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Entrapping Living Probiotics into Collagen Scaffolds: A New Class of Biomaterials for Antibiotic-Free Therapy of Bacterial Vaginosis

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A new concept of biomaterials for antibiotic-free therapy of bacterial vaginosis (BV) is here proposed. These biomaterials are obtained by entrapping two probiotic biofilms, viz., *Lactobacillus fermentum* (*Lf*) and *Lactobacillus acidophilus* (*La*) into scaffolds of self-assembled collagen fibers (col). An in-depth characterization and viability assays are performed on the resulting biomaterials. Results demonstrated that the collagen matrix plays a multifold role in improving the probiotic efficacy in a BV-simulated environment: i) it acts as a host to the formation of the probiotic biofilm, ii) it protects live probiotics during storage under harsh conditions, iii) it enhances the metabolic activity of entrapped probiotics thereby restoring the pH of BV-simulated microenvironment, and iv) it enhances the adhesion of probiotics to the simulated vaginal mucosa. These collective properties make these biomaterials as promising candidates for treating BV without antibiotics. In addition, the approach here presented can be adapted for the treatment of other complex microbial infections.

1. Introduction

Bacterial vaginosis (BV) is the most frequent cause of vaginal disorders in women of reproductive age. BV condition increases the risk of developing other pathologies, such as sexually transmitted infections,[1] including the human immunodeficiency virus,[2] and postpartum uterine infections.[3]

From a microbiology perspective, BV is characterized by the alteration of the normal balance of the vaginal microbiota. The health of the vaginal microbiota is believed to be maintained by lactic acid-producing organisms, such as *Lactobacilli*.[1] With a minute imbalance in the pH profile of the vaginal fluid (e.g., from pH 4 to 5 in a healthy vs unhealthy environment), these *Lactobacilli* are suppressed by the overgrowth of pathogenic bacteria, such as *Gardnerella vaginalis* and *Mobiluncus spp.*, which are present in small populations when healthy, resulting in BV in reproductive-age women.[4,5]

From a chemical point of view, the vaginal environment of a healthy woman is dominated by the lactic acid, the main metabolite of the *Lactobacillus* family. Other short-chain fatty acids (SCFAs), such as acetic, propionic, and butyric acids associated with other typically harmful bacteria are present at almost 100-fold lower concentrations.[4,5] The pH is therefore regulated by the lactic acid (pK_a = 3.80), which is found in healthy vaginal fluid at concentrations of the order of 120×10^{-3} M, leading pH values around 4. When BV develops, the decreasing population of *Lactobacilli* and the excessive growth of pathogenic bacteria modify the metabolite pattern: an increase of SCFAs and a dramatic decrease of lactic acid. In particular, acetic acid, the main component of SCFAs, can reach concentrations about 120×10^{-3} M from originally 2×10^{-3} M, which leads to an increase of pH up to the value of 5, since the pK_a of acetic acid (4.86) is higher than that of lactic acid. Therefore, the pH of the vaginal fluid moves from 4 (healthy) to 5 (BV) and worsening the disease condition over time. The pH change is, in fact, a key parameter used in the diagnosis of BV. For instance, the Amsel method diagnoses BV when the vaginal fluid has a pH higher than 4.5.[6]

BV has been traditionally treated with antibiotics, especially metronidazole, and more recently with new antibiotic-based therapies, as VivaGel.[7] However, a number of drawbacks in existing treatment modalities necessitate the development of new routes of therapy.[8] First, treatments based on antibiotics do not avoid recurrences. Second, the development of antibiotic-resistant bacteria due to the excessive use of antibiotics remains the biggest challenge faced by modern medicine.[9–13] Therefore, more effective long-term therapies that reduce our reliance on antibiotics are critically needed. Recent approaches have considered creams or gels containing probiotics of the

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Lactobacillus family.[14] While promising, the efficiency of this approach will depend on the adhesion and proliferation levels of the probiotic within the BV-compromised microenvironment. In particular, the high pH of vaginal fluids during BV tends to hamper *Lactobacilli* proliferation and subsequent therapy, leading to BV recurrence.

To overcome these challenges, we have developed a new strategy based on the use of a cytocompatible collagen-based matrix that protects probiotics and allows their effective localization and proliferation within the compromised vaginal fluids.

Collagen is the most abundant protein in mammals and the basic building block of extracellular matrices of different tissues. Collagen is considered an ideal natural polymer for tissue engineering due to its abundance and easy processing, flexibility, hydrophilicity, excellent biocompatibility and biodegradability and extremely low antigenicity.[15–17] Collagen matrix provides innate biological informational guidance to cells that elicits cell attachment and promotes chemotactic response.[18] Despite collagen scaffolds are being regularly designed and commercialized for tissue regeneration and drug delivery,[15,19] the use of collagen scaffolds to incorporate living probiotics, that proliferate and form biofilms has not been explored so far.

