

# High Efficiency Safety Profile for Recurrent Vaginitis and Biofilm Treatment

Sustainable system for the treatment of vaginal diseases, part I – pre-clinical laboratory study A holistic approach, to address the state of the vaginal biofilm using natural substances, enzymes and bacteria to stabilise the system. In the first set of experiments, we showed that yeast (*Candida albicans*) growth inhibition promoted by unique combination of *Lactobacilli* species and yeast-specific degradative enzymes. Part I of the research, based on sample received from the clinic taken from women of different ethnic groups who suffered from one or more symptoms as well as, healthy women. We understand that the main problem of the low successes of the recommended treatment is the biofilms. Vaginal swabbing to collect vaginal samples from women was carried out with the approval of the Helsinki Committee at an HMO clinic in Haifa and with informed consent obtained for each subject. Our first step in the research was to create a condition for the development of biofilm that will be similar to the source. We chose *Candida* as a representative pathogen. In this research we developed methods that enables us to prove under laboratory condition the following steps: A) to grow biofilms from vaginal biofilms samples of women from different ethnic groups. B) decomposed any kind of vagina biofilm of all the ethnic groups that we tested. C). eradication of pathogens originating from the biofilm that has been dismantled. D) construction of a new vaginal biofilm that will be sustainable and has a suppressive feature (inhibiting) integration of vaginal pathogens.

There are over 200 million annual cases worldwide of vaginitis.<sup>1,2</sup> FemLife is targeting the multi-billion-dollar worldwide women's health market for treating vaginal infections, while finding an innovative solution for recurrent vaginal infections.<sup>6,7,8</sup> FemLife's advanced local treatment aims to heal and improve vaginal health by creating healthy flora, with the aim of replacing other

drugs,<sup>9</sup> and can be used as prevention for the development of recurrent infections. The average prevalence in the world for vaginal infections in women for vaginal yeast (*Candida*) is about 27% and for Bacterial Vaginosis over 40%. The total number of recurrent and chronic vulvovaginal diseases in the world is over 50 million women every year. It is well known phenomena that each ethnic group is characterised by a different microbiome, different biofilm naturally developed in the vagina.<sup>3,4,5,8,9</sup>

## Materials and Methods

### *In vitro* Yeast and Lactobacilli Culture and Co-Culture and Viability Effects:

*Candida albicans* strains used in this study are listed in Table 1. Wild-type (WT-1) *Candida*

*albicans* was isolated from an infected woman in a Health Clinic,<sup>6</sup> in accordance with Helsinki protocols, and as described in<sup>12</sup> and confirmed using the Chromogenic medium – CHROMagar (CHROMagar *Candida*, France). Pure cultures were stored frozen in small aliquots (1ml) in glycerol (50% V/V). Strains were sub-cultured from freezer stocks on YPD plates containing yeast extract, peptone, dextrose and incubated at 37 °C overnight. Cultures were washed in saline (0.85g NaCl/1Li), counted, and diluted in saline for inoculation at a final concentration of 0.4 OD at 540 nm. *L. crispatus*, [as freeze-dried powder, 3.0x10<sup>10</sup> viable cells], *L. rhamnosus* [as freeze-dried powder 4.0x10<sup>10</sup> viable cells] *L. acidophilus* [as freeze-dried powder 6.0x10<sup>10</sup> viable cells] and *L. gasseri* [as

