate analysis (PCoA) combining the 16S sequence data from the pig and in vitro experiments is shown in **Figure 4**. As expected, a clear clustering of the microbiota by sample and by organ was observed; pig stomach/jejunum, pig colon/feces and cat and mouse fecal slurry generated well-defined clusters. In the in vivo experiments, the microbiota profile of samples recovered from the pills' collection channels closely resembled the profile of samples recovered from the surrounding intestinal lumen or feces (Table S1, Supporting Information). Based on this observation, we tested in in vitro experiment 3 to understand the extent to which the microbiota profile was impacted by material adhering to the surface of the pill, as opposed to material collected in the helical channels. To unequivocally demonstrate that the pills' osmotic pump actively aspirated material from the surrounding environment into the channel, pills from the in vitro experiment were briefly transferred from cat (or mouse) fecal slurry to mouse (or cat) slurry. Figure 4 shows that the samples recovered from the collection channels, designated with a circle in the figure and labeled "in" in the key, indeed originated from the fecal slurry in which the pills were immersed for 24 h. (triangles; "out"), and that the slurry from the opposite species did not contaminate the sample to a detectable extent. This is apparent from the tight clustering of the red (mouse) triangle with the circle of the same color and the green (cat) triangle with the green circles. The 16S sequence data show that the pills operate as intended and are capable of actively collecting samples from the surrounding matrix.

Primed pills fitted with enteric coating were also administered individually to eight adult rhesus macaques. In this experiment, we wanted to test the functionality of the pill in an animal model with a GI anatomy closely resembling that of humans. Of the eight pills gavaged to macaques, three were successfully recovered from the feces within 2 days of administration. For five animals, no pill was found in the feces. None of the missing pills were localized upon postmortem examination of the GI tract or computed tomography (CT) scanning, indicating that the pills were excreted but were missed when the feces were searched.

PCoA of 16S sequences from pills excreted from the primates as well as from matching fecal samples is shown in **Figure 5**. The position on the plot of the samples recovered from the pills' collection channels is clearly distinct from the feces collected directly from the ground or from feces adhering to the outside of the pills. These results indicate that the pills' channels contained sample collected from more proximal organs, consistent with the pills' ability to sample the entire GI tract.

We used linear discriminant analysis to identify operational taxonomic units (OTUs), which differ significantly in abundance between the pill's collection channels and the feces.[24] The three samples collected by the pills were highly enriched in Firmicutes

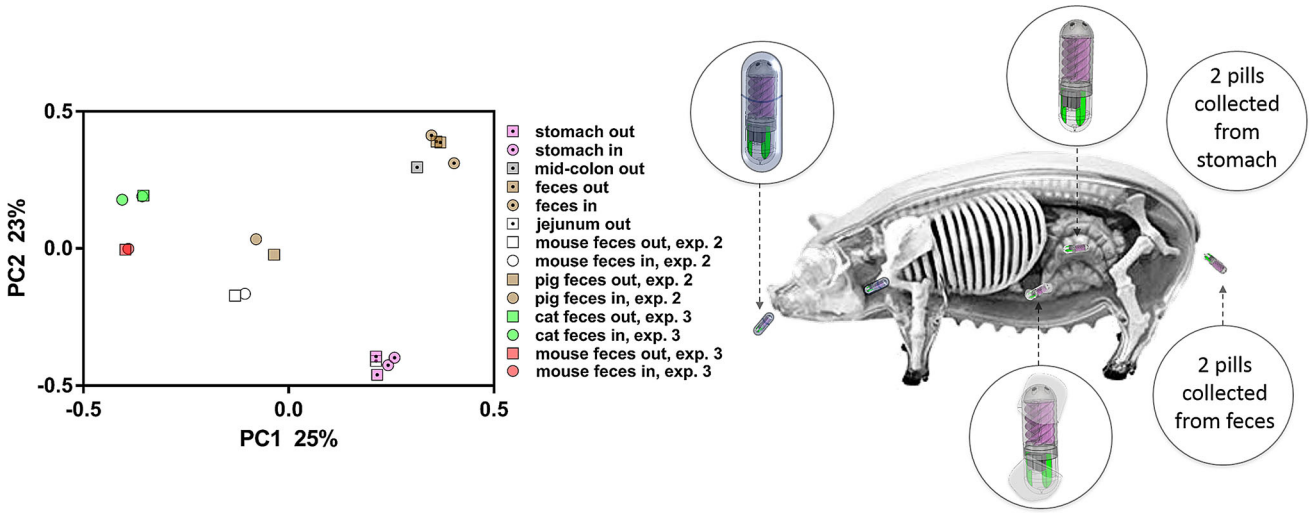

Figure 4. Principal coordinate analysis of bacterial populations sampled with pills. Results from two experiments in pigs and two experiments in vitro (experiments 2 and 3) are represented with dotted and empty symbols, respectively. Symbol shape indicates whether sample originated from the pill's collection channel (○, "in") or was collected from the matrix from which the pill was recovered (□, "out"). Color indicates organ for in vivo experiments and species origin of fecal sample for in vitro experiments. Two different mouse feces were used in experiment 2 and 3, explaining the distance between white and red datapoints.