This work explores the ability of collagen scaffolds (col) to protect two biofilm-producing probiotics of the genus *lactobacillus*, viz., *L. fermentum* (*Lf*) and *L. acidophilus* (*La*), resulting in hybrid materials, col-*Lf* and col-*La*, respectively, with enhanced healing properties against BV infections. Both materials correspond to biocomposites composed of collagen, probiotic, and the bacterial EPS (exopolysaccharides). col-*Lf* and col-*La* are able to restore the pH of a compromised simulated BV fluid to a healthy condition. Outstanding stability of these collagen-entrapped probiotics, combined with high adhesion to the vaginal mucosa and the intrinsic cytocompatibility of the collagen matrix, make these materials very promising for nonantibiotic BV therapy.

2. Results and Discussion

2.1. Collagen Matrix as a Scaffold for Probiotics

Type I collagen is a predominant protein in the human body and the major organic component of the bone extracellular matrix. In bone tissues, collagen triple helices (monomers) are self-assembled at the molecular level in a periodic staggered array resulting in large fibrils with a characteristic-banding pattern of 67 nm.[20] This self-assembly is driven by an increase in pH.[21] Taking inspiration from this process, we mixed an acidic solution containing collagen monomers with the probiotics (*Lf* or *La)* in phosphate buffered saline (PBS) buffer (pH 7.4) (Scheme SI1, Supporting Information). The instantaneous pH increase induces the collagen self-assembly into a high-density matrix of fibers with several hundred micrometers in length (**Figure 1**a,b), showing the characteristic D-banding pattern (67 nm). The fiber diameter ranges between 50 and 400 nm with an average diameter of about 250 nm (Figure SI1, Supporting Information). As it is evident from the scanning electron microscopy (SEM) micrographs (Figure 1), using this simple one-pot procedure, *Lf* (Figure 1c,d) and *La* (Figure SI2, Supporting Information) probiotics were successfully entrapped into the network of collagen fibers. Further, the presence of probiotics did not alter the pH-induced structure and assembly of the collagen fibers, which were similar to those obtained in PBS without bacteria (Figure 1a,b). Likewise, bacteria entrapment neither affected the diameter of the fibers (Figure SI1, Supporting Information).

In order to assess the stability of the collagen scaffold after entrapping the probiotics, we carried out a study of the collagen degradation (Experimental SI2, Supporting Information). The collagen release was monitored by the UV–vis absorption bands at 276 and 258 nm (from tyrosine and phenyl-alanine, respectively) and 205 nm (due to peptide bonds). After 24 h in PBS at 37 °C, the absorbance of the supernatant at 276 and 258 nm was negligible (Figure SI8, Supporting Information). From the absorption band at 205 nm, and using an extinction coefficient of 5.91 g^{-1} cm^{-1}, we determined that 3% w/w of collagen was released from the scaffold after 24 h. Likewise, the level of delivered probiotics from the collagen scaffold was directly counted in a Neubauer chamber (Experimental SI2, Supporting Information). A gradual release corresponding to the 15% of probiotics was observed during the first 6 h (Figure SI8, Supporting Information). This value kept constant after 24 h. Therefore, collagen degradation and probiotic release levels pointed out the stability of the material formed by entrapping probiotics into the collagen scaffold in PBS.

Interestingly, when both probiotic-containing collagen materials were incubated in an optimal probiotic medium, such as De Man–Rogosa–Sharpe (MRS), the bacteria successfully proliferated and produced biofilms, which decorated the collagen fibers (Figure 1e,f; and Figure SI2, Supporting Information). It is interesting to note that these kinds of bacteria excrete EPS. EPS play an important role in the formation of the biofilm, which is essential for bacteria to create a growth-conducive microenvironment in a substrate, in this case, the collagen fibers. The excellent integration of bacteria into the collagen matrix, witnessed by the formation of biofilms, is evident from Figure 1e,f, showing how bacteria adhere to collagen fibers through their EPS (Figure 1f; and Figure SI2, Supporting Information). These materials (col-*Lf* and col-*La*, hereafter) are in fact hybrid biocomposites formed by three constituents: the collagen matrix, the probiotic, and their bacterial EPS, being the latter the natural "glue" of the other two components. This class of biocomposites has never been reported.

The amount of EPS in the biocomposites col-*Lf* and col-*La* were quantified by a standard sugar test (see the Experimental Section). Values of 98 and 93.3 mg g^{-1} of collagen were obtained for col-*Lf* and col-*La*, respectively. Interestingly, the initial materials obtained by incubating probiotics and collagen in PBS contained much lower sugar levels (10 and 18.6 mg g^{-1}, respectively). The drastic increase of sugar in the final biocomposites col-*Lf* and col-*La* is certainly due to the biofilm formation in the optimum MRS medium, in agreement with SEM images.