| Symbol | Treatment                                             | Yeast CFU/ml         | Yeast SE (+)        | Lactobacilli CFU/ml  | Lactobacilli SE (+0) |
|--------|-------------------------------------------------------|----------------------|---------------------|----------------------|----------------------|
| A      | Yeast substrate + buffer                              | 0                    | 0                   | 0                    | 0                    |
| A      | Yeast substrate + buffer                              | 0                    | 0                   | 0                    | 0                    |
| B      | Lactobacilli medium + buffer                          | 0                    | 0                   | 0                    | 0                    |
| B      | Lactobacilli medium + buffer                          | 0                    | 0                   | 0                    | 0                    |
| C      | Yeast in a yeast + buffer medium                      | 1.2x10 <sup>5</sup>  | 177                 | 0                    | 0                    |
| C      | Yeast in a yeast + buffer medium                      | 1.1x10 <sup>5</sup>  | 181                 | 0                    | 0                    |
| D      | Lactobacilli in Lactobacilli medium + buffer          | 0                    | 0                   | 1.7x10 <sup>6</sup>  | 5.6x10 <sup>3</sup>  |
| D      | Lactobacilli in Lactobacilli medium + buffer          | 0                    | 0                   | 1.5x10 <sup>6</sup>  | 4.8x10 <sup>3</sup>  |
| E      | Yeast + Lactobacilli in substrate * + buffer          | 1.2x10 <sup>4</sup>  | 3.9x10 <sup>2</sup> | 1.6x10 <sup>5</sup>  | 1.5x10 <sup>3</sup>  |
| E      | Yeast + Lactobacilli in substrate * + buffer          | 1.0x10 <sup>4</sup>  | 4.1x10 <sup>2</sup> | 1.6x10 <sup>5</sup>  | 1.3x10 <sup>3</sup>  |
| F      | Yeast + Lactobacilli in substrate * + buffer + enzyme | 7.6x10 <sup>3</sup>  | 1.6x10 <sup>2</sup> | 1.0x10 <sup>5</sup>  | 1.8x10 <sup>3</sup>  |
| F      | Yeast + Lactobacilli in substrate * + buffer + enzyme | 7.4x10 <sup>3</sup>  | 1.7x10 <sup>2</sup> | 1.1x10 <sup>5</sup>  | 1.6x10 <sup>3</sup>  |
| G      | Yeast + Lactobacilli in substrate * + water + enzyme  | 7.55x10 <sup>3</sup> | 1.8x10 <sup>2</sup> | 1.11x10 <sup>5</sup> | 1.7x10 <sup>3</sup>  |
| G      | Yeast + Lactobacilli in substrate * + water + enzyme  | 7.4x10 <sup>3</sup>  | 1.6x10 <sup>2</sup> | 1.2x10 <sup>5</sup>  | 1.6x10 <sup>3</sup>  |

Table 1 describes the strains and conditions evaluated, T=0.

freeze-dried powder  $1.0 \times 10^{10}$  viable cells] were purchased from Wuhan Health-dream Biological Technology Co. Ltd. as also listed in Table 1 (together referred to herein as "lactobacilli"). Freeze-dried lactobacilli powders containing 109 CFU/gr were stored at 4 °C. A mixture of the bacteria were used, in equal ratio/concentrations per strain, and cultured on MRS plates (BD Difco™ Lactobacilli MRS Broth). Growth media was prepared in phosphate buffered solution (pH 5.8) or in water. Co-cultures were prepared with yeast and lactobacilli strains, as indicated in Table 1, where media for each organism (YPD and MRS) were included in a 1:1 ratio. Co-cultures were also further subjected to contact with a degradative enzyme mixture of B-(1-3) D glucanase, (Sigma G67138) at a final concentration containing 0.5 units/ml and Chitinase (Sigma C8241), was included at a final concentration of 0.6 units/ml, both in 0.05M Acetate, at pH 5.0. Data presented is the time zero mean value of 6 samples, assessed twice per condition tested, and Standard error of the mean was calculated (SE) in Table 1 and after a 17-hour incubation period in Table 2, presented hereinbelow. Table 1 describes the strains and conditions evaluated, at time "0" (T=0).

Yeast substrate = YDP liquid media, prepared in water or in buffer. Lactobacillus was grown in MRS liquid media, prepared in water or in buffer. CFUs were determined by plating on agar containing the respective media (1.8 % W/V). As is clear in Table 1, the strain designations (A–G), describe different culture conditions, where samples A and B are negative controls of freshly thawed seed lots which were not grown in culture; Samples C and D represent individual growth in appropriate buffered media conditions

| Infection status in each woman, at sample collection | Ethnic origin | #Sample |
|------------------------------------------------------|---------------|---------|
| Healthy                                              | European      | 1       |
| BV+CAN                                               | European      | 2       |
| BV+CAN                                               | European      | 3       |
| CAN                                                  | European      | 4       |
| HEALTHY                                              | Ethiopian     | 5       |
| BV                                                   | African/Asian | 6       |
| BV +GBS                                              | European      | 10      |
| CAN                                                  | European      | 11      |
| CAN + GBS                                            | European      | 12      |
| CAN + MYCOPLASMA HOMANIS                             | European      | 13      |
| CAN                                                  | European      | 14      |
| CAN                                                  | European      | 15      |
| CAN                                                  | African/Asian | 16      |
| CAN                                                  | European      | 20      |
| CAN                                                  | African/Asian | 21      |
| CAN                                                  | African/Asian | 22      |
| CAN + BV                                             | European      | 30      |
| CAN                                                  | European      | 31      |
| CAN + BV                                             | Asian         | 32      |
| CAN                                                  | Asian         | 33      |

