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Comparative identification of the *Lactobacillus spp.* from yogurt/buttermilk samples and human vaginal swabs

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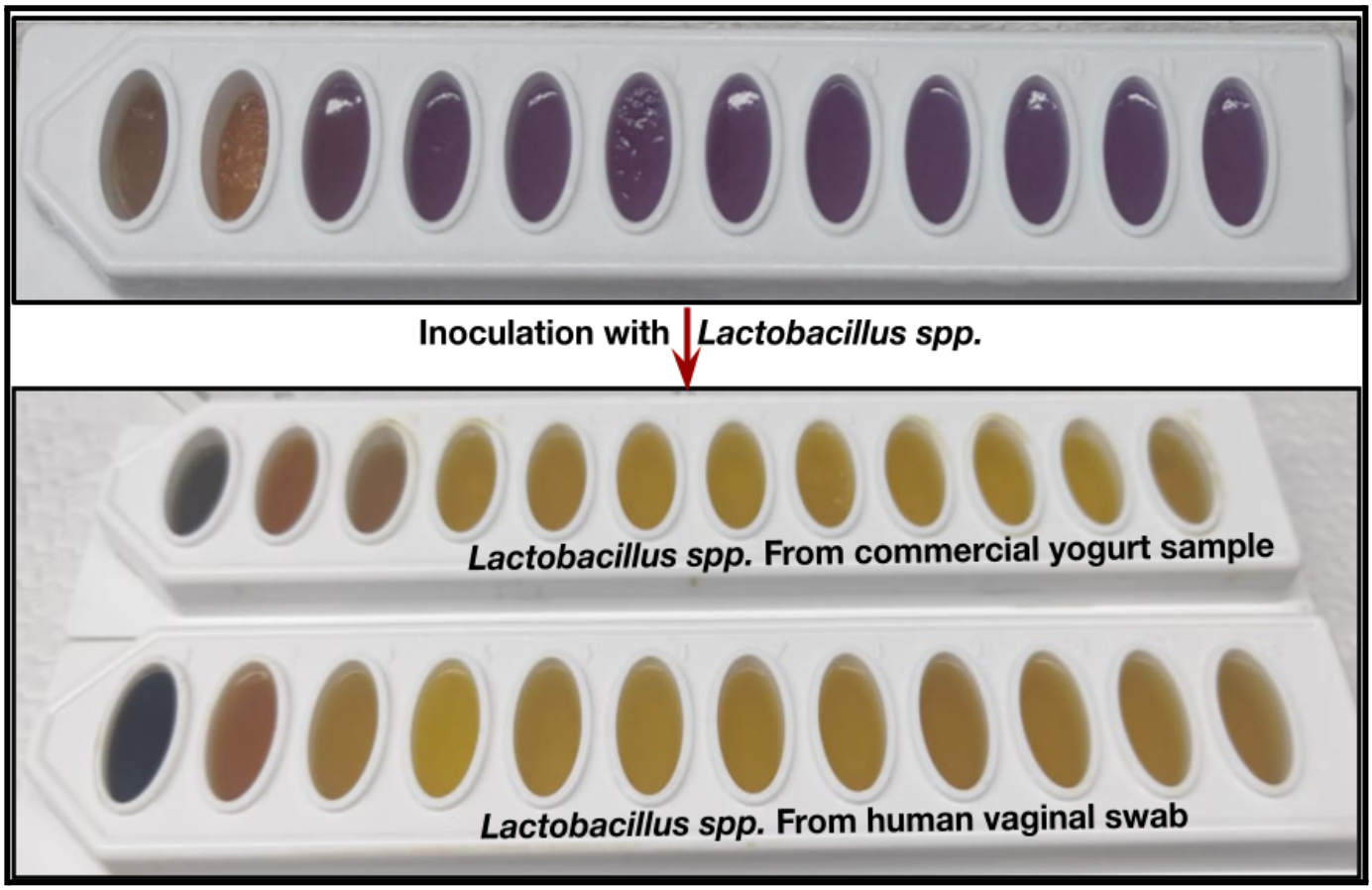
Keywords: *Lactobacillus*, yogurt, vaginal microbiome, fermentation, species identification, biodiversity.



Graphical abstract: *Lactobacillus spp.* identification using sugar fermentation technique.

