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# Emulsifier-modified sunflower oil-sunflower wax oleogel as growth modulator of probiotics

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ABSTRACT

The efficiency of oleogel as an oral delivery vehicle of probiotics depends on the chemical composition and gelator used. However, the gelators, which are surfactant in nature often interact with the probiotics and alter the therapeutic outcome. Keeping this perspective in mind, here we have developed oleogel of sunflower oil containing 5% (w/w) of sunflower wax and different emulsifiers, namely Span80 (S), Tween 80(T), stearyl alcohol (SA), and Span60 (SP), and checked their influence on probiotics in-vitro. Using confocal laser scanning microscopy, it was found that adding different emulsifiers changed the length and arrangement of the gelator network. SA and SP-modified oleogels, used at a concentration of 0.05% (w/v), demonstrated enhanced growth and metabolic activity of *Lactiplantibacillus pentosus*, which was employed as a model probiotic. Furthermore, the mucin adhesion test and scanning electron microscopy confirmed the negligible effect of those oleogels on the activity and morphology of the probiotic, respectively. When the secretome of such probiotics was applied to the colonic cell line, no negative effects were seen. This study implied that sunflower oil-sunflower wax oleogels modified using different emulsifiers can modulate probiotic growth.

## 1. Introduction

Probiotics have many benefits and have long been used for various medical conditions and general wellness. According to the Food and Agriculture Organization (FAO) and World Health Organization (WHO), probiotics are defined as living microorganisms whose consumption in adequate amounts can confer a number of health benefits. Probiotics are known for the enhancement of immunity [1], management and stabilization of gut mucosal barrier, lactose tolerance [2], prevention of diseases in the upper gastrointestinal tract (GIT) and coronary heart disease, prevention of colon cancer and usage in antibiotic resistance [3]. Many different kinds of probiotics are available, including *Lactobacillus, Bifidobacterium, Streptococcus,* yeast, and molds. Many of them are either anaerobic or facultative. Among them, Lactic acid bacteria (LAB) is a broad group of probiotic bacteria consisting of Lactobacillus species and Bifidobacterium species [4]. Most of these species are part of a normal gut microbiome; however, under diseased conditions, they often become outnumbered in the gut by other microbial species, or they change their biological role. Under such conditions, the external application of these microbes (probiotics) via the oral route is often found effective for treating the ailment. Presently probiotics are being offered as nutraceuticals in various forms, including, capsules, tablets, beads or powders [5], dairy and non-dairy drinks, dietary supplements, and infant formula [6]. However, the probiotics' performance depends on the preparation methods of the formulations (processing conditions and parameters) and the nature of the formulations. The processing of the formulation can seriously affect the viability of the probiotics as well as their efficacy [7]. The processing operations include drying, freezing, thawing, etc. For example, although spray drying is the most economical for processing probiotics, it leads to loss of viability due to high temperature, mechanical stress, dehydration, and osmotic stress [8]. On the other hand, the nature of the formulation itself has a crucial influence on the performance of the probiotics. The effectiveness of the probiotics hugely depends upon their storage and transport through the GIT, in

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which the formulation plays an important role. A good formulation ensures high on-shelf stability of the probiotics and ensures a directed delivery of the probiotics to the distal part of the GIT-like colon by protecting it from the harsh chemical environment of the early compartment of the GIT during transit. Presently a number of formulations of probiotics like yogurt, fermented drinks, capsules, milk, and viable spores are available in the market. Their performances are limited either because of the loss of viability/functionality during processing and storage or because of the non-protective nature of the formulation that causes loss of viability/functionality in the GI tract after administration. Globally scientists are looking for an advanced formulation for probiotics that can surmount the aforesaid limitation. Recently oleogel have emerged as a new kind of delivery system for drugs and therapeutics.

Oleogels are semi-solid systems where self-assembled networks of gelators physically immobilize continuous liquid phases. Structuring vegetable oil through oleogelation is a highly explored field where gelators self-assemble into a network that physically immobilizes liquid oil, effectively creating a gel that provides solid fat-like properties to edible oils [9]. Through this method, the formulated oleogels provide functional and textural properties to the oil and will be a healthier replacement for solid fat. Solid fat are continuously being replaced by food scientists across the globe in many food products like spreads, confectionery, and baked foods [10]. Since oils are not degraded in the stomach, and the oleogels structure can create a low oxygen environment by preventing gaseous transport, we hypothesized that an oleogel composed of oil and food-grade surfactants may serve as a good system for facultative probiotics. Based on this hypothesis, we have designed sunflower oil-based oleogel and checked its effect on the probiotics. Sunflower-derived fat (oil and wax) and fat-based formulations like spreads have been used for dietary consumption. Sunflower oil (SO) is widely preferred in diets and has gained importance due to the high content of oleic and linoleic acid that helps reduce cholesterol and prevent heart diseases [11]. Sunflower wax (SW) has been reported as an efficient oleogelator, which can form an oleogel with edible oils at a very low concentration. It comprises of wax esters (C38–C54, saturated, 66–69%), fatty acids (12–16%), fatty alcohols (11–13%), and hydrocarbons (6–7%) [12]. It has been observed that the architecture of the oleogel often impacts its thermal, mechanical, and other properties. The surfactant plays a crucial role in structuring the oil and modulating the physical nature of oleogels by influencing gel strength, which is further affected by factors like polymer molecular weight, solvent polarity, temperature, and surfactant concentration [13]. Here we have chosen four different surfactants, namely Span 80 (S), Tween 80 (T), stearyl alcohol (SA), and Span 60 (SP), to explore their role on the gel structure and its subsequent impact on the probiotics. All four surfactants have widely been used in the food industry and are safe for dietary consumption under permissible limits.

Here we have taken *Lactiplantibacillus pentosus (L.pentosus*) as a model probiotic to test our hypothesis. *L.pentosus* is a promising probiotic due to its strong growth and survival in harsh gastro-intestinal conditions, including low pH (1.5), high bile salt levels (up to 4%), and nitrate presence. It can auto-aggregate, co-aggregate with pathogens, adhere to epithelial cells, form biofilms, and ferment lactose and various prebiotics, supporting its resilience and probiotic potential [14]. We first checked the physicochemical characteristics of these formulations using different techniques like microscopy and Raman spectroscopy. Later we evaluated the effect of these oleogels on the probiotics. For this purpose, the probiotics were treated with different oleogels, and the viability and growth kinetics were measured. SEM and mucin adhesion also was done to observe the structural variation and adhesion ability respectively. The metabolic activity was tested through MTT, and metabolic growth. Finally, to understand the overall beneficial effect of the formulation, the secretome of the probiotics treated with oleogels was collected and administered to the colonic cell line, and its impact on the colonic cell line was measured in terms of cell viability, morphology, and ROS generation.

