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Investigation of phage and molasses interactions for the biocontrol of *E. coli* O157:H7

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Abstract

Nowadays, resistance in pathogens against antibiotics is one of the most critical health-threatening problems in the world. Therefore, finding new treatment methods to be used as an alternative to antibiotics has become a priority for researchers. Like phages, certain products containing antimicrobial components such as molasses are widely used to eliminate resistant bacteria. Molasses has a strong antimicrobial effect on the bacterial cell, and this effect is thought to be due to the breakdown of the cytoplasmic cell membrane and cell proteins of the polyphenols in molasses. In the present study, phages-molasses interactions were investigated to examine the effects arising from concomitant use. It was found that molasses samples increased the size of phage plaques by up to 3-fold, and MIC and $1/2 \times$MIC concentration of molasses increased the burst size of phages. Although no synergistic effect was found between the phage and the molasses, the antimicrobial activities of the components and the effect of molasses on phage activity were demonstrated.

Keywords: Bacteriophage, phage therapy, *Escherichia coli* O157:H7, synergism, molasses

Introduction

Foodborne illnesses caused by foods contaminated with toxic compounds and pathogenic microorganisms are still a major health problem despite the measures taken at all stages of food production. In recent years, the increase in food production, consumption, and wide supply chain have raised concerns about food safety (Hussain & Dawson 2013). Centers for Disease Control and Prevention (CDC) reported that 48 million people suffer from foodborne illnesses each year (CDC 2017). *Escherichia coli* O157:H7 (*E. coli* O157:H7), which is commonly found in the environment and foods as well as in the intestinal tract of humans and animals, is one of the most important bacteria that cause foodborne illnesses. Although many *E. coli* species are considered to be harmless, some strains may cause serious diseases such as diarrhea, bloodstream infections, respiratory tract diseases, and urinary tract infections (CDC 2018). The first *E. coli* O157: H7 outbreak occurred in 1982 as a result of the consumption of undercooked hamburgers and caused hemorrhagic colitis disease (Meira et al. 2017). Most *E. coli* O157:H7 outbreaks stem from consumption of raw, unpasteurized, uncooked, or undercooked foods (Huang 2010). Therefore, the interest in *E. coli* O157: H7 has been increased, and a number of studies have been performed for the detection and inhibition of this bacteria. In addition to antibiotics, strategies to combat this bacteria include the use of essential oils and phenolic acids (Meira et al. 2017; Mouatcho et al. 2017), peanut skin and grape seed extract (Levy et al. 2017), a combination of eucalyptus leaf oil and silver nanoparticles (Heydari et al. 2017), gamma radiation (Mohamed & El-Deen 2016), and organic acids (Almasoud et al. 2016). Although these methods can reduce *E. coli* O157:H7 levels to different degrees, new methods should be investigated.

Bacteriophages (phages in short) are the viruses which infect bacteria and cause their cells to become lysed by disrupting the metabolism of the bacterial cells (Sulakvelidze et al. 2001; Tayyarcan et al. 2018), and a number of virions are released with the lysis of the bacteria (Kazi & Annapure 2016). Phages can multiply as long as the host bacteria is present in the environment, which means that even a very small amount of phage is sufficient to reduce the bacterial count (Kazi & Annapure 2016; Oliveira et al. 2017). Bacteriophages were described by Félix d'Hérelle and Frederick Twort in the years 1915-1917 (Abedon et

al. 2011). Phage cocktail prepared by Felix d'Herelle was used for the treatment of soldiers who had dysentery during World War II (Chanishvili 2012). However, with the discovery of antibiotics, phage therapy gradually lost its importance (Lin et al. 2017). The emergence of the bacteria resistant to antibiotics brought about the post-antibiotics era, and phage therapy came to the fore once again (Rohde et al. 2018). New methods which can be used as an antibiotic alternative have been developed with the increase of antibiotic resistant bacteria in recent years (Soykut et al. 2019). Bacteriophage therapy which destroys pathogenic bacteria that cause infection in humans with the help of bacteriophages specific to these bacteria has become one of the most popular methods for bacterial inhibition as an antibiotic alternative (Abedon et al. 2011). Nowadays, phage therapy is used in various fields including treatment of infections in humans, veterinary medicine (Khairnar et al. 2013; Lim et al. 2012), food products, and equipment (Endersen et al. 2014; Sillankorva et al. 2012).

Molasses, which is a traditional food product, contains carbohydrates, organic acids, minerals, and certain vitamins (İbrahim 2015). It can be produced from several types of fruits such as carob, grape, apple, watermelon, sugar beet and sugarcane (Sengül et al. 2005) and obtained by boiling fruits with sugar (Yoğurtçu & Kamışlı 2006). Because of the substances in its composition, molasses is considered to be an important food product for human nutrition. In the literature, antimicrobial effect of molasses and similar products such as pomegranate and sugarcane molasses have been studied. In one of those studies, it was reported that pomegranate molasses has antimicrobial activity against *Salmonella* Typhimurium in ready-to-eat salads and parsley leaves (Faour-Klingbeil & Todd 2018). In another study, antimicrobial effect of sugar beet molasses was investigated, and it was found that sugar beet molasses has antibacterial effect against foodborne pathogen bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Escherichia coli*. Researchers have reported that the antimicrobial effect may be due to the polyphenols in molasses, and that polyphenols can cause disruption of cellular protein structures and change the physiological and morphological structure of cells (Chen et al. 2017).

There are also many other studies in which bacteriophages and products containing natural antimicrobial components were used against various pathogenic bacteria. There are various studies in the literature in which a possible synergism between phage and natural antimicrobial substances was investigated. In a study conducted by Oliveira et al. (2017), the synergistic effect of phage-honey combination was examined. According to their results, phage and honey U3 showed additional antimicrobial effect against *E. coli* after 12 hours compared to each individual component (Oliveira et al. 2017). Although such methods have been applied successfully, the increased prevalence of antibiotic resistance requires improvement of these methods and development of new approaches. One of these new approaches is using antimicrobial components together with phages and investigation of potential synergy between them. In this respect, several studies have examined the synergistic interactions of phages with different components to increase the efficiency of phages. In the phage synergism studies in the literature, synergism was mostly studied with antibiotics, but natural antimicrobial agents were not studied much. In a few studies; honey, chestnut honey, various plant extracts, bacteriocin and phenolic substances were studied to demonstrate phage synergism (Tayyarcan et al. 2019; Oliveira et al. 2017, 2018; Heo et al. 2018). Although phage synergism studies have been conducted before with substances containing antimicrobials, there is no such research with molasses. In this study, we examined the synergistic effect of molasses-phage because of the phenolic content and antimicrobial effect of molasses.

Although phage-antibiotic synergism studies are very common in the literature, there is no synergism study conducted on phage and foods with an antimicrobial content such as molasses. The aim of this work was to demonstrate the antimicrobial activity of molasses against *E. coli* O157:H7 and evaluate a possible synergy between phage and molasses, and eventually suggest a more effective method for pathogen inhibition.

Material and methods

Chemicals and media

Agar and CASO broth were obtained from Merck (Darmstadt, Germany). Glycerol that was used for bacterial and phage stock preparation was obtained from Merck Millipore Corporation (Massachusetts, ABD).

CASO Broth, CASO Agar (1.5%) and CASO Soft Agar (0.6%) were used for the growth of bacteria, phage propagation, and determination of phage titer as well as for the antimicrobial activity of molasses and to demonstrate phage-molasses synergism. Bacteria and phage stocks were prepared with glycerol to a concentration of 50% (v/v) and stored at -18 °C.

Bacteria and bacteriophages

E. coli O157:H7, which was previously used by Dogan et al. (2003) was kindly provided by Prof. Dr. İbrahim ÇAKIR. Commercial phage mixtures (Septaphage®) produced by George Eliava Institute of Bacteriophages, Microbiology and Virology (Tiblisi, Georgia) were used for the isolation of *E. coli* O157:H7 phages.

Isolation of phages

A commercial bacteriophage mixture called Septaphage® was used for the isolation of *E. coli* O157:H7 phages. Product information can be found in Supplementary Table 1. Firstly, 10 µL of phage mixture was transferred to sterile tubes. After 10 µL of *E. coli* O157:H7 was added to Eppendorf tube, this mixture was incubated for 10-15 min at room temperature for the adsorption of phages to bacteria. Sterile CASO broth was added to make a final volume of 1 mL, and the mixture was incubated at 37 °C for 18-24 h. After incubation, centrifugation was performed at 12 000 rpm for 5 min. Then, the supernatants were collected in another sterile tube, and the pellet was discarded. This procedure was repeated three times for increasing the titer of *E. coli* O157:H7 phages. The isolated phage was named as EliavaPhage3.

Molasses samples

Three different types of samples, *i.e.*, carob (*Ceratonia siliqua* L.) molasses - CM, white mulberry (*Morus alba* L.) molasses-MM and black mulberry (*Morus nigra* L.) syrup-BMS, were used in this study. Five different samples for each group produced by traditional methods were obtained from the local markets in Ankara, Malatya and Tekirdağ, Turkey. The master sample of each group (CM, MM and BMS) was prepared by mixing five different original samples in equal amounts. The samples were put in amber glass bottles and stored in a refrigerator at 4 °C and used in the analysis within a week.

