CHAPTER 5

To exploit *Lactobacillus* sp. and their culture free supernatant for future industrial usage.

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5.1. Introduction

Grigoroff in the year 1905 isolated the first probiotic strain *Lactobacillus bulgaricus* from yoghurt [569]. Eventually, studies on probiotics began and in the year 1907 Metchnikoff earned the Nobel prize for elucidating the benefits of Bulgarian yogurt. The term "probiotic" was defined by WHO in 2001 as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [570]. "Generally regarded as safe" (GRAS) these non-pathogenic live bacteria should have the ability to colonize humans, aggregate with pathogens, and produce antimicrobial compounds [571]. These bacteria produce inhibitory metabolites that prevent the growth of pathogens, supporting their own exponential growth through competitive inhibition. They also modify toxin receptors of human epithelial cells and reduce inflammation protecting the epithelial cells [572]. *Lactobacillus rhamnosus* strain GG a probiotic from the GI tract has been studied most widely for its beneficial effects on humans [573].

In the year 2021, International Scientific Association of Probiotics and Prebiotics (ISAPP) coined the term postbiotics as "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" [574]. Examples of postbiotics are absorbable metabolites such as SCFA's, bacteriocins, antimicrobial peptides, organic acids, vitamins, exopolysaccharides, and tryptophan produced by beneficial microbes [575]. These metabolites either directly have a positive effect on the host epithelial cells or indirectly benefit the host by inhibiting pathogens and helping the proliferation of beneficial microbes [576]. Reuterin and Nisin A are examples of potent antipathogenic postbiotics produced by *L. reuteri* and *Lactococcus lactis* subsp, which are thoroughly used in the food industry [577]. From the oldest times, the inclusion of probiotics/postbiotics in our diet has imparted benefits to gut health. Recently, their potential for improvement and maintenance of reproductive health has been highlighted.

This encourages the need to study and industrially utilize probiotics/postbiotics for reproductive health benefits.

5.2. Materials and Methods

5.2.1. Chemicals Used

- Lactose (HiMedia, India)
- Glycerol (HiMedia, India)
- Gelatin (HiMedia, India)
- Skim Milk (HiMedia, India)
- Saccharose (HiMedia, India)
- Calcium carbonate (HiMedia, India)
- Sodium-alginate (HiMedia, India)
- Carboxy methyl cellulose (HiMedia, India)
- Calcium chloride (HiMedia, India)
- *Lactobacillus* MRS broth (HiMedia, India)
- Nonwoven fabric 18gsm
- Nutrient agar (HiMedia, India)
- Potassium chloride (HiMedia, India)

5.2.2. Instruments Used

- Freeze Dryer, Labconco, USA
- Fourier Transmission Infrared Spectroscopy, Perkin Elmer, India
- Scanning Electron Microscope, JEOL, Japan
- Energy Dispersive X-ray, JEOL, Japan
- Centrifuge 5430 R, Eppendorf, Germany
- Q-Exactive Plus Biopharma-High Resolution Orbitrap, Thermo Fiseher Scientific, India

5.2.3. Lyophilization of *L. crispatus*

Lactobacillus crispatus was inoculated in 15 ml *Lactobacillus* MRS broth and grown anaerobically for 48 h at 37 ℃. The CFS was discarded and the bacterial cells were washed with PBS. The *L. crispatus* cells were resuspended in sterile lyophilization Media viz: (Media a- 5% lactose, 1% glycerol, 1.5% gelatin), (Media b -1% gelatin and 10% skim milk, 8% saccharose), and (Media c- 10% skim milk, 5% glycerol, 0.1% calcium

carbonate). The tubes were lyophilized and eventually stored at 12 ℃. The viability of the lyophilized powder was checked to assess the revival ability of *L. crispatus* [578, 579].

5.2.4. Encapsulation of *L. crispatus*

L. crispatus was added to sterile mixture of 2% sodium-alginate and 2% carboxy methyl cellulose (HiMedia, India) and homogenised on a magnetic stirrer for 15 min. The homogenate was added drop wise to 3% calcium chloride solution under agitation, using a sterile syringe with a flow rate of (90-100) drops per min. Small circular CMC-alginate beads with encapsulated *L. crispatus* cells were formed, 30 mins post agitation at room temperature the beads hardened. Control beads were prepared using the similar method with no *L. crispatus* cells. The beads were filtered using Whatman filter paper; the beads were washed twice with sterile PBS to remove the excess calcium chloride and stored in sterile distilled water overnight at -20 ℃. The beads were air dried the subsequent day and the following experiments were performed [580] [581].

5.2.4.1. Swelling and Deswelling of the CMC-alginate beads

The beads showed reduction in their size post freezing. The control CMC-alginate beads [508] and the beads with *L. crispatus* cells (PB) were weighed post drying at 0 h and dipped into 1ml PBS; weight of the beads was measured at an interval of 2 h till the beads reached their maximum solvent absorption capacity. Post reaching maximum absorption the beads were dried in open air at room temperature. The decreasing weight of the beads were measured at an interval of 12 h till it reached a constant weight [580].

5.2.4.2. Revival of Encapsulated L. crispatus

Bead PB was inoculated in 200 µl PBS buffer for 4 h, the entire inoculum with the beads was inoculated into *Lactobacillus* MRS broth of pH 7 and pH 4 and grown anaerobically for 72 h at 37 ℃. The growth was observed and gram's test was performed [582].

5.2.4.3. Physicochemical Characterization of the CMC-alginate beads

Fourier Transmission Infrared Spectroscopy (FTIR) (Perkin Elmer, India) analysis of bead CB and PB were conducted to study their functional groups; Scanning Electron Microscope (SEM) (JEOL, Japan) of bead CB and PB was performed to analyse the

surface and cross section of the beads; Energy Dispersive X-ray (EDX) (JEOL, Japan) analysis was done to study the elemental difference of the two beads [583].

5.2.5. CFS as an antibacterial spray

L. crispatus CFS the most potent extracellular metabolite was used as a spray to inhibit the five potential bacterial urogenital pathogen consortia. Two set of experiment was performed: In the first set, the control plate was unsprayed, whereas the test plate was sprayed once at 0 hours, the growth was observed post 12 hours. Whereas, in the second set, the control plate was unsprayed, whereas the test plate was sprayed at regular time interval of 2 hours. Growth of the consortium biofilm was monitored visually [584].

5.2.6. CFS as an additive on nonwoven fabric

5.2.6.1. Antimicrobial Property of treated fabric

The non-woven fabric (NWF) usually used for manufacturing sanitary napkin was cut into 1 cm (length × breadth) squares. The NWF was soaked in filtered *L. crispatus* CFS for 12 h aseptically at 37 ℃, followed by air drying for 3 h. The fabric was placed on nutrient agar plates inoculated with 10µl (1×10^7/100 µl) potential pathogens. The ZOI around the fabric was checked to see if the treated NWF was able to inhibit them [585, 586].

5.2.6.2. Physicochemical Analysis of non- woven fabric

FTIR analysis of CFS treated and non-treated fabric was conducted to study the functional groups of the fabric. SEM was done to assess the surface of the treated and non-treated fabric; EDX was also done to analyse the elemental differences between the two fabrics [587, 588].

5.2.6.3. Physicochemical characterization of non-woven fabric

Absorbent cotton 1.5 gms was pressed using heat, NWF was cut into 15 × 5 cm (l × b). The cotton was rolled into the non-woven fabric to make sample tampon. The tampons weighed 1.75 gm with length of 5 cm. The test tampons had treated NWF and control tampon had simple NWF[589, 590].

Sample tampons were dipped in 10 ml of distilled water for 30 min and hanged for 10 mins to release the excess distilled water, the maximal mass of the tampons was

measured. Further, the tampons were then centrifuged at 5,000 rpm for 5 min to release excess water and the minimal mass was measured as demonstrated by [591, 592].

The water absorbed by the tampon post was measured as the maximal water retention and water retained by the tampon post centrifugation at 5,000 rpm for 5 min was recorded as the minimal water retention[592].

Animal blood (1 ml) was pipetted on a petri plate and the tampons were placed and rolled over the blood; time required by the tampons to absorb the blood was checked as suggested by [593].

The pH of the non-woven fabric (test and control) was measured using the ISO3071 method; 1cm (l × b) fabric (test and control) were immersed in 0.01 M potassium chloride under constant agitation for 3 h at 37 ℃. pH of the potassium chloride was measured to indicate the pH of the fabric [592].

