Enhanced Storage of Anaerobic Bacteria through Polymeric Encapsulation

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ABSTRACT: Live microbes such as lactobacilli have long been used as probiotic supplements and, more recently, have been explored as live biotherapeutic products with the potential to treat a range of conditions. Among these microbes is a category of anaerobes that possess therapeutic potential while exhibiting unique oxygen sensitivity and thus requiring careful considerations in the formulation and storage processes. Existing microbial formulation development has focused on facultative anaerobes with natural oxygen tolerance; a few strategies have been reported for anaerobes with demonstrated oxygen intolerance, warranting novel approaches toward addressing the challenges for these

oxygen-sensitive anaerobes. Here, we develop a polymeric encapsulation system for the formulation and storage of *Bifidobacterium adolescentis* (*B. adolescentis*), a model anaerobe that loses viability in aerobic incubation at 37 °C within 1 day. We discover that this strain remains viable under aerobic conditions for 14 days at 4 °C, enabling formulation development such as solution casting and air drying in an aerobic environment. Next, through a systematic selection of polymer encapsulants and excipients, we show that encapsulation with poly(vinyl alcohol) (PVA) acts as an oxygen barrier and facilitates long-term storage of *B. adolescentis*, which is partially attributed to reduced generation of reactive oxygen species. Lastly, PVA-based formulations can produce oral capsule-loaded films and edible gummy bears, demonstrating its compatibility with both pharmaceutical and food dosage forms.

KEYWORDS: *polymeric encapsulation, probiotics, live biotherapeutic products, formulation, storage*

■ INTRODUCTION

Live microbes such as lactobacilli have been used as probiotics to confer health benefits for over a century.[1] They are often supplemented in food products (e.g., yogurt, cheese, fruit juice) for daily administration.[2] In the last two decades, increasing efforts have focused on developing live microbes for pharmaceutical applications, such as feces-derived spores, defined microbial consortia, and engineered probiotics; these microbes are regulated as live biotherapeutic products (LBPs) by the U.S. Food and Drug Administration.[3,4] Recent milestones in treating *Clostridioides difficile* infections highlight the potential of LBPs to provide a curative treatment for diseases while bypassing limitations of traditional therapeutic modalities such as antibiotic resistance.[5,6] Broadly, orally administered probiotics and LBPs modulate host physiology through various mechanisms, including (i) establishing colonization resistance to prevent pathogen infections,[7] (ii) regulating host immune systems to synergize with other therapeutics such as checkpoint inhibitors for cancer immunotherapy,[8] and (iii) performing critical metabolic functions in individuals with metabolic disorders.[9] As such, live microbes represent a unique modality with therapeutic potential toward addressing unmet clinical needs.
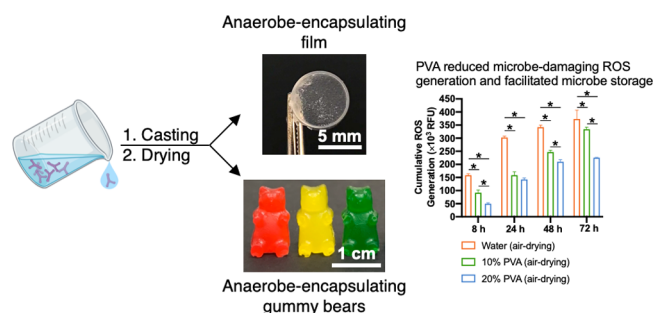
As viability is required for most microbes to perform their therapeutic functions, preserving microbial viability throughout formulation and storage is a crucial task. This is particularly challenging for a subgroup of anaerobes with high oxygen sensitivity, such as Bifidobacterium species for probiotic use[10] and Faecalibacterium species for most relevant LBPs.[11] These anaerobes either do not express, or minimally express, enzymes (e.g., superoxide dismutase, peroxidase) for detoxifying reactive oxygen species (ROS) (e.g., superoxide and peroxide),[12,13] leading to damage and microbial death under aerobic conditions.[14] Therefore, oxygen exposure must be carefully considered throughout formulation development for these anaerobes. Notably, current formulation approaches for microbes, such as lyophilization, air drying, and surface coatings, have been developed for facultative anaerobes,[15−18] which are naturally equipped to degrade ROS for survival;[19] few formulation approaches and possible underlying mecha-
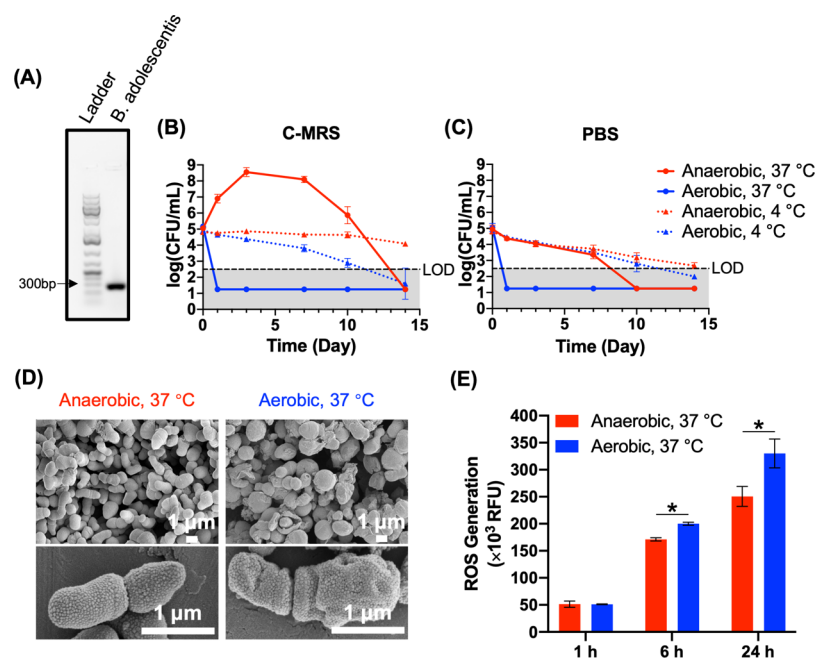
Figure 1. Oxygen sensitivity of *B. adolescentis*. (A) Confirmation of strain identity by PCR. (B) Bacterial viability in cysteine hydrochloride-supplemented MRS (C-MRS) under anaerobic (red) and aerobic (blue) conditions at 4 °C (dashed lines) and 37 °C (solid lines). (C) Bacterial viability in PBS under anaerobic and aerobic conditions at 4 and 37 °C. (D) SEM images of *B. adolescentis* in C-MRS with and without oxygen exposure for 24 h at 37 °C. (E) Time-course quantification of ROS generated from *B. adolescentis* culture in PBS. Each error bar represents the standard deviation ($n = 3$). Statistical analysis was conducted using Student's *t*-test (significant difference defined at $p < 0.05$). *: significantly different. LOD: Limit of detection.