The biocomposite was further characterized at the macroscale by Fourier transform infrared spectroscopy (FTIR) and thermogravimetric analysis (TGA), and at the molecular scale by synchrotron X-ray wide-angle total scattering (WAXTS) (Figure SI3, Supporting Information). FTIR spectra and TGA of col-*Lf* show the main fingerprints of collagen fibers along with some signals

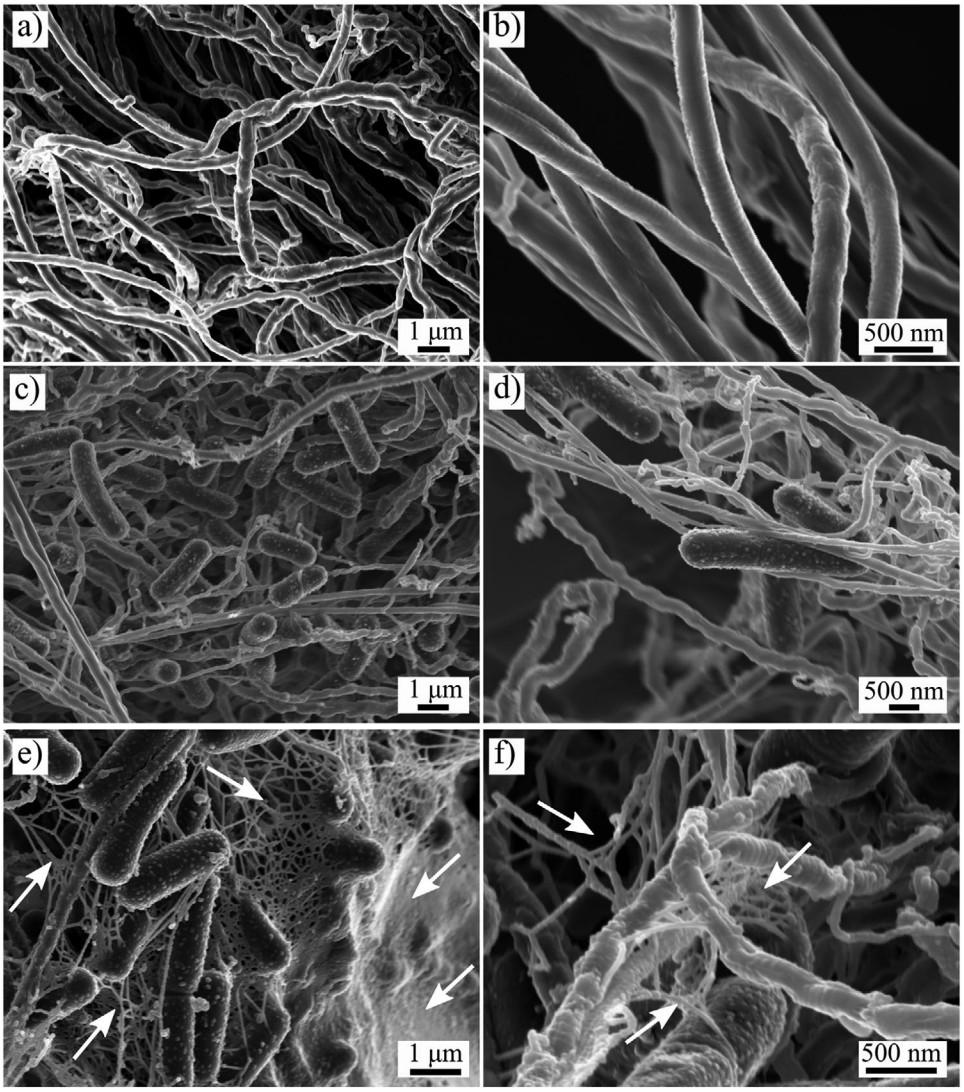

Figure 1. SEM micrographs of collagen fibers assembled in PBS (pH 7.4) in the absence a,b) and presence of the probiotic *Lf* c,d) and then, incubated in MRS to produce col-*Lf* e,f). Arrows indicate the EPS. Samples were fixed with cacodylate buffer and then stained with osmium tetroxide (1% v/v).

assignable to the presence of bacteria (Figure SI3b, Supporting Information). WAXTS patterns of collagen and col-*Lf* were similar (Figure SI3c, Supporting Information) with a broad peak centered at $q = 13.25$ nm^{-1} due to the diffuse scattering of the collagen fibers. The Bragg peak at $q = 5.46$ nm^{-1} corresponds to the equatorial molecule–molecule averaged separation (i.e., the intermolecular lateral spacing) of the collagen triple-helix. This peak results in a *d*-spacing of 1.15 nm (inset, Figure SI3c, Supporting Information), which is in good agreement with the corresponding *d*-spacing of collagen in dry nonmineralized biological tissues[22,23] and confirms that bacteria do not affect the macromolecular assembly of collagen fibers.

2.2. Viability of Probiotics in the Collagen Scaffold

Standard live/dead test based on SYTO9 (green)/propidium iodide (red) demonstrated that *Lf* and *La* bacteria were alive in the col-*Lf* and col-*La* materials (**Figure 2**; and Figure SI4, Supporting Information). As it is evident from Figure 2, the majority of *Lf* probiotics remain alive (green), with only a few dead bacteria found (red). Furthermore, side-view image (sum of successive x*y* sections) (Figure 2a) shows that the bacteria penetrated and invaded the entire collagen matrix to form unique hybrid biomaterials. Interestingly, the magnified top-view confocal image (Figure 2b,c) shows the alignment of bacteria along the collagen scaffold, suggesting the existence of specific recognition of the probiotic by collagen. Similar results were found for col-*La* as it is shown in Figure SI4 (Supporting Information).