Determination of some physicochemical characteristics of samples

Total soluble solids (TSS) were determined using ATAGO PAL-3 digital hand-held refractometer (ATAGO Co., Ltd, Japan) at 20°C. Density of CM, MM and BMS were found to be 0.56, 0.56 and 0.44 g/mL, respectively. Total titratable acidity (TTA) was determined by titration with 0.1 N NaOH sodium hydroxide to a phenolphthalein indicator end point and the results were expressed as g citric acid equivalents (CAE) per 100 g sample. Sugar content of the samples were determined using HPLC method (DIN-NORM-10758 1997) with slight modifications. The sugar analysis was conducted isocratically using HPLC performed on an LC-20A system (Shimadzu, Japan) with a refractive index dedector (RID-10A, Shimadzu). Samples were homogenized and 5 g of each was weighted, 40 mL of distilled water was added and mixed using magnetic stirrer. Then the sample was added to a flask containing 25 mL of methanol and the final volume was adjusted to 100 mL with distilled water, filtered through 0.45 µm syringe filter and injected to the HPLC system. A 10 µL of the extract was chromatographed on an InertSustain NH2 HPLC column (250 x 4.6 mm, 5µm; GL Sciences, Tokyo, Japan) using a mobile phase of acetonitrile:water (3:1). The temperature of the column oven was set at 30 °C and the flow rate was 2 mL/min. The stock standard solutions (100 g/L) of sucrose, glucose and fructose were prepared and used for the preparation of serial standard solutions (0.5-20 g/L) for the generation of calibration curves. The sugar contents of the samples were calculated using the calibration curves and the results were expressed as g sugar (glucose, fructose or sucrose) per 100 g sample. Peak detection and integration were done using LC Solutions (Shimadzu) program pack.

Folin-Ciocalteu method was used for determination of total phenolic (TP) content of the samples (Singleton and Rossi 1965). Firstly, 0.1 g sample was weighted and diluted using 10 mL of distilled water. Then, the solution was mixed using a multi-vortex (Multi Reax, Heidolph, Germany) and filtered through Whatman No 1 filter paper. The experiment was carried out according to the microscale protocol (Matić et al. 2017) as follows: Briefly, 20 µL of sample extract (or standard solution) and 1580 µL distilled water were mixed in a glass tube and 100 µL Folin–Ciocalteu reagent was added and mixed using a vortex (Reax top, Heidolph, Germany). Then, 300 µL Na_2CO_3 (200 g/L) solution was added and mixed again. The mixtures in the test tubes were incubated at 40 °C for 30 min in a water bath (WiseBath, Daihan Scientific, Korea). The absorbance of green-blue complex was read at 765 nm against a blank solution (prepared using 20 µL water instead of sample) with a UV-Vis spectrophotometer (UV-2600, Shimadzu). A calibration curve was generated by preparing several concentrations (10, 25, 50, 100, 250, and 500 mg/L) of standards from stock solution of gallic acid at a concentration of 1000 mg/L. This calibration curve was used for calculating the results. The results were expressed as mg gallic acid equivalents (GAE) per g sample. TP results were also given in dry weight (dw) basis as mg GAE/g dw.

Water activity (a_w) was directly determined using an AQUALAB 4 TE water activity meter, (METER Group, Inc. USA).

Determination of MIC and MBC

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of molasses samples were determined to reveal the antimicrobial effect clearly. Broth microdilution method was used to determine MIC and MBC (Balouiri et al. 2016). One hundred µL of the relevant dilution of molasses and 50 µL of bacteria at log-phase (app. 4×10^8 CFU/mL) were added to ELISA plate wells, and the final volume of the wells was adjusted to 250 µL with CASO broth so that the number of bacteria would be the same for all wells. For control groups, the bacteria without molasses and bacteria-free molasses were also added to

the wells. Microplates were incubated at 37 °C for 18-24 h. After incubation, the wells were screened for turbidity, then the samples were collected into the tubes and used for bacterial enumeration.

Effect of molasses samples on the size of phage plaques

The effect of molasses on phage plaque size was determined using a combination of agar diffusion and double layer agar methods. Briefly, 50 µL of molasses prepared at different concentrations (2×MIC - 1/4×MIC) was dropped onto the agar plates and left to dry at room temperature. Then, 150 µL of bacterial suspension (approximately 10^6 CFU/mL) and 100 µL of proper dilutions of phages (10^2-10^3 PFU/mL) were mixed in 4 mL of molten agar. Then, this mixture was overlaid on the agar plates and left to incubate at 37 °C for 18-24 hours. After incubation, plaque formation in the diffusion zones of molasses was examined and plaque sizes were measured in millimeter with a vernier caliper (Comeau et al. 2007b; Iqbal et al. 2020).

One-step growth curve assay

One-step growth curve experiment was performed both in the presence and absence of molasses (CM) to determine the latent period and burst size of EliavaPhage3. The experiment was designed as described by Suarez (2002) and Reyes-Gavilan (1990) (Clara et al. 1990; Suárez et al. 2002). Phage-bacteria-molasses mixture was prepared in such a manner that the final molasses concentrations would be MIC and 1/2×MIC and the multiplicity of infection (MOI) would be 0.1. This mixture was then incubated at room temperature for 15 min. After incubation, the mixtures were centrifuged at 11 529 x g for 5 min to remove non-adsorbing phages. The pellet was resuspended with CASO broth. Suspensions were incubated at 37 °C for 120 min. Samples were taken for 120 min, and the titer of phages was determined using double agar layer method.

Investigation of the interactions between phage and molasses samples

Antimicrobial activity of molasses, phage, and molasses-phage combinations were examined using time kill curve method (TKC) (Verma 2007). MOI value was adjusted to 0.1. Bacteria (10^7 CFU/mL), molasses:bacteria (MIC, 10^7 CFU/mL; $1/2 \times$MIC, 10^7 CFU/mL), bacteria:phage (10^7 CFU/mL bacteria, 10^7 PFU/mL phage), molasses, and phage:bacteria (MIC, 10^7 CFU/mL bacteria, 10^7 PFU/mL phage; $1/2 \times$MIC, 10^7 CFU/mL bacteria, 10^7 PFU/mL phage) samples were prepared in CASO broth and incubated at 37 °C for 5 h. During incubation, a 100 µL sample was taken into sterile tubes for 0, 30, 60, 90, 120, 240 and 300 min. Samples were centrifuged at 12 000 rpm for 5 min. Supernatants were taken to other tubes and used for the determination of phage titer. Bacterial cell pellets were resuspended in sterile saline solution (0.85%) and used for bacterial enumeration on EMB agar.

Statistical analysis

Statistical analysis of phage plaque sizes and bacterial count was performed using SPSS (IBM SPSS Statistic 23, New York, USA). One-way ANOVA and Duncan post-Hoc tests were used to compare the results, and p value <0.05 was considered statistically significant.

Time kill curve and one-step growth curve were conducted in triplicate, and standard deviation values were calculated using Microsoft Excel.

Results

In this study, phage isolation was carried out using *E. coli* O157:H7 as the host culture. The activities of the isolated phages were determined and the first ones to reach high titer among the phages with strong lytic activity were selected to investigate the effect of phage and phage-molasses combination on *E. coli* O157:H7.

Characterization of molasses samples

Some physicochemical characteristics of the samples were given in Table 1. TSS, TTA and fructose, glucose and sucrose contents of CM sample were determined as 75.20 %, 1.36 g CAE/100 g sample, 32.84 g/100 g, 26.79 g/100 g, 14.53 g/100 g, respectively. TSS, TTA and fructose, glucose and sucrose contents of MM sample were determined as 68.37%, 1.43 g CAE/100 g sample, 23.95 g/100 g, 20.70 g/100 g, 6.60 g/100 g, respectively. The results of the BMS sample can also be seen in Table 1. The TP content of BMS sample (1.73 mg GAE/g, corresponding to a value of 4.43 mg GAE/g dw) is significantly lower ($P<0.05$) than that of the other samples. This could be because of the heat treatment used in the production of the products. Polyphenolics, susceptible compounds to heat damage, are lost during various processing operations (Hojjatpanah et al. 2011).

Generally, there are some differences in the basic physicochemical characteristics among the samples. These differences in the composition of samples could be attributed to many factors such as the type and the composition of the fruit used as well as the production method and storage conditions of the molasses (Hojjatpanah et al. 2011; Salık et al. 2021).

Antimicrobial activity, MIC and MBC determination of molasses samples

Antimicrobial activity of three different molasses samples were evaluated with *E. coli* O157:H7 strain in liquid media, and it was seen that all molasses samples had antimicrobial activity against *E. coli* O157:H7 strain. The MICs of molasses samples were determined for the investigation of antimicrobial effect on *E. coli* O157:H7. MICs of CM, MM, and BMS were found to be 0.14, 0.14 and 0.11 g/mL, respectively (Table 2). MBCs of CM, MM and BMS were found to be 0.28, 0.28 and 0.22 g/mL, respectively.

Effects of molasses samples on the size of phage plaques

In order to evaluate the effect of molasses on phage plaque size, agar diffusion and double layer agar methods were used. As can be seen in Table 3, all molasses samples led to the enlargement of phage plaques. In addition, phage plaque sizes in the regions with and without CM sample are given in Figure 1. For CM,

the plaque size increased in 2×MIC, MIC, 1/2×MIC (p<0.05), while it remained constant in 1/4×MIC. It was also seen that increasing the concentration of MM and BMS samples increased the plaque diameter of *E. coli* O157:H7 phages (2×MIC, 1/2×MIC; p<0.05).

One-step growth curve

One-step growth curve experiment was carried out to determine the latent period and burst size of EliavaPhage3 in the presence and the absence of the molasses sample CM. CM was chosen for the time kill curve experiment since the best effect was observed in this sample. The burst size of the phage was found to be 264±32 phage per infected cell, and the burst size of the phage with molasses (at MIC value) was found as 575±41 phage per infected cell. Latent period was found to be 45 and 30 min in the absence of molasses sample and the presence of molasses sample, respectively. Phage burst size was found as 183±11 phage per infected cell without molasses, while it was found to be 581±52 phage per infected cell with molasses (at 1/2×MIC). The latent period was found to be 30 and 45 min in the absence of molasses sample and the presence of molasses sample, respectively. It is known that the burst size of phage may vary depending on the ambient conditions which the test is performed (Ellis & Delbrück 1939). According to these results, the presence of molasses increased the burst size of phage. The concentration MIC of molasses decreased the latent period of phage, whereas the concentration 1/2×MIC of molasses increased it.