nisms for storage enhancement have been described for anaerobes that face greater challenges in addressing ROS toxicity. Toward clinical translation, novel formulation and storage approaches are needed for anaerobes with demonstrated oxygen sensitivity.

Polymeric encapsulation has been used to formulate a variety of labile cargos for both pharmaceutical (e.g., bacteriophages) and food (e.g., micronutrients) applications to protect against environmental stressors including oxygen and heat.[20,21] Here, we apply this strategy to the formulation and storage of *Bifidobacterium adolescentis* (*B. adolescentis*), a model anaerobe that exhibits oxygen intolerance during growth[10,12] and has shown promise in alleviating metabolic diseases in preclinical models.[22,23] We first confirm its oxygen intolerance in aerobic media at 37 °C. We then discover that *B. adolescentis* shows extended survival in the same media at 4 °C, which enables formulation development in an aerobic environment. In our previous work, we have shown that microbe storage can be improved in sustained-release and mucoadhesive polymeric films formulated with bacteria-specific excipients.[18] Based on this established film fabrication platform, we demonstrate that encapsulation with PVA, a polymeric oxygen barrier,[24−27] significantly improves the survival of *B. adolescentis* during formulation and storage. As compared to lyophilization, a widely used approach to formulating probiotics and clinically investigated LBPs,[3,28] air-dried film formulations exhibit noninferior storage profiles, representing a promising approach for clinical applications. Importantly, we reveal that polymeric encapsulation enhances storage likely through reducing ROS generation. Additionally, we show that this formulation platform is compatible with both pharmaceutical (capsule) and food (gummy bear) dosage forms, underscoring its potential in pharmaceutical and food industries.

■ RESULTS AND DISCUSSION

Oxygen Sensitivity Assessment. We first confirmed the identity of this bacterial isolate with 16S-targeted, *B. adolescentis*-specific polymerase chain reaction (PCR). A single band around 280 bp was shown on agarose gel through standard electrophoresis (Figure 1A), consistent with the length of the expected amplification product for *B. adolescentis* and thus confirming the strain identity.[29] To validate its oxygen sensitivity at 37 °C, *B. adolescentis* (10^5 CFU mL^{-1}) was suspended in the growth media (cysteine hydrochloride-supplemented MRS (C-MRS)) or phosphate-buffered saline (PBS), followed by static incubation with or without oxygen exposure (Figure 1B and C). Complete viability loss was observed after 1 day of incubation in aerobic C-MRS and PBS at 37 °C (Figure 1B and C), demonstrating that *B. adolescentis* was intolerant to normal atmospheric oxygen levels at 37 °C. As a control, *B. adolescentis* exhibited an expected growth curve featuring exponential growth, stationary, and lag phases in C-MRS under anaerobic conditions at 37 °C (Figure 1B) and gradual viability loss over 10 days in PBS under anaerobic conditions at 37 °C (Figure 1C), likely a result of nutrient deprivation. We also analyzed oxygen sensitivity of *B. adolescentis* at 4 °C as temperature is more relevant to microbial formulation and storage processes. While oxygen exposure also led to faster viability loss relative to the anaerobic control, viability remained detectable under aerobic conditions for the entire 14 days in C-MRS and PBS at 4 °C. The temperature-dependent differences in viability loss are likely caused by a more rapid generation of ROS at higher temperatures, which has been observed in other organisms.[30,31] Importantly, moderate oxygen tolerance at low temperatures such as 4 °C may enable formulation development for anaerobes in an aerobic environment. To further characterize