2.3. Activity of Probiotics in the Collagen Scaffold

The collagen matrix not merely acts as a hosting scaffold for probiotics, but also provides them with additional protection

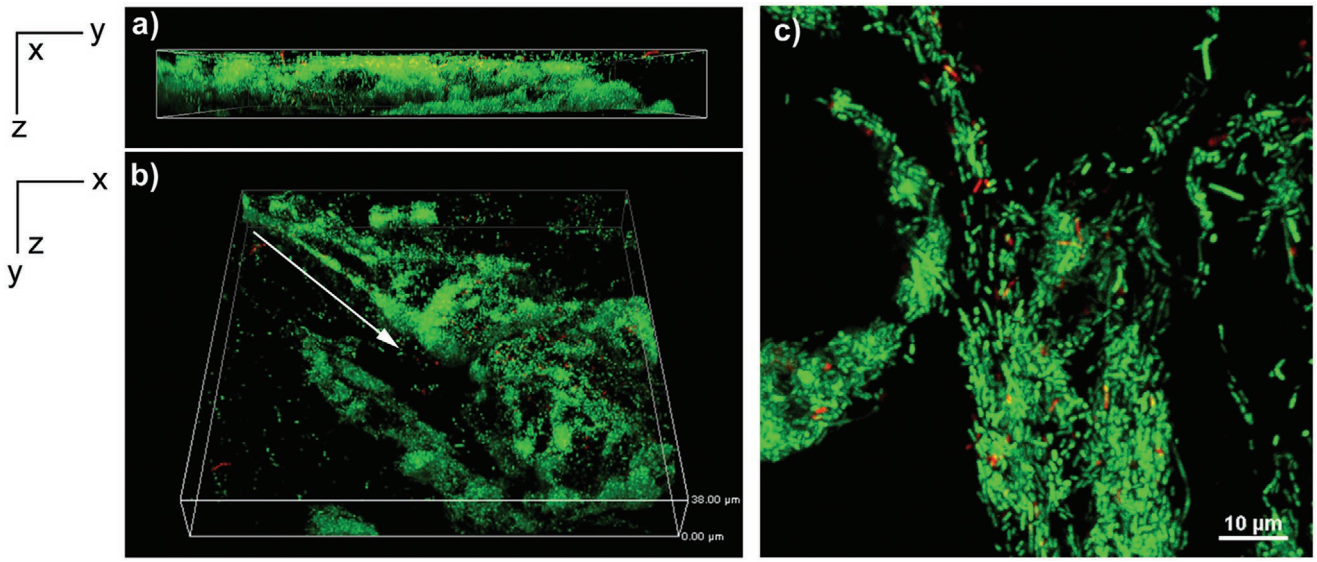

Figure 2. Confocal images of col-*Lf* stained with the SYTO9 (green) and propidium iodide (red) dyes. a) yz-section reveals that the bacteria penetrated and are fully integrated into the collagen matrix. b) 3D image ($318.20 \times 318.20 \times 38\ \mu m^3$) showing how bacteria are specifically aligned along the collagen fibers (arrow). c) A higher magnification image showing very few dead (red) bacteria (this image is a maximum intensity projection of 6 µm in depth).

during storage under harsh conditions (e.g., at 4 °C). Live bacteria excrete metabolites to the media and *lactobacilli*, in particular, excretes lactic acid, which regulates the pH at around 4. We measured the pH evolution to monitor the metabolic activity of the probiotics after storage under harsh conditions (4 °C). The pH evolution of fresh *Lf* and col-*Lf* (without thermal treatment) and after storage at 4 °C during 2, 4, and 8 weeks are shown in **Figure 3**a. Col-*Lf* induced a much higher drop of the pH in MRS media in comparison to that of *Lf* (Figure 3a), supporting that probiotics embedded within the collagen matrix retain their metabolic activity and confirming that the collagen matrix offers extra protection and enhances the shelf-life of the probiotics. Similar behaviors were observed for the col-*La*/*La* pair after 2 and 4 weeks of storage at 4 °C. However, col-*La* stored for 8 weeks showed much less activity (Figure SI5, Supporting Information).

In addition, we assessed the metabolic activity of stored col-*Lf* and col-*La* at 4 °C using an assay previously developed by our team, that correlate the reductive capacity of bacteria to their metabolic activity, using an electrochromic polyoxometalate (POM).[24] Once reduced, POM exhibits an absorption band in the UV–vis spectrum centered at 820 nm. The evolution of this absorption band after incubating aliquots from the supernatants of col-*Lf* or *Lf* cultures with an aqueous solution of POM was monitored (Figure 3b). The POM reduction activity in the MRS broth media is enhanced when the bacteria is embedded in the collagen matrix in comparison to those of free stored bacteria (Figure 3b). Final values of col-*Lf* stored for 2, 4, and 8 weeks were similar to those obtained for fresh samples (stored at room temperature). Similar behavior was observed for the col-*La*/*La* pair after 2 or 8 weeks. Samples of col-*La* after 2 months of storage showed much less capacity to reduce POM (Figure SI5, Supporting Information), in agreement with their inferior ability to reduce pH (cf., col-*Lf*) noted above (Figure SI5, Supporting Information).