## 2. Materials and methods

### 2.1. Materials

Commercial refined Sunflower oil (SO) (Fortune sunlight, Kutch, Gujarat, India), used for the oleogel preparation, was purchased from the local supermarket. Sunflower wax (SW) pellet was bought from Vijay Impex, Chennimalai, Tamil Nadu, India. Span80, pure stearyl alcohol, and sorbitan monostearate (Span60) were purchased from Loba Chemie Pvt. Ltd., Mumbai, India. Tween80 was bought from Himedia Laboratories Pvt Ltd., Mumbai, India, respectively. De Man, Rogosa, and Sharpe (MRS) agar and MRS broth were purchased from Titan Biotech Ltd. Dulbecco's modified eagle's medium (DMEM), antibiotic/antimycotic solution, Dulbecco's phosphate buffer saline (DPBS), fetal bovine serum (FBS) and MTT assay kit, was procured from Himedia, India.

### 2.2. Oleogel synthesis and characterization

#### 2.2.1. Oleogel synthesis

Oleogel of SO containing 5% (w/w) of SW was synthesized as per the previously mentioned protocol [15]. Four different emulsifiers, i.e., Span80 (S), Tween80 (T), stearyl alcohol (SA), and Span60 (SP), were chosen, and their respective stocks (0.1% w/w) were prepared in SO (Table 1). The final concentration of 0.015% w/w of emulsifier S and T and 0.05% w/w of emulsifier SA and SP were used according to the previously optimized data [16].In brief, precisely weighed SO and SW was taken in a beaker placed in a water bath (80 °C) for 20 min. The mixture was heated until all the wax was dissolved in the oil, and a homogeneous solution was formed. This clear solution was then kept at room temperature (25 °C) in the thermal cabinet for 30 min to ensure oleogel formation.

#### 2.2.2. Analysis of chemical composition of fatty acids through gas chromatography-mass spectrometry (GC–MS)

The raw material used for oleogel synthesis, i.e., SO and SW, were analyzed through GC–MS (Agilent Technologies GC 7890B - MS 5977A) for compositional information following esterification. For this, 0.5 g oil was combined with 5 mL of n-heptane in a 10 mL glass tube. Next, 200 μL of 2 M potassium hydroxide in methanol was added, and the mixture was shaken for 20 ss. The upper phase was then separated and analyzed by GC on a fused silica capillary column (100 $m \times 0.25$ mm $\times$ 0.2 μm) with an FID detector (Agilent 7890A). GC conditions for were as follows: injection volume, 1 μL; temperature program, 175 °C for 10 min, increase at 5 °C/min to 210 °C, then to 230 °C, holding at 230 °C for 15 min; detector temperature, 260 °C; injector temperature, 250 °C; carrier gas, $N_2$ at 1 mL/min; split ratio, 1:20; total run time, 58.5 min.

#### 2.2.3. Analysis of the microstructure of oleogels through confocal laser scanning microscopy

The microarchitecture of the oleogels was analyzed through confocal laser scanning microscopy (CLSM) (TCS SP8, Leica, Wetzlar, Germany). For staining the oleogels, 0.1% w/w of floral yellow 088 dye was added to the molten oleogels and placed on the coverslip. The samples were

**Table 1**
Composition of oleogles.

| Samples | SW (g) | SO (g) | Emulsifier Stock (g) |
|---|---|---|---|
| Control | 1.0 | 19.0 | 0.0 |
| S3 | 1.0 | 16.0 | 3.0 |
| T3 | 1.0 | 16.0 | 3.0 |
| SA10 | 1.0 | 9.0 | 10.0 |
| SP10 | 1.0 | 9.0 | 10.0 |

excited at a wavelength of 488 nm, and images were acquired at 515 nm. The size of the fiber structure was quantified through the ImageJ software

#### 2.2.4. Analysis of unsaturation in oleogels through Raman spectroscopy

Raman spectroscopy (LabRam HR, Horiba Jobin Vyon) of the oleogels was performed to confirm the unsaturation. The analysis was carried out by irradiating the oleogels with a laser of wavelength 633 nm, and the spectra were obtained in the range 200–3000 $cm^{-1}$.

#### 2.2.5. Analysis of thermal properties of oleogels using differential scanning calorimetry

The melting and crystallization capacities of the oleogels were evaluated using a differential scanning calorimeter (200 F3 DSC, Maia, Netzsch, Burlington, MA, USA) at a thermal scan rate of 5 °C/min. A perforated lid was used to seal a pan of aluminium with around 10 mg of oleogel for this testing. The melting profiles from 0 °C to 100 °C and the cooling profiles from 100 °C to 0 °C were recorded using a single program for each oleogel. The oleogels were kept at 100 °C for five minutes in the intervals between each run.

### 2.3. Study of the effect of oleogels on properties of L.pentosus

#### 2.3.1. Bacterial strain and growth environment

Probiotic *L.pentosus* was taken for the study. The strain was cultured in de Man Rogosa and Sharpe (MRS) broth (TM Media, Titan Biotech Ltd) or agar in aerobic conditions at 37 °C [17]. The culture was also stored in 20% glycerol at −80 °C for long-term storage. Apart from the control formulation, broth was taken in the studies for better comparative analysis. Broth and TCP were referred to MRS broth and DMEM media, respectively.

#### 2.3.2. The effect of oleogels on the survival and growth of L.pentosus

The effect of oleogels on the growth of probiotics was evaluated for 24 h at 37 °C under aerobic conditions. The overnight grown strain in broth culture was taken for the study. L.*pentosus* with an initial optical density of 0.01 were treated with 1 μg/ml,100 μg/ml and 10,000 μg/ml of different oleogels. The growth was monitored in a 96-well plate every hour through optical density measurement at 600 nm for 24 h with constant shaking at 200 rpm by taking reading through a BMG LabTech Microplate reader (POLARstar Omega, Germany). A set of blank controls containing MRS media with oleogels at each tested concentration, but without microbial inoculation, was included. These controls allowed us to measure and subtract any turbidity contribution originating solely from the oleogels.

#### 2.3.3. The effect of oleogels on the metabolic growth of L.pentosus

*L.pentosus* was grown in a similar manner as described above for 16 h. Cells were then pelleted at 12,000 g for 10 min. The supernatant was discarded, and the pellet was washed twice with 100 μl PBS. Cells were pelleted again, followed by incubation in 0.5 mg/ml MTT reagent (3-[4,5-dimethylthiazol-2yl]−2,5-diphenyl-tetrazolium bromide) for 30 min at 37 °C under constant shaking at 200 rpm. Cells were pelleted and further treated with 150 μl dissolution buffer (10 % SDS in 10 mM HCl) for 20 mins in the dark. Cells were pelleted to obtain the formazon crystals, and 100 μl of supernatant was transferred into the fresh plate. The plate reading was obtained at 570 nm.

#### 2.3.4. Analysis of mucin adhesion ability of probiotics

The probiotics were grown as mentioned in Section 2.3.2, for 16 h. For the mucin adhesion study, porcine gastric mucin-II (100 μg/ml) was added into the each well of a 96 well plate and incubated at 4 °C [18]. The wells were blocked with 0.2 ml PBS supplemented with 1% Tween 20 (PBST) for 1 h. Probiotic suspensions (150 μl) were added to each well and incubated for 1 h at 37 °C. The wells were washed with PBST and treated with 0.5% Triton-X solution to desorb the bound bacteria for two hours at 37 °C. 100 μl of the suspension was then taken, and OD was measured at 600 nm.