bacterial damage induced by oxygen exposure, we performed scanning electron microscopy (SEM) analysis and observed wrinkled and irregular morphology of the *B. adolescentis* cell wall after 1 day of oxygen exposure in aerobic C-MRS at 37 °C as compared to fresh *B. adolescentis* from anaerobic culture conditions (Figure 1D). These morphological changes have been observed on other bacteria in oxidative environments[32−34] and thus are a likely indicator of oxidation damage to *B. adolescentis*. We also quantified the ROS generation in *B. adolescentis* under aerobic and anaerobic conditions in PBS over time. A significantly higher generation of ROS was observed at 6 and 24 h of aerobic incubation at 37 °C as compared to anaerobic incubation (Figure 1E), supporting ROS-mediated damage on *B. adolescentis* during oxygen exposure. Together, these results demonstrated oxygen intolerance of *B. adolescentis* at 37 °C and implicated that reduction of oxygen interactions and ROS generation might improve the survival of *B. adolescentis* during and after formulation at 4 °C.

Screening of Polymer Encapsulants and Excipients for *B. Adolescentis* Storage. Our previous work demonstrated that polymeric encapsulation through solution casting and air-drying processes enabled facile manufacturing of multifunctional formulations (e.g., long-term storage, mucoadhesion, tunable release) for *Lactobacillus casei* ATCC 393 (*L. casei* ATCC 393).[18,35] While *L. casei* ATCC 393 is a facultative anaerobe that can grow in the aerobic environment, we have shown that encapsulation with poly(vinyl alcohol) (PVA), a biocompatible and oxygen-barrier material, improved its storage.[18] As such, we sought to evaluate polymeric encapsulation in formulating and preserving *B. adolescentis*, which is vulnerable to ROS-mediated damage and thus more likely to benefit from encapsulation. We screened polymer encapsulants for a 10 day storage period at 4 °C, including inulin, pullulan, and hyaluronic acid (HA) in addition to PVA as these polysaccharides have been reported as encapsulants for preserving other sensitive cargos.[21,36,37] To account for the differences in the initial loading across groups (Figure S1), we normalized viability at day 10 to initial loading at day 0 (Figure 2). We found that HA provided the largest survival benefit among all the tested polymer encapsulants, with PVA providing the second largest survival benefit. Given that traditional excipients such as sugars, amino acids, and proteins are necessary to ensure long-term storage according to our previous studies,[18] we also screened protective excipients

including polyols (glycerol), disaccharides (sucrose and trehalose), and composite excipients (skim milk containing lactose and whey). These excipients provide storage benefits likely using stabilizing lipids and proteins via hydrogen bonding.[21,38,39] We found that skim milk outperformed other excipients (Figure 2), potentially a result of the synergistic protective effects from disaccharides (lactose) and proteins (whey) found in skim milk. However, the combination of 12% skim milk and 2% HA did not produce solid films after air drying, which posed difficulties for downstream handling and eventual oral administration of the formulation. As such, we proceeded with combining PVA and skim milk to investigate the effects of polymeric encapsulation on the storage of *B. adolescentis*.

Encapsulation, Storage, and ROS Quantification of *B. adolescentis*. As our previous results showed that the storage improvement for *L. casei* ATCC 393 was dependent on the PVA content, we sought to evaluate the storage of *B. adolescentis* encapsulated in various amounts of PVA at 4 °C. As lyophilization represents a prevailing approach for formulating therapeutic microbes in the clinical trials,[3] we also incorporated lyophilized *B. adolescentis* for comparison. To confirm whether *B. adolescentis* was successfully encapsulated in the air-dried PVA matrix, we examined formulation morphology at the macroscopic and microscopic scales. Compared to a colorless and transparent PVA film from a 96-well plate template (Figure 3A), air-dried PVA formulations
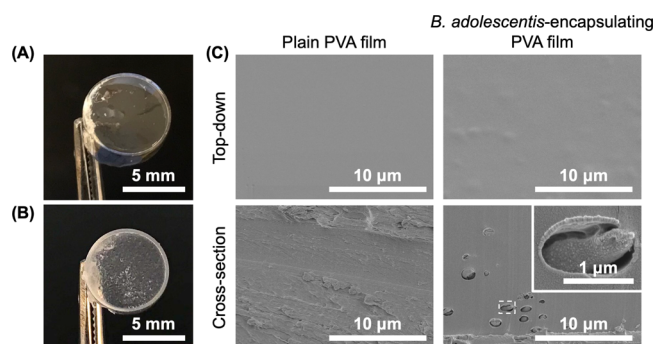
Figure 3. Imaging of *B. adolescentis* films. Camera images of (A) plain PVA film or (B) *B. adolescentis*-encapsulating PVA film that was fabricated from 20 wt % vol⁻¹ PVA and 12 wt % vol⁻¹ skim milk. (C) SEM images of a plain PVA film and a *B. adolescentis*-encapsulating PVA film without skim milk from the top-down and cross-section views.