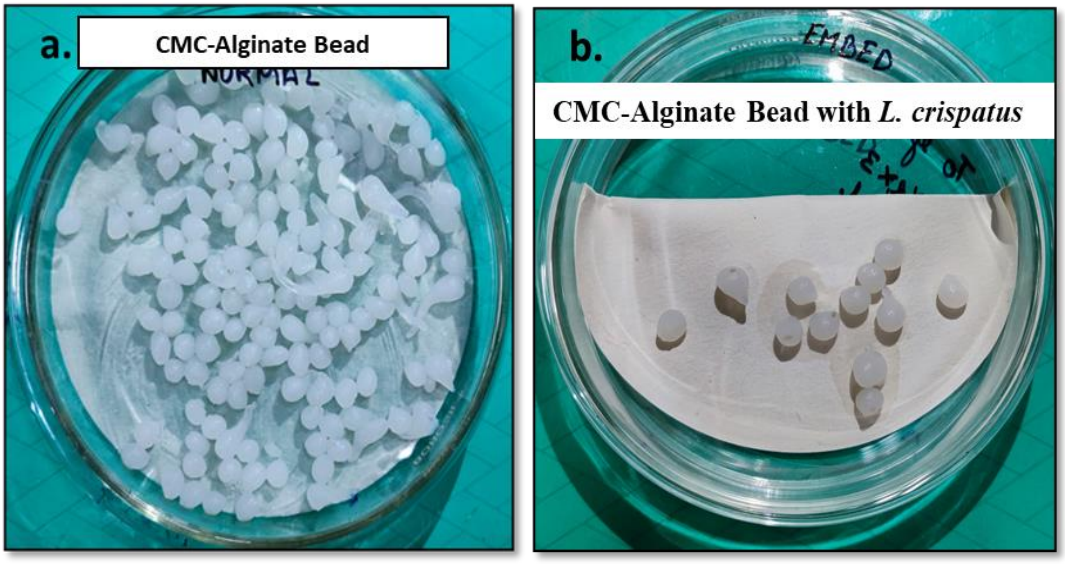
5.3. Result

Lyophilized *L. crispatus* in Medium A was grown at an interval of 3 months for a time period of 2 years. The isolate could easily grow in *Lactobacillus* MRS broth at 37 ℃ within 24-48 h of incubation. Gram's Test was done to visualize the bacterial cells. The bacteria post 48 h had ~2.1×10^7 number of cells per 100 µl (Fig.45).

Fig 45: Gram's test of 24-month-old lyophilized *L. crispatus* post growth

CMC- Alginate beads CB and PB showed similar spherical to oblong shape. Post hardening in $CaCl_2$ solution for 30 mins, the bead size ranged from 0.3-0.6 mm. Post preservation at -20℃, the beads size shrunk ranging from 0.2-0.3 mm. The surface of the beads prior to preservation was smooth, which post storage had turned rough (Fig 46).

Bead CB and PB absorbed PBS and swelled about 30% in their weight, showing their capacity to absorb solvents. After 8 h the beads reached their maximum absorbency (Table 16.a.). Beads were swollen for 6 h and kept at room temperature (25-28 ℃) for deswelling, after 36 h the beads reverted to their original shape (rough and wrinkly) and weight (Table 16.b.). The bead PB showed revival of *L. crispatus* within 48 h of incubation at pH 7 and pH 4 with ~ 2.4×10^8 number of cells. Gram test was done to confirm the growth (Fig 47). The EDX of both beads showed similar amount of oxygen, calcium, and antimony; but the amount of carbon was higher in bead PB due to the presence of bacterial cells (Fig. 48). Bead CB showed no growth in *Lactobacillus* MRS media post 48 h incubation at 37 ℃. SEM of entire and cross sectioned bead PB at 30x, 1000x, and 3000x showed the presence of *L. crispatus* cells embedded in the CMC-alginate bead. Bead CB at 3000x showed no presence of LAB cells (Fig. 49 and 50). The FTIR spectrum of bead CB showed the absence of any functional groups, but bead PB showed the presence of functional groups representing: carboxylic acid, aldehydes, and amino groups at (~1,600 cm^{-1}) and organic compounds and hydroxy acid groups at (~3,000 cm^{-1}) which may be due to the trapped LAB inside bead PB (Fig 51).



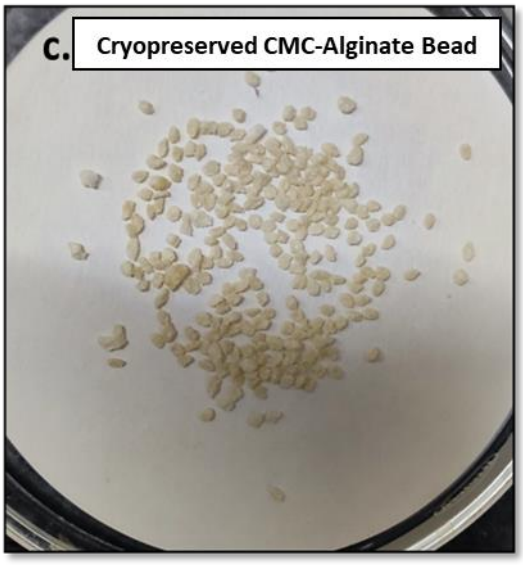


Fig 46: a. CMC-Alginate Bead (C), **b.** CMC-Alginate *L. crispatus* Bead (P), **c.** Cryopreserved CMC-Alginate Bead

Table 16: Physicochemical property of encapsulated and non-encapsulated Na-CMC Alginate beads **a.** Swelling of CMC-Alg Beads. **b.** Deswelling of CMC-Alg Beads.

h	CMC- Alginate (C)	CMC-Alginate + *L. crispatus* (P)
0	3.23 (± 0.178)	5.78 (± 0.190)
2	35.29 (± 1.15)	35.53 (± 0.636)
4	52.78 (± 0.800)	51.76 (± 0.798)
6	65.38 (± 1.185)	61.45 (± 0.606)
8	89.90 (± 0.185)	87.46 (± 0.351)

h	CMC- Alginate (C)	CMC-Alginate + *L. crispatus* (PB)
0	67.91 (± 0.456)	66.15 (± 1.059)
12	28.86 (± 1.152)	29.38 (± 0.745)
24	6.23 (±1.205)	5.7 (± 0.573)
36	3.13 (± 0.450)	3.17 (± 0.178)

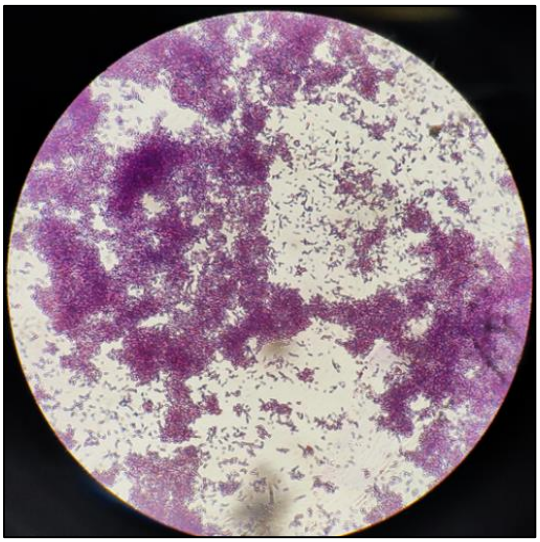
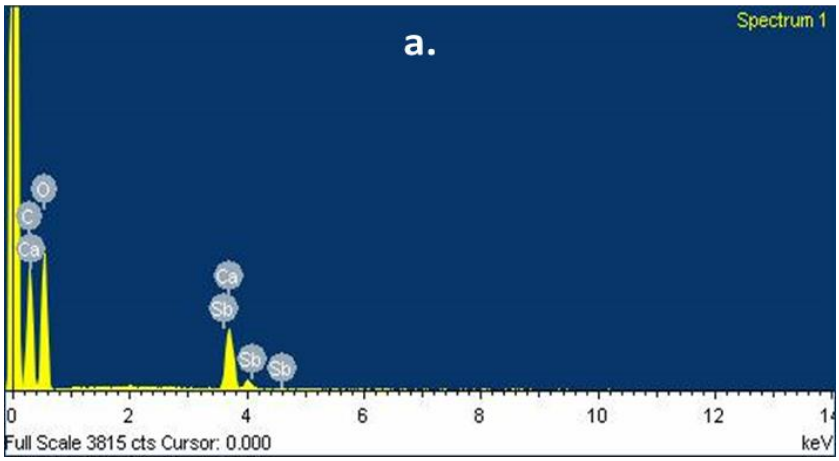


Fig 47: Gram's staining of *L. crispatus* encapsulated in Na-CMC alginate bead post growth