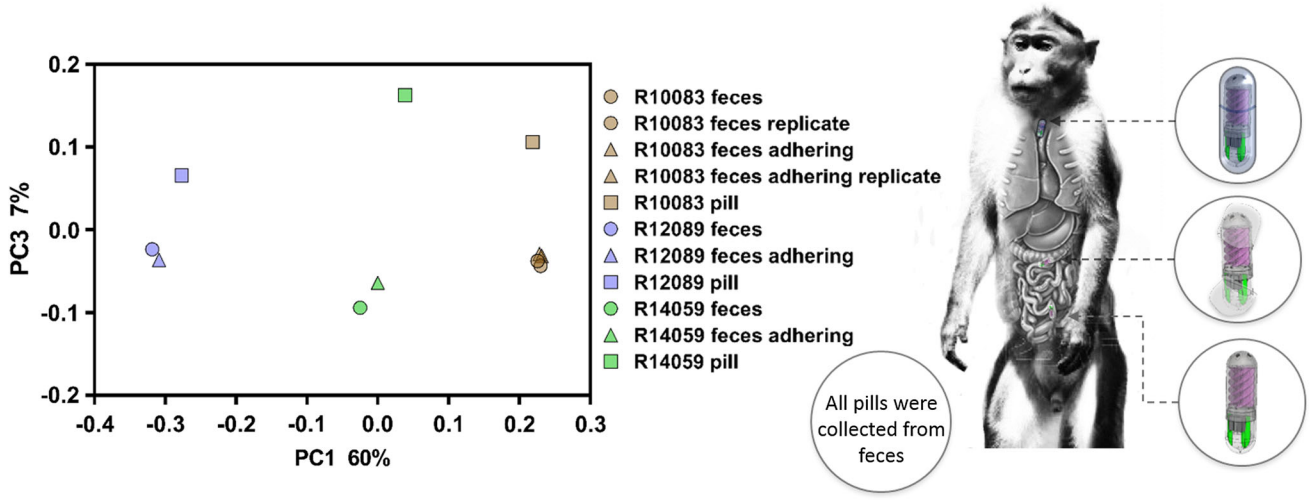

Figure 5. Principal coordinate analysis of bacterial microbiome sampled by pills orally administered to three macaques. Samples were extracted from the pills as described in the Experimental section. In addition, DNA extracted from the feces and from feces adhering to the outside of the pills were sequenced to assess to the extent the material sampled by the pills differed from the feces. The colors represent the animal as indicated in the key with a six-digit alphanumeric code. Symbol indicates origin of sample; square, pill's collection channels, circle, feces; triangles, and feces adhering to the outside of the pill. Identical symbols of the same color are technical replicates obtained by amplifying, barcoding, and sequencing a sample twice. The distance between replicates is a measure of technical variation, that is, variation introduced by sample processing and by sequencing.

belonging to the class Clostridia, order Clostridiales (Table S2, Experimental Section). Out of 32 OTUs significantly enriched in the pill samples, 21 (65.6%) belonged to this order, whereas in the fecal samples collected from the ground or adhering to the outside of the pills, only 1/9 OTUs (11.1%) belonged to this taxon. The association between Clostridia classification and sample origin is statistically significant (Fisher's exact test, $p = 0.003$). Because the small intestine is difficult to access, little information is available on microbial populations residing in more proximal sections of the GI tract. However, the genus *Clostridium* is known to be more abundant in the small intestine than in the colon.[25,26] The high abundance of Clostridiales OTUs in samples collected by the pills is consistent with active sampling as the pills travel through the macaques' GI tract. The complete taxonomic profile of the samples recovered from the pills, feces and fecal material adhering to the outside of the pills is shown in Table S3, Supporting Information.