Investigation of the interactions between phage and molasses samples

It was observed that molasses samples increased phage plaque size (Table 3). Therefore, the time-kill curve test was conducted to detect whether there was any synergistic effect. The MIC value of CM was found to be 0.14 g/mL against *E. coli* O157:H7. MIC and 1/2×MIC were used for time kill curve experiments. The antimicrobial effect of CM, phage, and their combination (phage and CM) were examined against *E. coli* O157:H7 through their incubation for 300 min at 37 °C as shown in Figure 2 and Figure 3. According to the results, at the concentration of 1/2×MIC, phage, molasses, and their combination reduced the bacterial

count in 5 hours by 7.24, 2.37, and 5.90 log CFU/mL, respectively (p<0.05). For MIC value, the results obtained at the end of 5 h was compared with the control (8.90 log CFU/mL), and it was found that the bacterial number of *E. coli* O157:H7 significantly decreased by 6.41, 4.75, and 4.38 log CFU/mL for phage, molasses, and the combination of them, respectively (p<0.05).

Discussion

Today, antibiotic resistance is one of the growing public health problems as several types of bacteria have developed resistance to antibiotics. Although phages are successfully used to combat antibiotic resistant bacteria, their effectiveness still needs to be improved. The use of phages with other antimicrobial compounds, especially with antibiotics, is among the most frequently studied topics. However, there are only a few studies in the literature involving the combination of phages and food products containing antimicrobial compounds. In this study, we evaluated the efficacy of a combination of phage and molasses to eliminate *E. coli* O157:H7. To the best of our knowledge, there is no study examining the elimination effect of phage-molasses combination against foodborne pathogenic bacteria.

The physicochemical characterization of CM, MM and BMS samples was carried out and the results were generally found to be compatible with the literature. TSS and TTA of CM were slightly higher than that of reported by Sengül et al. (2005) while they were agreeable with the findings of Tetik et al. (2011). The total phenolic content of CM along with invert sugar and sucrose amounts were also found to be in agreement with the findings of other researchers (Sengül et al. 2007; Akkaya et al. 2012). The results of MM used in the present study were generally consistent with the ones reported by the literature (Karataş and Şengül 2018; Salık et al. 2021). However, the TP content of BMS in the present study is higher than the one reported by Kamiloglu et al. (2013). The total acidity of BMS was found to be higher than that of the others. It is reported that the fruits of *M. nigra* (1.40 %) had higher acidity than *M. alba* (0.25%), which suggest that the acidity of the fruit used in the production of the molasses might be effective in the acidity of the final product (Ercisli and Orhan 2007).

The results obtained in the study showed that different molasses had variable antimicrobial activity against *E. coli* O157:H7. This variation could be attributed to the differences in the production methods as well as the fruits used in the production of white mulberry molasses and black mulberry syrup samples. Although *M. alba* and *M. nigra* belong to the same genus, their physiochemical parameters are known to be different (Jan et al. 2021). It is reported that there was a difference between black mulberry and white mulberry not only in terms of total phenolic and total organic acid content, but also in terms of phenolic substance and organic acid profiles (Gundogdu et al. 2011). Among the studied phenolics in the study of Gundogdu et al. (2011), chlorogenic acid was determined as the dominating phenolic in black mulberry and rutin as the one in white mulberry. Studies focusing on the antimicrobial activity of chlorogenic acid demonstrated the inhibitory effect of this phenolic against *E. coli* O157:H7 (Muthuswamy and Rupasinghe 2007; Santana-Gálvez et al. 2017). There are also significant differences in terms of titratable acidity, total phenolics, sugar contents among the samples which can be shown to address the differences in antimicrobial activity. Some studies suggested that polyphenols in sugar beet molasse can cause leakage of the cell (Chen et al. 2017). It is widely known that phenolic compounds of plant products can be used as antimicrobial agents against different pathogenic bacteria (Rauha et al. 2000; Pereira et al. 2007; Estevinho et al. 2008). Antimicrobial effect of phenolics could be related to cell membrane degradation and alteration of cell permeability (Campos et al. 2009). It was also reported that the antimicrobial action of phenolic acids was related with intracellular acidification caused by K+ efflux and cell membrane permeability might change and disturb the cell integrity due to the intracellular acidification (Campos et al. 2009; Borges et al. 2013).

The MIC and MBC values were obtained using broth microdilution method and it was found that MIC values ranged from 0.11 to 0.14 g/mL and MBC values ranged from 0.22 to 0.28 g/mL for *E. coli* O157:H7. These results revealed that the antimicrobial activity of molasse samples could be described as moderate to minimal and the effect of black mulberry syrup was higher than that of the others. It was argued that the raw material of the molasses (their phenolic content) and the process conditions may have an effect on the differentiation in the MIC and MBC values of the molasses samples (Chen et al. 2017). Although there are

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studies evaluated the antimicrobial activity of molasses in the literature, MIC and MBC of molasses samples are the first reported values obtained against *E. coli* O157: H7.

While examining the interaction between phage and molasses samples, the effect of molasses samples on phage plaque size was tested as a first step. In previous studies, it was reported that the increase in phage plaque size might be related with synergism (Comeau et al. 2007a; Kamal and Dennis 2015). In this study, we observed that all molasses samples enlarged the plaque size of *E. coli* O157:H7 phage. Also, CM sample was found to be the most effective in increasing the phage plaque size at MIC, 1/2×MIC and 1/4×MIC. CM increased phage plaque size approximately 2-fold at 1/4×MIC. The phage plaque size is generally related with the burst size of the phage, and one step growth curve experiments showed that molasses increased burst size at inhibitory and subinhibitory concentrations. Synergism studies in the literature have generally focused on phage-antibiotic synergism. On the contrary, we worked with molasses, which is one of the antimicrobial substances other than antibiotics and investigated how the burst size of phage changes with molasses. In the experiments, we observed that molasses increased phage plaque size and burst size at inhibitory and subinhibitory concentrations. Similar to our results, it was reported that black cumin (*Nigella sativa*) extract increase the plaque size of *S. aureus* phage (Tayyarcan et al. 2019). In literature, antimicrobial agents like antibiotics, essential oil and plant extracts have been used for enhancing the phage therapy applications. Most of the phage synergism studies have been conducted with antibiotics. Phage-antibiotic synergism has been studied by many research groups and different views have been put forward regarding the mechanism of the synergistic effect. It was observed that antibiotics increased the phage plaque size at subinhibitory concentrations (Kaur et al. 2012; Kamal and Dennis 2015). The presence of antibiotics is thought to be responsible for the changes in the cell structure and for facilitating phage maturation (Comeau et al. 2007a). Furthermore, elongation, filamentation, and cell cluster occur in bacterial cells in the presence of antibiotics, which facilitates the attachment of phage receptors to the cells (Kamal and Dennis 2015). Molasses may have a similar effect on the bacterial cell due to its antimicrobial effect. In some studies on the combined use of honey, which have high osmolarity like molasses, and phages, it was stated that

antimicrobial and antibiofilm activity increased when these combinations were used (Oliveira et al. 2018). Similarly, it may be possible that the molasses used in the study stresses bacteria by different mechanisms such as changing the osmotic pressure and thus renders them susceptible to phage infection. This may have been observed as larger plaques appear on the agar and increase in burst size. Although different mechanisms of action have been observed in studies conducted so far, the exact molecular mechanism of the effect of molasses on phage activity needs to be examined in detail.

Based on the change of burst sizes and MIC-MBC values, time-kill curve trials were conducted with CM in order to examine the effect of molasses samples on phage activity and whether there is a synergistic effect on bacterial elimination. However, although the enlargement of phage plaques in the agar medium was clearly seen, time-kill curve experiments in liquid medium did not show the same results. The antimicrobial effect of phages and molasses samples were determined separately, and then the changes in the antimicrobial effect in dual/combined use was investigated. Time-kill curve experiments were carried out to better understand the phage-molasses interactions in liquid media. According to the results obtained from time-kill curve experiments, phage, molasses, and phage-molasses combination caused a significant decrease in *E. coli* O157:H7 count. However, compared to the use of phage-molasses, the use of phage caused a greater decrease in the count of bacteria. In addition, it was observed that when molasses was used at MIC value concentration, phage titer decreased after 300 min. Also, it was seen that the phage titer decreased after 300 min when molasses was used at MIC, while the final titer of the phage increased at $1/2\times$MIC. This may be due to the antiviral effect of high-concentration-molasses against phage. Some studies in the literature mentioned the antiviral activity of phenolic compounds (Chávez et al. 2006; Lee et al. 2016; Li et al. 2017), and this may be associated with the decrease in phage titer at high molasses concentrations. Thus, combinations at concentrations below the MIC were found to be more effective, and it was observed that the molasses at MIC had a negative effect on the activity and titer of phage, while the use of molasses at lower concentrations reduced this adverse effect on phage. In addition, the phage-molasses combination at $1/2\times$MIC concentration was found to be more successful in eliminating bacteria than the phage-molasses

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combination at MIC concentration. This situation was also seen in the studies on synergism with antibiotics, and the positive results were obtained only when the antibiotic was tested with phages at subinhibitory concentrations (Comeau et al. 2007; Kamal & Dennis 2015; Kaur et al. 2012; Knezevic et al. 2013; Ryan et al. 2012).

It is possible to use components with antimicrobial content, including molasses, and phages to reduce pathogenic bacteria encountered in the food industry. Especially fresh or unprocessed foods are more likely to contain pathogenic bacteria, and it is important to prevent diseases caused by the consumption of such foods. Viazis et al. (2011) suggested the combined use of phage mixture and trans-cinnamaldehyde to reduce the number of *E. coli* O157:H7 in leafy green vegetables. They stated that the phage mixture (BEC8) and trans-cinnamaldehyde were effective in reducing the number of *E. coli* O157:H7 in leafy vegetables (Viazis et al. 2011). The use of such natural antimicrobial products to increase phage activity in the food industry can reduce the number of pathogenic bacteria in the food chain.

Phage therapy applications can be used in pre- and post-harvest processes of food as well as food contact surfaces. Phage therapy has crucial advantages against chemical disinfectants used in the food industry applications such as being specific to target pathogen, abundance in nature, having minimal or none harmful effect on natural microflora and minimal or none sensory effect on food (Vikram et al. 2020). Combination of phage and molasses can be used to reduce pathogenic bacteria in the food industry, but more comprehensive analyzes are required for optimizing the application.