These observations support that the collagen matrix assists in maintaining the core cellular activity of probiotics, especially that of *Lf*, even after their exposure to extended harsh storage conditions. The retention of high metabolic and cellular activities is a critical parameter for the successful use of probiotics in antibiotic-free therapies.

2.4. The Biocomposites Col-*Lf* and Col-*La* for BV Therapy: Adhesion and Activity

The adhesion of the probiotic to the vaginal mucosa is of paramount relevance to efficiently treat BV with probiotics. Indeed, if a biomaterial containing highly proliferating probiotics does not adequately adhere to the vaginal mucosa, it would not offer much value for BV therapy. The adhesion capacity of col-*Lf*/col-*La* and *Lf*/*La* to endothelial cells was evaluated with porcine mucin, i.e., heavily glycosylated proteins produced by epithelial tissues, which is a well-established adhesion model.[25,26] As shown in **Figure 4**a,b, the adhesion of col-*Lf* to mucin was sensibly higher than that of *Lf*, supporting the ability of collagen to enhance the adhesion of entrapped probiotics. The col-*La* formulation also exhibited a higher adhesion to mucin than the *La* probiotic alone (Figure SI6a, Supporting Information). On comparison of the adhesion of col-*Lf* and col-*La* to mucin, a higher signal corresponding to crystal violet was observed for col-*Lf*. This is likely due to the higher colony forming unit (CFU) value of col-*Lf* in comparison to that of col-*La*, as discussed above.

The typical adhesion model also includes a treatment with bovine serum albumin (BSA) after the immobilization of mucin. BSA blocks the remaining free sites of the protein-sensitive well plates after mucin immobilization. The experiments using BSA (Figure SI7, Supporting Information) showed no significant difference to that of the corresponding experiments without BSA (Figure 4b). This confirms that the

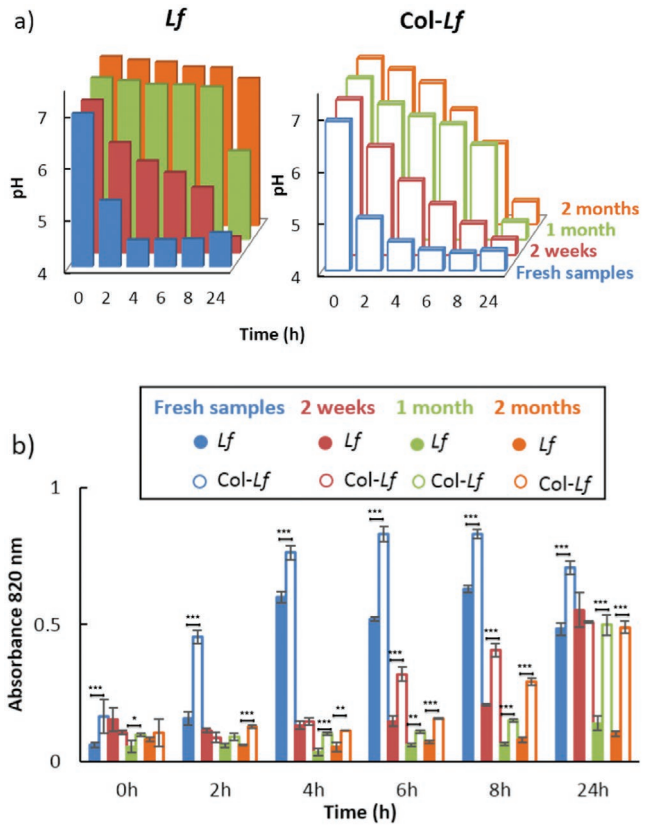

Figure 3. Bacterial activity after storage in harsh conditions (4 °C). a) Time evolution of the pH in 1/10 diluted MRS media containing *Lf* (solid bars) or col-*Lf* (open bars) after storage for 2 (red), 4 (green), and 8 weeks (orange) at 4 °C. The pH evolution of fresh samples (blue) is also shown. pH values for col-*Lf* and *Lf* were compared by Two-way Anova followed by a Bonferroni's test resulting in *p*-values below 0.05 at each time point. b) Time evolution of the POM reduction activity in 1/10 diluted MRS containing *Lf* (solid bars) or col-*Lf* (open bars) after storage for 2 weeks (red), 1 month (green), or 2 months (orange) at 4 °C. Evolution of the POM reduction activity of fresh samples (blue) is also shown. The A^{820} drop observed for the fresh sample at 24 h is due to the decrease of bacterial viability, and then its reducing capability, in the diluted MRS medium. Nondiluted MRS media cannot be used as it reduces POM. Statistical analysis was performed at each time point using Two-way Anova followed by a Bonferroni's test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

observed increase of absorbance is not due to the adsorption of collagen on the protein-biding plates, but to the adhesion of col-*Lf* on mucin.

A decisive step to confirm the functionality of col-*Lf* and col-*La* for BV therapy is their capacity to restore the healthy conditions from a simulant BV fluid pH 5.0. As noted from Figure 4c; and Figure SI6c (Supporting Information), both col-*Lf* and col-*La* were able to shift the pH 5 of the BV medium to that of healthy conditions, pH 4. Metronidazole, the genuine antibiotic used in the treatment of BV, was unable to drop the pH of the simulated-BV medium, even at a high dose (see the Experimental Section).