2.8.1. Toward Spatially Selective Sampling

The in vivo and in vitro studies validated the pills' functionality in sampling the gut environment with different anatomy and conditions. Our results show that the bacterial populations recovered from the pills' microfluidic channels closely resemble the bacterial population demographics of the microenvironment to which the pill was exposed. Most importantly, it showed the ability of the pill to sample the regions of the gut upstream of the colon with quite distinct microbiome populations compared with the feces. For a more spatially targeted sampling, one can immobilize the pills using an external magnet where more of the sample can be collected from a targeted region. We performed in vitro studies to validate the magnetic hold functionality for spatially selective sampling using colored dye as a model of GI fluid. The results reported in supplementary material successfully validate our hypothesis (see Figure S4 and Table S4, Supporting Information). Studies in animals with magnetic hold for spatially selective sampling will require large magnets, in vivo imaging and visualization, and other infrastructure—this is the basis for future work. One could also leverage existing methods for pill localization and tracking using the published method.[27,28]

3. Experimental Section

3.1. Ingestible Osmotic Pill Fabrication

The pill sampling head and the bottom salt chamber were 3D printed with a *Form 2* 3D printer (Formlab Inc., Somerville, MA, USA) using a high-temperature resin also provided by Formlab. The resin was translucent and became tough after curing in the FormCure photocuring device (Formlabs Inc.) for 60 min. A reverse osmotic semipermeable membrane (GE Osmonics flat sheet membrane, SterliTech Co., Kent, WA, USA) was used to construct the osmotic pump. The membrane comprises 5 μm-thick woven cellulose acetate fibers (see Figure 1e). A circle of 6 mm in diameter was cut from the membrane sheet using a laser cutter. Then, the membrane was placed on the back of the pill head. The high-temperature resin was cured on the periphery of the membrane to firmly affix the membrane in that position. A square neodymium magnet of 3 mm × 3 mm × 1.5 mm was placed in a specially designed cavity inside the salt chamber but isolated from the surrounding by high temperature resin (HTR). A green fluorescent dye (part number: 37943991, manufactured by DecoArt, Stanford, KY, USA) was added into two tube-like reservoirs in the salt chamber, dried, and sealed by drop casting with a small amount of the high-temperature resin added into the containers. The salt chamber was filled with calcium chloride

powder before the salt chamber and the sampling head were assembled together. The fabricated osmotic pill is shown in Figure 1b.

3.2. Sample Extraction

The inlet port that allows priming and extracting the collected sample after recovery from the feces was marked with a black dot. This port was directly connected to one of the helical channels. The four helical channels were connected at the bottom to the collection chamber. Sample recovery was achieved by applying suction at this port or by centrifugation of the pill in a head-down position. Either method extracted the sample out of the corresponding helical channel, the collection chamber, and then the other three helical channels in that sequence. A 100 μL pipette tip that easily fit into this marked inlet was used for this task. Extraction is achieved using a pipette or a syringe. The process is shown in Supplementary Video 1. The centrifugation procedure is described in a later section (Section 3.6)

3.3. Motile Bacteria Culture

Fluorescent wild-type *Bacillus subtilis* (strain SG67 GFP) cells were cultured by inoculating 5 mL of Cap Assay Minimal (CAM) motility medium with cells obtained from a frozen glycerol stock solution.[17] The cells were grown overnight at 37 °C while shaking at 250 rpm until the optical density reached $OD_{600} = 0.1$. These cells had $1\,\mu m \times 3\,\mu m$ elongated bodies and swam at a speed of $U = 40$–$65\,\mu m\,s^{-1}$.[17]

3.4. Imaging and Analysis

Epiluorescence imaging was performed on an inverted microscope (Nikon Ti-E) with a 10× objective to take snapshots of the cells in the samples with a Zyla sCMOS camera (Andor Technology). Image analysis was performed to enumerate the bacteria by intensity thresholding in ImageJ.[29]

3.5. Particle and Bacterial Concentration Measurement

To quantify the concentration of the bacteria/particles collected by the pill over time, these steps were followed: 100 μL of the sample extracted from the pill using a pipette were dispensed on a glass slide; two spacers of 100 μm height were placed on either edges of the glass slide; and another glass slide was placed on the top of the sample to confine the liquid; the sample was then imaged and the number of bacteria/particles on the surface was counted using the ImageJ software in the field of view. Three such images were taken over the entire glass slide. The average number of bacteria/particles per field divided by the visible area for the view and the particle concentration was expressed as number per mm^2.