#### 2.3.5. Morphological observation of oleogels-treated probiotics through scanning electron microscopy (SEM)

*L.pentosus* was grown for 16 h at 37 °C under constant shaking at 200 rpm. For SEM (SEM-Carl Zeiss, EDS-Oxford instruments) analysis, the treatment concentration for oleogel was 10,000 μg/ml. Post 16 h, the cells were pelleted down at 12,000 g, and the supernatant was discarded. The cells were washed and resuspended in 0.1 M phosphate buffer (pH=7.4) and placed on the glass slide coated with 0.01% agar. The cells were fixed with 2.5 % glutaraldehyde (in 0.1 M phosphate buffer) for 2.5 h, after which the excess glutaraldehyde was washed with the phosphate buffer. This was followed by cell dehydration through ethanol gradation. Before the imaging, the samples were sputter-coated with gold. Imaging was performed with SEM at an accelerating voltage of 2 kV.

### 2.4. Study of effects of probiotic cell-free supernatant on colon cells

#### 2.4.1. Collection of cell-free supernatant

The cells were grown similarly as in Section 2.3.4. After 16 h, the cells were pelleted at 15,000 rpm, and the cell-free supernatant was collected. The obtained cell-free supernatant was filtered through a 0.22 μm syringe filter prior to use with mammalian cells.

#### 2.4.2. Cell viability upon cell-free supernatant treatment

Firstly, the cell viability of the human colon adenocarcinoma cell line (HT29) upon treatment with probiotic secretome was studied. For this purpose, an MTT assay was conducted where cells were seeded in a 96-well plate at a cell density of $5 \times 10^3$. The cells were maintained in high glucose DMEM (10% FBS, 1% antibiotic/antimycotic solution) and were incubated overnight at 37 °C and 5% $CO_2$. Later the cells were treated with sterilized 10% of the secretome for 24 h. Post- incubation, MTT assay was performed as per the manufacturer's protocol. The absorbance reading was recorded at 595 nm using a multiplate reader (Multiskan Sky Thermo Fisher Scientific).

#### 2.4.3. Cell viability study using Calcein-AM staining

HT29 cells were seeded in a 96-well plate at a cell density of $5 \times 10^3$. Later the cells were treated with sterilized 10% of the secretome for 24 h. Post-incubation, cells were washed with PBS and treated with 1μg/ml Calcein-AM dye prepared in incomplete media. The cells were incubated with the dye for 30 min, and fluorescent microscopic (Axio observer 7 Carl Zeiss) images were obtained.

#### 2.4.4. Reactive oxygen species (ROS) production upon treatment of cells with secretome

ROS evaluation was done upon treatment of cells with secretome. The ROS production was estimated through the cell staining using 2,7′ dichlorofluorescein diacetate (DCFDA). Like the MTT assay, cells were incubated with 10% secretome for 24 h. Afterwards, the cells were washed with PBS and incubated with 15 μM DCFDA for 20 min in the dark. Then the cells were washed with a fresh medium and visualized under the microscope (Axio observer 7 Carl Zeiss) immediately and imaged.

#### 2.4.5. Statistical analysis

All the experiments were carried out in triplicate, and the data are expressed as mean ± *S*.D. (standard deviation). Student *t*-test was performed for the evaluation of the statistical significance of the data ($p < 0.05$).

## 3. Results and discussion

### 3.1. Compositional analysis of the fat used by GC–MS

In the present study, we have used commercially available Sunflower oil (SO) and sunflower wax (SW). GC–MS analysis was done to check the presence and relative abundance of key fatty acids and other minor components in the oil and wax. Data showed that SO and SW used contain a high percentage of β-sitosterol, which is common for SO and SW[19].The analysis also showed the presence of essential fatty acids such as palmitic, linoleic, stearic, and lignoceric acids [20]. The literature suggests the presence of different phytosterol, tocopherol, and Vitamin E, which support probiotic's health by reinforcing cell membranes. They are also known to support cholesterol metabolism, indirectly benefiting the environment in which probiotics thrive by contributing to a balanced lipid medium [20]. SO is rich in linoleic acid, an omega-6 polyunsaturated fatty acid that is metabolized by probiotics [21,22]. A list of abundant wax esters present in SW has also been shown in Table 2.

#### 3.1.2. Oleogel appearance

Oleogels composed of SO, SW, and different emulsifiers are shown in Fig. 1a as an inset of the micrographs. All the formulations used in the study form compact structures that were self-standing. The oleogel formulations, irrespective of the emulsifier type, appeared visually similar. All the formulations at room temperature appeared homogeneously opaque and white. The formulations were semi-solid, smooth and spreadable when touched with hands. Previous studies showed the appearance of similar smooth and homogeneous SW-based oleogels [23].

#### 3.1.3. Analysis of microstructure through CLSM

Understanding the microarchitecture of the oleogels is important in the context of their physical properties. It has already been noticed that use of different gelators can lead to different networks in the oleogels. Earlier studies showed that the use of different gelators like beeswax, rice bran wax, octadecanol, stearic acid, γ-oryzanol+β-sitosterol mixture, and ethylcellulose could lead to different structures and crystal networks upon gelation in SO [24]. In this study, we have tried to understand the microstructure of the formulated oleogels through CLSM using an oil-binding dye, fluorol yellow. The micrographs (Fig. 1a) of oleogels obtained through the CLSM displayed a fibrous network structure. SW crystals appeared as needle/fiber-like in the oleogel micrographs. This morphology is attributed to the presence of wax esters of SW, which are known to form a crystalline mesh in vegetable oil [25]. The oleogels (S80) formed from the incorporation of Span80 resulted in the formation of smaller crystals with an average length of 3.89 μm. However, the other oleogels showed comparable length crystals to that of control. As per the previous work, the long needle-like crystals contribute to a stronger crystalline network [26]. Interestingly, in stearyl alcohol containing oleogels (SA), the packaging of wax crystals appears more compact due to the presence of dense and longer wax crystals having an average length of 12 μm. This packaging improves the interactions among the crystals [27].