containing skim milk and *B. adolescentis* appeared white and opaque (Figure 3B). SEM analysis revealed lumps of bacterial shape at the film surface from the top-down view and visible *B. adolescentis* at the cross-sections for bacteria-loaded PVA films, while plain PVA films exhibited no bacterial features from either top-down or cross-section views (Figure 3C), confirming the encapsulation of *B. adolescentis* in the polymeric matrix. Formulations without PVA did not produce films. We observed that while all groups containing skim milk exhibited comparable viability upon drying, all PVA groups showed superior survival as compared to non-PVA counterparts at day 14 postdrying (Figure 4A). In addition, we compared the storage of 10% PVA and 20% PVA formulations in the presence of skim milk at 25 °C (Figure 4B), a harsher environment at higher temperature to accelerate viability loss and thus enable a more rapid evaluation of storage differences
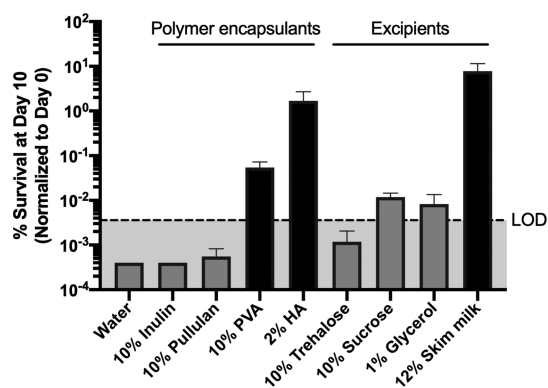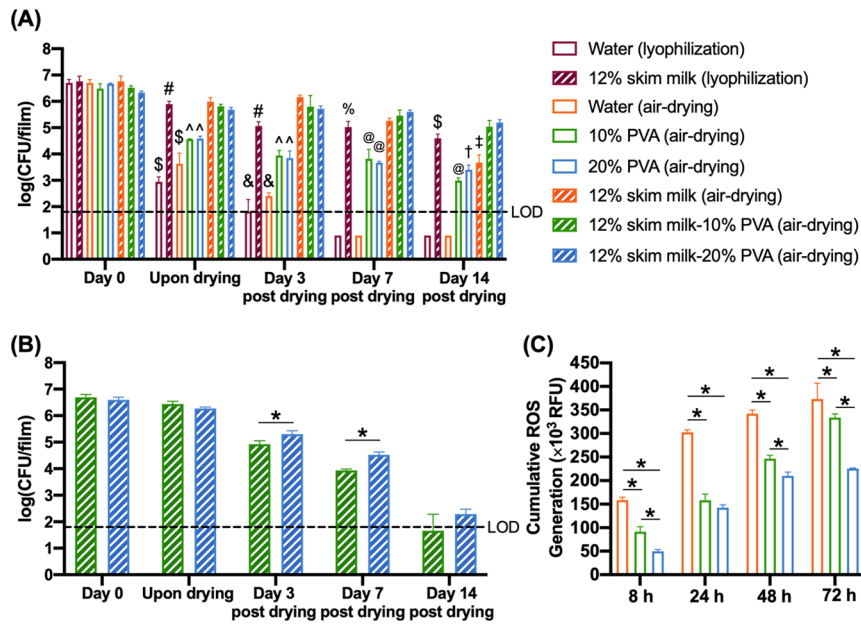
Figure 2. Screening of polymer encapsulants and excipients for the storage of *B. adolescentis* through the air-drying process. LOD: Limit of detection.