Table 3 describes the ethnicities of the women from which each sample number was derived, as follows

at the indicated CFU values, and Samples E, F and G describe co-culture conditions with the indicated strains in buffered

media alone (E), or in the presence of the degradative enzymes (F) or in the presence of the degradative enzymes in water. Table 2 describes the results of the culture conditions after 17 hours of incubation.

| Symbol | Treatment                                             | Yeast CFU/ml        | Yeast SE (+)      | Lactobacilli CFU/ml | Lactobacilli SE (+0) |
|--------|-------------------------------------------------------|---------------------|-------------------|---------------------|----------------------|
| A      | Yeast substrate + buffer                              | 0                   | 0                 | 0                   | 0                    |
| B      | Lactobacilli medium + buffer                          | 0                   | 0                 | 0                   | 0                    |
| C      | Yeast in a yeast + buffer medium                      | $4 \times 10^7$ A   | $8.4 \times 10^3$ | 0                   | 0                    |
| D      | Lactobacilli in Lactobacilli medium + buffer          | 0                   | 0                 | $1.2 \times 10^8$   | $2.1 \times 10^4$ A  |
| E      | Yeast + Lactobacilli in substrate * + buffer          | $2.1 \times 10^6$ B | $5 \times 10^3$   | $1.6 \times 10^5$   | $1.3 \times 10^3$ B  |
| F      | Yeast + Lactobacilli in substrate * + buffer + enzyme | $1.0 \times 10^3$ C | $1.6 \times 10^2$ | $1.2 \times 10^8$   | $3.6 \times 10^4$ A  |
| G      | Yeast + Lactobacilli in substrate * + water + enzyme  | $2.4 \times 10^3$ C | $1.6 \times 10^2$ | $2.5 \times 10^8$   | $3.7 \times 10^4$ A  |

Table 2 describes the results of the culture conditions after 17 hours of incubation.

Student's T test value of  $<0.05$  were achieved in pairwise comparisons as indicated, with respect to the indicated sample. In addition to assessing CFU changes, culture aliquots were taken for microscopic analysis of the yeast for viability effects. Comparing the data in Table 1 versus Table 2 shows that the culture conditions for growth of yeast alone or lactobacilli alone promoted a two-log increase in CFU within the 17-hours incubation period. When Yeast and lactobacilli were co-cultured in media alone, while the yeast CFU increased substantially, there was a clear growth inhibition of the lactobacilli, reducing the CFU by 1-log, compared to the T=0 culture

alone sample. Adding the degradative enzymes promoted lactobacilli growth with CFU levels comparable to lactobacilli alone culture values, while the Yeast CFU show a reduction, as compared to the same time point, when cultured in buffer alone. Comparable results were obtained whether the enzymes and strains were co-cultured in a buffered solution or in water. Samples were collected from the following women with different ethnicities, as indicated in Table 3 described further hereinbelow. Samples were taken from women of each ethnicity, some of whom were healthy, having no history of vaginal diseases in the last two years, some suffering from a vaginal fungal infection with Candida, some suffering from Bacterial Vaginosis and some suffering simultaneously from Candida infection and Bacterial Vaginosis.

Following sample collection swabs were placed in saline, mixed well, and stored under refrigeration at a temperature of 4–7°C, with aliquots in glycerol (10% V/V) also maintained at -20°C. 200 microliter aliquots of each sample were added to different wells in a 96-well ELISA plate, to assess different media/growth conditions and their impact on biofilm formation. Ten different media were tested for the creation of biofilm in the wells of Elisa plates. MRS broth (Sigma) 25 grams/l, pH 7.1 was found to support biofilm of all the samples of the different ethnic groups. Samples were incubated at 37 °C for 17 hours, in a humidified chamber using buffer Potassium – Phosphate 0.1M pH 7.1 in a total volume of 1 ml. Following incubation, wells were inspected for biofilm formation. Briefly, wells were washed and stained with Gentian Violet (1.5% solution in ethanol W/V), which was taken up by the biofilm. Dye is then washed in water, which removed unbound dye. An acetic acid solution (33%) was applied, and dye is then quantified by ELISA reader at 590 nm. Table 4 describes the OD values obtained in treated versus untreated samples 10–16 and provides the Student's T-test values as obtained, as well. Statistical significance was achieved for each of samples 10–33, as well.