**Table 2**
Major constituents present in sunflower oil and sunflower wax.

| % Abundance | Name |
|---|---|
| 53.07 | Phenol, 2,3,5,6-tetramethyl-(Durenol) |
| 0.43 | n-Hexadecanoic acid (Palmitic acid) |
| 0.97 | 9,12-Octadecadienoic acid (Z,Z) /Linoleic acid<br>Stearic acid |
| 0.16 | Eicosane |
| 0.30 | Tetracosanoic acid, methyl ester (Lignoceric acid) |
| 1.66 | Squalene |
| 8.46 | Vitamin E |
| 1.93 | Campesterol |
| 2.45 | Stigmasterol |
| 12.80 | β-Sitosterol |
| 2.14 | α−1-Sitosterol |
| 63.16 | Eicosanoic acid, ethyl ester |
| 0.57 | Nonadecanoic acid, ethyl ester |
| 24.62 | Hexadecanoic acid, ethyl ester |
| 3.87 | Octadecanoic acid, ethyl ester |
| 1.62 | Ethyl 13-methyl-tetradecanoate |

#### 3.1.4. Raman spectroscopy

Raman spectroscopy has commonly been used to study compounds constituting non-polar groups like fat. Different research groups have reported the characterization of oleogels using the Raman spectroscopy [28]. It majorly helps in telling the variations in the possible interactions among the oleogel components by monitoring deviations in the shape and frequency of the Raman bands precisely corresponding to molecular vibrations. The Raman spectra of all the oleogels are shown in Fig. 1b. The peak positioned at 1647 $cm^{-1}$ is attributed to the *cis* (C = C) stretching and can be corelated to the unsaturation of linoleic acid [29]. The scissoring of methylene groups is represented through the Raman peak at 1442 $cm^{-1}$[30]. Altogether the peaks at 1438 $cm^{-1}$ and 1647 $cm^{-1}$ appeared in the spectra for the unsaturation of the fatty acids of SW and SO and mainly reflect the Z and E unsaturation [31]. Taking C = C as a chemical signature, we checked its variation among different oleogels through Raman spectroscopy. We found that there were no observed variations in terms of peak positioning and peak intensity.

#### 3.1.5. Analysis of thermal properties of the oleogels through DSC

The respective thermogram related to the cooling and heating of these oleogels has been previously reported [15,16]. The changes in the physical state of oleogels are associated with the alteration in the endothermic or exothermic release process of oleogels. The SW-structured olive oil oleogels have been reported for melting temperatures in the range of 58–63 °C [32]. In this study, the control sample showed $T_m$ at 62 °C (Fig. 1c(i)). The formulations S80 and T80 showed a rise in the $T_m$ from control. For all the oleogels, the crystallization temperature ($T_c$) was 60 °C, irrespective of the emulsifiers. The difference in the melting and crystallization temperature is defined as the degree of supercooling, and the same has been represented in (Fig. 1c (ii)). Crystallization occurs under supercooling conditions [33]. A higher degree of supercooling indicates quicker nucleation induction and secondary crystallization of wax crystals [34]. This might result in smaller crystals or crystal defects in the structuring. The same was evident from the respective micrograph (Fig. 1a) of S80 and T80, which displayed a discontinuous network structure.

### 3.2. Effect of oleogels on L.pentosus growth properties

#### 3.2.1. Growth of probiotics in the presence of oleogels

Since probiotics are sensitive to the components of the delivery system, it is important to check the effect of these oleogels on the survival of the probiotics. The *L.pentosus* is an important probiotic of genus lactobacillus. Here we have checked the growth of *L.pentosus* in the presence of the oleogels. Earlier studies have evaluated survival of *L. reuteri* within the organogel matrix formulated using beeswax, hydrogenated rapeseed oil, and fumed silicon dioxide [35]. The growth kinetics (Fig. 1a,b,c) were obtained after culturing *L.pentosus* with 1 μg/ml, 100 μg/ml, and 10,000 μg/ml of different oleogels for 24 h. The visual inspection of the graphs showed a similar overall trend in the growth curves, consisting of the lag phase (up to 5–7 h), exponential phase (up to 16 h), and stationary phase. The initial growth OD of probiotics remained lower and similar at 1 μg/ml and 100 μg/ml of oleogels, as seen in Fig. 1 (a and b). However, the presence of particulate material at the highest concentration of oleogel might have resulted in a higher initial OD (Fig. 1c) value compared to the same at lower concentrations. The experimental data of the growth curves were fitted to

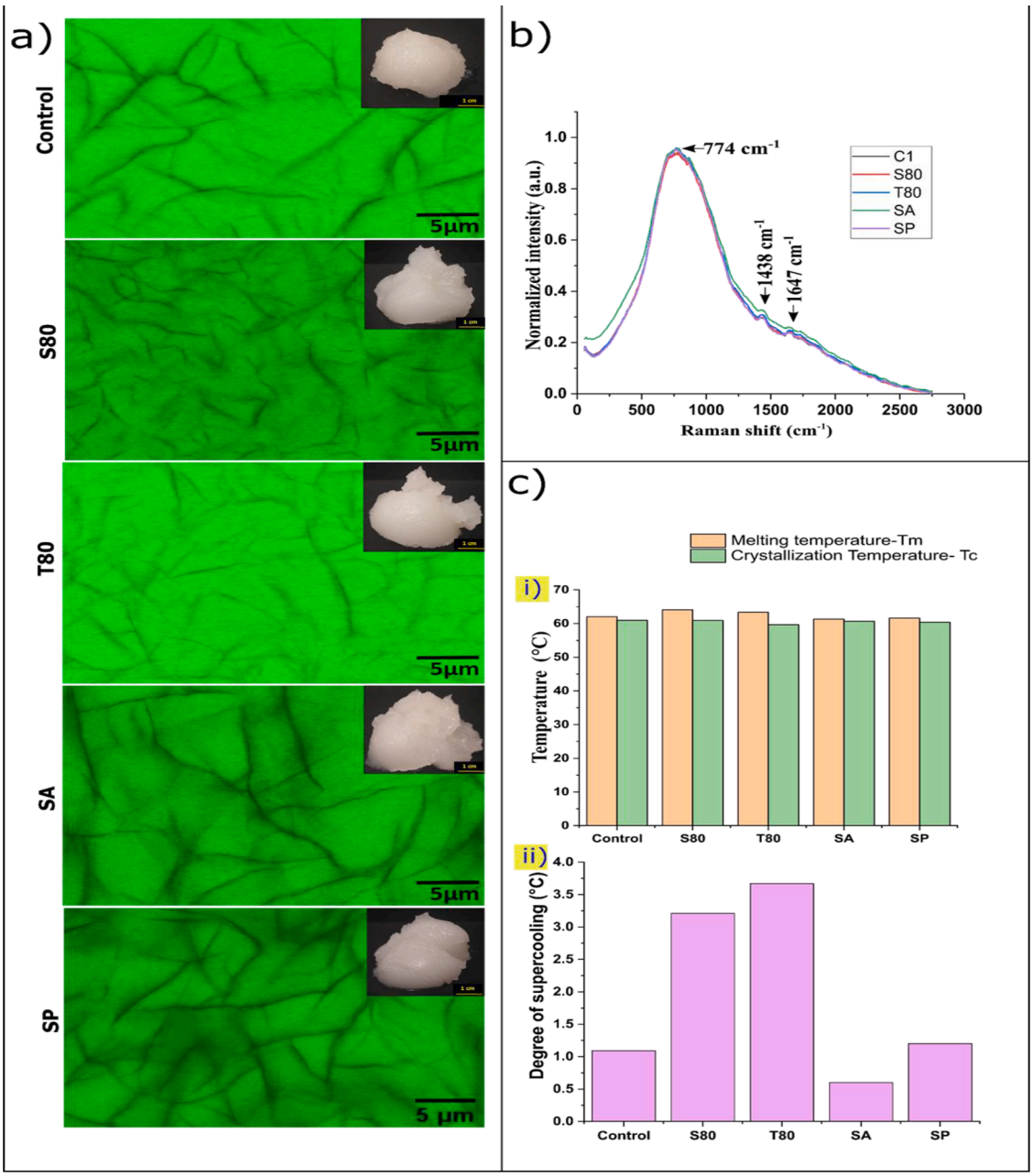


**Fig. 1.** Oleogel characterizations: a) CLSM images displaying fiber network inside the oleogels (magnification:40x); b) Raman spectra of the oleogels containing emulsifiers obtained after excitation at 633 nm laser; c) Data obtained from DSC thermogram: i) Melting and crystallization temperature; ii) Degree of supercooling.