These results point out that, even if the simulated BV media (pH = 5) diffuses and contacts with entrapped probiotics, these did not reduce their activity and were capable to restore the pH to the physiological value (pH = 4). This pH shift is certainly due to the excretion of lactic acid by the probiotic bacteria, confirming that the bacteria remain alive and active once entrapped into the collagen matrix, as discussed above. Interestingly, the drop in pH with *Lf* and *La* without collagen was much slower and did not reach the healthy pH value of 4.0 (Figure 4c; and Figure SI6c, Supporting Information).

Two observations are worth highlighting from these results: i) the two materials, col-*Lf* and col-*La*, were capable of shifting the pH from the unhealthy BV medium (pH 5) to the healthy vaginal conditions (pH 4); and ii) the ability to reduce the pH of col-*Lf* or col-*La* is higher than those of *Lf* or *La*. Furthermore, we have shown that the capacity of probiotics to overcome the hostile BV medium and restore the physiological pH requires that the entrapment of the probiotics within the collagen scaffold. In fact, when we tested a mixture of collagen and probiotics, instead of the biocomposite col-*Lf*, the pH was not totally restored (Figure 4c). The mixture of the independent components failed to reach the physiological pH.

3. Conclusions

We have developed a new class of biomaterial-based formulation for BV therapy consisting of collagen as a cytocompatible matrix for effectively entrapping probiotic bacteria in a simple one-step synthesis. The high promise of this approach is shown through entrapping two BV-relevant probiotics, *L. fermentum* and *L. acidophilus*. Other probiotics may also be easily entrapped following the same procedure. Once *L. fermentum* and *L. acidophilus* are entrapped in the collagen fiber network, they remain alive, proliferate, and show enhanced metabolic activity. Importantly, these entrapped probiotics (col-*Lf* and col-*La*) are capable of acclimatizing and proliferating in a hostile BV environment, whereas the respective nonentrapped bacteria, *Lf* and *La*, do not show credible performance. Through proliferation under BV conditions, these entrapped probiotics are able to restore the pH of the BV medium (pH 5) to a healthy pH of around 4. The restoration of healthy pH conditions should consequently promote further proliferation and activity of healthy vaginal microbiota via suppressing unhealthy bacteria. In addition to the stability that the collagen matrix confers to the entrapped probiotics, col-*Lf* and col-*La* exhibit excellent adhesion to mucin, sensibly higher than those of *Lf* and *La*. The combination of these properties makes these biomaterials ideal candidates for the treatment of BV, as they overcome the two critical limitations of the current probiotic-based therapies: high adhesion to the vaginal mucosa and protection of the probiotics in the hostile BV environment.

4. Experimental Section

Reagents, Bacteria Sources, and Collagen: High purity reagents were purchased from Sigma-Aldrich. All solutions were prepared with ultrapure deionized water (18 MΩ cm). *Lf* (CECT5716) powder was obtained from Biosearch Life S.A. *La* (CECT903, Moro 1900) was purchased from CECT (Colección Española de Cultivos Tipo). MRS medium was purchased from Thermo Scientific. Type I collagen was

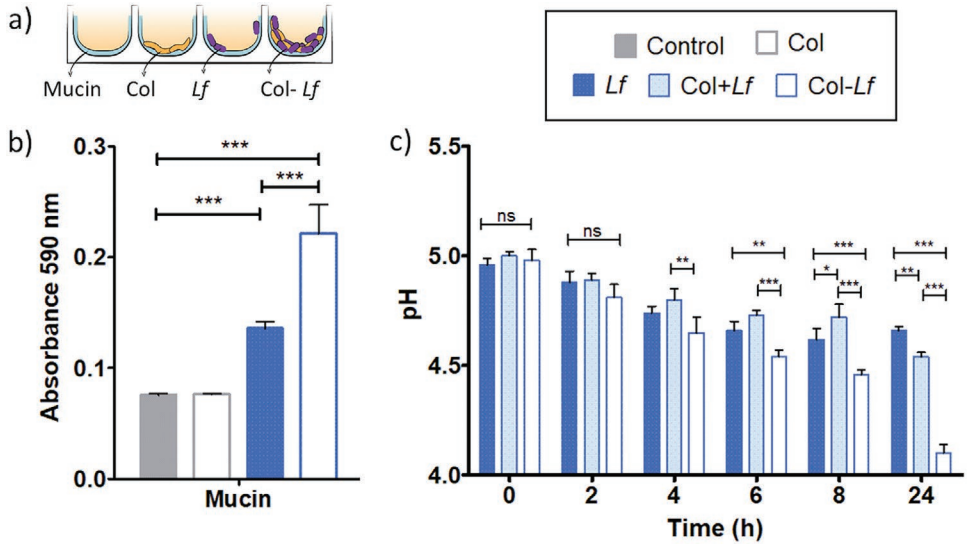

Figure 4. a) Schematic representation of the adhesion experiments. b) Evaluation of the adhesion of *Lf* (solid blue bar) and col-*Lf* (open blue bar) to immobilized mucin. Absorbance at 590 nm correlates with the amount of bacteria adhered to mucin. Absorbance values of controls (Mucin and Mucin + col) are also included. Statistical analysis was performed using One-way Anova followed by a Bonferroni's test (*** $p < 0.001$). c) pH evolution of simulated BV fluid (initial pH 5.0) in the presence of a mixture of collagen and Lf (col+*Lf*), col-*Lf* and *Lf*. Statistical analysis was performed at each time point using Two-way Anova followed by a Bonferroni's test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

extracted from equine tendon using the standardized manufacturing method of OPOCRIN S.p.A. (Corlo di Formigine, Modena, Italy).[27]

