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Antimicrobial properties of ethylene vinyl alcohol/epsilon-polylysine films and their application in surimi preservation

Muriel-Galet, V; López-Carballo, G; Gavara, R; Hernández-Muñoz, P*

Packaging Lab, Instituto de Agroquímica y Tecnología de Alimentos, IATA-CSIC, Av. Agustín Escardino 7, 46980 Paterna, SPAIN, Phone: +34-963900022, Fax: +34-963636301
Email: phernan@iata.csic.es

ABSTRACT

Polymer films based on ethylene vinyl copolymers (EVOH) containing a 29% (EVOH29) and a 44% molar percentage of ethylene (EVOH44), and incorporating ε-polylysine (EPL) at 0, 1, 5 and 10% were successfully made by casting. The optical properties and the amount of EPL released from the films to phosphate buffer at pH 7.5 were evaluated, films showing great transparency and thoseEVOH29 copolymer releasing a greater amount of EPL. The antimicrobial properties of the resulting films were tested *in vitro* against different foodborne microorganisms and *in vivo* in surimi sticks. With regard to the antimicrobial capacity tested *in vitro* in liquid medium at 37 °C and 4 °C against *Listeria monocytogenes* and *Escherichia* coli over a period of 72 h, films showed a considerable growth inhibitory effect against both pathogens, more notably against *L. monocytogenes*, and being EVOH29 more effective than EVOH44 films. At 37 ºC total growth inhibition was observed for EVOH 29 films incorporating 10% EPL against both microorganisms whereas the copolymer EVOH 44 did show total inhibition against *L. monocytogenes* and the growth of *E. coli* was reduced by 6.64 log units. At 4 ºC no film was able to inhibit completely bacterial growth. Scanning electron microscopy micrographs showed corrugated cell surfaces with blisters and bubbles, and collapse of the cells appearing shorter and more compact after treatment with EPL. Finally, the films were successfully used to increase the shelf life of surimi sticks. The results show the films developed have great potential for active food packaging applications.

Keywords: EVOH, epsilon-polylysine; antimicrobial polymer films; *L. monocytogenes*; *E. coli*

1. INTRODUCTION

Ready-to-eat products with an extended shelf life, stored at refrigeration temperatures (4 °C) and consumed without further cooking have been implicated in various outbreaks of microbial food-borne illness. *Listeria monocytogenes* and *Escherichia coli* have been implicated in many outbreaks, most commonly due to post-processing contamination (Tzschoppe et al. 2012; Muriel-Galet et al. 2012a).

Listeriosis is a food-borne illness that affects susceptible populations, such as the young, the elderly, pregnant women and immunocompromised individuals (YOPIs). *L. monocytogenes* is considered a pathogen of major concern. It has a long incubation period, which makes it difficult to identify the food that is contaminated with *L. monocytogenes* and causes illness (Huss et al. 2000). *E. coli* is found in a wide variety of foods, causing food-borne disease outbreaks (Buchanan and Doyle 1997; Mead and Griffin 1998). A great number of cases can be severe and they are sometimes fatal.

Imitation crab meat (surimi) is a paste prepared from mechanically deboned, washed (bleached) and stabilized flesh of fish (FAO 2005). Surimi is one of the most promising approaches to obtain value-added food products from low-cost fishery products (Venugopal and Shahidi 1995). Surimi is used in the preparation of cooked ready-to-eat products which are sensitive to microbial contamination with diverse Gram positive and Gram negative bacteria after processing, e.g. *L. monocytogenes* (Miya et al. 2010). This microorganism is a serious threat to food safety in processing plants, being a very ubiquitous bacterium that can grow in many chilled food products. Proper refrigeration temperature below 4ºC, handling, preparation and service under Good Manufacturing Practices (GMP) are strategies that can prevent the cross-contamination of surimi products (Kaneko et al. 1999; Park 2014).

The increase in food-borne illness outbreaks has intensified research on antimicrobial packaging technologies (Suppakul et al. 2003), with a particular interest in the use of natural antimicrobial agents, including essential oils and their components, organic acids, enzymes, peptides, etc. Epsilon-polylysine (EPL) is a natural cationic linear homopolymer compound of 25–35 residues of L-lysine connected between the ε-amino and α-carboxyl groups (Shima and Sakai 1977; Shima et al. 1984). It is produced from aerobic fermentation by *Streptomyces albulus*, a non-pathogenic microorganism (Hiraki et al. 2003). EPL is characterized as being edible, water soluble, stable at high temperatures and of low environmental impact because of its biodegradability. The antimicrobial activity of EPL depends on electrostatic interaction with the cell surface of microorganisms, leading to distortion of the outer membrane and producing abnormal distribution of the cytoplasm (Shima et al. 1984). EPL has an isoelectric point of 9.0, and the optimum pH range to exert its antimicrobial activity is between 5 and 8. EPL is non-

toxic to humans and has been approved as a food additive in Japan at a concentration of 1000–5000 ppm for sliced fish or fish sushi and at a concentration of 10–500 ppm for the preservation of rice, soup and vegetables (Hiraki 1995, 2000). EPL has been classified as GRAS (Generally Recognized as Safe) by the Food and Drug Administration (FDA) (FDA 2004). In recent years, EPL has been used in a wide range of industrial applications, as a food preservative (Zinoviadou et al. 2010; Chang et al. 2010), emulsifying agent (Chang et al. 2012), etc. Information regarding the antimicrobial activity of EPL when it is incorporated into a film matrix is limited, finding studies with edible coatings (Unalan et al. 2011; Zinoviadou et al. 2010), however, no studies have been found in the bibliography regarding the development of antimicrobial films by incorporating EPL in conventional polymer matrices used in food packaging.

Ethylene-vinyl alcohol (EVOH) copolymers are approved for food contact applications and have been used as matrices for the development of active packaging systems (Muriel-Galet et al. 2012b; Muriel-Galet et al. 2012a; Muriel-Galet et al. 2013a; Lopez de Dicastillo et al. 2011; Lopez-de-Dicastillo et al. 2010; Lopez-de-Dicastillo et al. 2012). Because of their hydrophilic nature, EVOH films have a great potential to be used as carriers and release matrices of bioactive agents. These polymers can protect the agent during storage in a dry environment and trigger their activity on exposure to a humid environment, in this case created by the packaged food product (Aucejo et al. 2000).

In this context, the present study intends to show the potential of EVOH copolymers to develop antimicrobial films for food packaging applications incorporating the antimicrobial compound EPL in EVOH films containing a 29% and a 44% molar percentage of ethylene. The ability of the films to inhibit the growth of *L. monocytogenes* and *E. coli* was evaluated *in vitro*. Finally, the effectiveness of the films was tested in a real food, surimi sticks. This product is an ideal medium for bacterial growth because it has high water and nutrient contents and limited shelf life.

2. MATERIALS AND METHODS

2.1. Materials

Ethylene vinyl alcohol copolymer (EVOH) possessing a 29 and a 44% ethylene molar content was kindly provided by The Nippon Synthetic Chemical Company (Osaka, Japan); 1-propanol was purchased from Sigma (Madrid, Spain). EPL, from Chisso Corporation (Yokohama, Japan), was kindly provided by Goddard Research Group (Amherst, USA). Imitation crab meat (surimi) was purchased from a local market, labelled as preservative-free and containing 2.5 g of total fat, 12.9 g of total carbohydrate, and 8.4 g of protein.

2.2. Film Preparation

EVOH films were prepared as described in a previous work (Muriel-Galet et al. 2012b). Briefly, EPL was dissolved in distilled water at 1, 5 and 10% (g/100 g dry polymer). EVOH 29 was dissolved in a 1-propanol:EPL water mixture at 80 °C and EVOH 44 in a 2:1 (v:v) 1-propanol:EPL water mixture at the same temperature. The solution was stirred for 30 min using a magnetic stirrer. After that time, 5 mL of each film-forming solution was extended over a glass plate using an extension bar, and placed in a drying tunnel equipped with a 2500 W heat source for 10 min until it was completely dry. Films prepared without EPL were used as controls. Film thickness was measured using a Mitutoyo micrometer (Osaka, Japan) and had an average value of 15 ± 2 µm. Finally, the films were stored in glass desiccators containing silica gel at 22 °C prior to use.

2.3. Optical properties

Film colour was measured with a Konica Minolta CM-3500d spectrophotometer (Konica Minolta Sensing Inc., Osaka, Japan) set to D65 illuminant/10° observer angle. The film specimen was placed on the surface of a standard white plate. The instrument's software, SpectraMagic NX, was used to acquire the colour data and to display them in the CIELAB colour space.