the modified Gompertz model (Eq. (1)). The obtained parameters from this model have specific meanings within the bacterial growth curve.

$$D_t = D_0 + A\exp\left\{-\exp\left[\left(\mu\frac{e}{A}\right)(\lambda - t) + 1\right]\right\} \quad (1)$$

Where $D_t$ is the OD value at time t; $D_0$ is OD value at time 0; A is defined as the increase in the OD from $D_0$ to the maximum OD value; μ is the maximum growth rate ($h^{-1}$), and λ is the duration of lag phase. Almost every parameter obtained through the Gompertz model for all the concentrations remained the same, except for a few, as shown in Table 3. There was a 0.78-fold decrease in the μ value for SA2 with respect to the broth. Additionally, at the highest concentration, there was a significant rise (1.20 fold) in the μ value of C3 from the broth. Interestingly S3, and SA formulations have shown rapid attainment of the log phase compared to the broth. The growth profile of the bacteria under the influence of different oleogels after 16 h was further presented in Fig. 2 (d,e, and f) for better comparison. Not much variation was observed in the growth at a concentration of 1 (Fig. 2d) and 100μg/ml (Fig. 2e) of different oleogels. However, at a 10,000 μg/ml concentration (Fig. 2f), control, T80–3, and SP-3 showed a significant 1.97 and 1.07-fold rise in the growth w.r.t to the broth. The explanation can be given in the case of T80 considering that Tween 80 can change the nutrient availability by changing the size of nutrient particles in the media. Earlier it was shown that the inclusion of Tween 80 in growth media reduced the mean size of the nutrient particle and the poly-dispersity index [36]. This may result in the media having homogenously sized smaller particles, which might explain the significant rise in T80 growth. The rise of growth in T80 can be attributed to the establishment of Lactic acid bacteria that utilize Tween 80 as a carbon source [37,38]. For the case of SP, being a hydrophobic emulsifier (similar HLB value to Tween 80), may have similarly reduced the mean size of nutrient particles.

**Table 3**
Parameters obtained through the Gompertz modeling of the probiotic growth curve.

| Sample | Factors | | |
|---|---|---|---|
| | A | μ ($h^{-1}$) | λ (h) |
| **Broth** | 0.60 ± 0.01 | 0.10 ± 0.01 | 7.30 ± 0.12 |
| **C-1** | 0.62 ± 0.01* | 0.11 ± 0.01 | 6.60 ± 0.18* |
| **C-2** | 0.59 ± 0.01 | 0.09 ± 0.01 | 6.69 ± 0.48 |
| **C-3** | 0.57 ± 0.01 | 0.12 ± 0.01* | 7.16 ± 0.15 |
| **S-1** | 0.60 ± 0.01# | 0.11 ± 0.01* | 7.50 ± 0.26# |
| **S-2** | 0.61 ± 0.01# | 0.11 ± 0.01*,# | 7.33 ± 0.25 |
| **S-3** | 0.58 ± 0.02 | 0.10 ± 0.01# | 6.30 ± 0.24*,# |
| **T80–1** | 0.61 ± 0.01 | 0.09 ± 0.01 | 6.75 ± 0.50 |
| **T80–2** | 0.65 ± 0.02# | 0.10 ± 0.01 | 7.04 ± 0.16 |
| **T80–3** | 0.64 ± 0.01*,# | 0.11 ± 0.01 | 6.82 ± 0.27 |
| **SA-1** | 0.59 ± 0.01# | 0.08 ± 0.01*,# | 6.72 ± 0.19* |
| **SA-2** | 0.62 ± 0.02 | 0.08 ± 0.01* | 6.63 ± 0.33* |
| **SA-3** | 0.54 ± 0.01*,# | 0.09 ± 0.01*,# | 7.25 ± 0.20 |
| **SP-1** | 0.62 ± 0.01 | 0.09 ± 0.01 | 6.85 ± 0.35 |
| **SP-2** | 0.64 ± 0.01*,# | 0.10 ± 0.01 | 6.91 ± 0.32 |
| **SP-3** | 0.59 ± 0.01 | 0.11 ± 0.01 | 6.97± 0.31 |

* in supercript dispalyed statistically significant ($p \leq 0.05$) values w.r.t broth.
# in superscript was placed for significantly different($p \leq 0.05$) values w.r.t respective concentration of control (i.e. C-1,C-2, and C-3).

### 3.2.3. Metabolic activities of probiotics

Bacterial metabolism is often influenced by the content of the culture media. One way of checking such variation is to check the expression of the marker proteins expressed constitutively. In microbiology investigations, the conversion of tetrazolium salts to colored formazans is frequently utilized as a measure of cell [39]. It has been shown in the previous work that the electron transport chain (ETC), which transfers electrons from intracellular donors (mostly NADH), results in MTT reduction [40]. However, the ETC's reduction sites vary depending on the experimental circumstances. Here, MTT assay was performed to get an idea about the metabolically active cells after 16 h of incubation with the oleogels, and the data is shown in Fig. 3 (a,b, and c). At the lowest used concentration (Fig. 3a), MTT absorbance was found 1.1 fold higher in S80–1, T80–1, and SA-1 than in broth. At a concentration of 100 μg/ml (Fig. 3b) of oleogels, increased metabolic activity in comparison to broth occurred in the case of SP-2. Other oleogel formulations at this concentration showed similar metabolic activity to the broth's. However, at the highest concentration (Fig. 3c), the metabolic activity lowered in S80–3 (0.92-fold). Again, the metabolic activity was 1.4-fold and 1.1-fold higher in SA-3 and SP-3 from the broth, respectively. Oleogels may act as a physical barrier protecting the probiotics from the external environment. The presence of stearyl alcohol (i.e., SA3) might have resulted in the better stabilization of this physical barrier due to the presence of the –OH group, which can interact with the aqueous phase [41]. As per the previous literature, Span 60 has been reported to prevent fat aggregation and narrowing particle size distribution of fat droplets, which may also assist in better exposure to probiotics [42]. Further, the metabolic rate per bacteria was calculated using the growth and metabolic activity data (Fig. 3d, e, and f). This parameter is important as cell responses to treatment are not always limited to the ability to replicate, and the inability to reproduce does not always mean loss of viability. The metabolic rate per bacteria was again higher in S80–1, T80–1, and SA-1. At the highest concentration (Fig. 3f) of oleogels, SA-3 showed a higher metabolic rate per bacteria suggesting their beneficial effect on the probiotics' metabolic growth. Few changes have been observed in the metabolic activity and metabolic rate per bacteria when control was compared with emulsifier-modified samples, and the same has been highlighted in the figure. However, these results were inconsistent. Combining the growth and metabolic growth parameters, we found that formulation SA-3 and SP-3 may assist in the viability of *L. pentosus*.