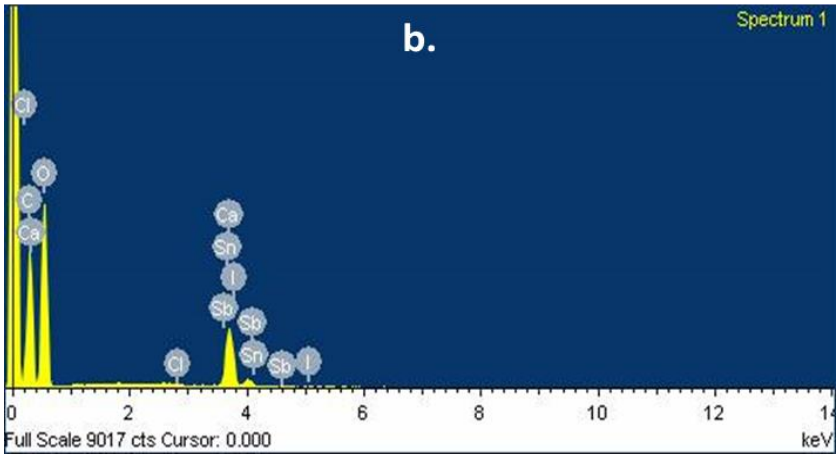
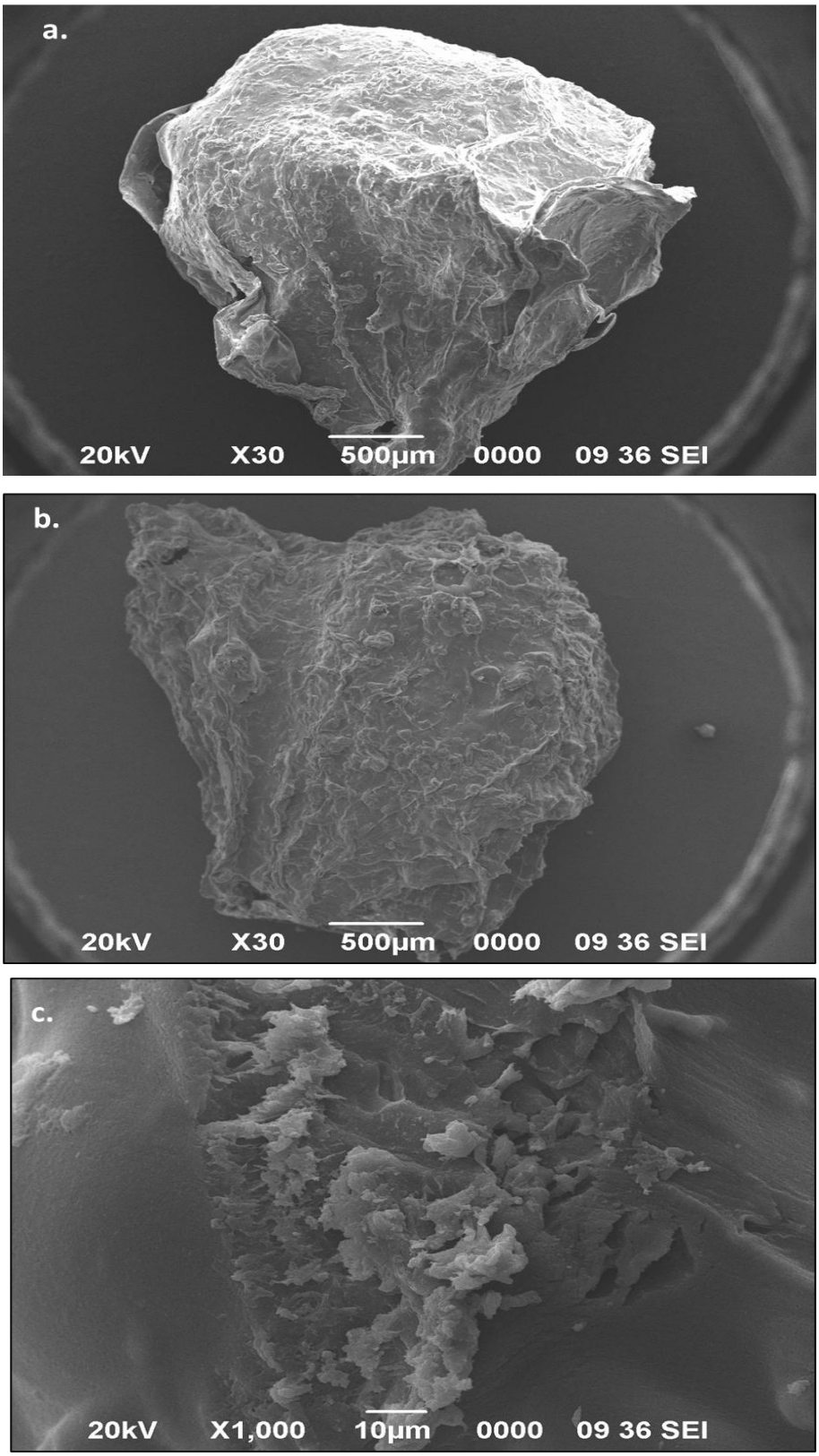


Fig 48: a. Energy Dispersive X-Ray of CMC-Alginate Bead **b.** Energy Dispersive X-Ray of *L. crispatus* CMC-Alginate Bead


a.
20kV X30 500µm 0000 09 36 SEI
b.
20kV X30 500µm 0000 09 36 SEI
c.
20kV X1,000 10µm 0000 09 36 SEI

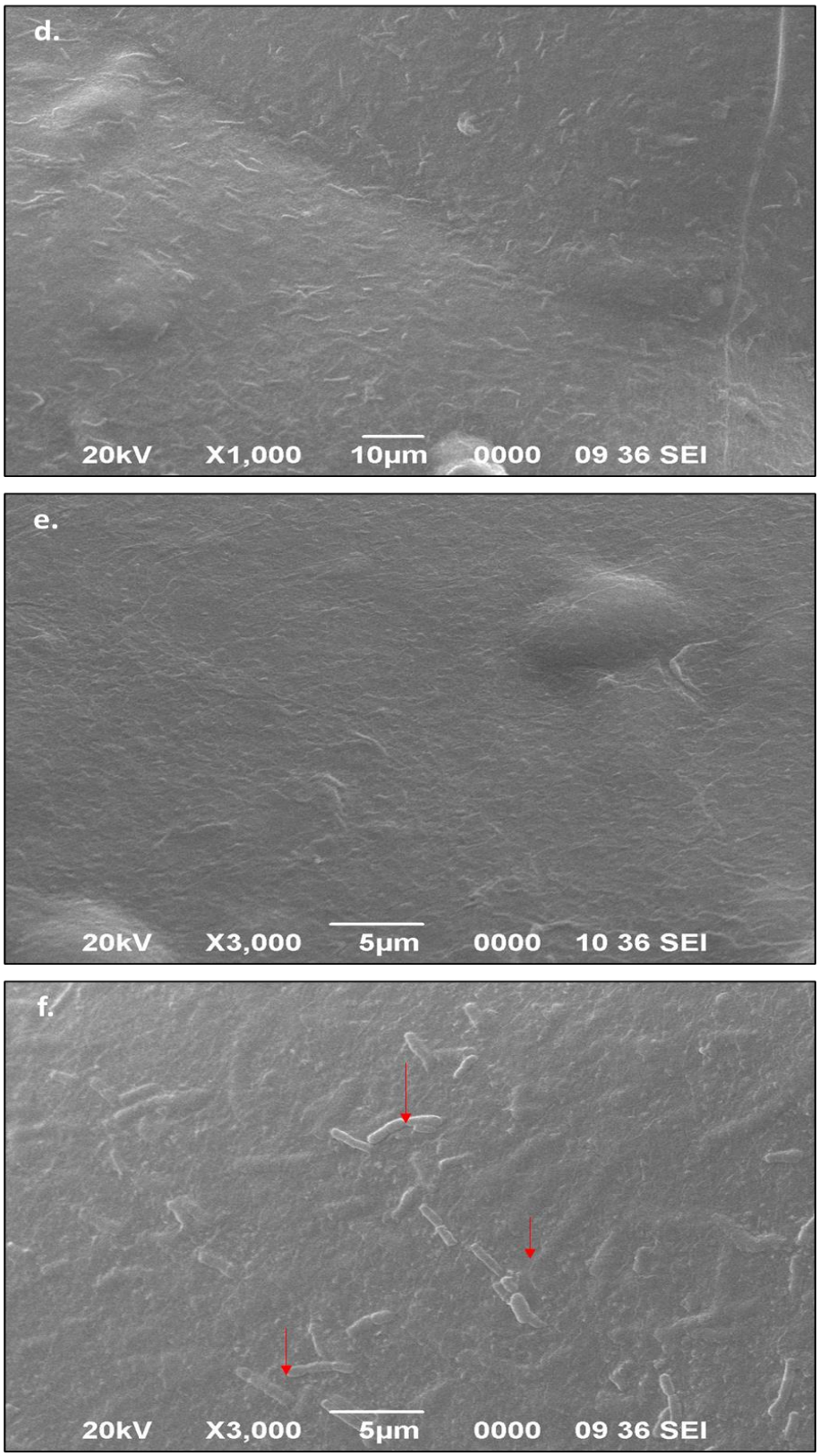
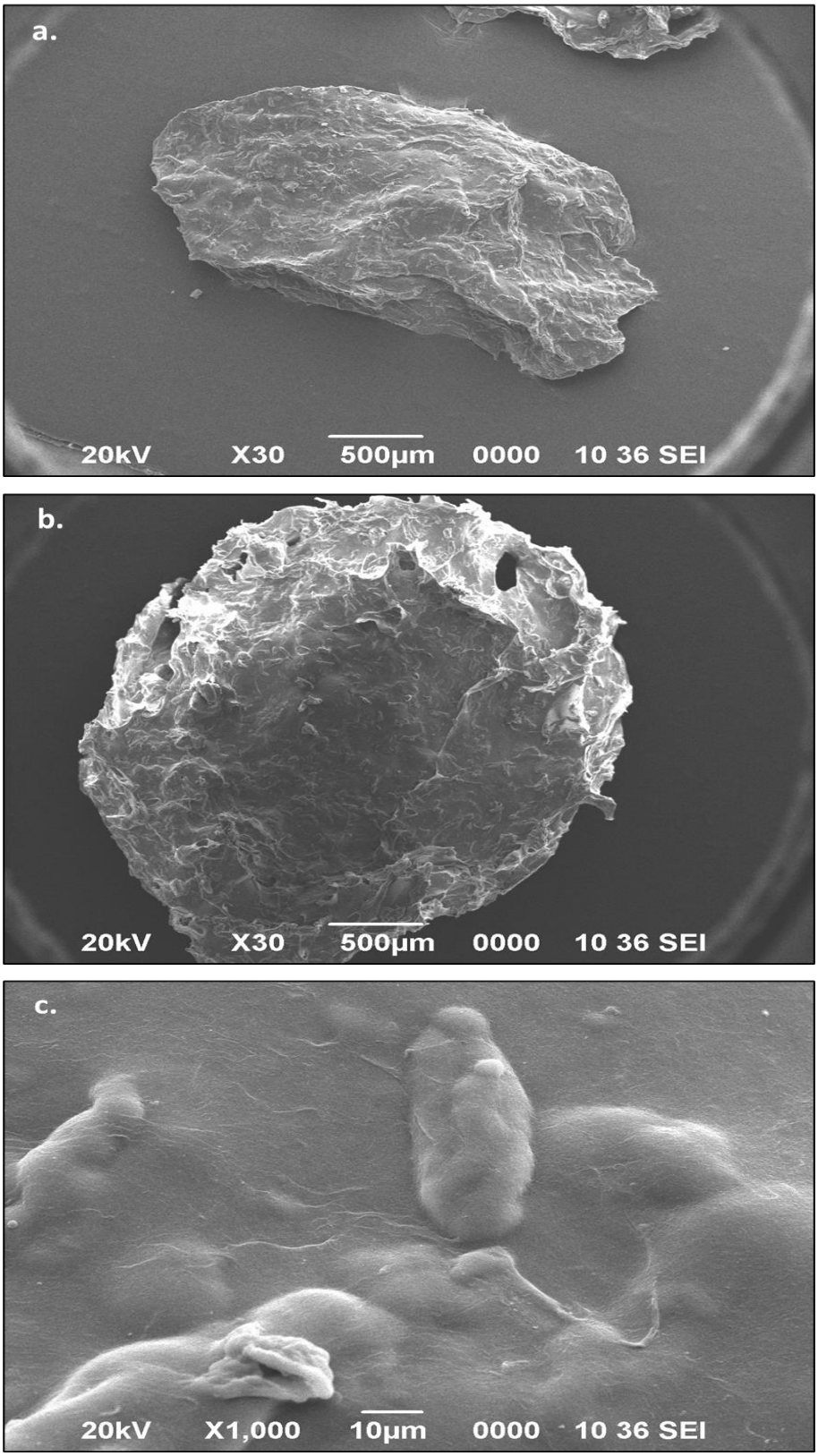