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Acquiring stem cells is a difficult task especially using the canonical invasive methods. Considering the endless applications of stem cells in biomedical sciences a search for non-invasive methods is always in progress. As a part of these efforts, we plan to isolate and propagate the endometrial stem cells from the human menstrual waste. However, the menstrual waste has to pass through the vaginal canal that has a typical microbiome which may affect the potency of the endometrial stem cells. In this study, we used vaginal swabs to explore the human vaginal microbiome primarily focused on the identification of the predominant bacteria, the *Lactobacillus spp.* diversity. As a natural control we used the yogurt/buttermilk samples to compare the bacterial diversity. Fermentable sugars were used to identify various *Lactobacillus spp.* in both samples. Our results suggest that both samples show a similar profile with significant diversity in the *Lactobacillus spp.* present in them. The sugar fermentation patterns were similar if not the same in both samples indicating that the effect of this bacterial microbiome would be cumulative in nature which further warrants the species identification using the 16S rRNA sequencing along with the LC/MS-based metabolite profiling in future.

In terms of ecology every organism is interdependent on other organisms for its growth and survival. Sometimes this association could be mutualistic and thus beneficial to both ends or it could be the other way around. Just like that, the human body and several microorganisms also co-evolved to be interdependent on each other [1]. Microorganisms present in the human body contribute to the human microbiome, which exist in mutualistic interaction with each other. Over decades many studies established its importance in protecting the host from various diseases at cellular level [2]. With most emphasis given on intestinal and gut microbiome studies, little is known about the importance and effects of vaginal microbiome at molecular level [1]. Vaginal microbiome also harbors several species of bacteria as determined by 16s rRNA sequencing and contributes to 9% of total human microbiota [3, 4, 5]. It gives protection against diseases like STD's, bacterial vaginosis, etc. [4]. Vaginal microbiome is highly variable and the species composition differs between individuals and also changes during the lifetime of an individual, but when compared to other habitats in the body it is considered to have higher stability [11]. Several factors like hormonal levels contribute to changes of vaginal microbiome in an individual, with pregnant women having predominance of *Lactobacillus spp*., *Actinomycetales, Clostridiales*, and *Bacteroidales* and non-pregnant women having predominance of *Lactobacillus spp. Actinobacteria, Prevotella, Veillonellaceae, Streptococcus, Proteobacteria, Bifidobacteriaceae, Bacteroides* and Burkholderiales [6, 7].

Lactobacillus rich human vaginal microbiota is seen in reproductive age women with *L. iners, L. crispatus, L. gasseri* and *L. jensenii* being usually predominant [4, 7-9]. It would be an interesting question to answer how this shift in species composition is seen during the lifetime of an individual. *Lactobacillus spp.* are gram-positive, rod-shaped and anaerobic, non-spore forming bacteria [10]. *Lactobacillus* produces lactic acid which results in low pH of around 4.5 in the surrounding niche [12]. Thus it is shown to have a protective role against opportunistic pathogens by indirectly triggering the immune system [12, 13]. They also produce antimicrobial proteins called bacteriocins which affect the growth of other bacteria [5, 14]. Loss of *Lactobacillus spp* leads to decrease in pH levels in vagina and loss of protective barrier [15]. This eventually leads to increased abundance of facultative bacteria such as the *Gardnella spp*. causing bacterial vaginosis [15, 16]. Thus *Lactobacillus* plays a critical role in vaginal health that may have consequences on delivered babies' immune system compared to babies born through cesarean section.

The 16s rRNA sequencing has greatly enhanced our understanding of microbial species in the vaginal microbiome however its high cost as a limiting factor makes it less accessible [17-19]. Therefore culture dependent ways of identifying and characterizing species still serve the purpose. As a part of our project as mentioned in our previous report, we want to analyze the effects of the vaginal microbiome and metabolome on menstrual blood derived stem cells [20]. Microorganisms that are present in the vaginal canal may have influence on the quality and survival capabilities of the endometrial stem cells that are drained out during the menstrual cycles. It is important to understand their effect on these stem cells before proceeding to further differentiation experiments. Our aim is to use culture dependent methods such as fermentation of different sugars to identify the species of *Lactobacillus* genus present in the vaginal swab using the commercial yogurt samples as control. Commercially different species of *Lactobacillus* are used to produce industrial products by the process of fermentation by utilizing sugars in the medium [21]. Different species are capable of using different sugars. The end product of the metabolism by utilizing these sugars will result in the pH change which can be detected by an indicator or a color change when phenol red broth is used [22]. By using this approach, it is possible to approximately estimate the *Lactobacillus spp.* composition present in the sample. As a preliminary study to identify the presence of *Lactobacillus spp.* in yogurt/buttermilk and vaginal microbiome using culture based technique, we collected the samples and cultured them followed by identification of *Lactobacillus spp* present in sample with 12 tests namely esculin hydrolysis, catalase test, xylose, cellobiose test, arabinose test, maltose test, galactose test, mannose test, melibiose test, raffinose test, sucrose test, trehalose test using phenol red broth.