### 3.2.4. Study of the morphology of probiotics after incubation with oleogels using SEM

The effect of the oleogels on the cellular morphology of *L.pentosus* was investigated through SEM (Fig. 4a). Bacteria treated with the oleogels containing highest emulsifier were chosen for this study. The cells exhibited a rod-shaped morphology consisting of a smooth surface which is in accordance with the literature [43]. Morphological changes like disruption and deformation, a sign of cellular stress are not observed [44]. For further analysis, 50 cells were taken from different micrographs, and a ratio of length to width was measured in each cell (Fig. 4b). The length of the cells varied from 0.8 μm to 3.5 μm, and the

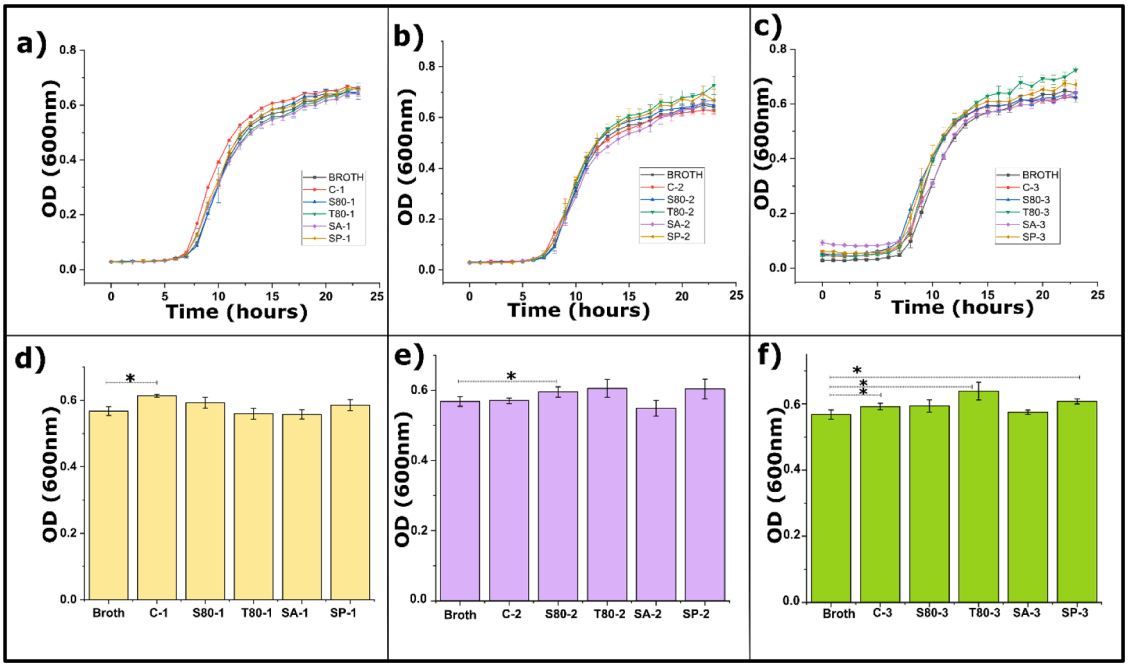


**Fig. 2.** Kinetics of probiotic growth and survival upon (a) 1 μg/ml (b) 100 μg/ml (c) 10,000 μg/ml of oleogel treatment; Probiotic growth at 16 h (d) 1 μg/ml (e) 100 μg/ml (f) 10,000 μg/ml. Data were expressed as Mean±*S*.D..

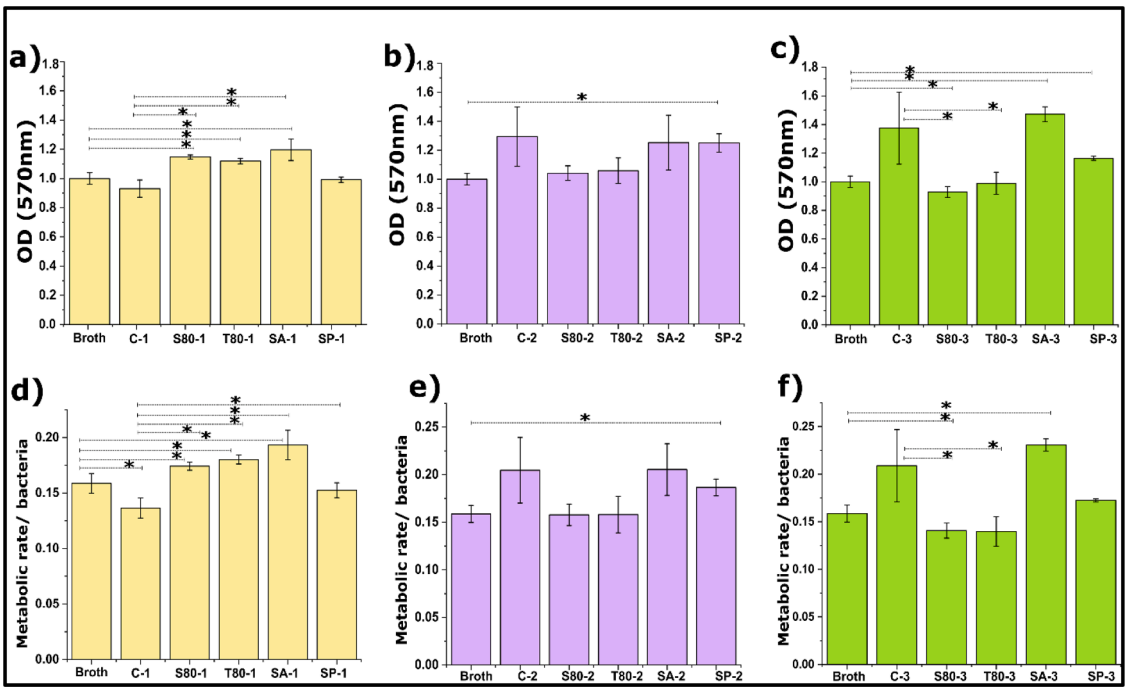


**Fig. 3.** Metabolic activity of probiotic upon incubation with oleogels (a) 1 μg/ml (b) 100 μg/ml (c) 10,000 μg/ml; Metabolic rate per bacteria at (d) 1 μg/ml (b) 100 μg/ml (c) 10,000 μg/ml oleogel concentration. Data were expressed as Mean±*S*.D.

width ranged from 0.5 μm to 0.8 μm [45]. No significant difference in the obtained length-to-width ratio among the oleogel-treated cells. This indicated that the cells integrity and morphology were maintained even when they were grown with oleogels prepared with different emulsifiers.