Fig 49: Scanning electron microscopy of Na-CMC-alginate bead surface

a. 30x magnification of CB **b.** 30x magnification of PB **c.** 1000x magnification of CB **d.** 1000x magnification of PB **e.** 3000x magnification of CB and **f.** 3000x magnification of PB with red arrows pointing the *L. crispatus* cells.


a.
20kV X30 500µm 0000 10 36 SEI
b.
20kV X30 500µm 0000 10 36 SEI
c.
20kV X1,000 10µm 0000 10 36 SEI

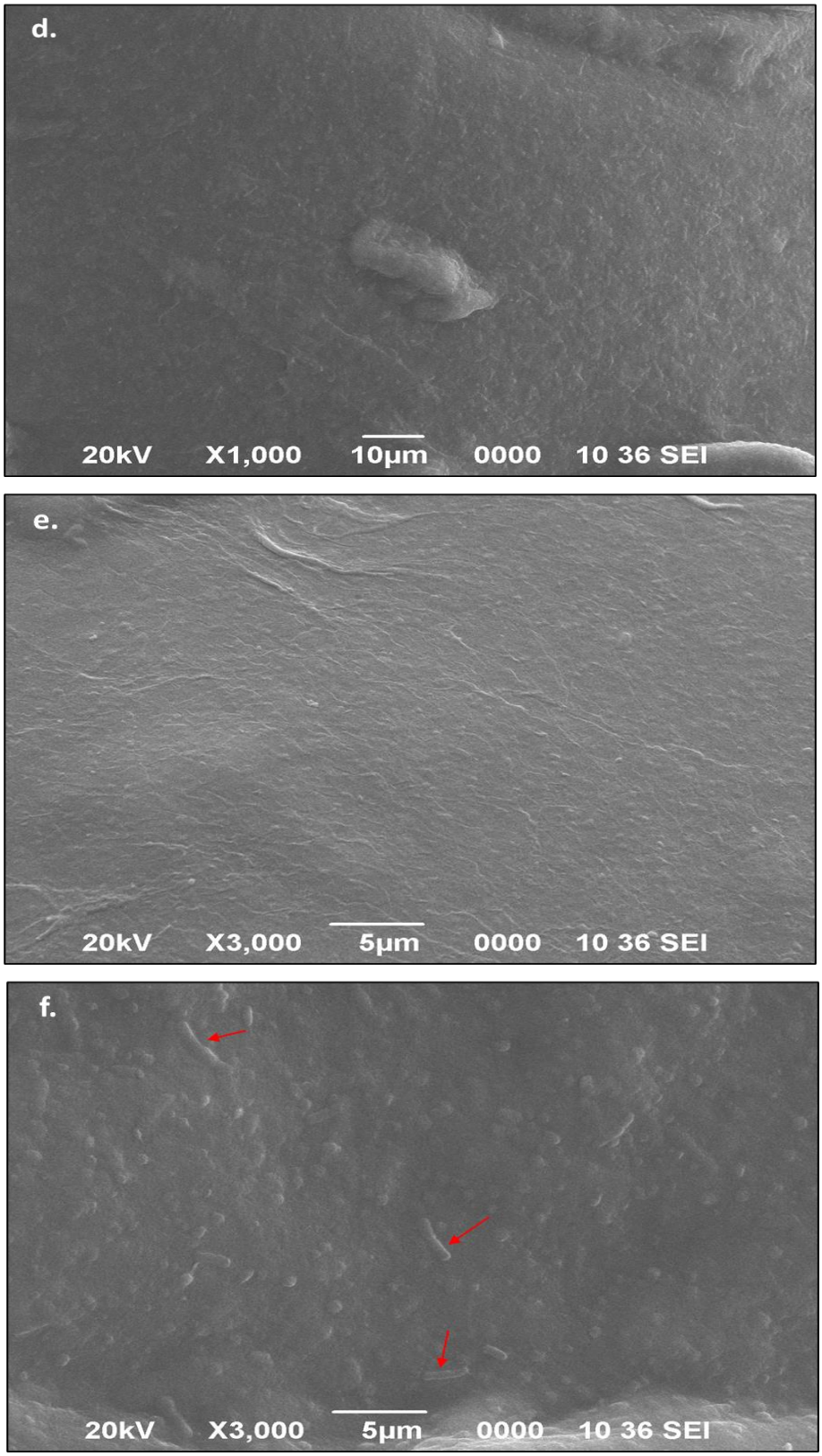


Fig 50: Scanning electron microscopy of Na-CMC-alginate bead cross section **a.** 30x magnification of CB **(b)** 30x magnification of PB **(c)** 1000x magnification of CB **(d)** 1000x magnification of CMC-Alg PB **(e)** 3000x magnification of CB and **(f)** 3000x magnification of PB with red arrows pointing the *L. crispatus* cells.