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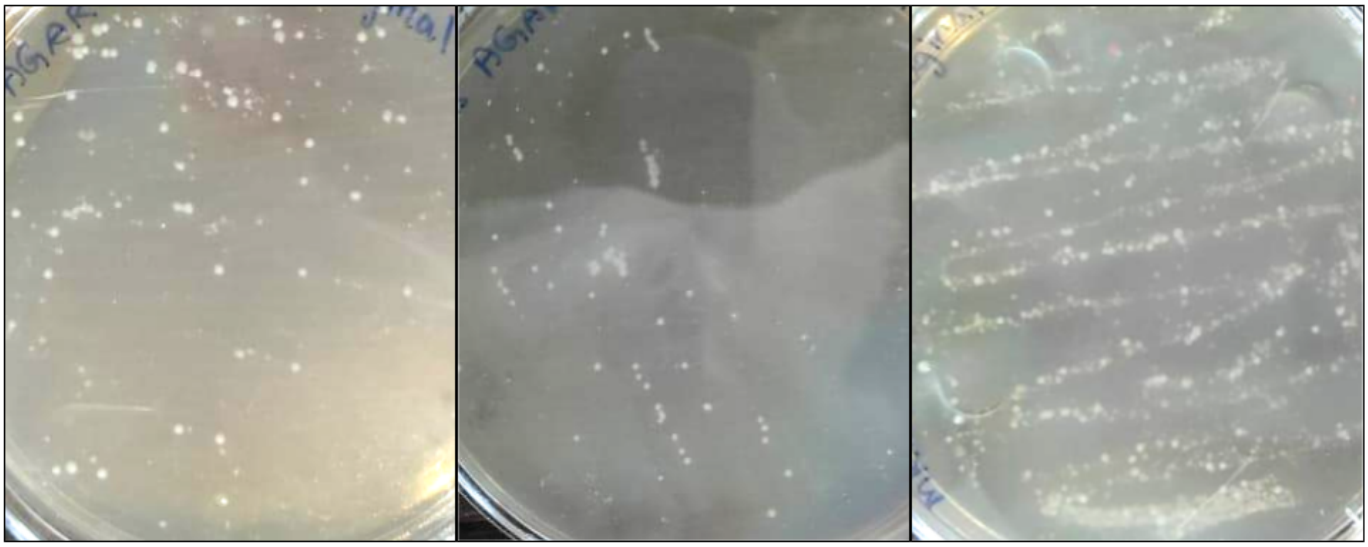

Figure 1. *Lactobacillus* from vaginal swabs on MRS-Agar plates.

Materials & Methods:

Preparation of MRS agar plates: MRS agar (From HiMedia) was used to selectively grow *Lactobacilli*. Followed by autoclaving at 15lbs for 15 minutes the media was poured into 5 sterile petri plates. Buttermilk sample was streaked onto one plate and was labeled. Upon consent from a healthy volunteer, a vaginal sample was collected using a sterile swab and streaked onto three plates with MRS agar within less than 15 minutes from the time of sample collection. One plate was used as a negative control without streaking any samples. All 5 plates were incubated at 37℃ overnight.

Preparation of MRS broth: A volume of 50 ml MRS broth was prepared. After autoclaving, 5ml of the MRS broth was taken into 3 sterile 15ml culture tubes and a loopful of culture from the MRS agar plates was inoculated into two tubes containing broth while the third tube was not inoculated to serve as a negative control. All three tubes were kept in an incubator at 37°C overnight. Turbidity is seen after 24 hours of incubation in both inoculated tubes while the negative control remained transparent.