To further extend these studies, where using the 1X enzymatic mixture, robust biofilm degradation was readily seen that was statistically significant, it was of interest to discern if better results could be obtained, using a more concentrated enzymatic solution. Thus, biofilms were prepared from samples 20–23, as indicated, and treated with 1X dose or 2X doses of

| T- test, pairwise comparison, Treated vs | Average OD following incubation, following enzymatic treatment | Average OD following incubation, no enzymatic treatment | Sample # |
|------------------------------------------|----------------------------------------------------------------|---------------------------------------------------------|----------|
| 1.43E-20                                 | 0.101                                                          | 0.309                                                   | 10       |
| 1.34E-07                                 | 0.023                                                          | 0.099                                                   | 11       |
| 1.34E-08                                 | 0.09                                                           | 0.136                                                   | 12       |
| 4.13E-12                                 | 0.021                                                          | 0.145                                                   | 13       |
| 5.24E-07                                 | 0.06                                                           | 0.112                                                   | 14       |
| 6.75E-19                                 | 0.092                                                          | 0.211                                                   | 15       |
| 1.62E-07                                 | 0.095                                                          | 0.162                                                   | 16       |
| 8.53E-25                                 | 0.118                                                          | 0.329                                                   | 30       |
| 1.63E-34                                 | 0.074                                                          | 0.303                                                   | 31       |
| 1.58E-34                                 | 0.105                                                          | 0.459                                                   | 32       |
| 1.96E-20                                 | 0.107                                                          | 0.307                                                   | 33       |

Table 4: OD at 590 nm results and statistical significance for enzymatic degradation of biofilms formed from samples 10–33.

| Sample # | OD Following incubation, no enzymatic treatment | OD Following incubation, 1x enzymatic treatment | OD Following incubation, 2x enzymatic treatment | T- test, 1X Treatment vs Untreated | T- test, 2X Treatment vs Untreated | T- test, 2X Treatment vs 1X Treatment |
|----------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|------------------------------------|------------------------------------|---------------------------------------|
| 20       | 0.941                                           | 0.371                                           | 0.341                                           | 6.08E-24                           | 2.15E-25                           | 0.023431                              |
| 21       | 0.258                                           | 0.182                                           | 0.111                                           | 0.003219                           | 1.14E-09                           | 0.004109                              |
| 22       | 0.658                                           | 0.563                                           | 0.318                                           | 7.6939E-08                         | 3.97E-08                           | 0.099759                              |
| 23       | 0.237                                           | 0.084                                           | 0.046                                           | 1.32E-09                           | 4.75E-15                           | 0.000834                              |

Table 5: Biofilm degradation – Comparative results between 1X dose or 2X doses of enzymatic mixture (two times concentration of the enzymes) to the untreated control.

| Sample # Original | CFU/ml Sample # After dilution 1:100 | CFU/ml Samples # Original | CFU/ml following enzymatic treatment Diluted | CFU/ml following enzymatic treatment Original |
|-------------------|--------------------------------------|---------------------------|----------------------------------------------|-----------------------------------------------|
| 10                |                                      | 10 <sup>6</sup> x9.88     |                                              | 2.4x10 <sup>5</sup>                           |
| 10                | 1.4x10 <sup>4</sup>                  |                           | 2.3x10 <sup>2</sup>                          |                                               |
| 11                |                                      | 10 <sup>6</sup> x3.1      |                                              | 10 <sup>5</sup> x8.3                          |
| 11                | 1.2x10 <sup>4</sup>                  |                           | 7.5x10 <sup>2</sup>                          |                                               |
| 12                |                                      | 10 <sup>7</sup> x1.6      |                                              | 10 <sup>5</sup> x8.8                          |
| 12                | 1.1x10 <sup>5</sup>                  |                           | 8.2x10 <sup>2</sup>                          |                                               |
| 13                |                                      | 10 <sup>7</sup> x1.7      |                                              | 10 <sup>5</sup> x9.7                          |
| 13                | 6/3x10 <sup>5</sup>                  |                           | 6.7x10 <sup>2</sup>                          |                                               |
| 14                |                                      | 10 <sup>6</sup> x1.56     |                                              | 10 <sup>4</sup> x5.1                          |
| 14                | 1.2x10 <sup>4</sup>                  |                           | 3.3x10 <sup>2</sup>                          |                                               |
| 15                |                                      | 10 <sup>6</sup> x3.12     |                                              | 10 <sup>5</sup> x5.7                          |
| 15                | 1x10 <sup>4</sup>                    |                           | 6.2x10 <sup>2</sup>                          |                                               |
| 16                |                                      | 10 <sup>6</sup> x7.55     |                                              | 10 <sup>5</sup> x5.8                          |
| 16                | 5x10 <sup>4</sup>                    |                           | 4.1x10 <sup>2</sup>                          |                                               |