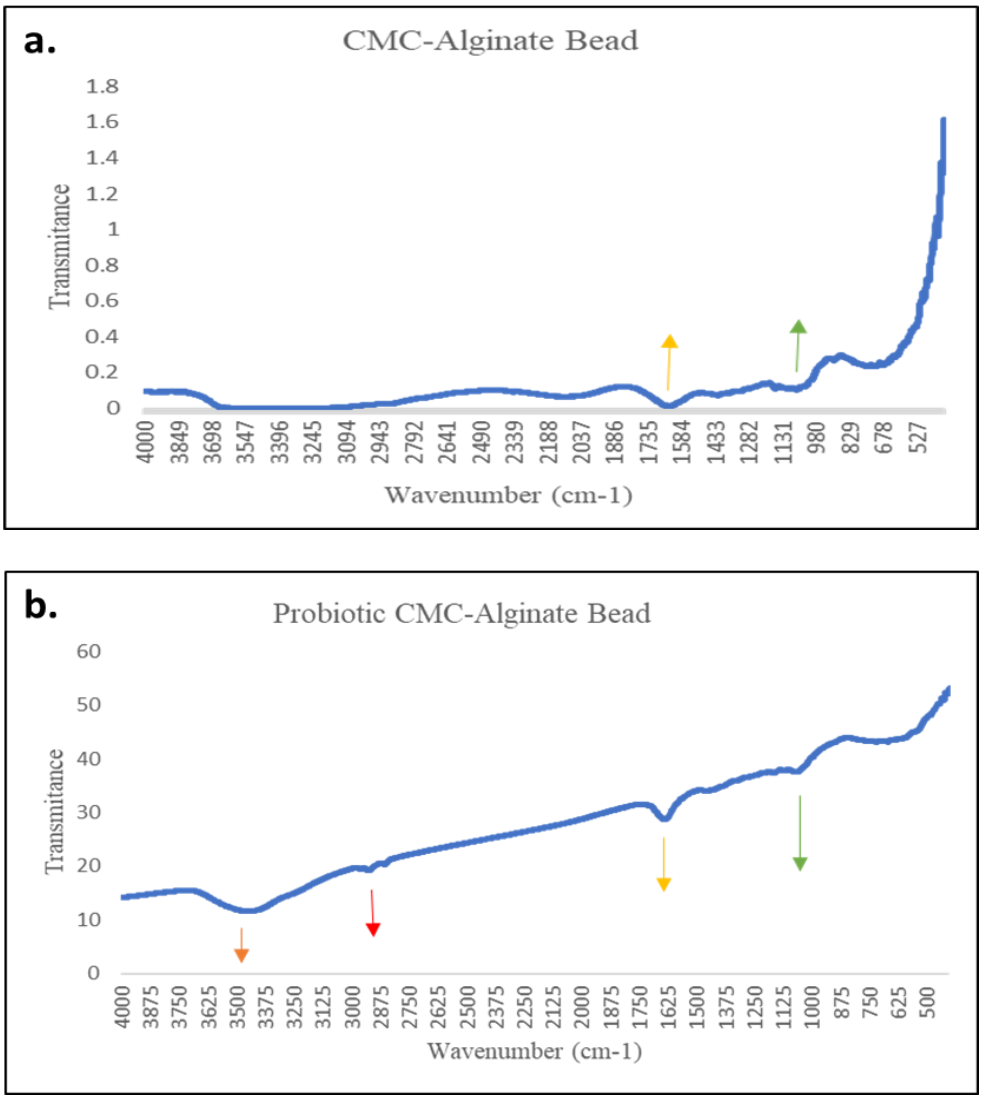


Fig 51: Fourier Transmission Infra-Red of CMC-Alginate Bead. **a.** FTIR of CB, **b.** FTIR of PB.

The CFS of *L. crispatus* inhibited the pathogen consortium from over-growing. A single spray of CFS inhibited the biofilm formation of the pathogen consortia (Fig. 52). The spray of CFS at an interval of 3 hours completely inhibited the growth of any pathogen till 12 hours (Fig. 52). The treated fabric inhibited all the pathogens, showing its potential to be used as an antibacterial additive on the non-woven fabric (Fig. 53). The ZOI in mm was measured and noted on (Table 17). The SEM of non-woven fabric (treated and non-treated) showed similar view upon magnification at 30x. At 500x, the fabric showed uneven surface and swollen portions, indicating the absorption of the CFS (Fig. 54). The EDX of treated fabrics showed the presence of silicon, sodium, potassium, and titanium, which might be constituents of the CFS (Fig. 55). The FTIR plot showed a wider peak at

(~3000 and~1631 cm^{-1}) showing the presence of carboxylic acid, aldehydes, amino groups, hydroxyl acids, and organic matter in the treated fabric (Fig. 56). The test and control sample tampons showed similar absorption and retention capacities. The treated NWF however showed a lower pH of ~ (4-5) (Table 18).

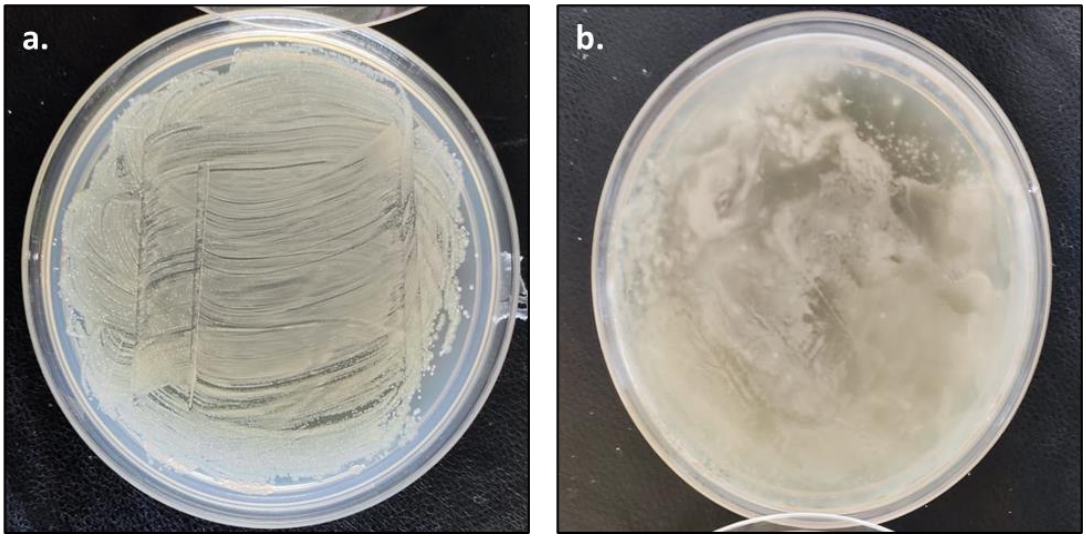
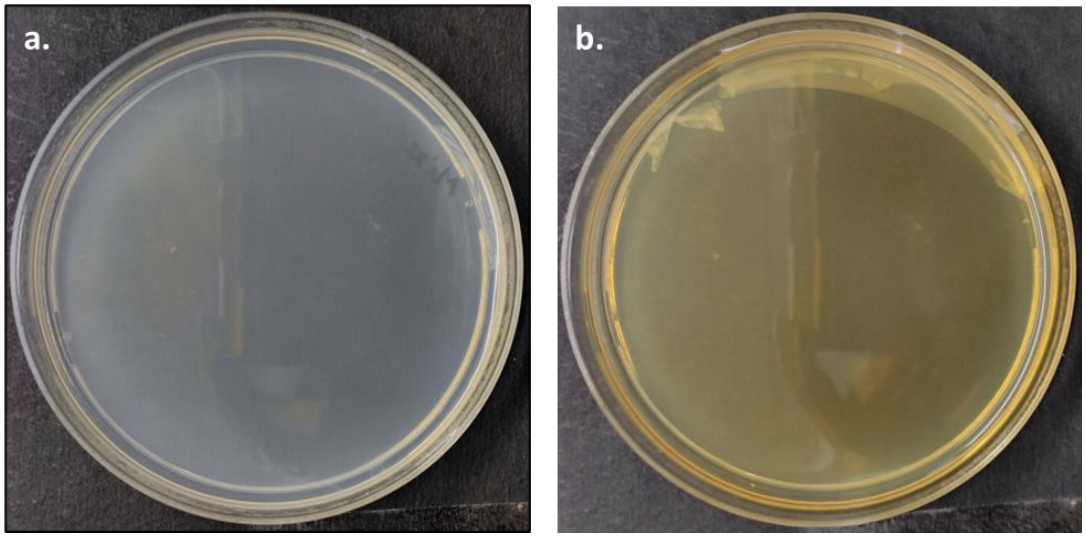
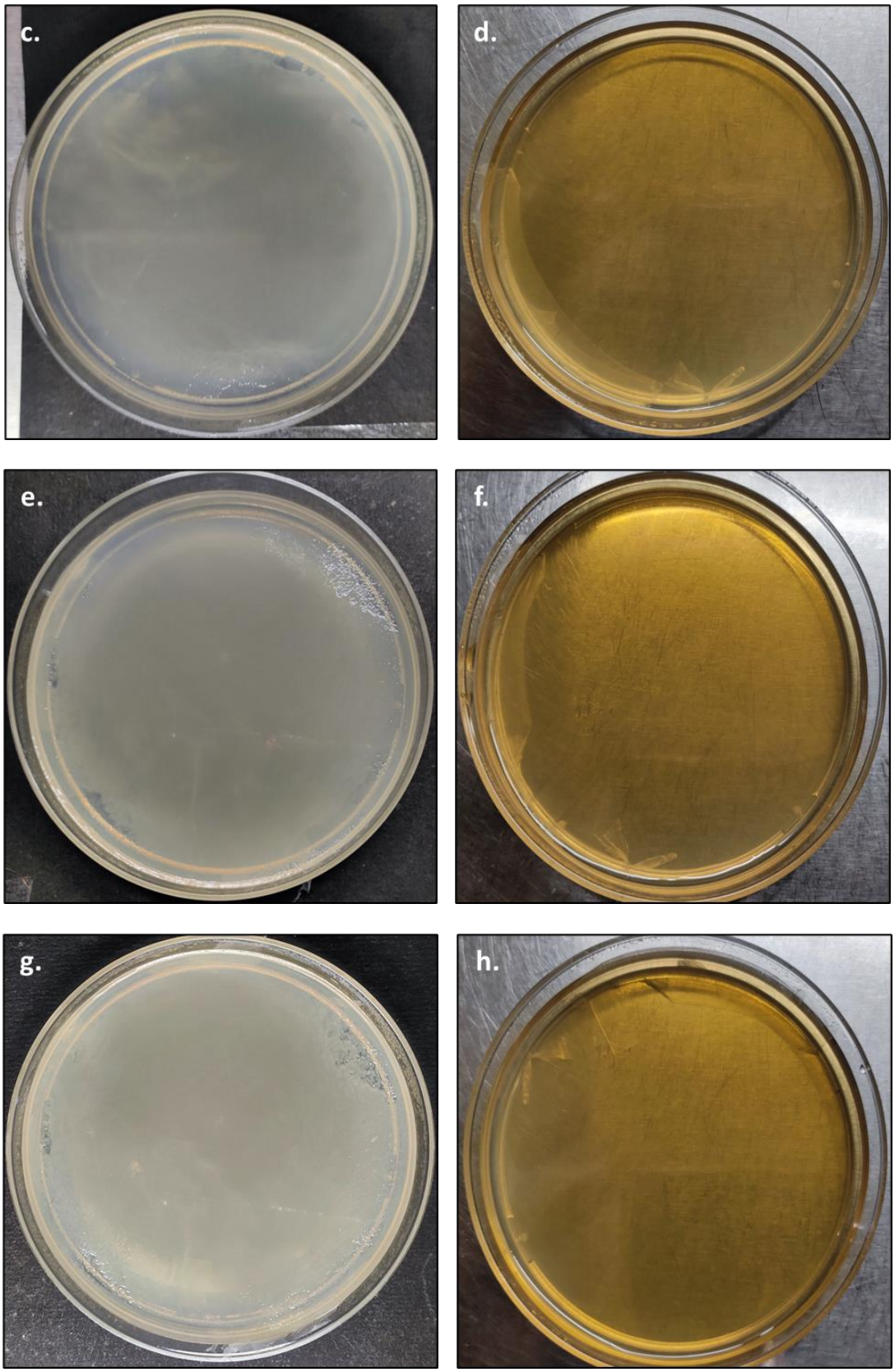