Lactobacillus species identification using samples: Lactobacillus spp. identification strips are purchased from Himedia Laboratories. Each strip contains 12 wells with media in each well along with sugars of different types in each well. Therefore 12 tests which corresponds to namely esculin hydrolysis, catalase test, xylose test, cellobiose test, arabinose test, maltose test, galactose test, mannose test, melibiose test, raffinose test, sucrose test, trehalose test can be performed at a time using single strip. Into the 12 wells of one strip 50 µl of sample from broth is inoculated and covered with a lid and placed in an incubator at 37℃ overnight.

Catalase test: The Catalase test is performed by taking 2 ml of 3% H_2O_2 into a microcentrifuge tube.To this tube a loopful of culture is inoculated from the HiLacto kit incubated at 37℃.

Results and Discussion:

Bacterial colonies: The MRS Agar plates inoculated with the buttermilk and human vaginal swab samples gave multiple colonies while the control plate did not have any colonies. The plate with the buttermilk sample resulted in a lawn of bacterial colonies that overlapped undistinguishably (data not shown) while the plates with the human vaginal swabs resulted in a few to many colonies that were well separated and clearly distinguishable from each other. As shown in Figure 1, three plates were streaked with the human vaginal swabs which yielded a different number of colonies on each. In order to identify the *Lactobacillus spp.* diversity, colonies from all 3 plates shown in Figure 1 were considered for the sugar fermentation test.

Lactobacillus spp. identification test: Three multicolored, multiwell strips containing different sugars were considered among which, one was used as a negative control without any bacterial inoculation. The other two strips were inoculated with the *Lactobacillus* culture obtained from buttermilk and the human vaginal swab each. As shown in Figure 2, the initial colors of all wells changed in the two strips that were inoculated with *Lactobacillus* culture while the negative control did not change the colors. These color changes were then analyzed using the standard reference chart given by the manufacturer for species identification.

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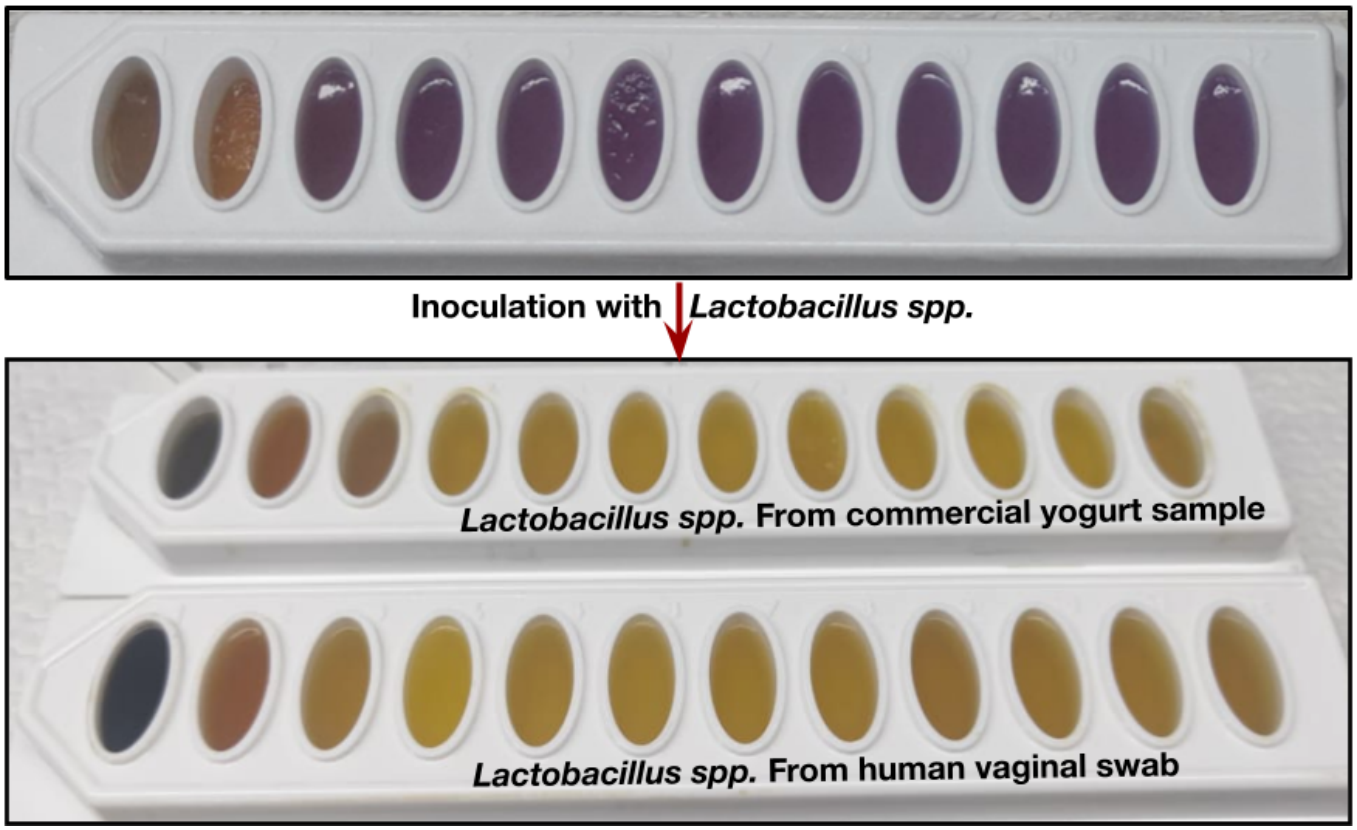