We investigated the antimicrobial effect of molasses samples on *E. coli* O157:H7, which is extremely pathogenic for humans, and the antimicrobial effect of phage and molasses on *E. coli* O157:H7 together and separately. The obtained results showed that molasses samples increased the phage plaque size significantly on agar media. The one step growth curve experiments showed that both molasses concentrations of MIC and $1/2 \times$MIC increased the burst size of phage. However, only MIC concentration of molasses decreased the latent period of phage. The time-kill curve test revealed that there was no synergism between the phage

and the molasses. Although phage plaque size and burst size increased, time-kill curve test results showed that the phage titer decreased in the presence of molasses. Phages were found to be more successful in reducing the number of bacteria than phage-molasses combination. These results offer an alternative to the use of phages against food pathogens with products containing natural antimicrobial ingredients that can be included in the food content safely. However, further studies are needed to better understand the interaction between phage and molasses samples by increasing the number of phages and molasses types. The detailed chemical composition of the molasses samples might also be determined in order to better understand this interaction.

Acknowledgements

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Figure captions

**Figure 1.** Effect of CM molasses sample on plaque size of EliavaPhage3

a) $2\times$MIC,10^{-5}; b) $2\times$MIC, 10^{-6}; c) MIC, 10^{-5}; d) MIC, 10^{-6}; e) $1/2\times$ MIC, 10^{-5}; f) $1/2\times$ MIC, 10^{-6}
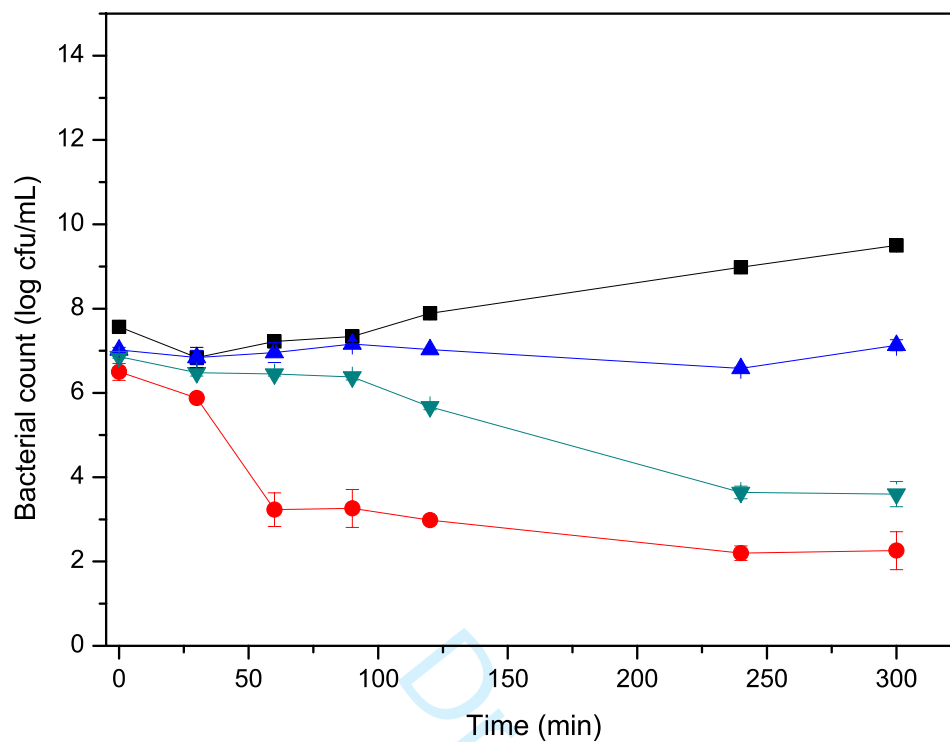
Figure 2. Time kill curve in the presence of molasses at MIC concentration

■ Bacteria, ● Bacteria + Phage, ▲ Bacteria + Molasses, ▼ Bacteria + Molasses + Phage

Figure 3. Time kill curve in the presence of molasses at $1/2\times$ MIC concentration
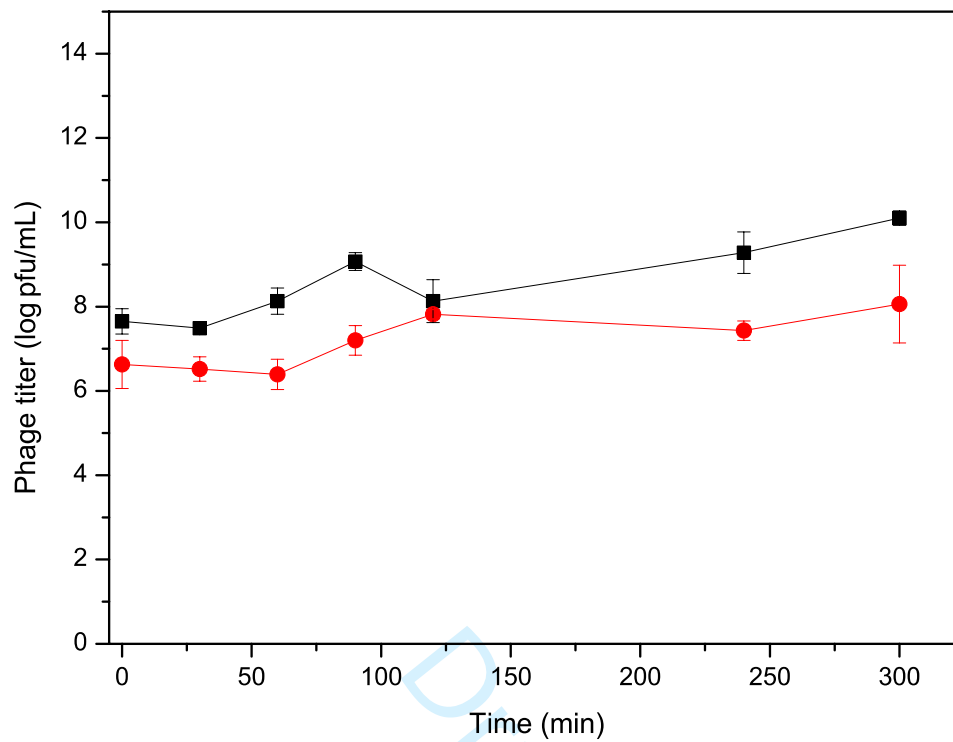
■ Bacteria, ● Bacteria + Phage, ▲ Bacteria + Molasses, ▼ Bacteria + Molasses + Phage

Figure 4. Change of phage titer in the presence of molasses at MIC concentration

■ Bacteria + Phage, ● Bacteria + Molasses + Phage

Figure 5. Change of phage titer in the presence of molasses at $1/2\times$ MIC concentration

■ Bacteria + Phage, ● Bacteria + Molasses + Phage

1 **Tables**

2 **Table 1.** Some physicochemical characteristics of samples

Sample Code [1]	TSS (%)	a_w	Density (g/mL)	TTA (g CAE/100 g sample)	TP (mg GAE/g)	Sugar content (g/100 g sample)		
						Fructose	Glucose	Sucrose
CM	75.20±0.0 a	0.70±0.0 c	0.56	1.36±0.02 c	11.73 ± 0.62 a	32.84 ±0.57 a	26.79 ±0.50 [a]	14.53 ±0.15 [a]
MM	68.37±0.06 b	0.79±0.0 b	0.56	1.43±0.02 b	10.95 ± 0.72 a	23.95 ±0.61 b	20.70 ±0.56 [b]	6.60 ±0.06 [b]
BMS	38.13±0.06 c	0.93±0.0 a	0.44	2.36±0.03 a	1.73 ± 0.25 b	17.78 ±0.37 c	16.68 ±0.299 [c]	< LOQ

3 [1] CM, Carob Molasses; MM, White Mulberry Molasses; BMS, Black Mulberry Syrup

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8   **Table 2.** MIC and MBC values of molasses samples

Molasses Samples	MIC (g/mL)	MBC (g/mL)
Carob Molasses	0.14	0.28
Mulberry Molasses	0.14	0.28
Black Mulberry Syrup	0.11	0.22

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11   **Table 3.** Effect of molasses samples on phage plaque size.

Molasses Samples	Phage Plaque Size (mm) $\pm$ SD			
	2×MIC	MIC	1/2× MIC	1/4× MIC
Carob Molasses	1.59 $\pm$ 0.25	1.61 $\pm$ 0.28	1.51 $\pm$ 0.14	1.06 $\pm$ 0.13
Mulberry Molasses	1.52 $\pm$ 0.19	1.62 $\pm$ 0.13	1.09 $\pm$ 0.20	0.97 $\pm$ 0.19
Black Mulberry Syrup	1.57 $\pm$ 0.21	1.12 $\pm$ 0.23	0.93 $\pm$ 0.17	0.65 $\pm$ 0.35