The species *Lf* and *La* are included in the QPS (Qualified Presumption of Safety) lists published by the EFSA (European Food Safety Authority) and FDA (Food and Drug Administration). These lists include the microbial species considered safe for use in humans due to their consumption history. Likewise, FDA approved medical devices based on collagen for a wide range of applications in tissue regeneration.

Entrapment of Lactobacilli Bacteria in the Collagen Matrix: *La and Lf* were separately inoculated in 100 mL of MRS broth and incubated for 24 h at 37 °C under continuous stirring (180 rpm). Subsequently, bacteria were collected by centrifugation (10 000 g, 5 min), washed with saline solution (0.9% NaCl in water) and resuspended in PBS (pH 7.4) to obtain a bacteria suspension with 2×10^9 CFU mL^{-1}. Then, 3 mL of bacteria suspension was mixed with 1 mL of collagen solution (1.5 mg mL^{-1}, pH 3.0) triggering to the formation of a 3D matrix of self-assembled collagen fibers in the presence of the probiotic. The collagen matrices with the entrapped bacteria were maintained for 30 min at 37 °C under continuous stirring (180 rpm) and then, washed with 10 mL of saline solution (0.9% NaCl in water) to remove the nonentrapped bacteria.

Preparation of Col-Lf and Col-La Biocomposites: The collagen matrices with the entrapped bacteria were then incubated in 10 mL of MRS broth for 6 h at 37 °C. The resulting materials, referred to as col-*Lf* and col-*La,* were washed with saline solution and stored until further ex situ characterizations.

Field Emission Scanning Electron Microscopy (FESEM): Collagen matrices (with and without probiotics) and Col-*La* and Col-*Lf* biocomposites were fixed in 1 mL of cacodylate buffer (0.1 M, pH 7.4) containing 2.5% of glutaraldehyde at 4 °C. After 24 h, the samples were washed 3 times (30 min at 4 °C) with cacodylate buffer. The sample was stained with osmium tetroxide solution (1% v/v) for 2 h in the dark and then repeatedly rinsed with Milli-Q water to remove the excess of osmium. Samples were then dehydrated at room temperature with ethanol/water mixtures of 50%, 70%, 90%, and 100% v/v for 20 min each, being the last concentration repeated three times and dried at the CO_2 critical point. Finally, dehydrated samples were mounted on metal stubs with conductive adhesive, covered with a thin carbon film and analyzed using a FESEM (Zeiss SUPRA40V, Center for Scientific Instrumentation, University of Granada). The protocols used for the ex situ characterization of the biocomposites (FTIR, synchrotron X-ray diffraction and Thermogravimetric analysis) are described in detail in Experimental S1 (Supporting information).

EPS Quantification: The standard phenol-sulfuric method was used for EPS quantification.[28] Samples were immersed in 1 mL of saline solution containing with 500 µL of phenol (5% in water), and 2.5 mL of sulfuric acid and maintained in a water bath at 30 °C for 20 min. Absorbance of each sample was measured at 490 nm. EPS concentration was determined using the absorption coefficients obtained from a calibration curve and expressed in mg of sugar g^{-1} of collagen. Calibration curves were performed using a mixture of dextran and levan, the most characteristics components of the EPS excreted by lactobacilli. Each sample was tested in triplicate.

In Vitro Viability Assay: Bacterial viability in col-*Lf* was qualitatively evaluated with *Bac*Light Bacterial Viability kit (Thermo-Fisher) following manufacturer's instructions. This assay consists in combining membrane impermeable DNA-binding stain, i.e., propidium iodide (PI), with membrane-permeable DNA-binding counterstain, SYTO9, staining dead and live and dead bacteria, respectively. Confocal laser scanning microscopy images were collected with a Nikon Eclipse Ti-E A1 microscope equipped with 60x oil immersion objective. Images were analyzed with NIS Elements software. For acquiring SYTO 9 signals, 488 nm laser and 505–550 nm emission filter was used. For PI, 561 nm laser and 575 nm long-pass emission filter was used. The reconstruction of the tridimensional image was obtained with 39 z-sections.