Fig 52: Growth of potential bacterial pathogen consortia post single spray of CFS **a.** Treated **b.** Untreated




c.
d.
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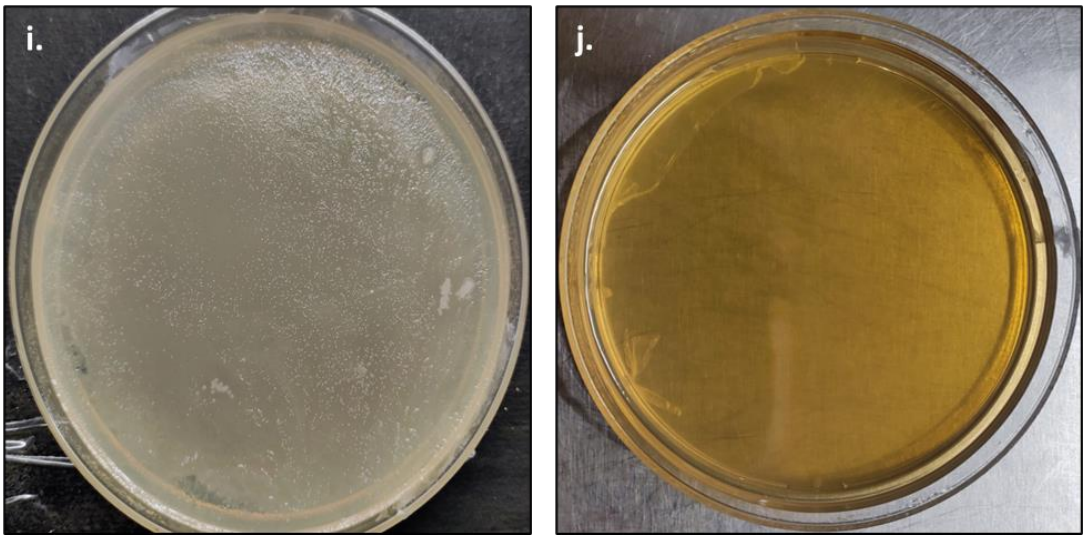
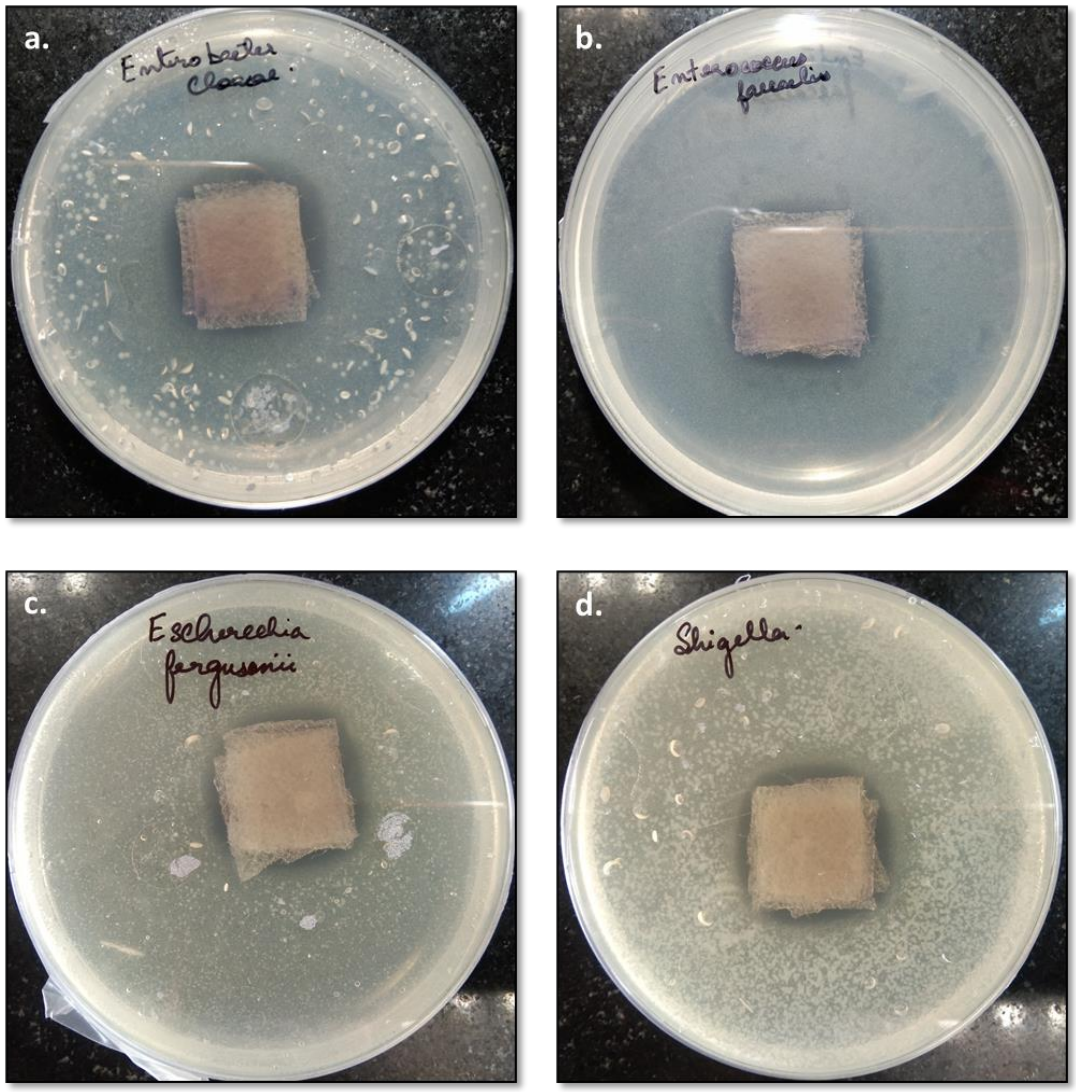


Fig 53: Growth of potential bacterial pathogen consortia with regular spray of CFS **a.** 3 hr Untreated **b.** 3hr treated **c.** 6hr Untreated **d.** 6hr Treated **e.** 9hr Untreated **f.** 9hr Treated **g.** 12hr Untreated **h.** 12hr Treated **i.** 24hr Untreated **j.** 24hr Treated



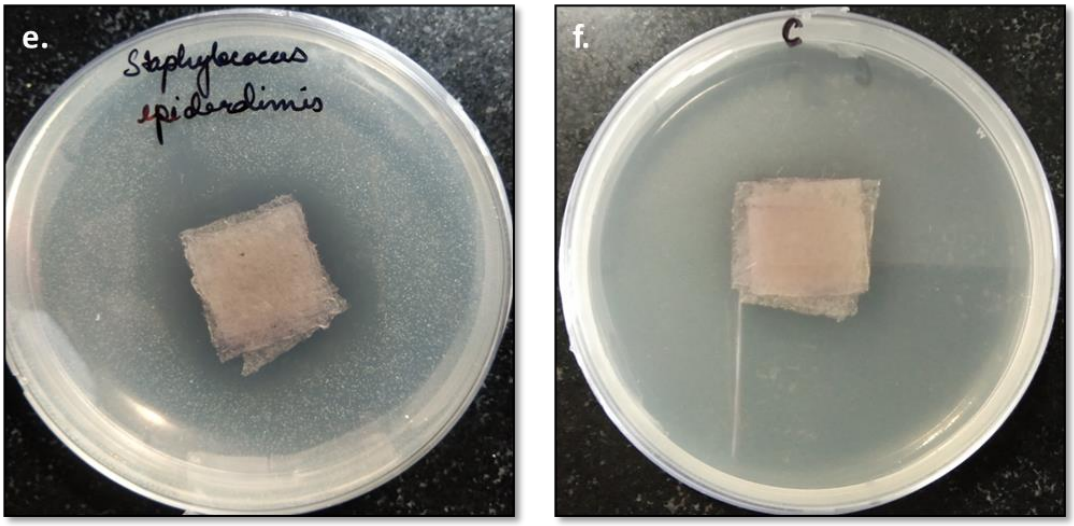


Fig. 54: Inhibition of potential urogenital pathogens by *L. crispatus* CFS treated non-woven fabric **(a)** *E. cloacae,* **(b)** *E. faecalis,* **(c)** *E. fergusonii,* **(d)** *Shigella sp.,* **(e)** *S. epidermidis,* **(f)** Control.