The parameters L^* (black (0) to white (100)), a^* (green (−) to red (+)) and b^* (blue (−) to yellow (+)) were obtained and the polar coordinates, the chroma C^* and the hue angle $h°$ were calculated. Eight measurements of each sample were taken, and three samples of each film were evaluated. All the samples were selected with the same thickness to reduce the effect of thickness on the colour parameters.

2.4. Quantification of EPL migration from EVOH films

The amount of EPL capable of migrating out of the polymer matrix was quantified using the bicinchoninic acid assay (BCA). A sample of 0.25 g of each film (control, and incorporating 1, 5 and 10% of EPL) was immersed in 10 mL of pH 7.5 phosphate buffer for 24 hours at 37 °C. After that time, 100 µL of each sample was put in contact with the working reagent. The absorbance was measured at 562 nm (Kang et al. 1996; Uchida et al. 1993) using a POLARstar Omega multi-detection microplate reader (Biogen Cientifica S.L., Madrid, Spain). A standard curve of bovine serum albumin was used to calculate protein mass per film sample area.

2.5. Antimicrobial tests

2.5.1. Strains

Gram-positive bacterium *Listeria monocytogenes* CECT 934 (ATTCC 19114) and Gram-negative bacterium *Escherichia coli* CECT 434 (ATCC 25922) were selected because of their relevance to imitation crabmeat (surimi). Strains were obtained from the Spanish Type Culture Collection (CECT Valencia, Spain) and stored in Tryptone Soy Broth (TSB), purchased from Scharlab (Barcelona, Spain), with 20% glycerol at −80 °C until needed. For experimental use, the stock cultures were maintained by regular subculture on slants of Tryptone Soy Agar (TSA) from Scharlab (Barcelona, Spain) at 4 °C and transferred monthly. Before each experiment a loopful of each strain was transferred to 10 mL of TSB and incubated at 37 °C for 18 h to obtain early-stationary phase cells.

2.5.2. Antimicrobial activity of EPL against L. monocytogenes and E. coli

The antimicrobial activity of EPL was tested in sterile TSB to study the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) against *L. monocytogenes* and *E. coli*. To do so, serial dilutions of 1000 ppm of EPL in peptone water were made. Previously, 100 µL of cell cultures of each microorganism in stationary phase, with an optical density of 0.9 at 595 nm, was diluted in 10 mL of TSB and incubated at 37 °C until exponential phase, corresponding to an optical density of 0.2 at 595 nm (10^5 CFU/mL). Optical density was measured with a UV-Vis spectrophotometer (Agilent 8453 Spectroscopy System) using TSB as blank. 100 µL of each microorganism in exponential phase was inoculated in each test tube with 100 µL of EPL solution at concentrations ranging between 10 and 150 µg/mL. Tubes with 100 µL of peptone water were used as control. Turbidity at 595 nm was determined after 24 and 72 h (M100-S18 2008). The lowest EPL concentration that inhibited the pathogen microorganisms was recorded as the MIC. The MBC was the lowest concentration at which bacteria failed to grow in TSB and were not culturable after spreading 100 µL onto 15 mL of culture medium TSA. Tests were performed in triplicate.

2.5.3. Antimicrobial activity of EVOH films containing EPL against L. monocytogenes and E. coli

Antimicrobial activity of EVOH films with EPL was tested in liquid media at 37 °C. This temperature was chosen because it is the optimal growth temperature and it is a standard method to evaluate the activity of antimicrobial films. For this purpose, 0.25 g of EVOH films cut into pieces measuring 1.5 cm^2 (EVOH 29 and EVOH 44), without and with 1, 5 and 10% EPL, was added to a glass tube containing 10 mL of TSB. Then 100 µL of microorganism in exponential phase was transferred to the samples and incubated at 37 °C for 24 h. Depending on the

turbidity of the tubes, serial dilutions with peptone water were made and plated in Petri dishes with 15 mL of TSA culture medium. Colonies were counted after incubation at 37 °C for 24 h.

2.5.4. Scanning electron microscopy observations

After the microorganisms had been in contact with EVOH films at 37 °C for 24 h as described above, the samples were centrifuged and resuspended twice in saline solution (0.8% NaCl). The suspension was filtered on a 0.2 mm Nuclepore Track-Etch Membrane (Whatman, UK) and the membranes were dehydrated in graded alcohols (30%, 50%, 70%, 90% and 100%). SEM observation of *L. monocytogenes* and *E. coli* was carried out, working at 5–10 kV (HITACHI S 4100)

2.5.5. Antimicrobial activity of EVOH films containing EPL over time

Next, the effect of EVOH with EPL films on the growth of *L. monocytogenes* and *E. coli* over time was studied. Bacterial growth experiments were performed at two temperatures, 37 °C and 4 °C, and they lasted 72 hours. For this purpose, 100 µL of exponential phase microorganism was inoculated into tubes with 10 mL of TSB. 0.25 g of EVOH films cut into pieces measuring 1.5 cm^2 (EVOH 29 and EVOH 44), without and with 1, 5, and 10% EPL, was added to each tube and incubated at the corresponding temperature. Aliquots containing 100 µL were removed from the solution at 0, 1, 3, 6, 24, 48 and 72 hours and serial dilutions with peptone water were made and plated in Petri dishes with 15 mL of TSA culture medium. Colonies were counted after incubation at 37 °C for 24 h. Experiments were performed in triplicate.

2.5.6. Antimicrobial activity of EVOH films incorporating EPL in surimi microbiota

Refrigerated surimi sticks were purchased in a local market. Individual pieces (ca. 25 g) were wrapped with EVOH 29 and EVOH 44 films containing 10% of EPL. Samples without film, and samples wrapped with EVOH 29 and EVOH 44 films without EPL were prepared as controls. All the surfaces of the food were in contact with the films. Samples were stored at 4 °C for 6 days.

The effect on the surimi microbiota of being covered with film was evaluated on days 1, 3 and 6. For this purpose, at appropriate times, surimi samples were transferred aseptically in a sterile stomacher bag, diluted with 25 mL of peptone water (Scharlab, Barcelona, Spain) for 3 min using a Stomacher (IUL S.L., Barcelona). Serial dilutions in the same saline solution were plated on specific media (Scharlab, Barcelona, Spain) under the following culture conditions: a) Violet Red Bile Glucose agar (VRBG) for total enterobacteria, incubated at 37 °C for 48 h; b) Man, Rogosa and Sharpe agar (MRS) for lactic acid bacteria, incubated at 25 °C for 5 days; c) Nutrient Agar (NA) for total aerobic bacteria, incubated at 37 °C for 48 h; d) Nutrient Agar

(NA) for total aerobic psychrotrophic bacteria, incubated at 10 °C for 10 days; e) Plate Count Agar (PCA) for total aerobic count, incubated at 30 °C for 48 h; f) King B agar for *Pseudomonas*, incubated at 30 °C for 48 h. The counts were performed in triplicate.

2.5.7. Antimicrobial activity of EVOH films incorporating EPL on surimi inoculated with L. monocytogenes and E. coli

For this study, surimi sticks were inoculated with a diluted overnight culture (100 µL; 10^5 CFU/mL) of *L. monocytogenes* and *E. coli*. The inoculums were separately dispersed on the food surface with a sterile pipette. The subsequent procedure was similar to that described in the previous section. The antimicrobial activity of the films against inoculated microorganisms was evaluated on days 1, 3 and 6, as mentioned above. For this purpose, serial dilutions were made and plated on selective media: Palcam Listeria Selective Agar for *L. monocytogenes* (Scharlab, Barcelona, Spain) and Brilliant Green agar for *E. coli*. Colonies were counted after incubation at 37 °C for 24 h. Samples were analysed in triplicate.

2.6. Statistical analysis

One-way analyses of variance were carried out using the SPSS®189 computer program (SPSS Inc., Chicago, IL, USA). Differences in pairs of mean values were evaluated by the Tukey test for a confidence interval of 95%. Data are represented as mean ± standard deviation.

3. RESULTS AND DISCUSSION

In this work, EPL was successfully incorporated in EVOH films (EVOH 29 and EVOH 44) at 1, 5 and 10% and the films, produced by casting, were transparent and without discontinuities, presenting a thickness of approximately 15 ± 2 µm.