Bacterial Activity of Col-Lf and Col-La after Storage at 4 °C: Collagen-probiotic biocomposite and the corresponding controls (naked probiotics) were stored at 4 °C for 2 weeks, 1 month, and 2 months. After this time, samples and controls were separately added to 10 mL of MRS broth media and incubated at 37 °C and 180 rpm for 1 h. Then, all samples were washed twice (3000 g, 5 min) with saline solution (0.9% NaCl in water). Subsequently, 10 mL of 1/10 diluted MRS broth media were added and incubated at 37 °C and 180 rpm. 1/10 diluted MRS broth was used to avoid autoreducing ability of nondiluted MRS broth. Aliquots of 1 mL were sequentially collected from each of the cultures' supernatants at scheduled times: 0, 2, 4, 6, 8, and 24 h. Aliquots were centrifuged (3000 g, 5 min) to remove any residual bacteria. The fermentation activity of the encapsulated living materials and the corresponding controls were monitored by pH measurements. To confirm the higher viability of collagen bacteria systems with respect to nonentrapped bacteria, a procedure was applied developed by the

consisting on correlating the bacterial proliferation to its reductive capacity against the electrochromic POM, $[P_2Mo^{VI}_{18}O_{62}]^{6-}$).[24] 190 µL of each aliquot were added to a well containing 10 µL of a 10×10^{-3} M solution of POM. Samples were left in dark at room temperature. Then, they were irradiated with UV light (365 nm) for 10 min and then the absorbance was measured in a Tecan's NanoQuant plate reader. Same experiments were performed on materials stored at room temperature (fresh samples). In addition, controls of free col were used to confirm that this material itself is not able to decrease pH or reduce POM. Each aliquot was tested in triplicate.

In Vitro Assay of Bacterial Adhesion: The adhesion of col-probiotics and probiotics was assayed according to the method previously reported with slight modifications.[25,26] In brief, the wells of Maxisorp plates (Nunc, Roskilde, Denmark) were incubated overnight at 4 °C with 100 µL of a 5 mg mL^{-1} solution of porcine mucin (Sigma-Aldrich) in PBS pH 7.4. After immobilization, wells were washed three times with PBS and blocked with 2% BSA in PBS for 1 h. Bacterial pellets from 5 mL fractions of bacterial culture were collected by centrifugation (6000 g, 5 min), washed three times with PBS and resuspended in 5 mL of PBS. Upon removal of the BSA solution and washing three times the wells with PBS, the bacterial suspension (100 µL, 10^8 CFU mL^{-1}) was added. In the case of collagen-bacteria matrix samples, 25 µL of collagen (50 mg mL^{-1} in 0.1 M acetic acid) were added. The plate was incubated with *Lf* or *La* on an orbital shaker at 180 rpm for 2 h at 37 °C. Nonadhered bacteria were removed by washing the wells three times with 100 µL of PBS, and the samples were fixed at 60 °C for 20 min. Afterward, the fixed cells were stained with crystal violet (100 µL per well, 0.1% solution) for 45 min at room temperature. Finally, the wells were washed twice with 150 µL of PBS and the stain bound to the bacteria was dissolved in 50 µL of citrate buffer (pH 4.3) for 1 h. The absorbance was measured at 590 nm using the microtiter plate reader (NanoQuant). Results were expressed as the mean of triplicates with a total volume of 200 µL, after collecting four replicates of 50 µL in one well. Controls consisted of mucin and mucin-collagen, and samples were mucin-*Lf,* mucin-*La,* mucin-col-*La,* and mucin-col-*Lf*, blocked or not with BSA.

Bacterial Activity of Col-Lf and Col-La in a Simulant BV Fluid: The collagen-probiotic samples and controls were separately added to 10 mL of simulant BV fluid and incubated at 37 °C and 180 rpm. 1 L of BV fluid, prepared according to the protocol previously reported[29] with slight modifications, contains NaCl, 3.51 g; KOH, 1.40 g; $Ca(OH)_2$, 0.222 g; bovine serum albumin, 0.018 g; urea, 0.4 g; glycerol, 0.16 g; glucose, 10 g; lactic acid, 2 g; and acetic acid, 1 g. The pH was of 5.00. Aliquots of 1 mL were sequentially collected from each of the cultures' supernatants at scheduled times: 0, 2, 4, 6, 8, and 24 h. Aliquots were centrifuged (3000 g, 5 min) to remove any residual bacteria. The pH of the entrapped living materials and the corresponding controls was measured to demonstrate the capacity of restoring the pH of the media. Two experiment controls were performed: first, a positive control, which consisted of a mixture of assembled collagen with the bacterial suspension (*Lf*) and second, a saturated solution of the antibiotic metronidazole. The procedure for the first control was the same that used for collagen-probiotic samples. For the second control, metronidazole powder (100 mg) was directly added to 10 mL of simulant BV fluid and incubated at 37 °C. The concentration of the antibiotic was close to saturation (11 mg mL^{-1}).[30] The pH of the mixture was 5.20 ± 0.10 and kept constant for 24 h. Each experiment was tested in triplicate.

Statistical Analysis: Results are expressed as average ± standard deviation as error bars ($N = 3$). Statistical comparisons were performed using one-way or two-way ANOVA followed by Bonferroni's post hoc test using GraphPad Prism software (version 6.0). Differences between data sets were considered significant at $p < 0.05$.

Supporting Information

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

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

antibiotic-free therapy, bacterial vaginosis, collagen, collagen scaffolds, probiotic bacteria

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