12   [*] Plaque size of control samples is 0.57 $\pm$ 0.22 mm

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18   **Figures**
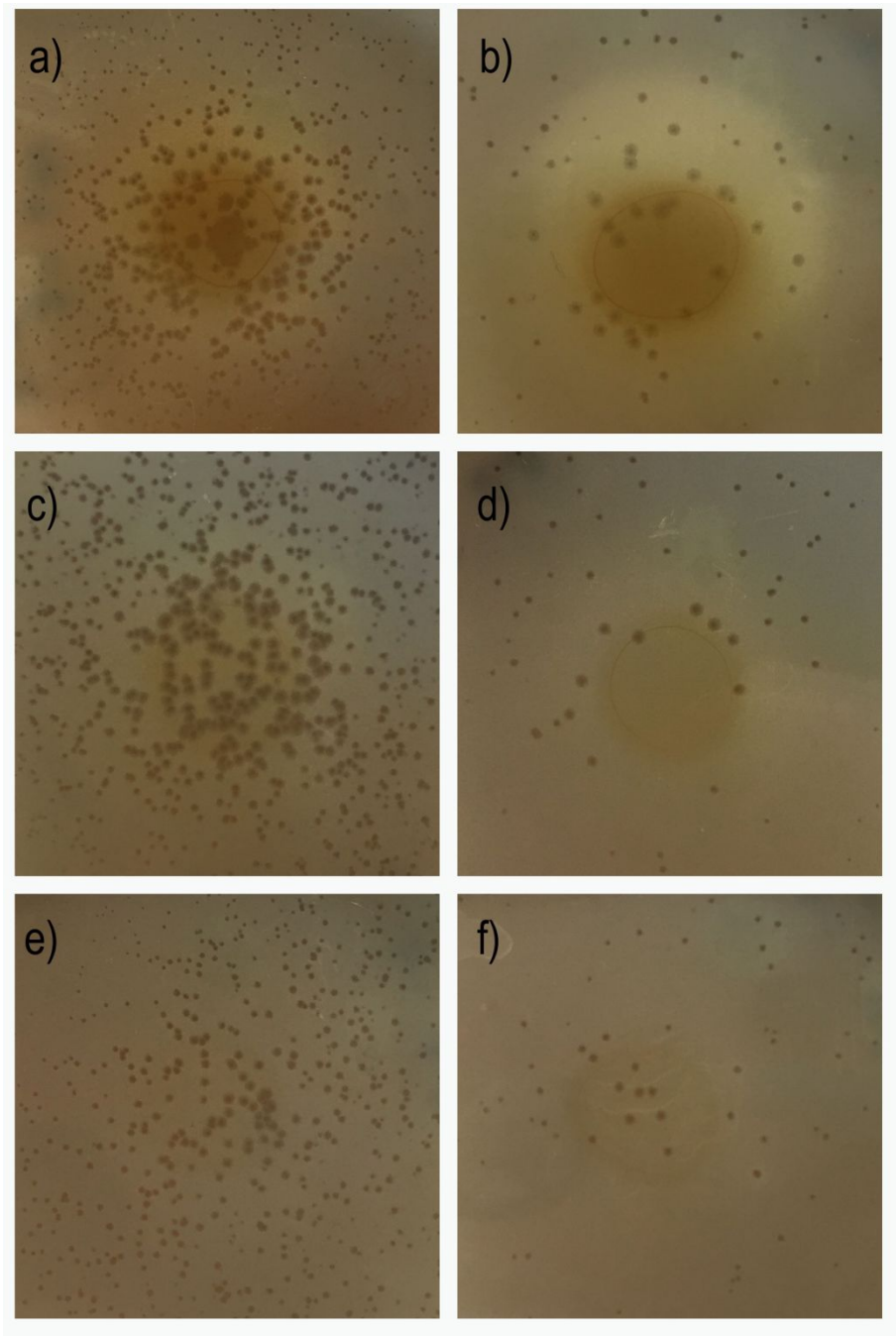
19

20   **Figure 1.** Effect of CM molasses sample on plaque size of EliavaPhage3

21   a) 2×MIC,10^{-5}; b) 2×MIC, 10^{-6}; c) MIC, 10^{-5}; d) MIC, 10^{-6}; e) 1/2× MIC, 10^{-5}; f) 1/2× MIC, 10^{-6}

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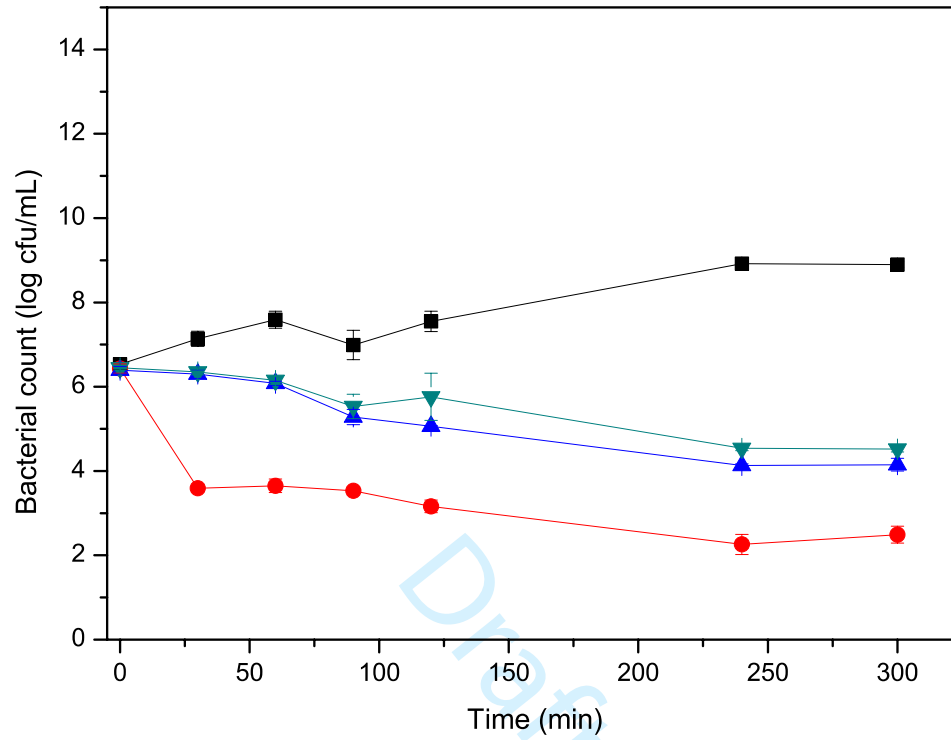
Figure 2. Time kill curve in the presence of molasses at MIC concentration

26 ■ Bacteria, ● Bacteria + Phage, ▲ Bacteria + Molasses, ▼ Bacteria + Molasses + Phage

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Figure 3. Time kill curve in the presence of molasses at 1/2×MIC concentration

30 ■ Bacteria, ● Bacteria + Phage, ▲ Bacteria + Molasses, ▼ Bacteria + Molasses + Phage

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Figure 4. Change of phage titer in the presence of molasses at MIC concentration

37 ■ Bacteria + Phage, ● Bacteria + Molasses + Phage

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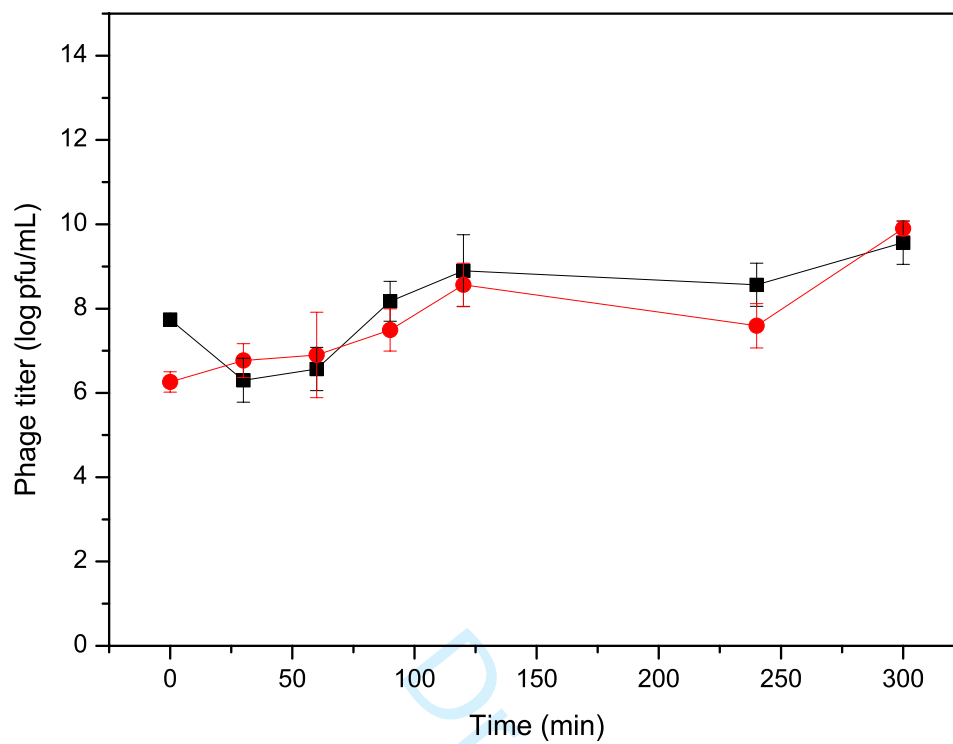
Figure 5. Change of phage titer in the presence of molasses at 1/2×MIC concentration

47   ■ Bacteria + Phage, ● Bacteria + Molasses + Phage

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1 **Tables**

2 **Table 1.** Some physicochemical characteristics of samples

Sample Code [1]	TSS (%)	a_w	Density (g/mL)	TTA (g CAE/100 g sample)	TP (mg GAE/g)	Sugar content (g/100 g sample) Fructose	Glucose	Sucrose
CM	75.20±0.0 a	0.70±0.0 c	0.56	1.36±0.02 c	11.73 ± 0.62 a	32.84 ±0.57 a	26.79 ±0.50 [a]	14.53 ±0.15 [a]
MM	68.37±0.06 b	0.79±0.0 b	0.56	1.43±0.02 b	10.95 ± 0.72 a	23.95 ±0.61 b	20.70 ±0.56 [b]	6.60 ±0.06 [b]
BMS	38.13±0.06 c	0.93±0.0 a	0.44	2.36±0.03 a	1.73 ± 0.25 b	17.78 ±0.37 c	16.68 ±0.299 [c]	< LOQ

3 [1] CM, Carob Molasses; MM, White Mulberry Molasses; BMS, Black Mulberry Syrup

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Table 2. MIC and MBC values of molasses samples

Molasses Samples	MIC (g/mL)	MBC (g/mL)
Carob Molasses	0.14	0.28
Mulberry Molasses	0.14	0.28
Black Mulberry Syrup	0.11	0.22

Table 3. Effect of molasses samples on phage plaque size.