### 3.2.5. *Probiotic adhesion to mucin*

A large number of health-promoting effects of probiotics arises from their adhesion to the mucosal surface for colonization and persistence [46]. The ability of some probiotic strains to inhibit pathogen colonization is connected to their ability to adhere to the intestinal mucus. The basic model used to evaluate the adhesion capability of the probiotics is through the quantification of the immobilization of probiotics on the mucin-coated surface [47]. The mucus layer of the GIT is primarily composed of glycoproteins, i.e., mucin. It protects the host cells from foreign antigens and supports the gut microflora with habitat and nutrients. Thus, adhesion of probiotics to mucin is crucial for their functional characteristics. Fig. 4c (i, ii, and iii) represents the mucin adhesion activity of the probiotics post-treatment with 1 μg/ml, 100 μg/ml, and 10,000 μg/ml of oleogel, respectively. We did not observe any variation in the trend in all these cases. The mucin activity was mostly similar except for S80–2 and C-3, where probiotics displayed increased mucin adhesion.

## 3.3. *Effects of cell-free supernatant derived from oleogel-treated probiotics culture on colon cell*

### 3.3.1. *Effect of cell-free supernatant on colon cell proliferation*

The cell-free supernatant of the cultured probiotics contains protein and metabolites that *L.pentosus* releases and forms the secretome. This secretome is essential for the physiological function of the gut epithelium as it holds an important role in cell-to-cell interactions [48]. Recently, the effect of 157 gut bacteria's secretome on the proliferation rates of five colorectal cancer cell lines (CRC), including HT-29, was assessed. The researchers have shown that the effects of gut bacteria on cell growth (both promoting and inhibiting) depend on the mutational landscape of the cells as well as the functional content of the bacteria, including any substances released or linked to their cell walls [49]. Probiotics enhance the intestinal barrier by adhering to the intestinal wall and stimulating mucus production by intestinal epithelial cells (IECs). By digesting dietary fiber to produce short-chain fatty acids (SCFAs), probiotics and pathogenic bacteria work together to decrease intestinal pH, preventing pathogenic bacteria from growing and proliferating in the gut by fighting with them for food and energy and for niche occupancy[50]. Both the large and small intestines may benefit from the generated SCFA by increasing cell proliferation and differentiation. This study evaluated the probiotic secretome for its role in the human colon adenocarcinoma cell line HT29. The effect of probiotic secretome on the HT29 cell viability was evaluated by MTT assay. It is observed that the secretome obtained after incubation of probiotics in broth is having a negative impact (0.8-fold decrease) on the proliferation of HT-29 cells compared to TCP. However, there was a recovery in the proliferation of cells treated with secretome obtained from an emulsifier incorporated oleogels treatment of probiotics. This recovery was quantified, and it was found that there was a 1.3, 1.1, and 1.2-fold increase in ($p \leq 0.05$) cell proliferation in T80–3, SA-3, and SP-3, respectively, compared to the broth. The secretome obtained from the probiotics treated with different emulsifiers (S80–3, T80–3, SA-3, and SP-3) showed no difference in the viability of the HT29 cells, as the viability appeared similar to TCP (Fig. 5a). However, the secretome viability was reduced (0.79 folds) significantly from TCP in case C-3. The effect of the cell-free supernatant on the colon cell viability was also checked using calcein-AM staining. Viable cells convert calcein-AM into calcein through an esterase enzyme which emits green fluorescence. The treatment of cells with secretomes resulted in no obvious change (Fig. 5b) in the fluorescence of cells w.r.t to TCP (data not shown).

### 3.3.2. *Effect of cell-free supernatant of reactive oxygen species (ROS) production*

Since ROS is a hallmark of oxidative stress and can trigger controlled cell death processes, we analyze ROS generation to explain the observed effects of the probiotic secretome on the viability of HT29 cells. The results demonstrated minimal fluorescence intensity, indicating a low level of ROS production. The weak green fluorescence signal suggests