Figure 2. Post fermentation analysis of *Lactobacillus spp*.

The color changes are as shown in Table 1. Positive result in well no. 1 is seen as the color change from yellow to Black which indicates that esculin hydrolysis has happened. Positive result in wells no. 3-12 is seen as the color change from purple to yellow which indicates fermentation of sugars present in the media of each well (Figure 2). These color changes can only identify groups of *Lactobacillus spp*. that have the capability to ferment that particular sugar. For further delineating the individual species, one has to choose the 16S rRNA sequencing in combination to the above mentioned fermentation results.

As shown in Table 1, a panel of 36 *Lactobacillus spp.* were identified from the sugar fermentation test. Just to name a few among the species that were identified contain: *L. collinoides, L. ferninotoshensis, L. frumenti, L. mucosae, L. panis, L. thermotolerans, L. diolivovans, L. hammesii, L. hilgardii, L. ingluviei, L. oris, L. paracollinoides, L. spicheri, L. suebicus, L. vaccinostercus, L. gastricus,* etc.

The Catalase test is performed as described in materials and methods briefly, the culture from well no.2 is taken using a sterile pipette tip and placed into the microcentrifuge tube. As shown in Figure 3, the effervescence (formation of bubbles) is not observed in the tubes with *Lactobacillus* taken from the plates (Figure 2). However, effervescence was seen in the control tube containing a random laboratory bacterial culture that was not *Lactobacillus* (Figure 3). This test further confirms the presence of *Lactobacillus spp*.

Conclusion and Future directions:

Taken together, these results showed that *Lactobacillus* species are present in both the samples (buttermilk and human vaginal swab) after streaking the sample collected on MRS plates. To know the type of *Lactobacillus spp*. we did a preliminary study by initially identifying the *Lactobacillus* species that might be present in the buttermilk sample by 2 conventional biochemical and 10 carbohydrate fermentation tests. However we also believe that 16S rRNA sequencing will serve the purpose of species identification more accurately. Therefore our future direction is to do the sequencing taking vaginal microbiome samples from different age groups and to identify how species composition varies among the population. These studies followed by metabolite profiling using LC-Mass spectrometry/NMR spectroscopy and their testing on stem cell culture will give us an understanding about the type of metabolites that might show an effect on the viability or potency of Menstrual Blood derived stem cells.