Molasses Samples	Phage Plaque Size (mm) ± SD			
	2×MIC	MIC	1/2× MIC	1/4× MIC
Carob Molasses	1.59 ± 0.25	1.61 ± 0.28	1.51 ± 0.14	1.06 ± 0.13
Mulberry Molasses	1.52 ± 0.19	1.62 ± 0.13	1.09 ± 0.20	0.97 ± 0.19
Black Mulberry Syrup	1.57 ± 0.21	1.12 ± 0.23	0.93 ± 0.17	0.65 ± 0.35

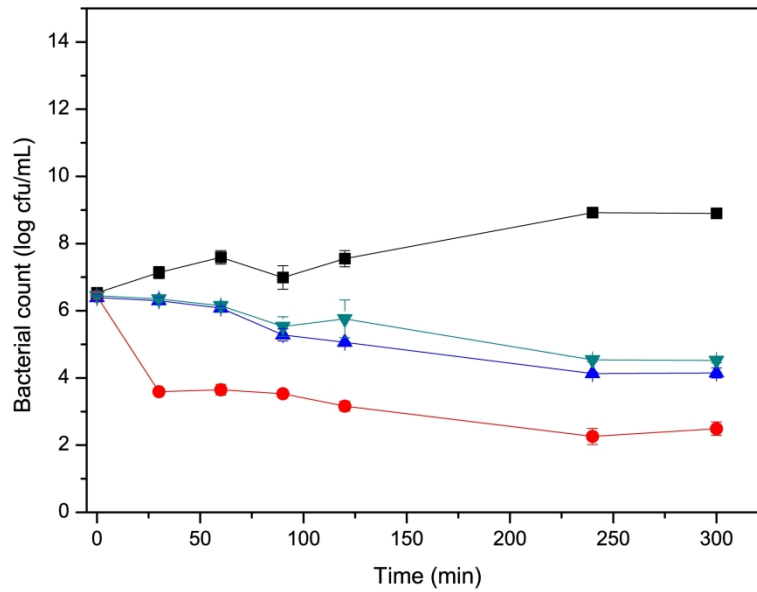* Plaque size of control samples is 0.57 ± 0.22 mm