3.1. Optical properties

Colour coordinates L*, a*, b*, chroma (C*) and hue (h^o) of films made from EVOH 29 and EVOH 44 without and with 1, 5 and 10% of EPL are given in Table 1. Incorporation of the antimicrobial agent did not affect the luminosity of either of the copolymer films, since in all the samples the L* values are similar to those obtained for the control (Table 1), without significant differences. Colour coordinates a* and b* presented values close to –1 and 1 respectively for both films, EVOH 29 and EVOH 44. The addition of EPL increased the absolute value of both coordinates when EPL was added at the higher concentration, with significant differences appearing between samples and control. Colour intensity given by the chroma (C*) parameter increased with the concentration of EPL but films maintained their original light yellow-green

tone given by the hue (h^o) parameter. Significant differences were only found in C* for films containing 10% EPL were compared with control films.

3.2. Quantification of EPL migration from EVOH films

The amount of protein that had migrated after 24 h at 37 °C in pH 7.5 phosphate buffer was determined with the BCA assay. Table 2 shows the results obtained for EVOH 29 and EVOH 44 films incorporating 1, 5 and 10% EPL. The value of the protein released into the phosphate buffer increased with the concentration of EPL in the films, being 47.43 µg/mL for 1% and 95.90 µg/mL for 10% with EVOH 29. For EVOH 44, the amount that migrated was 42.87 µg/mL for films with 1% EPL and 71.46 µg/mL for films with 10% EPL. It can be observed that films with a higher percentage of ethylene retained a greater amount of EPL. This behaviour has been observed previously for the antimicrobial LAE (Muriel-Galet et al. 2013b). The lower swelling achieved for EVOH 44 films in liquid media could explain the results obtained.

3.3. Antimicrobial activity of EPL against *L. monocytogenes* and *E. coli*

The antimicrobial activity of EPL was tested against *L. monocytogenes* and *E. coli*. The growth of *L. monocytogenes* and *E. coli* was inhibited by EPL at concentrations in TSB of 23 and 40 ppm, respectively. In another study, a lower MIC value was reported for *L. monocytogenes* (Brandt et al. 2010), but according to various authors EPL inhibits the growth of both Gram-positive and Gram-negative bacteria and the minimum inhibitory concentration is below 100 ppm (Hiraki et al. 2003; Shima et al. 1984). Differences in methodology, media composition and bacterial strains may be responsible for the different values. The MBC values obtained were 70 and 90 ppm for *L. monocytogenes* and *E. coli*, respectively. The antimicrobial effect of EPL is attributed to electrostatic absorption onto the cell surface of the microorganism, where it interacts with the bacterial membranes (Ho et al. 2000). The difference in the MIC values between the Gram-positive and Gram-negative bacteria might derive from different cell surface conditions of the bacteria tested. Gram-negative microorganisms have an increased defence system and are less susceptible to antibacterial action than Gram-positive microorganisms. They have an outer membrane surrounding the cell wall that restricts the diffusion of compounds (Adams and Moss 2008).

3.4. Antimicrobial activity of EVOH films containing EPL against *L. monocytogenes* and *E. coli*

The antimicrobial activity of the EVOH films was tested against *L. monocytogenes* and *E. coli*. Tables 3 and 4 present the results for EVOH 29 and EVOH 44 films, respectively. The EVOH 29 films with 1% EPL produced a 4.09 log reduction in the growth of *L. monocytogenes* and a

2.74 log reduction for *E. coli*. Films with 5% EPL produced a reduction of 6.09 log against *L. monocytogenes* and 5.58 log for *E. coli,* and films with 10% EPL produced total inhibition against both microorganisms tested. As can be seen in Table 4 (EVOH 44 films), the viable counts for all microorganisms decreased with 1% EPL, being 1.39 for *L. monocytogenes* and 0.52 for *E. coli*. Films containing 5% EPL caused a growth reduction of 4.97 log against *L. monocytogenes* and 3.94 for *E. coli*, and EVOH 44 films with 10% EPL produced total inhibition only against *L. monocytogenes* and produced a 6.64 log reduction in the growth of *E. coli*.

The results show that the antimicrobial activity was greater for EVOH 29 than for EVOH 44 films, and *E. coli* appears to be less susceptible to the antimicrobial effect of EPL. This lower antimicrobial effect of EVOH 44 films was expected; as noted in the migration assay of EPL carried out with BCA, the amount of EPL that migrated from EVOH 44 films was always lower than the amount that migrated from EVOH 29 films.

3.5. Scanning electron microscopy observations

SEM was performed on bacteria exposed to EVOH 29 and EVOH 44 films with 10% EPL, to study the morphological changes resulting in the membrane structure. The micrographs show that bacteria exposed to the antimicrobial films displayed considerable morphological alterations in comparison with control bacteria. Figs. 1A and 1C show micrographs of control *L. monocytogenes* and *E. coli*, respectively, with bacteria presenting a smooth surface and characteristic rod shape. Figs. 1B and 1D show bacteria that have been exposed to the antimicrobial films, and it can be observed that the bacteria are seriously damaged, presenting an irregular rough surface with blisters and bubbles and the collapse of the bacteria compared with the control. In the case of *L. monocytogenes* the alterations are more obvious.

The images reveal that EPL leads to dramatic changes in the cell membrane. This hypothesis is consistent with the results obtained by Shima et al. (1984), which show that the mechanism of action of EPL on bacterial growth is the electrostatic adsorption onto the cell surface of microorganisms, increasing the membrane permeability and causing an abnormal distribution of cytoplasm.

3.6. Antimicrobial activity of EVOH films containing EPL over time

3.6.1. Bacterial growth studies at 37 °C

Fig. 2 shows the growth curves at 37 °C of *L. monocytogenes* and *E. coli* exposed to EVOH 29 films containing 1, 5 and 10% EPL for a period of 72 hours. Fig. 3 shows the results obtained with EVOH 44. As can be seen in both figures, in the absence of antimicrobial films the bacteria

grow to values of 9 log at 37 °C; owing to temperature conditions and presence of nutrients in the liquid media, the bacteria exhibited optimal growth. Fig. 2A shows that the antimicrobial activity of EVOH 29 films against *L. monocytogenes* increased with the concentration of EPL in the film. Films with 1% EPL presented 3 reductions after 3 hours in contact with the bacteria, maintaining these values throughout the 72-hour period studied. EVOH 29 films with 5 and 10% EPL produced a more rapid decrease in bacterial growth at short times. After 1 hour these films caused reductions of 1.84 and 2.89 log compared with the control, and the maximum inhibition degree was reached after 6 hours. Films containing 5% EPL produced a 6 log reduction and films with 10% EPL caused total inhibition, and these values remained constant until the end of the experiment.

Antimicrobial activity of EVOH 29 films against *E. coli* over a 72-hour exposure period is given in Fig. 2B; for films with 1% EPL the inhibition was 2.5 log after 6 hours and this value was maintained until the end of the experiment. After 1 hour, films with 5 and 10% EPL produced reductions of 1.5 log and 2.5 log, respectively, in comparison with the control. Films with 5 and 10% EPL also reached the maximum inhibition degree after 6 hours of being in contact with the bacteria, and this value remained constant, with 3 log reductions for films with 5% EPL and total inhibition for films with10% EPL.

Figs. 3A and 3B show the results obtained when working with EVOH 44 instead of EVOH 29 against *L. monocytogenes* and *E. coli*, respectively. EVOH 44 films with 1% EPL showed a 2 log reduction after 3 hours in contact with *L. monocytogenes*, maintaining these values throughout the period of time studied. Films with 5 and 10% EPL reached the maximum inhibition degree after 6 hours, producing a 5 log reduction when 5% EPL was incorporated and total inhibition with 10% EPL. These results were maintained until the end of the experiment.

Fig. 3B shows the growth curve of EVOH 44 films against *E. coli*. No inhibition was observed with 1% EPL. The maximum inhibition degree achieved for films with 5% EPL was 4 log reductions, and films with 10% EPL produced a reduction of 7 log. As mentioned above, the antimicrobial activity against Gram-negative bacteria was lower and total inhibition was only observed with EVOH 29 films incorporating 10% EPL. EVOH 29 proved to be more effective than EVOH 44, releasing EPL to the media and then inhibiting bacterial growth.

3.6.2. Bacteria growth studies at 4 °C

Bacterial growth studies were also carried out at a temperature of 4 °C to test the effectiveness of the films at refrigeration temperatures in order to simulate storage conditions in the

consumer's refrigerator. The antimicrobial activity of EVOH 29 and EVOH 44 films against *L. monocytogenes* and *E. coli* at 4 °C overtime is shown in Figs. 5 and 6 for each copolymer.