3.6. In Vitro Experiments with Live Bacteria and Fecal Slurries

Experiments in which pills were immersed in suspensions of live bacteria were conducted to assess the ability of the pill's osmotic pump to draw bacteria into the collection channel. To assess the effect of time on the ability of the osmotic pump to aspirate from the environment, a live suspension of each of the following bacterial species was prepared: *E. coli* strain K12, *B. subtilis* strain 168 BFA, and *L. rhamnosus* (BEI Resources, strain LMS2-1, cat # HM-106). *E. coli* and *B. subtilis* were grown overnight in LB broth (Fisher BioReagents, cat. # BP1426-500) and *L. rhamnosus* in MRS broth (Fluka Analytical, cat. # 69966). The concentration of the colony forming unit (CFU) of each culture was estimated by plating tenfold serial dilutions on agar plates. Bacterial suspensions were prepared in phosphate-buffered saline at a concentration of $10^8\,CFU\,mL^{-1}$ and kept in motion with a magnetic stirrer. Pills without enteric coating were immersed sequentially for 4 h in each suspension as follows: pill 1, 4 h each in *E. coli*, *B. subtilis*, and *L. rhamnosus* and pill 2 and pill 3, 8 h in *E. coli* followed by 4 h in *L. rhamnosus*. To recover the content of the collection channel, each pill was introduced with the channel opening facing down into a 1.5 mL microcentrifuge tube and spun at $11\,000 \times g$ for 5 min. The pill was removed, and the entire volume of approximately 200 μL recovered from the pill was processed for DNA extraction. The same sample recovery method was used in all in vitro and in vivo experiments described below.

A second in vitro experiment was conducted to confirm that the uptake of sample requires the action of the osmotic pump. Standard pills with $CaCl_2$ in the salt chamber as shown in Figure 1b and pills lacking salt were immersed in parallel in fecal slurry. No enteric coating was used. The pills were kept in motion on a magnetic stirrer in 200 mL glass beakers containing fecal slurry. The experiment was conducted at room temperature.

A third in vitro experiment was undertaken to further assess the function of the pill when it is immersed in fecal slurry. Specifically, we investigated the level of contamination of the sample recovered from the collection channels with material adhering to the outside of the pills. Fecal slurry was prepared as described above for the second experiment, namely by homogenizing a volume of approximately 5 mL of feces in 500 mL of water. Two pills were immersed in each of the two slurries (cat or mouse) and retrieved after 24 h. Thereafter, each pill was briefly immersed in the opposite slurry (mouse or cat), rinsed with distilled water, and dried.

3.7. Experiments in Pigs and Macaques

Weaned pigs approximately 3 weeks of age were purchased from the Cummings Veterinary School campus and housed in pens according to IACUC guidelines (protocol number G2018-03 Tufts University). The pills' osmotic pump was activated with distilled water and the collection channels primed with water. The pills were enclosed in an enteric capsule (Size 00 white empty enteric coated capsules from CapsulCN International Co., Ltd, China) immediately prior to oral administration. The pill was inserted into a pill "gun" to introduce the pill directly into the pig's esophagus, preventing regurgitation (see Figure S5, Supporting Information). Two experiments were conducted in pigs. In experiment 1, two pills were recovered from the stomach of the pig post-euthanasia. In experiment 2, one pill was recovered from the feces (see Figure S6, Supporting Information).

Experiments in nonhuman primates (see Figure S7, Supporting Information) were conducted at the Biomedical Primate Research Center (BPRC) in Rijswijk, The Netherlands. Eight healthy rhesus macaques (*Macaca mulatta*) were selected from a group of animals that received a physical examination for a nonrelated scientific project (permit AVD5020020186346). This specific part was approved by the Animal Welfare Body (IvD 014A) and consisted of six males and two females, aged between 4 and 8 years. All animals were born and raised in the BPRC colony. Upon selection for studies, the animals were pair housed in the experimental facility in primate cages of $1 \times 2 \times 2$ m provided with bedding and enrichment. The animals were fed with commercial monkey pellets supplemented with vegetables and fruit, and drinking water was provided ad libitum. For the physical examination, the animals were sedated with $10\,\mathrm{mg\,kg^{-1}}$ ketamine hydrochloride. The pill was manually placed into the pharynx of the animal and guided from the upper part of the esophagus to the stomach with the help of a feeding tube. The animals were examined for 48 h and four times per day for stool passage, and all fecal materials were collected and palpated. Four pills were recovered from the fecal material of four animals (two males, 4 and 8 years old, two females, 6 and 8 years old) within the first 24 h. The remaining animals were followed for another 48 h, and fecal material was collected and checked, but no pills were found. The animals from which no pills were recovered were euthanized after 2 months as part of the scientific project for which they were assigned. During the necropsy, no pills were found, and the GI tract showed no significant findings. Furthermore, an additional CT scan of one of the animals just before necropsy did not show any evidence of the remains of a pill. It can be assumed that the unrecovered pills were passed through the stools by the animals but were missed during the checking of the fecal material.