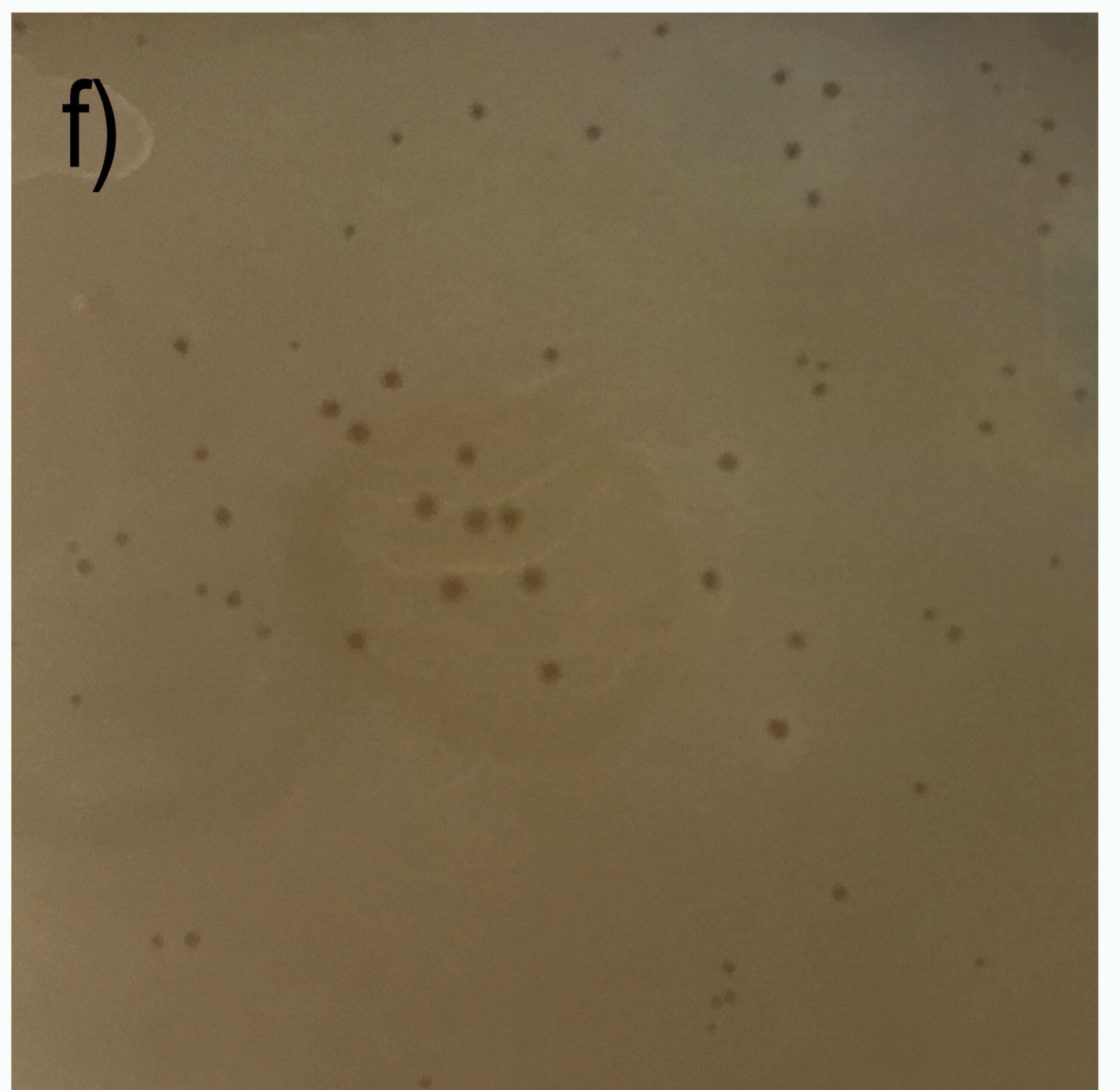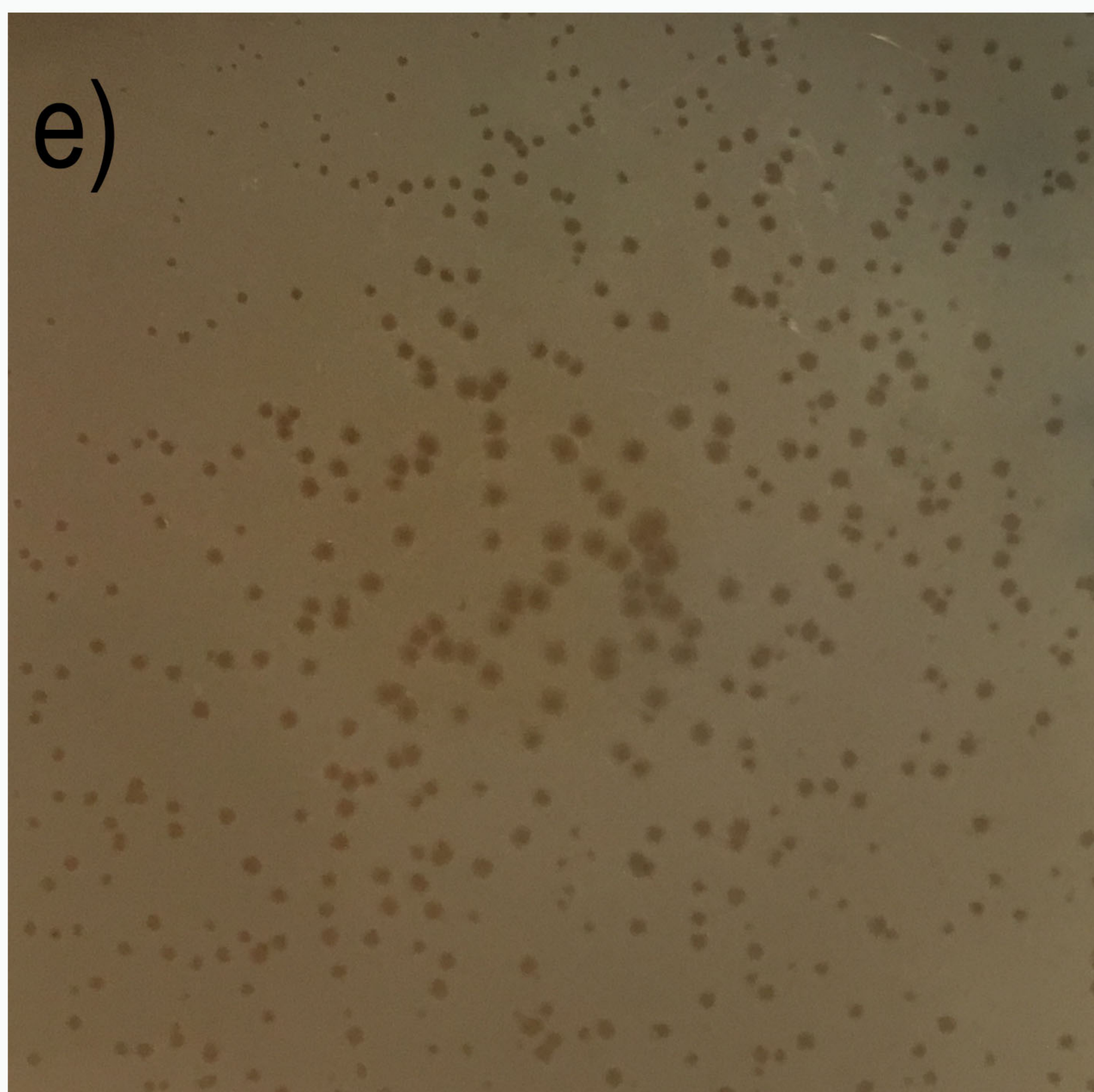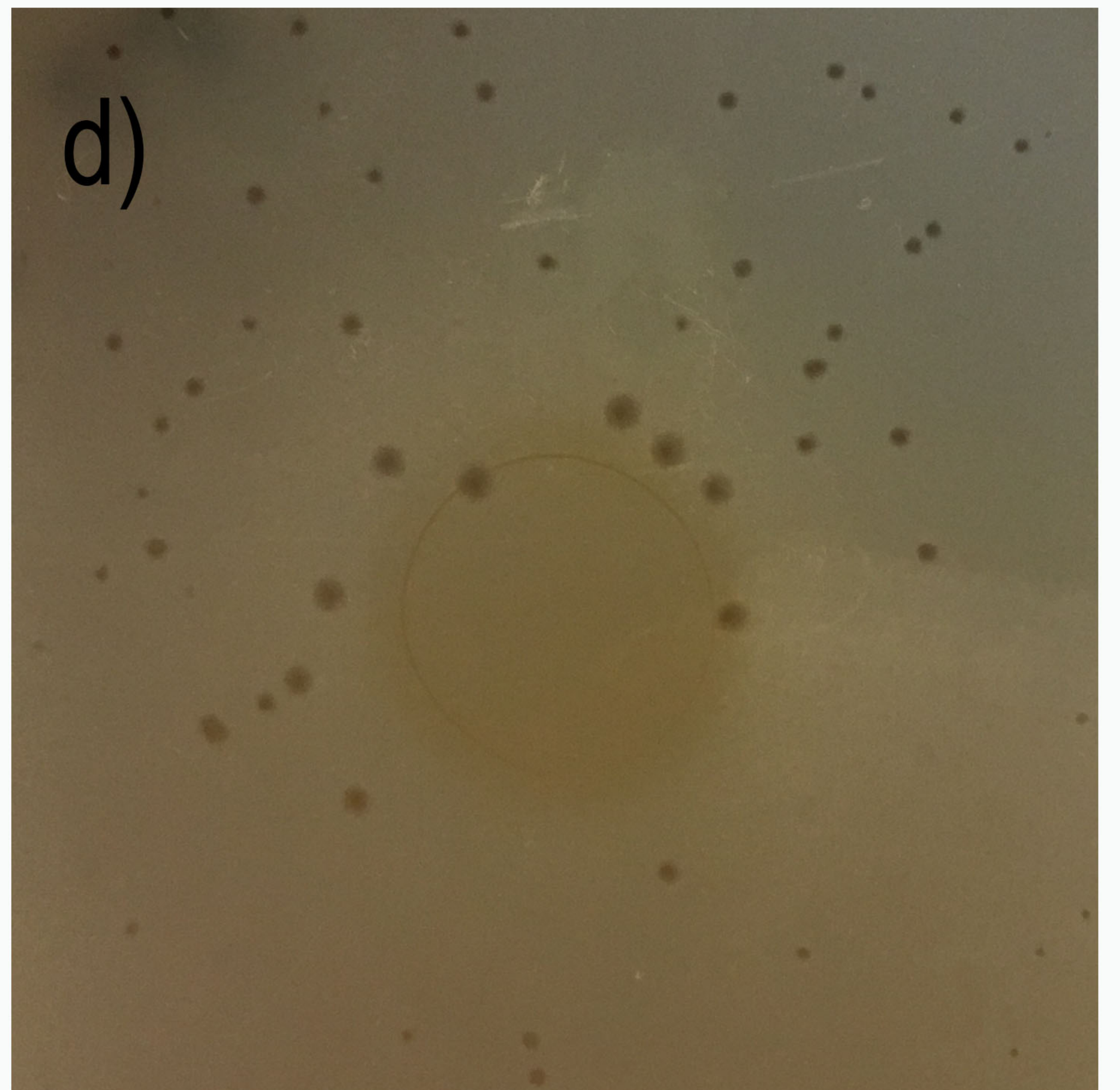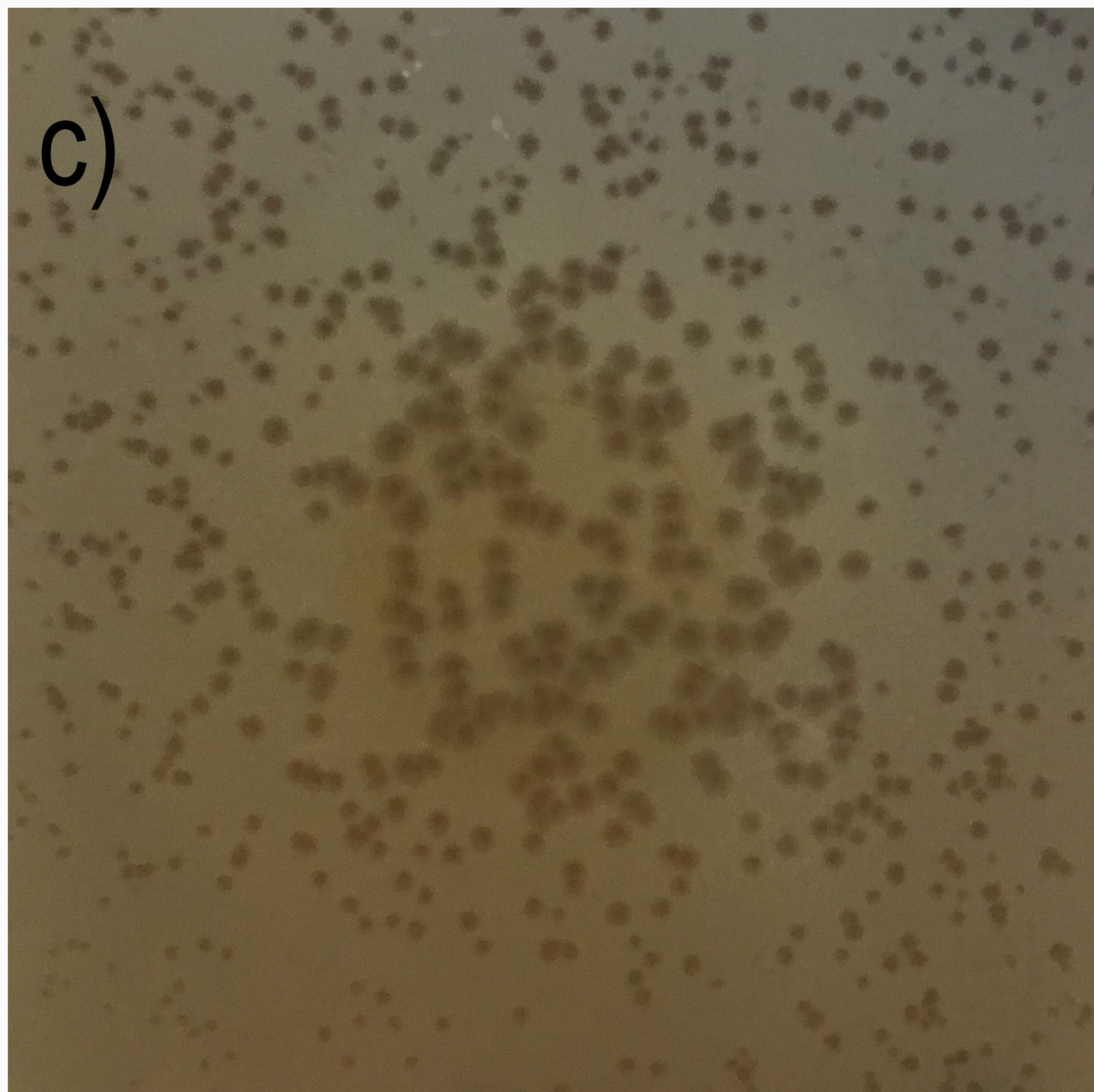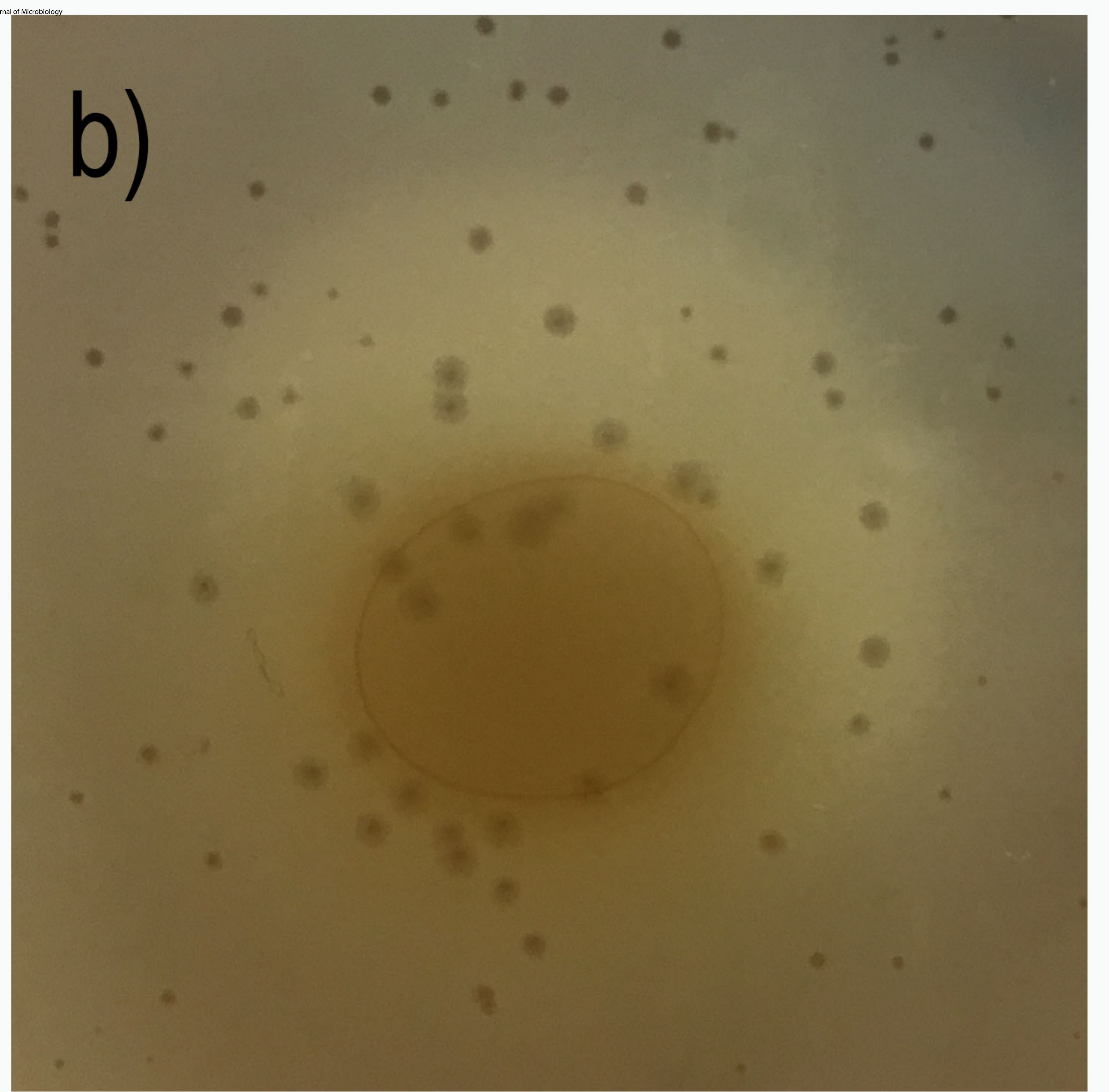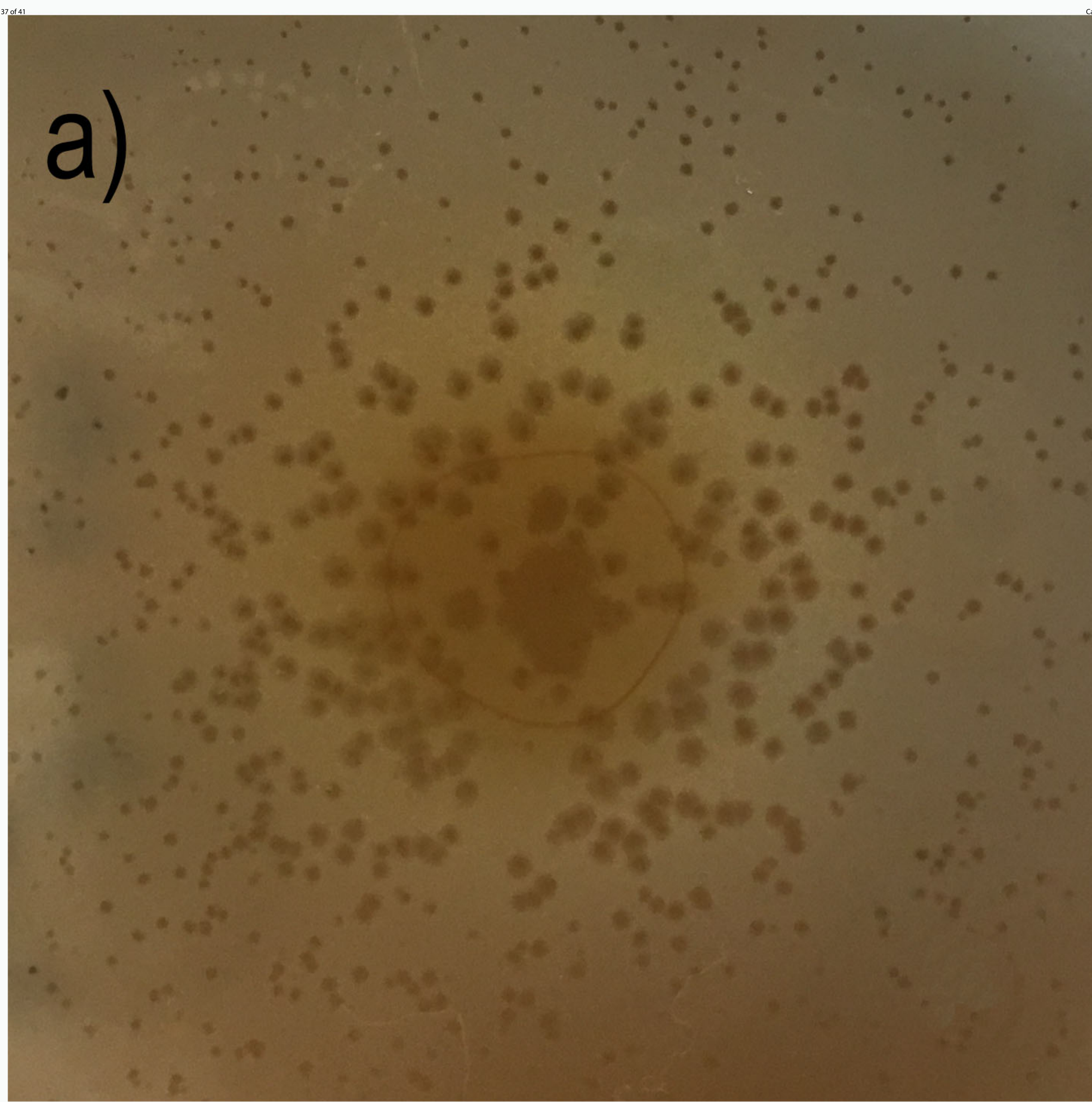
Figure 2. Time kill curve in the presence of molasses at MIC concentration
■ Bacteria, ● Bacteria + Phage, ▲ Bacteria + Molasses, ▼ Bacteria + Molasses + Phage
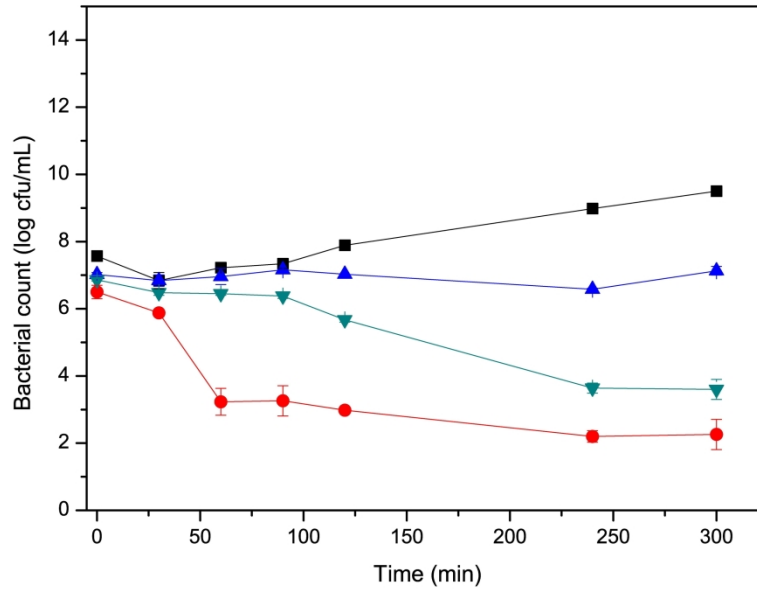
287x201mm (300 x 300 DPI)