Table 17: Zone of inhibition (mm) of CFS treated non-woven fabric on potential bacterial pathogens

	CFS NWF (mm)	
	L	B
E. cloacae	0.7	0.6
E. fergusonii	0.7	0.5
E. faecalis	0.5	0.4
S. epidermidis	1.3	1.2
Shigella sp.	0.7	1

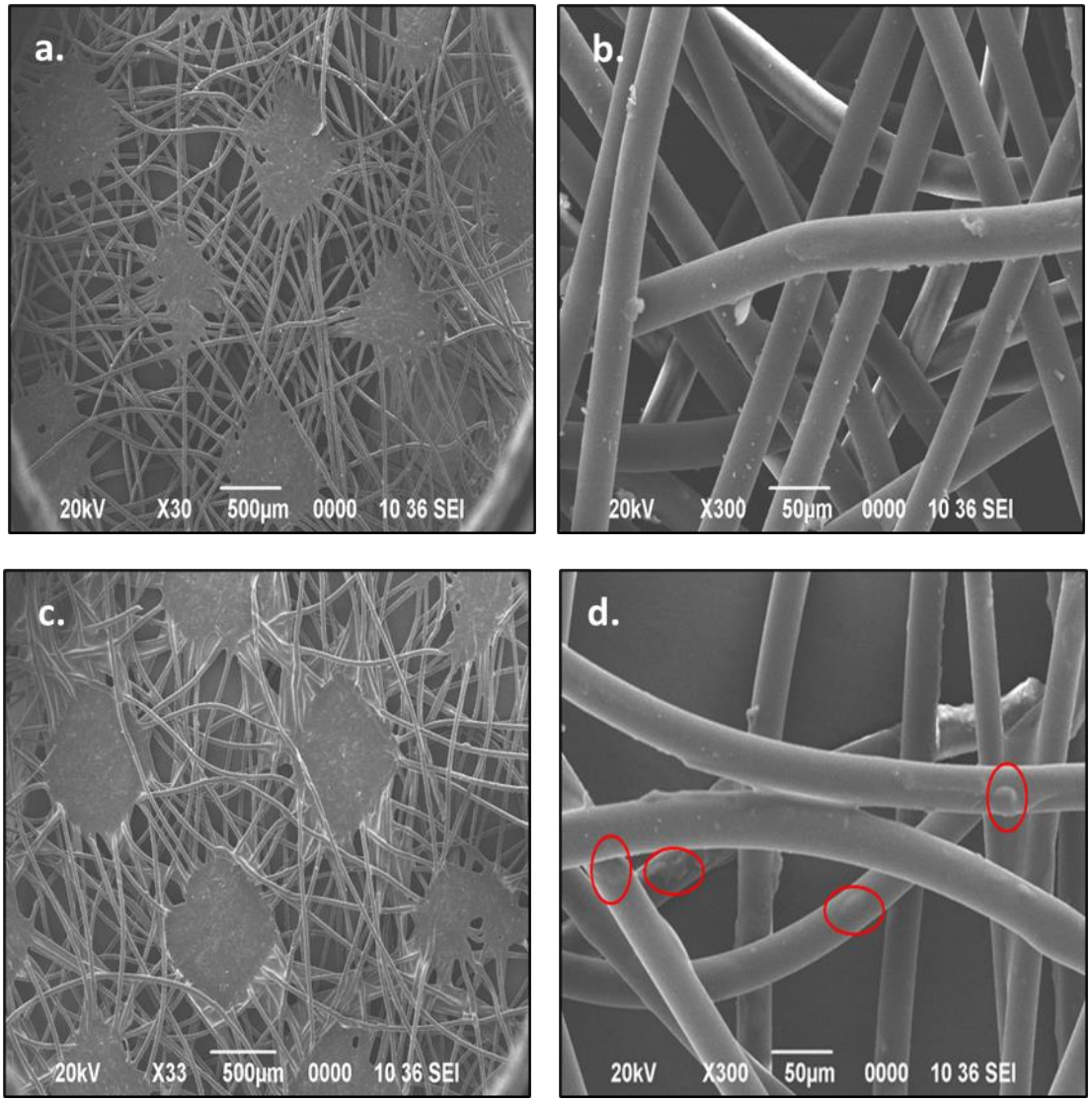


Fig 55: Scanning Electron Microscopy of non-woven fabric **a.** 30x magnification of non-woven fabric, **b.** 300x magnification of non-woven fabric, **c.** 30x magnification of non-woven fabric treated with CFS, **d.** 300x magnification of non-woven fabric treated with CFS with marked area of absorption.

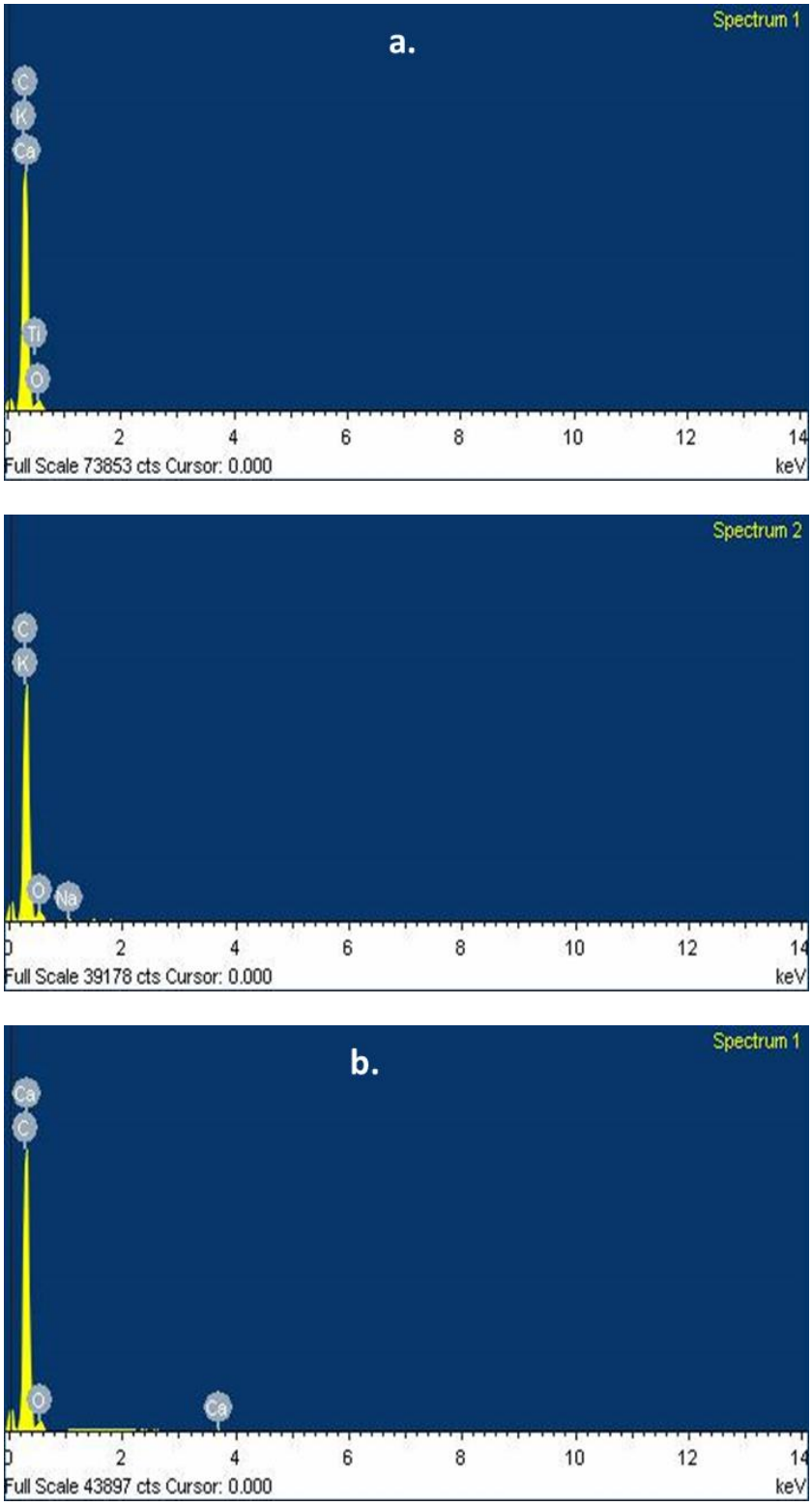


Fig 56: Energy Dispersive X-Ray non-woven fabric **a.** CFS treated non-woven fabric **b.** non-woven Fabric