L. monocytogenes is a psychrotrophic pathogen whose ability to survive and multiply at low temperatures is demonstrated in Figs. 4A and 5A. As can be observed, bacteria reached values of 7.5 log after 72 hours of exposure to control films in liquid media. In contrast, refrigeration temperature significantly reduced the growth of *E. coli*. As can be seen in Figs. 4B and 5B, the control sample maintained bacterial counts around 6 log throughout the experiment. Films with 1% EPL showed a reduction of ca. 2 log and 1.5 log for EVOH 29 and EVOH 44, respectively, against *L. monocytogenes* compared to the control. However, no inhibition was observed against *E. coli*, which may be due to slow release of EPL to the medium, being below the minimum inhibitory concentration value. Considering that the amount of protein migrated from films with 1% EPL in phosphate buffer at 37 °C for 24 h is 47.43 μg/mL and 42.87μg/mL for EVOH 29 and EVOH 44, respectively, and taking into account that in the current experiment the release temperature is 4 °C, it can be expected that the release of the antimicrobial would occur more slowly and the films would have less effectiveness. Furthermore, films incorporating a greater amount of EPL were not able to inhibit completely the growth of all the microorganisms tested. Thus, EVOH 29 and EVOH 44 films with 10% EPL gave similar reduction values against *L. monocytogenes* and *E. coli*: reductions of 5.5 log and 4.8 log against *L. monocytogenes* were observed with EVOH 29 and EVOH 44, respectively, and 4 log and 3.5 log against *E. coli*.

It can be concluded from this experiment that the greatest bacterial inhibition was achieved at 37 °C, and films made from copolymers with lower ethylene content were more effective. However, when working with a refrigeration temperature of 4 °C, differences in bacterial inhibition caused by both copolymers were less acute. Moreover, a slower release of EPL is expected at 4 °C, which results in a limited concentration of antimicrobial in the medium, and thus a decrease in the effectiveness of the films.

3.7. Antimicrobial activity of EVOH films containing EPL on surimi microbiota

Once the antimicrobial effectiveness of the EVOH films had been assessed at refrigeration temperatures, a new study with real food was carried out. EVOH 29 and 44 films with 10% EPL were chosen because these films presented great antimicrobial activity. Surimi sticks were individually wrapped with these films and stored at 4 °C for 6 days. Samples without film and samples wrapped with film without EPL were prepared as controls. The samples were subjected to microbiological analysis on days 1, 3 and 6 of refrigerated storage. The results of microbiological counts of the surimi samples are shown in Table 5. It must be pointed out that

no differences were found between unwrapped samples and samples wrapped with film without EPL (data not shown).

On the first day of storage no growth of Enterobacteriaceae, lactic acid bacteria, psychrotrophic bacteria, total aerobic count and *Pseudomonas* bacteria was observed in any sample (data not shown), and no growth of Enterobacteriaceae was detected during the extended refrigerated storage period studied. No growth of these bacteria is considered as an index of fish quality, which is related to storage in ice, washing and evisceration (Zambuchini et al. 2008). These results confirm that the surimi samples were made under Good Manufacturing Practice (GMP), ensuring the quality of the products.

Lactic acid bacteria are commonly found in seafood products, and bacterial counts tend to increase during extended refrigerated storage. As can be seen in Table 3, lactic acid bacteria were found in the control sample after three days of refrigerated storage. EVOH films containing 10% EPL were capable of inhibiting growth of lactic acid bacteria in surimi sticks during the period of time monitored.

Psychrotrophic bacteria are able to grow at refrigeration temperatures and responsible for the aerobic spoilage of fish stored at refrigeration (Gunlu and Koyun 2013), and in this experiment their proliferation was observed after the third day of storage Bacterial growth was also observed for samples wrapped with active films: samples wrapped with EVOH 29 incorporating 10% EPL presented a 1.45 log inhibition at the third day and a 2.16 log inhibition at the end of storage compared with the control; when wrapped with EVOH 44, a 1.07 log reduction was achieved at the third day of storage and a 1.71 log reduction at the end of the storage period.

Total aerobic bacterial counts appeared in the control samples at the third day of storage (2.34 log) and the aerobic population increased by ca. 1 log at the end of the storage period. A bactericidal effect was observed when surimi sticks were wrapped with EVOH 29 containing 10% EPL during the storage time, whereas when EVOH 44 films were used microbial aerobic bacterial growth was observed at the 6^{th} day of storage. With regard to *Pseudomonas* proliferation in the surimi samples, Table 3 shows that bacterial growth was detected in control samples on the third day of storage. EVOH 29 films with 10% EPL exerted total inhibition on day 3, whereas a 0.68 log reduction was achieved with EVOH 44 compared with the control. On day 6 of storage, 1.43 log and 1.00 log reductions were found with EVOH 29 and EVOH 44 with 10% EPL, respectively, compared with the control.

It has also been demonstrated in previous studies that count between 2.00 log and 4.00 log were found in total aerobic plate and total psychrophilic bacteria (Coton et al. 2011; Singh and Balange 2005).

It is worth pointing out that no growth of yeast and moulds was observed in any sample during the storage period.

It can be concluded from this experiment that the active films developed provide inhibition of lactic acid bacteria and total aerobic count, whereas the psychrotrophic and *Pseudomonas* counts decreased with respect to the control by the end of storage at 4 °C, when the concentration of the agent on the surimi surface is expected to be higher.

3.8. Antimicrobial activity of EVOH films incorporating EPL on surimi inoculated with *L. monocytogenes* and *E. coli*

Surimi sticks were surface inoculated with *L. monocytogenes* and *E. coli*, wrapped with EVOH 29 and EVOH 44 films with 10% EPL, and stored for 6 days at 4 °C. Samples without film and samples wrapped with EVOH 29 and EVOH 44 films without EPL were prepared as controls. With regard to bacterial growth in control samples and in samples wrapped with EVOH with EPL, no significant differences were found. Fig. 6 shows the inhibitory effect of EVOH 29 with 10% EPL on *L. monocytogenes*: after 1 day of storage, a reduction of 1.76 log was observed; this value was maintained until the 3rd day of storage and increased to 2.76 log reductions at the end of the storage period. Regarding the samples contaminated with *E. coli*, the reduction was ca. 1 log during the entire storage time.

Fig. 7 shows the inhibitory effect of EVOH 44 with 10% EPL against *L. monocytogenes* and *E. coli*. *L. monocytogenes* was reduced by ca. 1 log during the entire storage period. On the other hand, *E. coli* reduction increased at the end of storage, on the 6th day of storage. The total food-borne bacteria counts of surimi wrapped with the active samples showed a significant decrease in comparison with the control sample.

Comparing the results obtained above with the *in vitro* test with TSB, it can be observed that a higher concentration of EPL would be necessary to produce total inhibition against *L. monocytogenes* and *E. coli*. The antimicrobial activity of the films was probably reduced because of an interaction of the antimicrobial agent with some components of the food matrix, reducing its availability to kill bacteria. Moreover, the kinetics of release and the amount of EPL released to the media may change when assays are made with a solid food instead of a liquid medium. This behaviour has also been reported in previous works. Geornaras et al. (2007) demonstrated that the antimicrobial activity of epsilon-polylysine decreased against *L. monocytogenes* when was tested in six food products compared with the results obtained *in vitro* assays carried out in broth liquid media. The authors also showed that the antimicrobial effect of

active films on surimi products was higher against *L. monocytogenes* than against *E. coli*, as it has been shown throughout this work.

The direct addition of antimicrobial agents into surimi sticks was also studied by other authors (Li et al. 2012; Ting et al. 1999). Direct incorporation of the antimicrobial into the food produces an immediate reduction of bacterial populations but this may not prevent the recovery of injured cells or the growth of cells that were not destroyed by direct addition if residues of the antimicrobial are rapidly depleted (Chi-Zhang et al. 2004). Therefore, antimicrobial active films are an excellent technology to extend food shelf-life, providing a continuous antimicrobial effect on the food during extended exposure.

An inadequate consumer knowledge on how to store ready-to-eat food at home, at the right refrigerated temperature, has led to higher risks of *L. monocytogenes* growth (Gambarin et al. 2012). *L. monocytogenes* and *E. coli* are pathogens commonly detected in ready-to-eat products because of their wide distribution in food factories, especially affecting interior surfaces of equipment that are complicated to clean, water and utensils. Thus the chances of surimi recontamination with these food-borne bacteria after post-process procedures are very high. Low temperature control during processing, shipment and storage may not be sufficient to control bacterial growth adequately. Therefore, to increase food safety and extend the shelf life of ready-to-eat surimi products during storage time, it is necessary to complement the post-processing action to control the growth of pathogens *L. monocytogenes* and *E. coli*.