Figure 4. Storage and ROS quantification of *B. adolescentis*. (A) Bacterial viability in air-dried and lyophilized formulations stored at 4 °C. (B) Bacterial viability in air-dried formulations stored at 25 °C. (C) ROS quantification for *B. adolescentis*-containing formulations through the air-drying process up to 72 h when all formulations were dried. Figure legends indicate the predried formulation components and the corresponding drying method for each group. Each error bar represents the standard deviation (*n* = 3). Statistical analysis at each timepoint was conducted using Student's *t*-test (for B) or one-way analysis of variance (ANOVA), followed by post hoc Tukey's honestly significant difference (HSD) test for pairwise comparison (for A and C) (significant difference defined at $p < 0.05$). $: significantly different from all other groups. #: significantly different from all skim milk-free groups. ^: significantly different from water (lyophilization), water (air drying), and all skim milk-containing groups. &: significantly different from 12% skim milk (air drying) and skim milk-free groups. @: significantly different from 12% skim milk (lyophilization) and skim milk-free groups. %: significantly different from 10% PVA (air drying), 20% PVA (air drying), and 12% milk−20% PVA (air drying). †: significantly different from 12% skim milk (lyophilization), 10% PVA (air drying), 12% skim milk−10% PVA (air drying), and 12% skim milk−20% PVA (air drying). ‡: significantly different from 12% skim milk (lyophilization), 10% PVA (air drying), 20% PVA (air drying), and 12% skim milk (air drying). *: significantly different. LOD: Limit of detection.
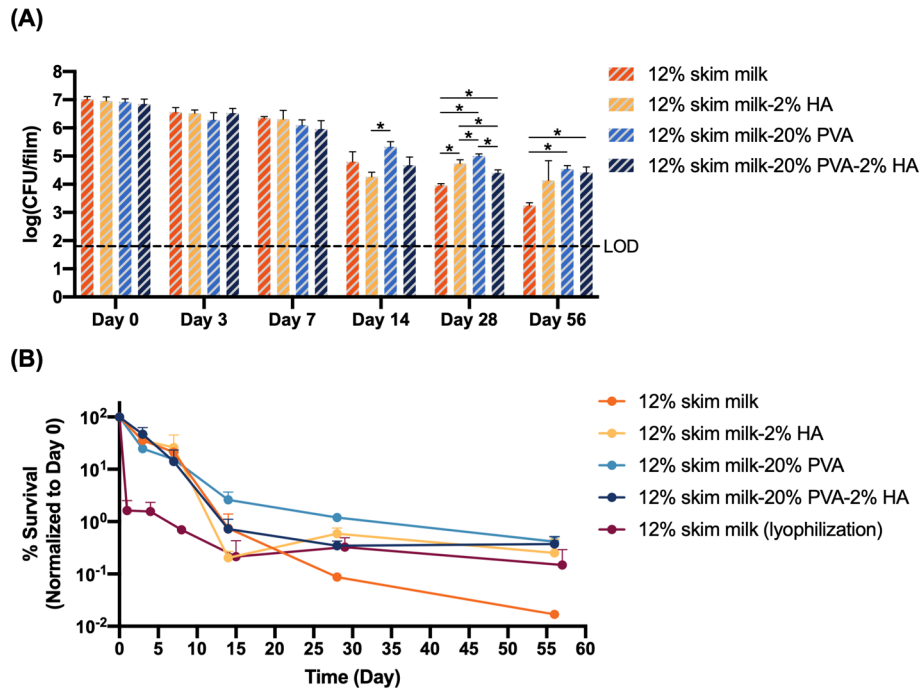

Figure 5. Long-term storage of *B. adolescentis* in skim milk-containing formulations at 4 °C. (A) Viability of *B. adolescentis* in skim milk-containing formulations over 56 days 4 °C. (B) Survival curves of *B. adolescentis* in skim milk-containing formulations at 4 °C. Each error bar represents the standard deviation (*n* = 3). Statistical analysis at each time point was conducted using one-way ANOVA, followed by post hoc Tukey's HSD test for pairwise comparison (significant difference defined at $p < 0.05$). *: significantly different. LOD: Limit of detection.

as a function of formulation characteristics. We found that 20% PVA film formulation exhibited higher survival at day 3 and day 7 as compared to 10% PVA. These results suggested that the incorporation of a higher amount of PVA into skim milk enhanced storage for *B. adolescentis* mainly after formulations were dried. Notably, in the presence of skim milk, 10 and 20% PVA film formulations provided noninferior storage than lyophilized formulation throughout the entire study (Figure 4A), highlighting the potential of air-dried polymeric encapsulation toward formulating clinically relevant microbes.

To uncover the underlying mechanisms of polymeric encapsulation for enhanced storage, we quantified ROS generation as a function of the PVA content. We observed a significant reduction in the ROS level with increasing PVA concentrations during the air-drying process (Figure 4C), indicating that higher polymer content decreased ROS generation. As no validated assays are available to quantify ROS generation in solid microbial formulations to our knowledge, we were not able to directly compare ROS generation over time after formulations were dried. However, the generation of ROS during air-drying of formulations was consistent with their storage profiles (Figure 4B), suggesting that the inclusion of PVA improves the survival of *B. adolescentis* by reducing ROS generation. While previous studies showed that metabolic activities could affect ROS generation[40] and PVA was able to serve as a nutrient source for certain bacteria,[41] we found that PVA did not support the growth of *B. adolescentis* as a carbon source (Figure S2), indicating that ROS generation is unlikely correlated to nutrient availability under this specific condition. As such, this trend is potentially explained by the increase in viscosity associated with higher PVA concentrations[42,43] or due to the denser polymer networks of higher PVA concentrations, thus reducing oxygen diffusion into the formulation. Indeed, previous literature indicates that solid PVA-based films can reduce oxygen permeation,[24−27] likely impeding the interaction of oxygen with encapsulated payloads and alleviating oxidation, while the quantitative evidence requires future assay development that allows quantification of ROS generation in dried forms. We also attempted to compare ROS generation as a function of PVA content in the presence of skim milk; however, skim milk generated high background signals (Figure S3A and S3B). Overall, these results suggested that polymeric encapsulation could improve the storage of *B. adolescentis*, and the improved viability was inversely correlated to the generation of ROS.