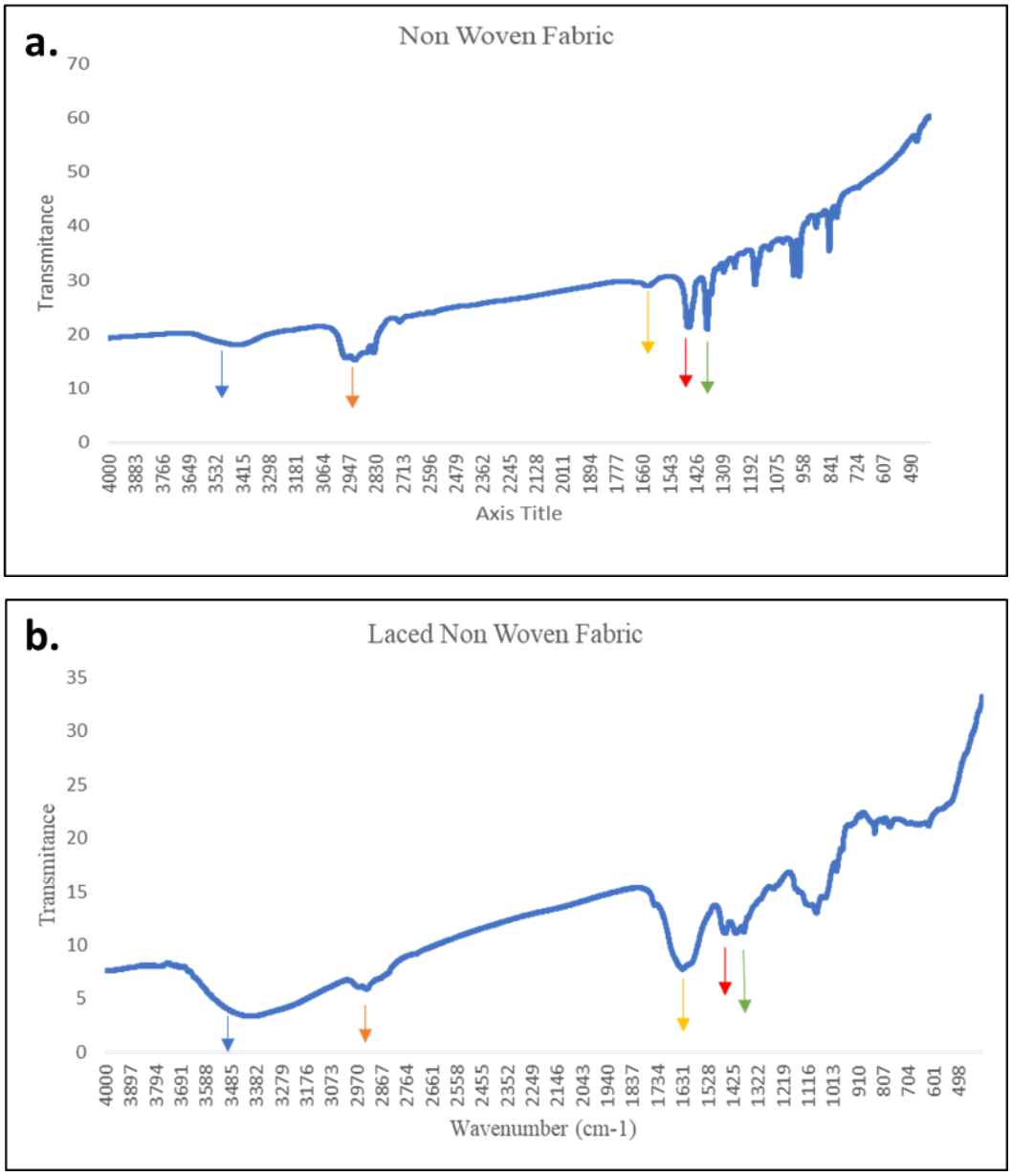


Fig. 57: Fourier Transmission Infra-Red of non-woven fabric **a.** non- woven fabric, **b.** CFS treated non-woven fabric

Table 18: Physicochemical property of treated and untreated non-woven fabric.

a.

Physicochemical	Normal Tampon	Treated Tampon
Maximal Water Retention	0.46 µl (± 0.152)	0.20 µl (± 0.051)
Minimal Water Retention	7.38 ml (± 0.374)	7.40 ml (± 0.479)
Maximal Mass	11 gm (± 0.143)	10.86 gm (± 0.283)
Minimal Mass	5.9 gm (± 0.323)	6.7 gm (± 0.718)
pH	6.69 (± 0.161)	4.36 (± 0.1)

b.

Blood	Normal	Treated
1ml	15	15
2 ml	15	15
3 ml	15	20
4ml	15	20
5 ml	20	25
6ml	25	25
7ml	25	30

5.4. Discussion

Probiotic that improves digestive problems and inflammation in the GI tract are abundantly available in the market in comparison to vaginal probiotics [96]. The Indian market has a range of probiotics without vaginal specific *Lactobacillus* strains. Yeast Guard (*Bacillus coagulans*), Garden of Life RAW Probiotics Vaginal Care (*Lactobacillus* sp and *Saccharomyces* sp), Culturelle Women's 4-in-1 Protection (*Lactobacillus*

rhamnosus GG), Ritual Synbiotic + Probiotic Capsules (*Bifidobacterium animalis* ssp. Lactis and *Lactobacillus rhamnosus*), HUM Nutrition Private Party Probiotic Capsules (*Lactobacillus rhamnosus, Lactobacillus acidophilus,* and *Lactobacillus reuteri*), Klean Athlete Probiotic Capsules (*Bifidobacterium longum* and *Lactobacillus rhamnosus*), Bio-Kult Pro-Cyan (*Lactobacillus acidophilus* and *Lactobacillus plantarum*), RenewLife Women's Probiotic Capsules (*Lactobacillus reuteri* and *Lactobacillus rhamnosus*), Ora Organic Lady Bugs Prebiotic and Probiotic Capsules (*Lactobacillus acidophilus* and *Lactobacillus plantarum*), Seed DS-01 Daily Synbiotic (*Lactiplantibacillus plantarum* and *Bifidobacterium lactis*), Olly Probiotic and Prebiotic Gummies(*Bacillus coagulans*), Women Multiculture Probiotic Supplement by Florajen- with strains of (*L. Acidophilus* LA-14, *L. Acidophilus* NCFM, *L. Rhamnosus* HN001) are few examples. Vaginal *Lactobacillus* specific probiotic is rarely found in the market: Good Down There (*L. crispatus, L. gasseri, L. jenseni,* and *L. rhamnosus*), Nutricelebrity Nutriflora Probiotics for Women (*L. crispatus, L. gasseri, L. acidophilus, L. acidophilus, L. rhamnosus, and Bifidobacterium lactis*) and Azo Complete Feminine Balance Daily Probiotic (*L. crispatus, L. gasseri, L. jenseni*, and *L. rhamnosus*) are a few examples. Probiotics available in the market show improperly identified microbial strains with weak revival capacity of the organism [594, 595].

The vaginal LAB population increases in abundance when women are ovulating or pregnant [407]. However, during menstruation, hormonal shifts in women change the microbial flora of VC to have less population of LAB [567, 596]. As a result, there is an increasing risk of infection during this period. Encapsulation and lyophilization techniques have been regularly used to preserve the probiotics in a dormant state for prolonged industrial usage [597, 598]. The lyophilized LAB had a revival potency of up to 24 months. In this regard, the LAB in their lyophilized form might be used in sanitary products or as push-in tablets to inhibit urogenital infections[599, 600]. This would not just discourage potential pathogenicity during menses but also help in repopulation of LAB in VC. *L. crispatus* encapsulated CMC-alginate bead displayed both solvent absorptive capacity and bacteria revival potential. These beads with *L. crispatus* encapsulation can be employed in sanitary suppositories to provide better protection from pathogens and enhance the absorptive capacity of the suppositories [580, 601]. Alternative to super-absorbent polymer, these beads are an environment friendly and healthier choice [602].

Products of postbiotic blends against vaginal infections are rarely available in the Indian market [603]. The only well-known vaginal care product available in the market is vaginal wash which has a detergent base. This product has been widely used by the general population since the last decade. But high amount of lauryl sulfate in the product may cause harmful effects on the vaginal soft tissue and natural beneficial microflora [604, 605]. These products discourage the overall growth of any microorganism on the vaginal microenvironment and their use is not recommended when the VC has inflammation.

The CFS produced by the LAB may be used as a vaginal spray/ vaginal wipe for selective inhibition of potential pathogen over-growth. The proper imbibition of natural organic acid produced by the LAB provides an antibacterial property to the fabric. Thus, the CFS could be used as an additive on sanitary wipes and napkin fabrics to inhibit the growth of common potential pathogens to some extent without compromising the absorption and retention capacity of the sanitary suppositories [606, 607]. Sanitary suppositories with probiotic formulations are emerging as a new trend and will soon be adopted by the masses [590].

The use of probiotic and postbiotic formulations provide an economic, eco-friendly, safe approach in the bio-medical field without any hazardous outcomes [527] and thereby needs a wider acceptance by the local population of developing countries to impart an extra edge of health benefit to the women population.