3.8. Molecular Biology Methods

Following recovery of the content of the collection channels, DNA was extracted in a QiaCube instrument using the QIAamp PowerFecal DNA kit (QIAGEN, cat. 12830-50) according to the manufacturer's protocol. DNA was eluted in 50 µL elution buffer and stored at −20 °C. A previously described PCR protocol was used to prepare 16S V1V2 amplicons libraries for 16S amplicon sequencing.[30] The quality of the 16S amplicons was assessed by agarose gel electrophoresis. Amplicons were pooled in approximately equal molar ratio, size selected with a Pippin Prep instrument (Sage Science, Beverly, MA) and sequenced in an Illumina MiSeq sequencer at the Tufts University Genomics Core (tucf.org) using a single-end, 300-nucleotide strategy. To control for technical variation introduced during library preparation and sequencing, each library included two replicates of two randomly chosen samples. Replication involved processing duplicate fecal samples, amplified and barcoded individually.

Bacterial DNA was quantified by real-time PCR using the same V1V2 PCR protocol as used to construct the 16S libraries. PCR was performed in a Roche LightCycler instrument. Crossing point (C_t) values, i.e., the number of temperature cycles required to reach a predetermined concentration of the PCR product, were determined from the PCR amplification curves using the instrument's software. To convert C_t to relative DNA concentration (C), C_t values obtained from a tenfold serially diluted DNA sample were regressed on the log(C). The DNA concentration of this solution was determined using a Qbit 3.0 fluorometer (Invitrogen Life Technologies). The linear regression equation determined from these data was

$$C_t = -4.811 \cdot \log(C) + 15.32 \quad (3)$$

which was used for converting C_t to DNA concentration (C).

3.9. Bioinformatics Analysis

Bioinformatics analysis of 16S sequences was performed in *mothur* essentially as described.[30–32] Briefly, random subsamples of 5000 sequences per sample were processed using a sequence processing pipeline designed to generate pairwise-weighted UniFrac distances between samples.[33] Distance values were entered into a distance matrix and visualized by PCoA. The PCoA plot was then computed in GenAlEx.[34] Sequences were taxonomically classified using the method described by Wang et al.[35] in *mothur* (see studies by Schloss et al. [31]). The minimum bootstrap value for taxonomic assignment was set at 70%.

To convert relative 16S sequence abundance values for each bacterial species obtained in the in vitro experiment 1 to relative abundance of bacteria, relative sequence abundance data were normalized against the number of rRNA operons present in the genome of each bacterial species. This calculation was necessary because the number of rRNA operons varies by species and sometimes also between strains of the same species. The number of operons obtained from the University of Michigan rrnDB database at https://rrndb.umms.med.umich.edu/ is 7 for *E. coli* K12, 10 for *B. subtilis* 168 BFA, and 5 for *L. rhamnosus* LMS2-1.[36]

DNA Sequence Accessions

16S DNA sequence data were deposited in the European Nucleotide Archive under accession numbers PRJEB30052 (Pig experiment and in vitro experiment 3) and PRJEB32383 (Primate experiment and in vitro experiment 2).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

S.S. invented the concept of the pill. H.R.N and S.S conceptualized the design of the pill and cowrote the manuscript. H.R.N fabricated the pills and performed in vitro and some ex vivo studies. H.R.N ran the simulations and prepared figures for the manuscript. G.W. led the animal studies effort and cowrote the manuscript. S.T. supported the animal studies in pigs. I.K and J.A.M.L performed in vivo animal studies in monkeys. B.C.M.O. and G.W. performed in vivo studies in pigs and ex vivo experiments and analyzed and interpreted the animal studies. H.R.N. and A.S. performed the magnetic hold test experiment. A.D., J.G., and H.R.N. performed in vitro sampling studies with motile bacteria. A.S. also helped prepare some figures. All authors discussed the results and commented

on the manuscript. Office of Naval Research (ONR) primarily sponsored this work under the grant N0014-16-1-2550 (Program manager: Dr. Linda Chrisey). B.C.M.O and G.W. were supported by the National Institute of Allergy and Infectious Diseases (grant # 5R21AI125891). A.D and J.G would like to acknowledge the support of National Science Foundation (CBET-1511340, CAREER-1554095). The authors thank Boudewijn Ouwerling and Don Girouard for expert assistance with animal experiments and Dr. Albert Tai and the Tufts Genomics Core Facility staff for high-throughput sequencing.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

biomedical devices, lab-on-a-chip, microbiomes, microfluidics, smart pills

Received: June 17, 2019
Revised: July 14, 2019
Published online: August 8, 2019

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