To explore and optimize the long-term preservation of *B. adolescentis* with the polymeric encapsulation approach, we included HA in the formulation and evaluated potential synergistic effects with PVA and skim milk in a 56-day storage study at 4 °C, as HA demonstrated the highest survival for polymer encapsulants during storage in the initial screening experiment (Figure 2). All groups exhibited minimal viability loss (<1 log) during the first week of storage (Figure 5A). PVA-containing groups exhibited enhanced survival as compared to the skim milk group without polymer encapsulants at day 28 and day 56, indicating that encapsulation with PVA improved long-term preservation. PVA encapsulation also provided long-term storage benefits without skim milk (Figure S4A and S4B). As the molecular weight and degree of hydrolysis of PVA have been shown to influence film properties such as tensile performance and water vapor permeation,[44] these molecular parameters also likely affect oxygen diffusion in the polymeric matrix, thus warranting further investigation to optimize storage profiles. We observed that while the combination of HA and skim milk significantly improved survival at day 28, the addition of HA into a PVA-skim milk formulation did not further improve storage. These results suggest that additional protectants do not always translate to storage benefits, thus necessitating additional strategies to further improve storage. We also compared the long-term storage of air-dried formulations to a lyophilized formulation in skim milk. We found comparable survival of the lyophilized formulation (Figure S5) with all polymer-encapsulated, air-dried formulations (Figure 5B), corroborating the potential of air-dried films as a next-generation formulation for probiotics or LBPs. Increase in the initial colony-forming unit (CFU) loading on a per-film basis and further optimization on the mass and type of excipient, drying process parameters (e.g., drying rate), and humidity control during storage will likely improve bacterial viability to meet clinically relevant dosing over long-term preservation.

The current microbe-based formulations intended for human use fall into two major categories: LBPs as pharmaceuticals regulated as therapeutics[45] and probiotics regulated as dietary supplements.[46] As such, we sought to evaluate the compatibility of our approach with common pharmaceutical- and food-based formulations. Previously, we demonstrated that PVA-based films were compatible with and readily loaded into standard human oral capsules through film folding.[18] Here, we fabricated films in smaller templates (96-well plates as compared to 24-well plates[18]), which enabled loading into capsules without manual folding (Figure 6A), simplifying formulation processing of the oral dosage form. A 00-sized capsule allowed for the loading of 40 films, enabling over 10^8 CFU loading per oral capsule (Figure 6A). Additionally, up to 40 capsules are used clinically to achieve one oral dose;[47] as such, the combined film-capsule dosage form is viable to meet a clinically relevant dose, such as 10^9 CFU.[48] Separately, as each film can encapsulate different microbes of interest, this film-capsule system potentially enables delivery of microbial consortia, which have demonstrated therapeutic potential in treating conditions such as pathogenic infections,[7] colitis,[49] and cancer.[8] To explore the compatibility of PVA-based formulations with food, we sought to fabricate air-dried PVA formulations as bear-shaped gummy candies (gummy bears), which are commonly used to deliver supplements such as vitamins.[50] Food coloring was incorporated into the formulation (containing *B. adolescentis*, 20% PVA, and 12% skim milk) to mimic the appearance of commercial gummy bears. Formulations were cast three times into a silicone mold on three consecutive days, respectively, to increase the thickness of the dosage form. Gummy bears were harvested when they were dried and readily removed from the silicone mold without losing structural integrity. Freshly harvested gummy bears were mechanically stable on a supporting substrate (Figure 6B). Approximately 1 log reduction in the CFU occurred upon drying as compared to the initial loading on a per-gummy-bear basis (Figure 6C), similar to the survival when formulated as films (Figure 3A). To demonstrate the edibility of these formulations, we compared the dissolution rate of these PVA-based gummy bears to gelatin-based, commercial gummy bears at 37 °C. They exhibited no significant difference in the dissolution rate (Figure 6D), indicating that the PVA-based gummy bears could be digested at a comparable rate as gelatin-based gummy
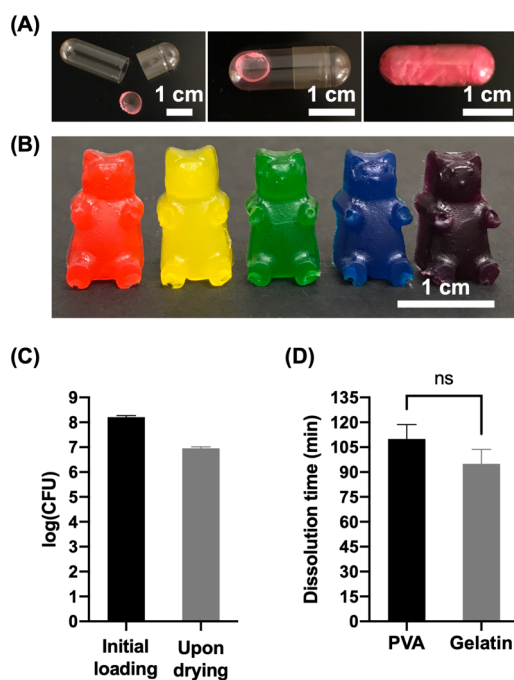
Figure 6. Compatibility of the PVA-based formulation with pharmaceutical and food dosage forms. (A) Left: an empty 00-sized oral capsule and one rhodamine-dyed, *B. adolescentis*-encapsulating film. Middle: loading of one rhodamine-dyed, *B. adolescentis*-encapsulating film into a 00-sized oral capsule. Right: loading of 40 rhodamine-dyed, *B. adolescentis*-encapsulating films into a 00-sized oral capsule. (B) Air-dried gummy bears fabricated from 20 wt % vol^{-1} PVA, 12 wt % vol^{-1} skim milk, *B. adolescentis*, and food coloring. (C) CFU loading in the PVA-based gummy bears before and after the air-drying process. (D) Dissolution time of PVA-based and gelatin-based (commercial) gummy bears in PBS at 37 °C. Each error bar represents the standard deviation ($n = 3$). Statistical analysis was conducted using Student's *t*-test (significant difference defined at $p < 0.05$). ns: not significant.