4. CONCLUSIONS

Films made from EVOH 29 and EVOH 44 copolymers incorporating several amounts of EPL were successfully developed by casting, being continuous and transparent. Their optical properties represented changes with respect to the control samples only at the higher concentration of EPL (10%), increasing significantly but slightly the chroma. The amount of EPL capable of migrate from the copolymer films to liquid medium was quantified revealing that films possessing a lower percentage of ethylene released a greater amount of EPL. Bacterial growth studies carried out at 37 and 4 ºC with *L. monocytogenes* and *E. coli* in the presence of the films incorporating EPL showed that a greater bacterial inhibition was achieved at 37 °C and EVOH 29 were more effective than EVOH 44 films inhibiting bacterial growth. *In vivo* experiments carried out with surimi sticks inoculated with pathogen bacteria and wrapped in EVOH 29 and EVOH 44 with 10% of EPL, produced a reduction in the microbial load thus increasing the microbiological shelf life of the product at refrigeration temperatures. Therefore, the results obtained in this work provide strong evidence of the antimicrobial effect of active EVOH films on the survival of *L. monocytogenes* and *E. coli* "*in vitro*" and inoculated into surimi sticks stored under refrigeration temperatures. It was also concluded that *E. coli* was

more resistant against EPL than *L. monocytogenes*. The present study shows that active packaging is a non-thermal preservation technology which could be implemented to improve the microbiological stability of ready-to-eat surimi-derived products.

5. ACKNOWLEDGEMENTS

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LEGENDS TO FIGURES

Fig.1. Scanning electron micrographs of *L. monocytogenes* cell A) CONTROL and B) 10% EPL. *E. coli* cell C) CONTROL and D) 10% EPL.

Fig. 2. Growth control study with EVOH 29 control, 1, 5 and 10% EPL. A) *L. monocytogenes* at 37 °C and B) *E.coli* at 37 °C.

Fig. 3. Growth control study with EVOH 44 control, 1, 5 and 10% EPL. A) *L. monocytogenes* at 37 °C and B) *E. coli* at 37 °C.

Fig. 4. Growth control study with EVOH 29 control, 1, 5 and 10% EPL. A) *L. monocytogenes* at 4 °C and B) *E. coli* at 4 °C.

Fig. 5. Growth control study with EVOH 44 control, 1%, 5% and 10% EPL. A) *L. monocytogenes* at 4 °C and B) *E. coli* at 4 °C.

Fig. 6. Antimicrobial activity of EVOH 29 films with 10% EPL on surimi sticks inoculated with (A) *L. monocytogenes* and (B) *E. coli*.

Fig. 7. Antimicrobial activity of EVOH 44 films with 10% EPL on surimi sticks inoculated with (A) *L. monocytogenes* and (B) *E. coli*.

Fig-1A

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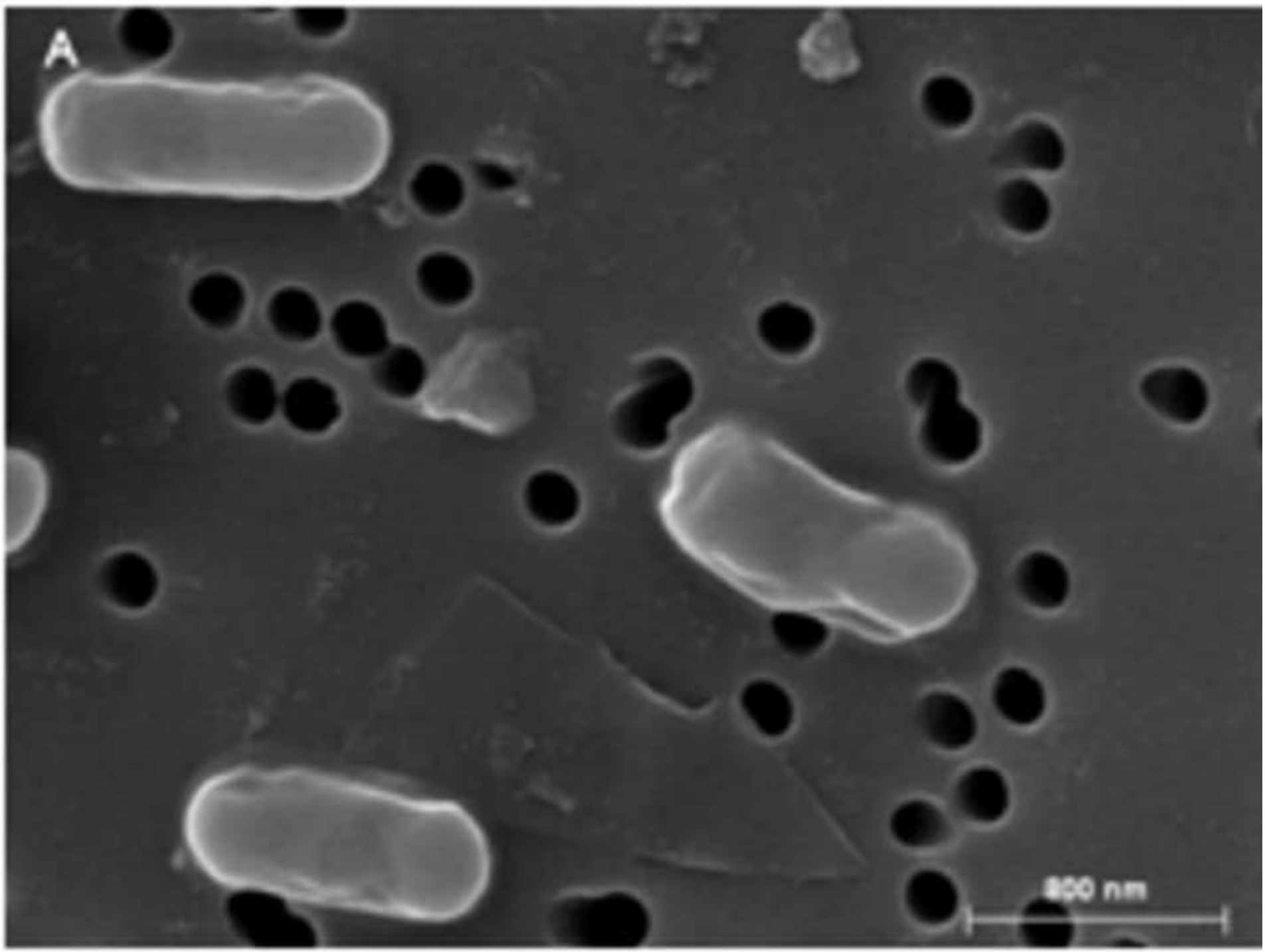