Test	Species that might be present in both the samples
Esculin hydrolysis	*L.collinoides, L.ferninotoshensis, L.frumenti, L.mucosae, L.panis, L.thermotolerans.*
Catalase	*Lactobacillus species are present.*
Xylose	*L.collinoides, L.diolivovans, L.ferintoshensis, L.hammesii, L.hilgardii, L.ingluviei, L.mucosae, L.oris, L.panis, L.paracollinoides, L.spicheri, L.suebicus, L.thermotolerans, L.vaccinostercus.*
Cellobiose	*L.frumenti, L.gastricus, L.hammesii,*
Arabinose	*L.acidifarinae, L.antri, L.brevis, L.buchneri, L.collinoides, L.diolivorans, L.durianis, L.ferintoshensis, L.hammesii, L.ingluviei, L.oris, L.panis, L.parabuchneri. L.reuteri, L.orossiae, L.suebicus, L.thermotolerans, L.zymae, L.vaccinostercus.*
Maltose	*L.acidifarinae, L.antri, L.brevis, L.buchneri, L.collinoides, L.diolivorans, L.ferintoshensis, L.fermentum, L.frumenti, L.gastricus, L.hammesii, L.hilgardii, L.kefiri, L.lindneri, L.malefermentans, L.mucoase, L.oris, L.panis, L.parabuchneri, L.paracollinoides, L.parakefiri, L.pontis, L.reuteri, L.rossiae, L.sanfranciscensis, L.spicheri, L.suebicus, L.vaccinostercus, L.vaginalis, L.zymae*
Galactose	*L.acidifarinae, L.antri, L.collinoides, L.diolivorans, L.ferintoshensis, L.fermentum, L.frumenti, L.gastricus, L.hammesii, L.oris, L.panis, L.parabuchneri, L.parakefiri, L.reuteri, L.suebicus, L.vaginalis*
Mannose	*L.frumenti, L.gastricus, L.hammesii, L.ferintoshensis, L.vaginalis, L.panis*
Melibiose	*L.antri, L.brevis, L.buchneri, L.collinoides, L.diolivorans, L.fermentum, L.frumenti, L.gastricus, L.kefiri, L.oris, L.panis, L.parabuchneri, L.paracollinoides, L.reuteri, L.vaginalis*
Raffinose	*L.antri, L.fermentum, L.frumenti, L.gastricus, L.oris, L.panis, L.parabuchneri, L.reuteri, L.vaginalis*
Sucrose	*L.antri, L.fermentum, L.frumenti, L.gastricus, L.ferintoshensis, L.vaginalis, L.ingluviei, L.pontis, L.kunkeei, L.mucosae, L.oris, L.panis, L.parabuchneri L.reutieri*
Trehalose	*L.gastricus, L.ferintoshensis, L.frumenti, L.hammesii*

Table 1. List of *Lactobacillus spp.* vs. sugar fermentation.

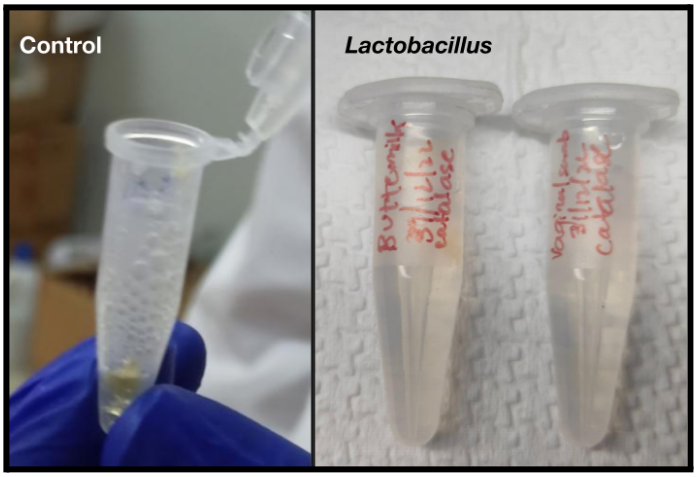

Figure 3. Catalase effervescence test for bacteria.

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Conflict of interest: The authors declare no conflict of interest in this study. However, this research article is an ongoing project currently at TCABS-E, Visakhapatnam, India.

Author contributions: S.A. supervised K.G., P.S., L.G. and L.S. in all experiments. K.G. and P.S. performed the species identification of vaginal swabs. L.G. and L.S. performed the species identification of yogurt/buttermilk samples. R.S.Y. is the principal investigator who designed the project, trained S.A. in experiments, secured required material for the project, provided the laboratory space and facilities needed. S.A. and R.S.Y. wrote and edited the manuscript.

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Full figure legends:

Figure 1. *Lactobacillus* colonies obtained from the vaginal swabs of the volunteer on the MRS Agar plates. Total number and distribution of colonies from plate to plate shows variation. Similar plate with the *Lactobacillus* culture obtained from the yogurt/buttermilk sample (not shown here) gave a lawn of colonies that were undistinguishable.

Figure 2. Display of the multicolored, multiwell strips containing sugars before and after incubation with the *Lactobacillus* cultures that were obtained from the human vaginal swab and yogurt/buttermilk.

Figure 3. Effervescence in a random control sample (left panel) is seen but not in the *Lactobacillus spp*. samples taken from either the buttermilk or the human vaginal swab (right panel).