bears after oral administration. Altogether, these results highlight that this PVA-based formulation is suitable for both pharmaceutical and food applications.

CONCLUSIONS

While certain anaerobes with therapeutic potential are naturally vulnerable to ROS under oxygen exposure,[10,11] few strategies and underlying mechanisms are reported to improve viability for these anaerobes during formulation and storage. Here, we demonstrate a material-based approach toward addressing the challenges of formulating oxygen-sensitive anaerobes and provide mechanistic insights into the storage improvement, motivating future research into the development of formulations for clinically relevant, oxygen-sensitive anaerobes. We show that *B. adolescentis* exhibits extended survival at 4 °C as compared to rapid viability loss at 37 °C under aerobic conditions. This discovery opens possibilities of formulating classical oxygen-sensitive anaerobes at temperatures lower than that for their growth conditions and specifically allows for encapsulation in polymer-based formulations in the presence of oxygen using a straightforward, air-drying technique. We further demonstrate that encapsulation with polymers enhanced the storage of *B. adolescentis* beyond that of universally applied protectants such as skim milk, likely by impeding the generation of ROS in polymeric formulations. In

addition, the PVA-based formulation is compatible with pharmaceutical and food dosage forms, highlighting its possible applications for improving probiotic and LBP storage in both pharmaceutical and food products.

EXPERIMENTAL SECTION

Materials. *B. adolescentis* in this study, originally isolated from a human stool sample, was a kind gift from UNC Microbiome Core at the University of North Carolina at Chapel Hill. PVA (87−90% hydrolyzed, 30−70 kDa), inulin from chicory (I2255), pullulan from *Aureobasidium pullulans* (P4516), and oligonucleotide primers were purchased from Sigma-Aldrich (Missouri, USA). Glycerol, sucrose, agarose, trehalose, rhodamine B, GelRed DNA Stain, GeneRuler 1 kb Plus DNA ladder (SM1331), PBS, and DeMan-Rogosa-Sharpe (MRS) broth were purchased from Thermo Fisher Scientific (Massachusetts, USA). MRS agar, M9 minimal salts, and skim milk powder were purchased from Becton, Dickinson and Company (New Jersey, USA). HA sodium salt from *Streptococcus equi* (J66993) and cysteine hydrochloride were purchased from Alfa Aesar (Massachusetts, USA). Capsules were purchased from Torpac Inc. (New Jersey, USA). Gummy bear silicone molds and food coloring were purchased through Amazon (Washington, USA). Commercial gummy bears (Haribo Gummi Candy) were purchased from CVS Pharmacy (Rhode Island, USA).

Methods. *Strain Identification.* The PCR mixture (50 μL) was prepared with a pair of *B. adolescentis*-specific primers [CTCCAGTTGGATGCATGTC (BiADO-1), CGAAGGCTTGCTCCCAGT (BiADO-2), 0.4 μM each primer],[29] 1 μL of bacterial pellets, and DreamTaq Green PCR Master Mix (#K1081, Thermo Fisher Scientific, USA) according to the instruction manual. The PCR was conducted in a thermocycler (Bio-Rad, UK). The amplification protocol included 1 cycle of 98 °C for 3 min, then 30 cycles of 98 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s, and finally 1 cycle of 72 °C for 10 min. Amplification products were analyzed through standard gel electrophoresis in a GelRed-stained agarose gel (1 wt % vol^{-1}).

Bacterial Culture and Viability Quantification. *B. adolescentis* was inoculated into cysteine hydrochloride (6.5 wt % vol^{-1})-supplemented MRS broth (C-MRS) and grew statically in the anaerobic chamber at 37 °C in 50 mL conical tubes. Before use, bacterial culture (OD$_{600}$ = 0.6−0.8) was centrifuged anaerobically at 4000 rpm for 10 min at room temperature, and the pellet was washed once in sterile PBS. OD$_{600}$ values were read using a GENESYS 30 visible spectrophotometer (Thermo Scientific, California, USA) after background subtraction of bacteria-free media. To quantify bacterial viability, aqueous samples were serially diluted, drop-plated (10 μL) on C-MRS agar, incubated anaerobically at 37 °C for 24−48 h, and enumerated for CFUs. Three to thirty CFUs were considered as countable. CFU counts below 3 were plotted as such. Samples without detectable CFU are shown as one-half of the LOD value.