Fig-1B

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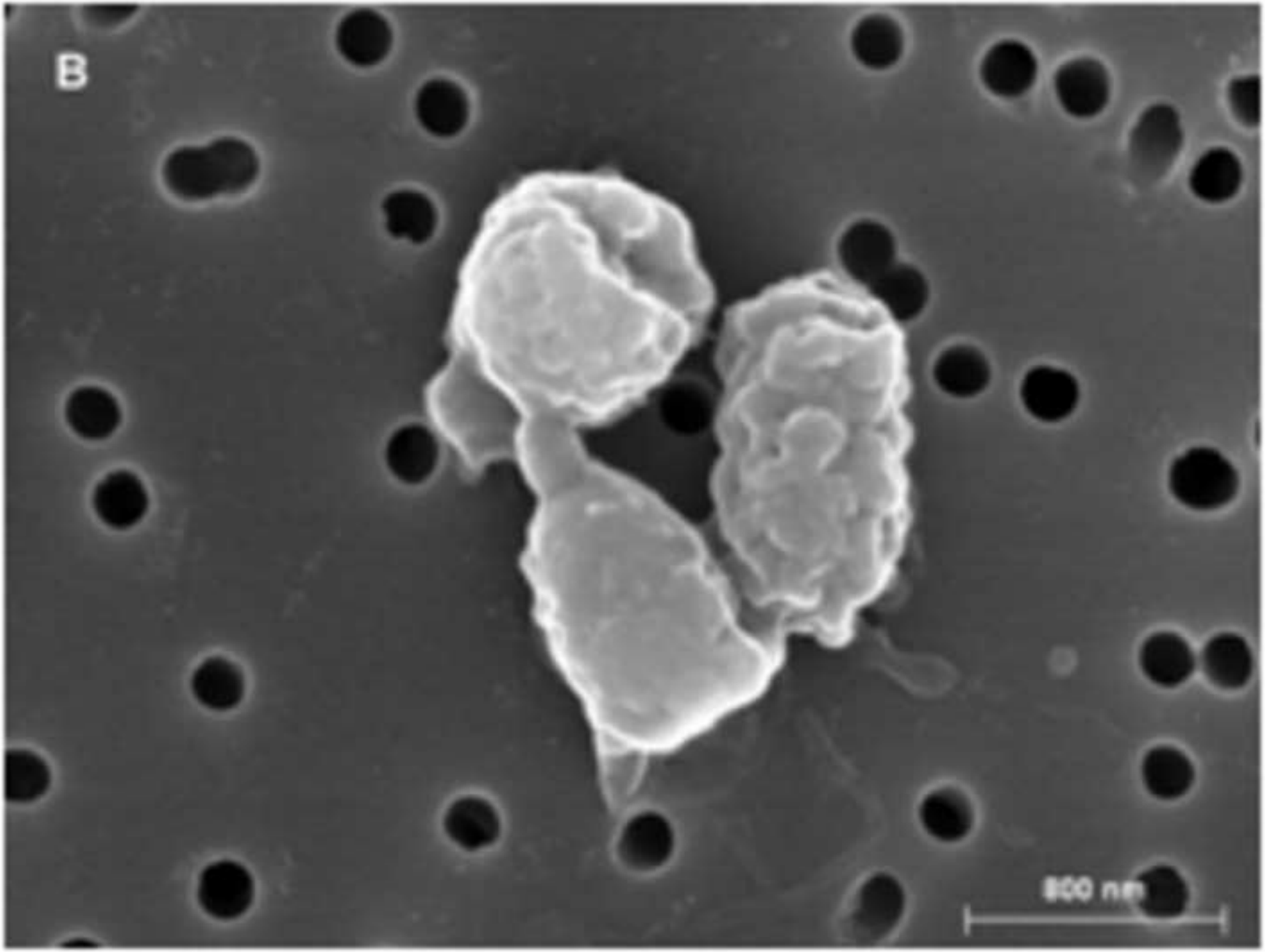

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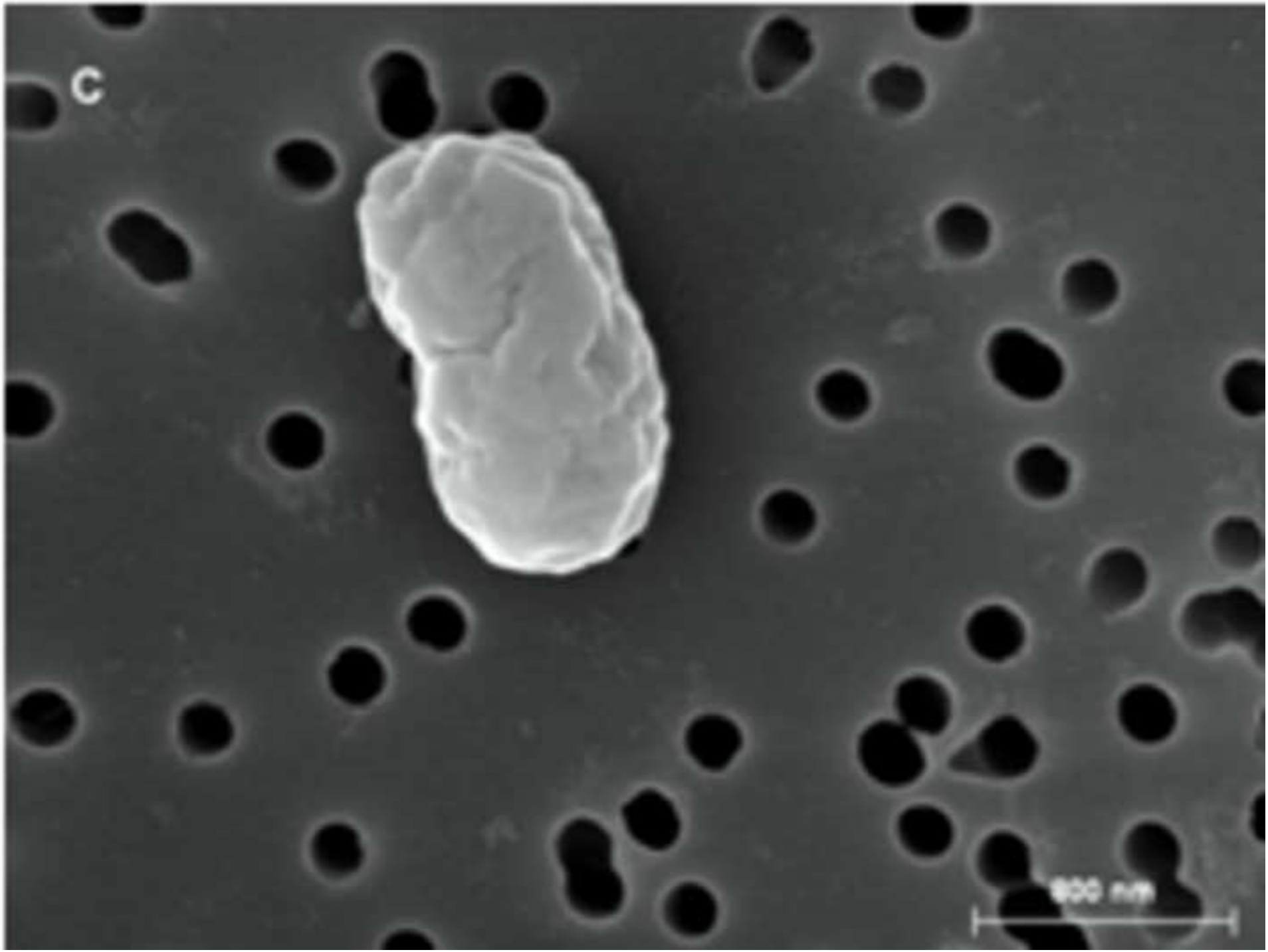

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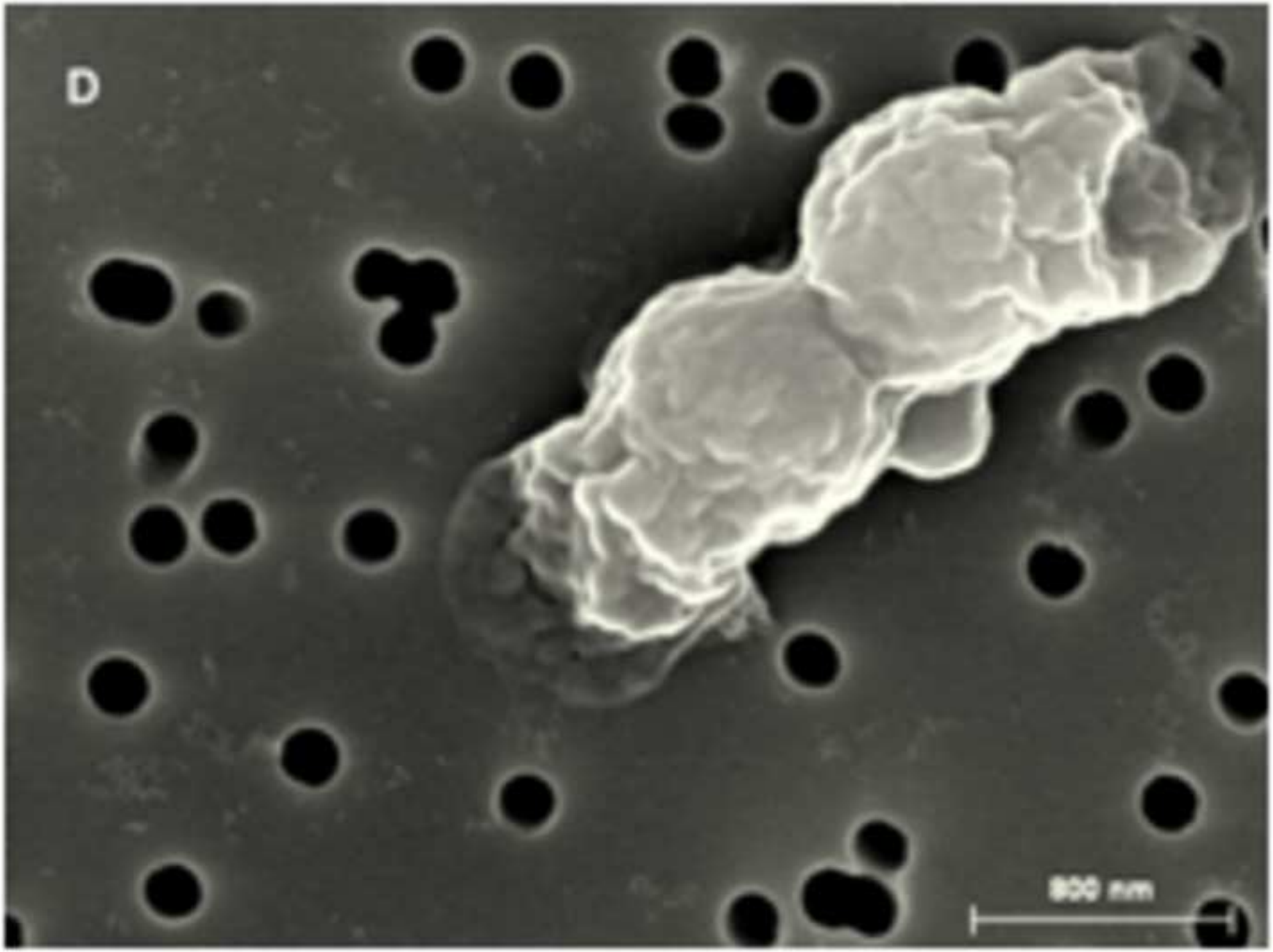