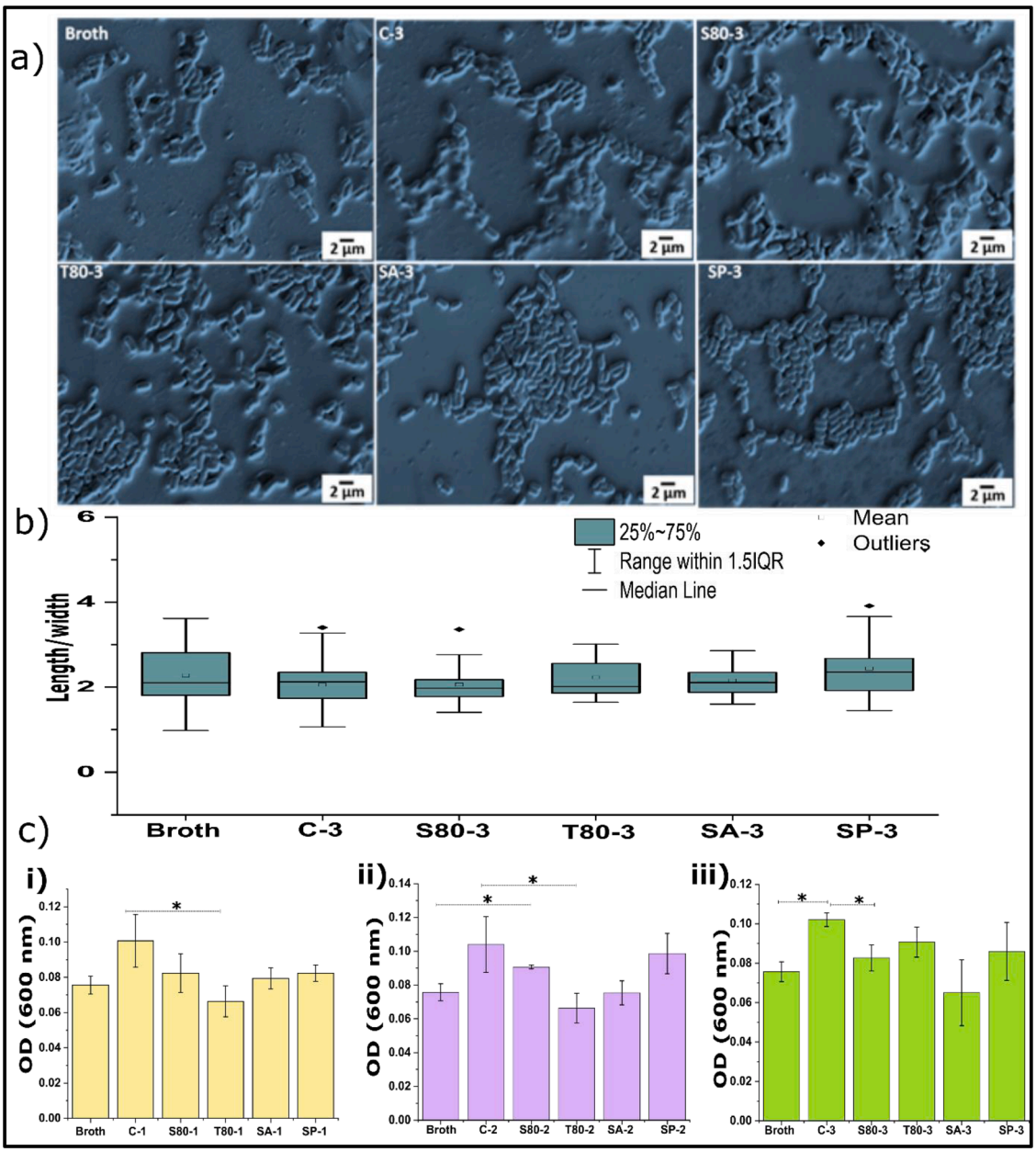


**Fig. 4.** a) SEM analysis (magnification: 5 KX) to observe probiotic morphology treatment with 10,000 µg/ml oleogel concentration; b) Quantification of length/width ratio of probiotics. Analysis was done through ImageJ software for 50 cells per samples using SEM; c) Role of oleogels on mucin-adhesion of the probiotics. Cells were treated with (i) 1 µg/ml (ii) 100 µg/ml, and (iii) 10,000 µg/ml of oleogels. The broth is referred to MRS broth. Data were expressed as Mean±SD. For these studies, probiotics were treated with oleogel for 24 h.

that incubation of these cells with the secretome (Fig. 5c) obtained from the treatment of different oleogel formulations probiotics has no significant impact on the production of ROS.

## 4. Conclusion

The current study is focused on exploring the effects of different emulsifier-modified oleogels on probiotics. We observed probiotics treated with oleogels (made using different emulsifiers) exhibit variable proliferation *in-vitro*. No detrimental effect was observed when the secretome of such probiotics was applied to the colonic cell line. Interestingly we did not observe any change in mucin adhesion properties or the cellular morphology of the probiotics treated with different oleogels. The variable impact of the treatment may be attributed partly to the chemical nature of the different emulsifiers used and partly because of the variation in crystal length and arrangement. It was found that emulsifier-incorporated oleogels showed consistent results when a comparison was made with broth or TCP. However, the results obtained compared with oleogel formulation without emulsifier in different studies (i.e., control) were not indicating any definite trend. We observed that SA and SP-containing formulations have the maximum positive effect on growth and viability among different emulsifiers.

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This research received no external funding.

## Data availability

Data are available from the corresponding author upon request.

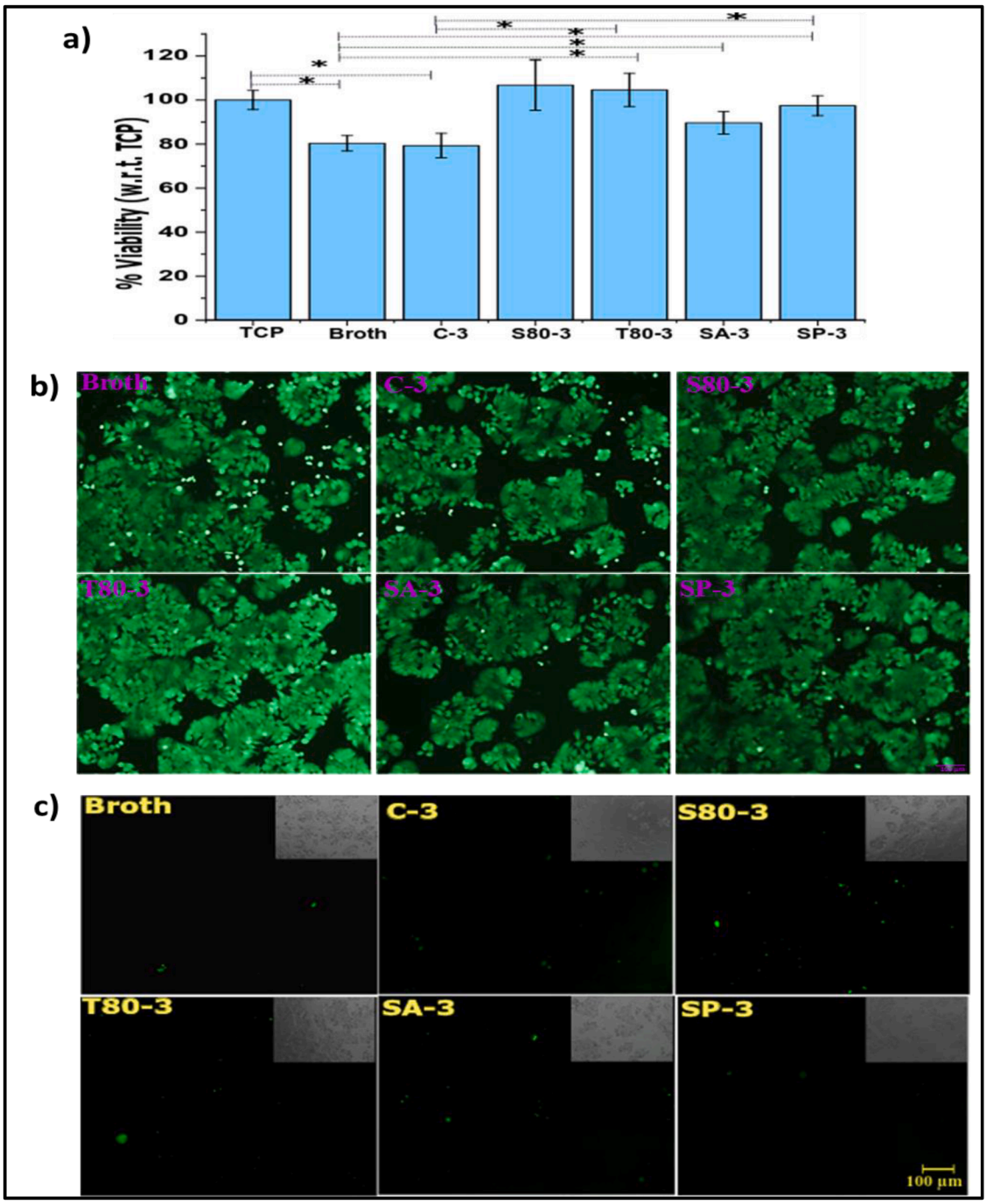


**Fig. 5.** Effect of probiotic secretome on HT-29 cells post 24 h: a) Cell viability through MTT assay; b) Study of cell viability by Calcein-AM staining; c) Micrograph for ROS production through DCFDA staining. Magnification: 10X. Broth and TCP are referred to as MRS broth and DMEM media, respectively.

## CRediT authorship contribution statement

**Deepti Bharti:** Writing – original draft, Methodology, Data curation. **Senthilguru Kulanthaivel:** Writing – review & editing, Data curation. **Prashant Mishra:** Writing – review & editing, Visualization, Resources. **Neha Jain:** Resources, Conceptualization. **Kunal Pal:** Writing – review & editing, Supervision. **Indranil Banerjee:** Writing – review & editing, Validation, Supervision, Investigation.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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