Oxygen Sensitivity Assessment. *B. adolescentis* (1 × 10^5 CFU mL^{-1}) was inoculated in sterile PBS or C-MRS, followed by static incubation under four conditions: (i) anaerobic atmosphere, 37 °C, (ii) aerobic atmosphere, 37 °C, (iii) anaerobic atmosphere, 4 °C, and (iv) aerobic atmosphere, 4 °C. Viability was evaluated over time by drop-plating, as described above.

SEM Analysis. To examine changes in bacterial morphology after oxygen exposure, *B. adolescentis* was cross-linked with glutaraldehyde (2.5 vol % vol^{-1} in PBS) under shaking for 2 h at room temperature, followed by treatment of each ethanol solution (50, 70, 90, and 100 vol % vol^{-1} in water) for 20 min. Bacteria were pelleted at 4000 rpm for 10 min between treatments. Final pellets were resuspended in acetone and pipetted onto SEM stubs until complete solvent evaporation for SEM imaging. To evaluate encapsulation of *B. adolescentis* in films, cross-linked *B. adolescentis* (4 × 10^9 CFU mL^{-1}) were added into the PVA solution (20 wt % vol^{-1}), followed by solution casting, as described above. Films were sectioned with a razor blade to display cross-sections and then adhered to carbon tape on an SEM stub for imaging.

ROS Quantification. Cellular ROS detection assay kit (#ab186027, Abcam, UK) was used to evaluate ROS generation with and without oxygen exposure at 37 °C. The bacterial suspension (100 μL) in PBS was mixed with 100 μL of the ROS assay solution according to the instruction manual in 96-well plates and then incubated in the normal atmosphere or the anaerobic chamber at 37 °C. At indicated time points, samples were subjected to fluorescence reading at excitation/emission = 520 nm/605 nm. To quantify ROS generation during the air-drying process at 4 °C, a modified instruction manual was implemented. Specifically, reconstituted ROS red dye in dimethyl sulfoxide (DMSO) from the same assay kit was added in each formulation at 1:500 volume ratio ($V_{(ROS\ Red\ Dye)}:V_{(Formulation)}$), and then, the mixture was cast into 96-well plates. At indicated time points, water was supplemented into each well to reach 200 μL as the total volume and fully mixed with the samples, followed by fluorescence reading, as described above.

Formulation Fabrication and Dissolution. *B. adolescentis* (0.2−1 × 10⁸ CFU mL⁻¹) was mixed in solubilized polymer and excipient solutions to obtain prefabricated formulations. For air-dried formulations, the prefabricated formulations (50 μL per well) were cast into 96-well plate templates, followed by air drying at 4 °C in a cold room until drying. Air-dried formulations were stored at 4 or 25 °C, as indicated in each experiment. Rhodamine B (20 μg mL⁻¹) was added to the formulation for visualization. For lyophilized formulations, the prefabricated formulations (50 μL per tube) were aliquoted into microcentrifuge tubes, snap-frozen in liquid nitrogen, and then immediately transferred to a benchtop lyophilizer for overnight drying. At indicated time points, lyophilized and air-dried formulations were dissolved in 200 μL of sterile water for viability quantification, as described above. To fabricate gummy bears, 1, 0.4, and 0.1 mL of the freshly prepared, prefabricated formulation [containing *B. adolescentis*, PVA (20 wt % vol⁻¹), skim milk (12 wt % vol⁻¹), and food coloring] was cast into individual silicone molds on three consecutive days, respectively. Gummy bears were harvested 7 days after the third casting and then homogenized in 13 mL of sterile water for viability quantification, as described above. Bacterial viability in the prefabricated formulations was used as controls. To compare the dissolution rate of PVA-based and commercially available gelatin-based gummy bears, each gummy bear was incubated in PBS at the same mass:volume ratio (64 mg of gummy bear per mL PBS) at 37 °C under shaking. Dissolution time was recorded when each individual gummy bear was completely dissolved.

Statistical Analysis. Experiments were performed in triplicate, and data were presented as mean ± SD unless otherwise noted. CFU data were log-transformed unless otherwise noted. Statistical significance was evaluated using Student's *t*-test, parametric one-way ANOVA, and post hoc Tukey's HSD test in Prism (version 9.0.2, GraphPad Software, LLC), as noted in figure captions. α = 0.05. Significant difference was defined as *p*-value <0.05.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.1c11785.

> Raw CFU data for the screening of polymer encapsulants and excipients, bacterial growth in PVA solution, ROS quantification of formulations, and raw CFU data for long-term storage of air-dried and lyophilized *B. adolescentis* (PDF)

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Notes

The authors declare the following competing financial interest(s): K.Q. and A.C.A. are inventors on a patent application filed by the University of North Carolina at Chapel Hill on aspects of the work presented here.

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