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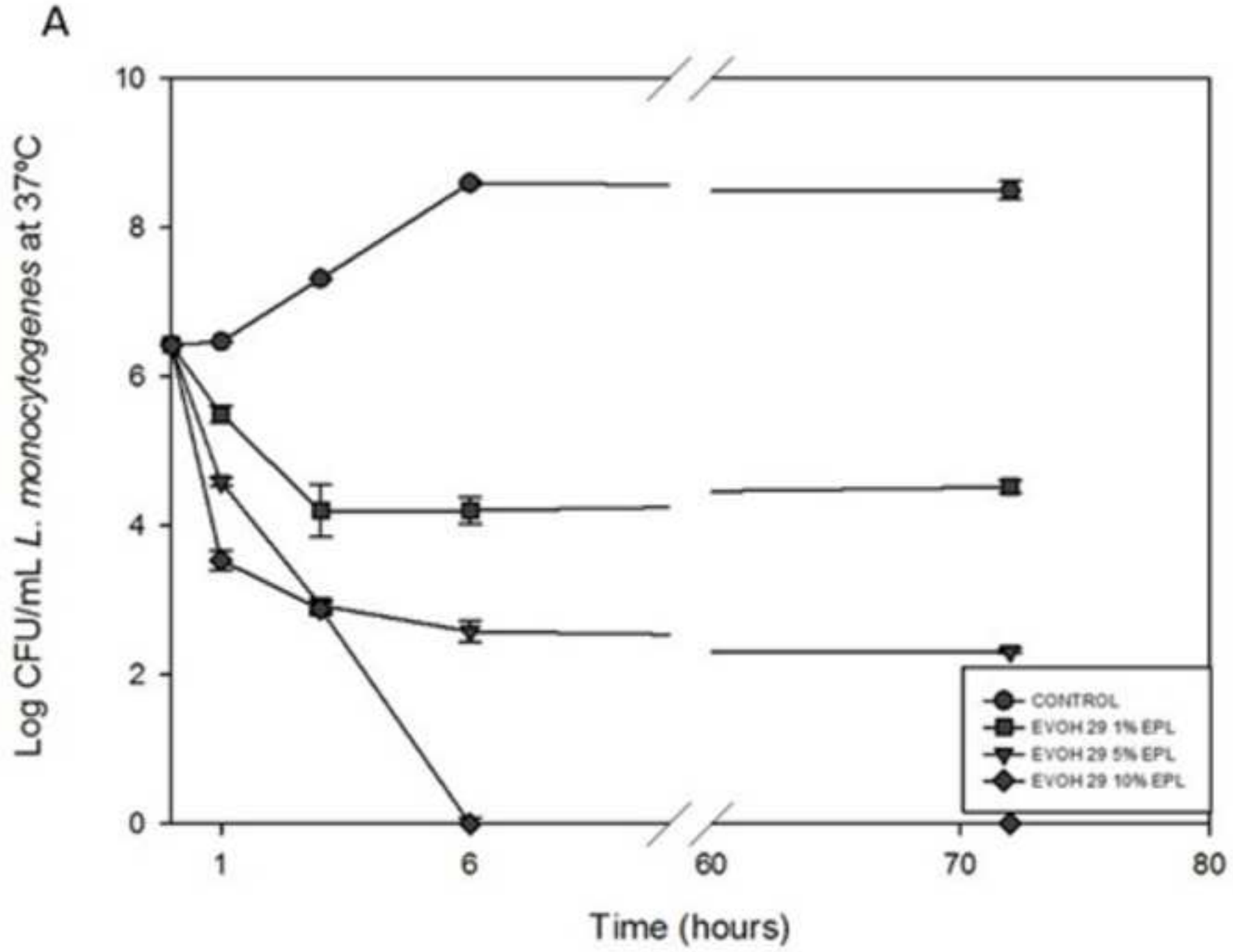

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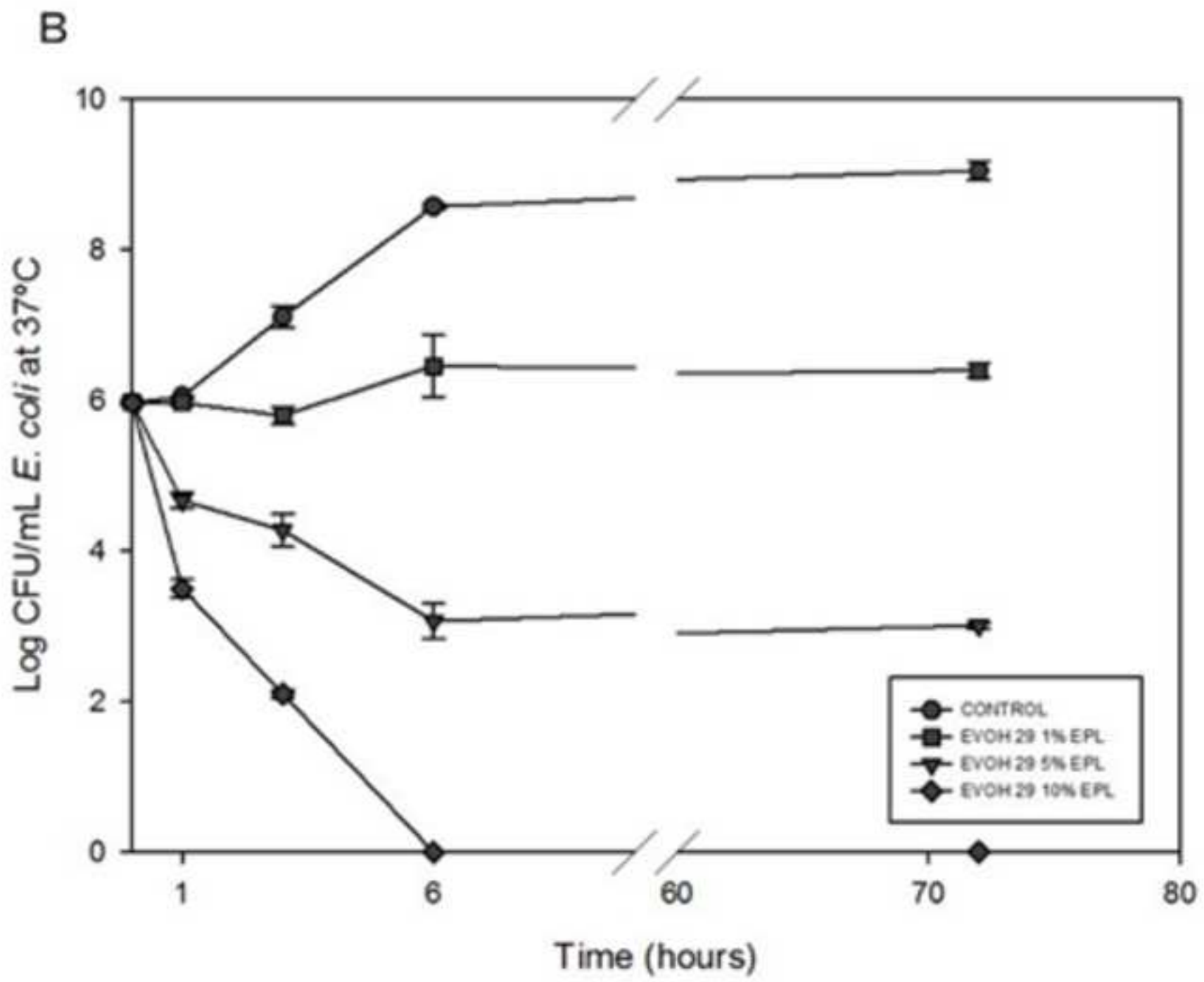