Table 6: Yeast CFUs following second enzymatic treatment: original population and after dilution of the original population 1:100

| Yeast CFU/ml obtained, second enzymatic treatment + lactobacilli combination | Yeast CFU/ml obtained, second enzymatic treatment only | Yeast CFU/ml obtained in untreated samples | Sample #   |
|------------------------------------------------------------------------------|--------------------------------------------------------|--------------------------------------------|------------|
| $5 \times 10^3 \pm 64$                                                       | $9 \times 10^6 \pm 2.1 \times 10^3$                    | $1 \times 10^9 \pm 3.6 \times 10^5$        | 32         |
| $22 \pm 1$                                                                   | $1.2 \times 10^2 \pm 7.2$                              | $8 \times 10^5 \pm 4.1 \times 10^2$        | Diluted 32 |

Table 7: Yeast growth following enzymatic treatment +/- lactobacilli co-culture. The results in the table are the mean of 5 replications for each sample.

| Candida CFU/ml recovered | Lactobacilli CFU/ml recovered | Description of Treatment                |
|--------------------------|-------------------------------|-----------------------------------------|
| 0.0                      | $6.3 \times 10^8$             | Following simple incubation of sample 1 |
| 0.0                      | $1.5 \times 10^3$             | Sample 1 post enzymatic treatment I     |
| 0.0                      | $8.6 \times 10^9$             | Sample 1 post enzymatic treatment II    |

Table 8: CFU recovery in samples exposed to both enzymatic treatments:

enzymatic mixture and compared to untreated samples. The results are provided in Table 5.

The results shown in Table 5 demonstrate that in these samples, as well, the enzymatic treatment produced a statistically significant destruction of the biofilms formed, in 1X and 2X concentration. Comparing the more concentrated mixture showed an improved effect over the original enzymatic mixture concentration, which achieved statistical significance in three out of the four samples assessed here.

When the candida population was high, around 107/CFU ml, 10 percent of the population survived the treatment. But if the population before the treatment was around 105CFU/ml, only 0.1 percent of the population survived the treatment reduction of 3 logs units after one treatment.

These results were further extended to show the effects of lactobacilli co-culture in the above system. Biofilms were subjected to enzymatic treatment to liberate the yeast component, then further cultivated and exposed to the second yeast-specific degradative enzyme treatment. Parallel samples were then cultivated with and without lactobacilli addition to the culture media containing the degradative enzymes. Table 7 presents the results obtained.

As can be seen from the table, enzymatic treatment reduced yeast CFU by almost 3 logs, whereas having lactobacilli present resulted in a surprising almost 6-log reduction in CFUs in native and diluted samples.

*Yeast growth inhibition promoted by unique combination of Lactobacilli species and*

*yeast-specific degradative enzymes in newly developed culture methods.*

#### Materials and Methods Mimicking Human Vaginal Organ Culture in an Infection Model:

Human vaginal flora samples were isolated and prepared as described in Example 2. In order to re-capitulate formation of biofilms *in vivo*, a culture method was developed. Toward this end, dialysis bags (Sigma- Dialysis tubing cellulose membrane, Typical molecular weight

cut-off = 14,000. Supplied in rolls, dry average flat width 33 mm (1.3 in.) were purchased, filled with media and flora from samples 6, 20 and 30 were independently inoculated into multiple dialysis bags.

Biofilm formation was allowed to proceed by cultivating the dialysis bags under low oxygen conditions at 37°C. Recovery of the biofilm was via saline wash of sonicated bags and CFUs assessed.

Biofilms of parallel sample inoculated dialysis bags were subjected to the biofilm degradative enzyme mixture containing Lipase, Deoxyribonuclease I, and B-(1-3) glucanase (hereinafter referred to as "treatment 1"), as described in Example 2, to assess the breakdown of the biofilm to liberate the biofilm components. CFUs were determined from these samples to demonstrate the efficacy of the enzymatic treatment on biofilm degradation.