Figure 3. Time kill curve in the presence of molasses at 1/2× MIC concentration
■ Bacteria, ● Bacteria + Phage, ▲ Bacteria + Molasses, ▼ Bacteria + Molasses + Phage
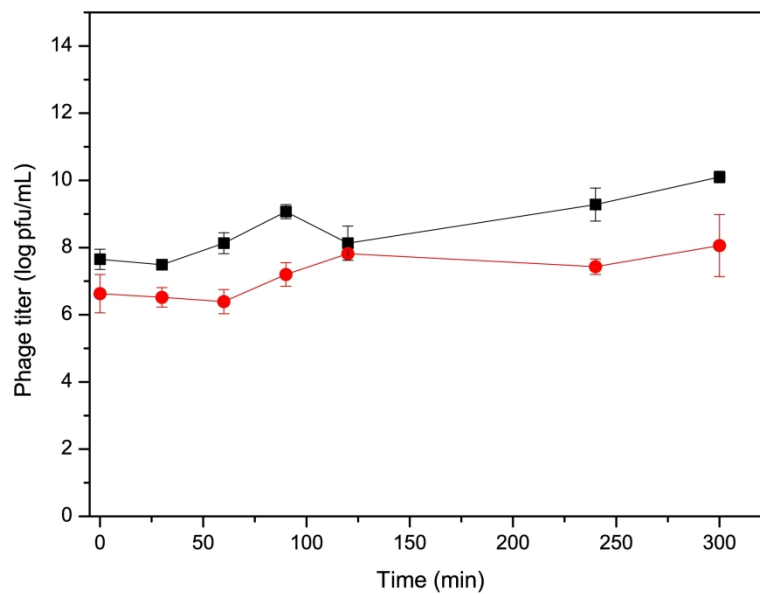
287x201mm (300 x 300 DPI)

Figure 4. Change of phage titer in the presence of molasses at MIC concentration
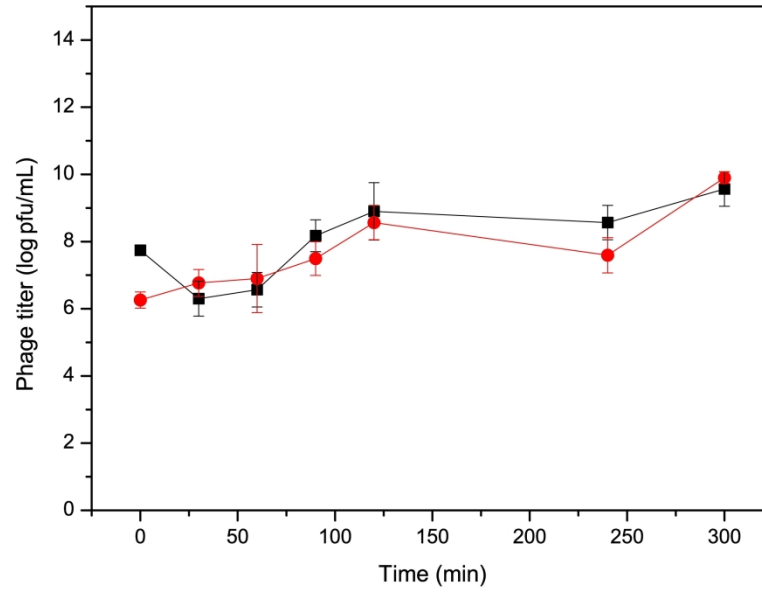■ Bacteria + Phage, ● Bacteria + Molasses + Phage

287x201mm (300 x 300 DPI)

Figure 5. Change of phage titer in the presence of molasses at 1/2× MIC concentration
■ Bacteria + Phage, ● Bacteria + Molasses + Phage

287x201mm (300 x 300 DPI)