To further assess the impact of the second Candida eradicating enzymatic treatment specifically targeting yeast growth the second enzymatic treatment containing B-(1-3) D glucanase, and Chitinase (hereinafter, referred to as "treatment 2") was applied as listed in Example 2 to

| Candida CFU/ml recovered | Lactobacilli CFU/ml recovered | Description of Treatment                         |
|--------------------------|-------------------------------|--------------------------------------------------|
| $2.6 \times 10^8$        | $3 \times 10^2$               | Following simple incubation of sample 30         |
| $3 \times 10^4$          | $1.3 \times 10^3$             | Sample 30 post enzymatic treatment I             |
| 0                        | $7.9 \times 10^9$             | Sample 30 post enzymatic treatment II            |
| $1.8 \times 10^8$        | $3.1 \times 10^3$             | Following simple incubation of sample 6          |
| $1 \times 10^9$          | $3.6 \times 10^3$             | Duplicate simple incubation of sample 6          |
| $1.3 \times 10^4$        | $5 \times 10^3$               | Sample 6 post enzymatic treatment I              |
| $1.6 \times 10^4$        | $4.6 \times 10^3$             | Duplicate, Sample 6 post enzymatic treatment I   |
| 0                        | $4 \times 10^9$               | Sample 6 post enzymatic treatment II             |
| 22                       | $5.8 \times 10^9$             | Duplicate, Sample 6 post enzymatic treatment II  |
| $2 \times 10^7$          | $2 \times 10^6$               | Following simple incubation of sample 20         |
| $4.9 \times 10^4$        | $3.1 \times 10^4$             | Duplicate simple incubation of sample 20         |
| $1.4 \times 10^3$        | $3 \times 10^6$               | Sample 20 post enzymatic treatment I             |
| 10                       | $4.5 \times 10^5$             | Duplicate, Sample 20 post enzymatic treatment I  |
| 16                       | $7.5 \times 10^8$             | Sample 20 post enzymatic treatment II            |
| 0                        | $1 \times 10^9$               | Duplicate, Sample 20 post enzymatic treatment II |

Table 9: CFU recovery in additional samples exposed to both enzymatic treatments:

another parallel sample inoculated dialysis bag, with concomitant introduction of the four lactobacilli species, delivered as dry powder, as also described in Example 2 (*Acidophilus*, *Crispatus*, *Rhamnosus*. *Gasserii*) further supplemented with media, where following incubation, samples were then washed and plated for CFUs, on parallel agar plates containing supportive media for yeast and lactobacilli growth, respectively.

The enzymatic treatments 1 and 2 were applied to the dialysis bags delivered in a Pullulan capsule (LFA USA, Empty Pullulan Capsules – Size #0 Clear). The capsule was loaded in the dialysis bag via syringe, containing a cut end to expand the bore. Pullulan serves as a carbon source for the lactobacilli, which liberates its contents upon degradation.

## Results

Following the positive results obtained in table 7, it was of interest to create an *in vitro* culture system that more closely mimics *situ* conditions. Toward that end a system was developed to prepare biofilms isolated from patients using dialysis bags and modified culture conditions, to assess independent growth of *Candida* and lactobacilli and to assess the effect of the enzymatic treatments on as well.

Table 8 describes the results obtained in assessing Sample 1, sample taken from healthy women. In the table, CFUs of lactobacilli and candida as a function of the treatment applied to the model system.

Table 8 demonstrates that the biofilm of healthy women is typically rich in lactobacilli, without appreciable candida.

Samples 30, 6 and 20 were similarly probed and Table 9 describes results obtained in assessing Sample 30, Sample 6 and Sample 20, CFUs of lactobacilli and candida as a function of the treatment applied to the model system.

It is clear that while at the outset, significant candida was recovered from the biofilms in each sample, and far fewer cultivable lactobacilli species were cultivatable, following treatment to disrupt the biofilm, candida CFUs precipitously dropped, while lactobacilli CFUs rose.

Completing the exposure to the second degradative enzymatic treatment almost abrogated candida presence, while

| Sample & treatment Description           | Lactobacilli CFU/ml | Candida CFU/ml      | % Of the Control Lactobacilli | % Of the Control Candida |
|------------------------------------------|---------------------|---------------------|-------------------------------|--------------------------|
| Following simple incubation of sample 4  | 3.5x10 <sup>3</sup> | 1.4x10 <sup>8</sup> | 100.0                         | 100.0                    |
| Sample 4 post enzymatic treatment I      | 4.8x10 <sup>3</sup> | 1.5x10 <sup>4</sup> | 137.1                         | 0.1                      |
| Sample 4 post enzymatic treatment II     | 4.9x10 <sup>9</sup> | 11                  | 1.4x10 <sup>7</sup>           | 0                        |
| Following simple incubation of sample 5  | 1.2x10 <sup>3</sup> | 3x10 <sup>9</sup>   | 100.0                         | 100.0                    |
| Sample 5 post enzymatic treatment I      | 1.5x10 <sup>3</sup> | 6.3x10 <sup>4</sup> | 125.0                         | 0.021                    |
| Sample 5 post enzymatic treatment II     | 5x10 <sup>8</sup>   | 6x10 <sup>2</sup>   | 4.2x10 <sup>7</sup>           | 0.0                      |
| Following simple incubation of sample 22 | 8x10 <sup>3</sup>   | 5x10 <sup>6</sup>   | 100.0                         | 100.0                    |
| Sample 22 post enzymatic treatment I     | –                   | –                   | –                             | –                        |
| Sample 22 post enzymatic treatment II    | 1.6x10 <sup>9</sup> | 30                  | 1.9x10 <sup>6</sup>           | 0.0                      |

Table 10: Influence of additional lactobacilli carbon sources on lactobacilli growth in the model system

supporting robust lactobacilli CFUs in this model system.

To further extend these studies, it was of interest to determine if additional modifications to the assay system could further support positive lactobacilli growth.

Toward this end, 3 additional samples were assessed as above, including establishing the biofilms from Samples 4, 5 and 22 in the dialysis bags containing MRS and YDP broth at a 1:1 V/V ratio. Following addition of the first enzymatic biofilm degradative mixture containing Lipase, Deoxyribonuclease I, and B-(1-3) glucanase, the second candida enzymatic degradative mixture containing B-(1-3) D glucanase, and Chitinase mixture was applied, in the presence of the lactobacilli mixture, with the media provided being supplemented with inulin and Pullulan to serve as lactobacilli carbon sources.

Table 10 provides the results of these studies assessing candida and lactobacilli CFUs, respectively, recovered as described hereinabove, with media enriched lactobacilli growth.

Candida growth declined substantially after the first enzymatic treatment and was almost undetectable following the second enzymatic

treatment, as is evident in columns 2 and 4 of the table. A concomitant increase in lactobacilli reconstitution is seen following the second enzymatic treatment with lactobacilli spp.

In table 10, candida growth declined substantially after the first enzymatic treatment and was almost undetectable following the second enzymatic treatment, as is evident in columns 2 and 4 of the table. A concomitant increase in lactobacilli reconstitution is seen following the second enzymatic treatment with lactobacilli spp.

To further extend the above studies, biofilms were prepared and assessed in the *in vitro* model system probing effects on Sample 3, assayed as 4 replicates for each treatment. Average CFUs for lactobacilli and *Candida* are provided in Table 11 following each treatment, as follows:

Treatment C as listed in the table refers to samples where a biofilm was prepared, with no further treatment applied to same (i.e. no enzymatic degradation exposure is provided). Treatment I comprised the first enzymatic biofilm degradative mixture containing Lipase, Deoxyribonuclease I, and B 1-3 glucanase, as described hereinabove. Treatment II comprised the second candida enzymatic degradative mixture containing

| test T   | test T   | test T   | test T | Candida             | Lactobacilli        | Treatment |
|----------|----------|----------|--------|---------------------|---------------------|-----------|
| IY-IIY   | CY-IY    | IB-IIB   | CB-IB  |                     |                     |           |
|          |          |          |        | 3.2x10 <sup>6</sup> | 3.5x10 <sup>4</sup> | C         |
|          |          |          |        | 2.5x10 <sup>2</sup> | 2.5x10 <sup>2</sup> | I         |
|          |          |          |        | 17                  | 5.3x10 <sup>8</sup> | II        |
| 2.18E-07 | 6.47E-06 | 6.71E-06 | 0.005  |                     |                     |           |

Table 11: Statistical significance for all treatments

B-(1-3) D glucanase, and Chitinase mixture, applied in the presence of the lactobacilli mixture of species, wherein the media provided is supplemented with the carbon sources inulin and Pullulan for use by the lactobacilli.

Statistical significance was assessed using the Student's T-test, in pairwise comparisons between CB-IB, representing lactobacilli CFUs compared in samples treated per C conditions versus those subjected to Treatment I, IB-IIB, compares lactobacilli CFUs post Treatment II versus Treatment I, and CY-IY relates to pairwise comparisons of candida CFUs compared in samples treated per C conditions versus those subjected to Treatment I, and IY-IY relates to pairwise comparisons of candida CFUs compared in samples post Treatment II versus Treatment I.

It is noted that each treatment showed statistical significance in terms of the impact of the treatment on both Candida and lactobacilli growth.

## Discussion

In this research we achieved our six goals:

- I. Grown any kind of vaginal biofilm from natural source of different women of different ethnic groups.
- II. We were able to decompose those different biofilms.
- III. Eradication of pathogens originating from the biofilm that had been dismantled.
- IV. We developed a method based on two capsules for two-step treatments of vaginal infections of any type based on natural substances (enzymes and additives). and focusing on the acute area of recurrent vaginal infections, according to the steps described.
- V. Construction of a new vaginal biofilm composed of mixture of lactobacilli spp. applying with two special carbon sources that can be utilized by the lactobacilli and not by the pathogens. The new biofilm, will be sustainable and has a suppressive feature (inhibiting) integration of vaginal pathogens.

- VI. This treatment will be easy to use by friendly applicator.

Scientifically, we developed a new method for growing biofilms from common clinic vagina samples that are sent to the diagnostic laboratories. Quantitative method was developed for estimations biofilms using our stained with Gentian Violet as described earlier followed by using Eliza reader to quantify the intensity of the colour that represent the amount of the biofilm. We found the suitable enzymes to break the biofilms as well as to controlling the candida – a common pathogen in the vagina's system. We invented a mimic tool made of dialysis tub, that served as a vagina model for the laboratory experiment with the uses of Sonicator that enabled us to estimate microflora that creates biofilm including pathogens.

The most important innovation is the creation of the two capsules for treatment I and treatment II. – the enzymes, the carbon sources and the mixture of the lactobacilli spp.

# ADCA



## STEAMING SOLUTIONS FOR ALL INDUSTRIES

### Steam traps



### Pressure regulators



### Control valves



### Pipeline ancillaries



### Special equipment



As demonstrated herein, an inexpensive treatment regimen using specific combinations of readily available enzymes and lactobacilli strains can be used as an effective therapy to limit growth of yeast and other vaginal flora components to favor lactobacilli growth. Some unique aspects to our invention include the ability to assess BV and/or candida treatment regimens in female subjects, and offer the promise of reconstitution of vaginal flora without need for antibiotic therapy, which in turn helps reduce the known global burden of antibiotic resistance.

We developed a model system to specifically assess vaginal flora from affected patients having the potential to form biofilms, in terms of the sensitivity of the flora to our unique combination therapy.

Our newly developed system provided a means to qualitatively and quantitatively assess growth of pathogenic flora, the impact of our treatment regimen on the pathogenic components and concomitant positive impact on the growth of protective lactobacilli species.

Some unique aspects to our invention include the ability to assess BV and/or candida treatment regimens in female subjects, and offer the promise of reconstitution of vaginal flora without need for antibiotic therapy, which in turn helps reduce the known global burden of antibiotic resistance.

Furthermore, repeat treatments, as needed would not be expected to be reduced in efficacy, since the enzymatic treatment specifically targets components critical for the survival and niche creation of the pathogenic organisms.

### Summary

The two treatments effect on vagina pathogens:

#### Before the treatment:

present of biofilm composed of bacteria mixture and fungi including pathogens covered by extracellular matrix: polysaccharides, lipids, DNA and proteins which protect them from the common medicines.

#### After the first treatment (capsule I):

the biofilm matrix is broken down by the enzymes, the microflora that was a part of the biofilm separates to free cells.



#### After the second treatment (capsule II):

the pathogens were attacked by enzymes that broke their cell walls, as a result, their population dramatically decreased. The lactobacilli complex that was inserted with the enzymes and with two carbon sources (that can't be utilised by the pathogens). Under this enrichment conditions, the lactobacilli spp. Created within a short period time new sustainable suppressive biofilm towards the pathogens.

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