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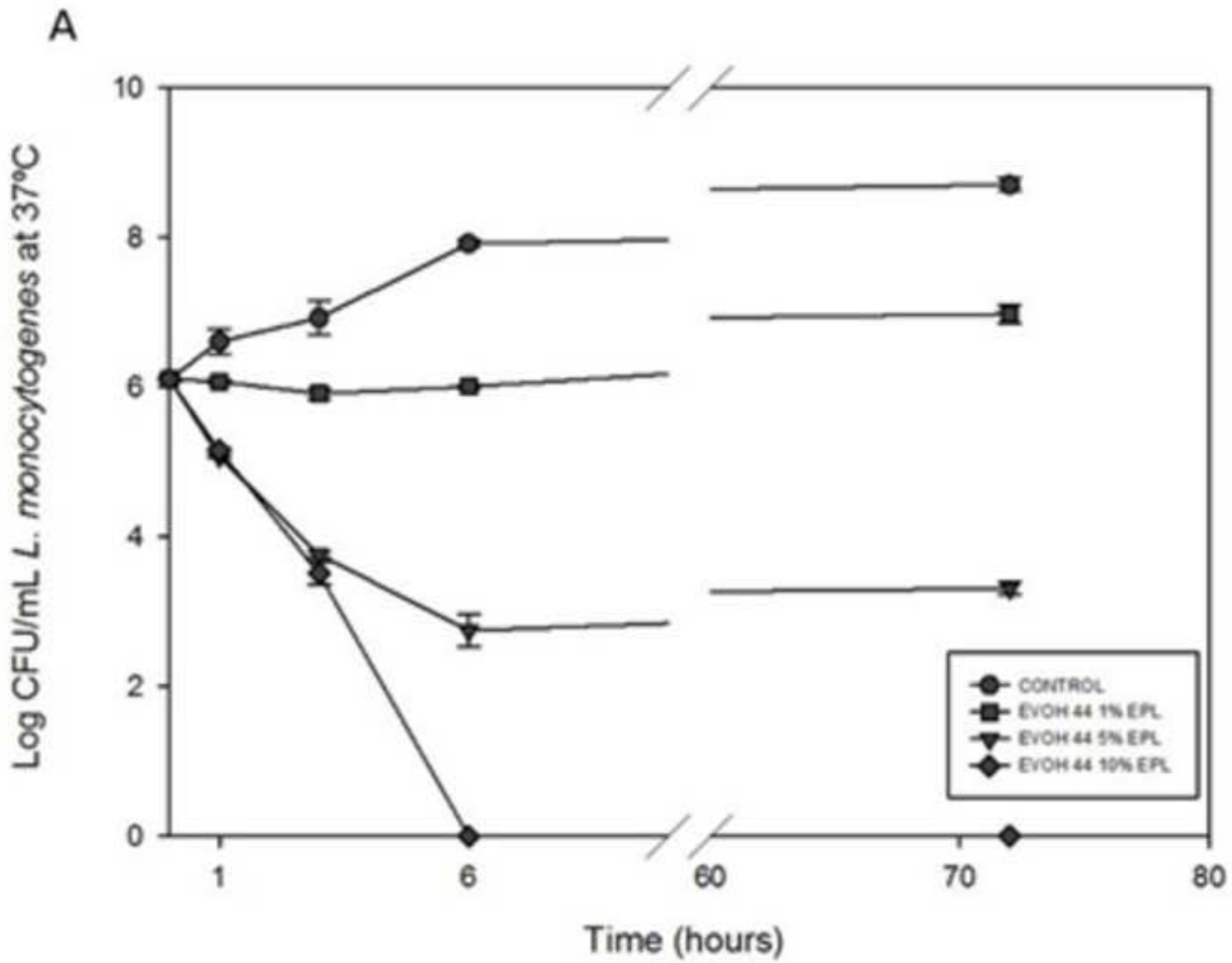

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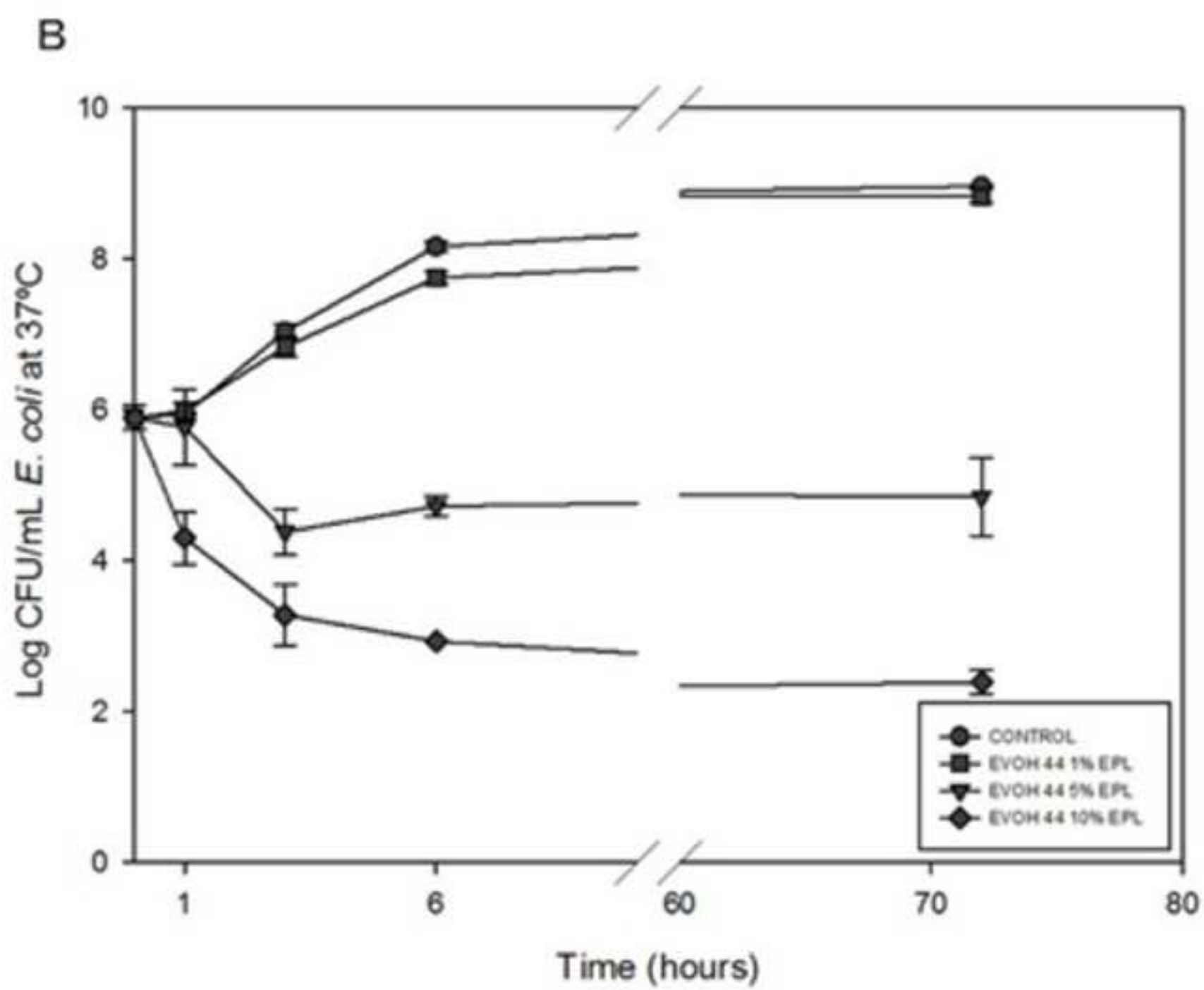

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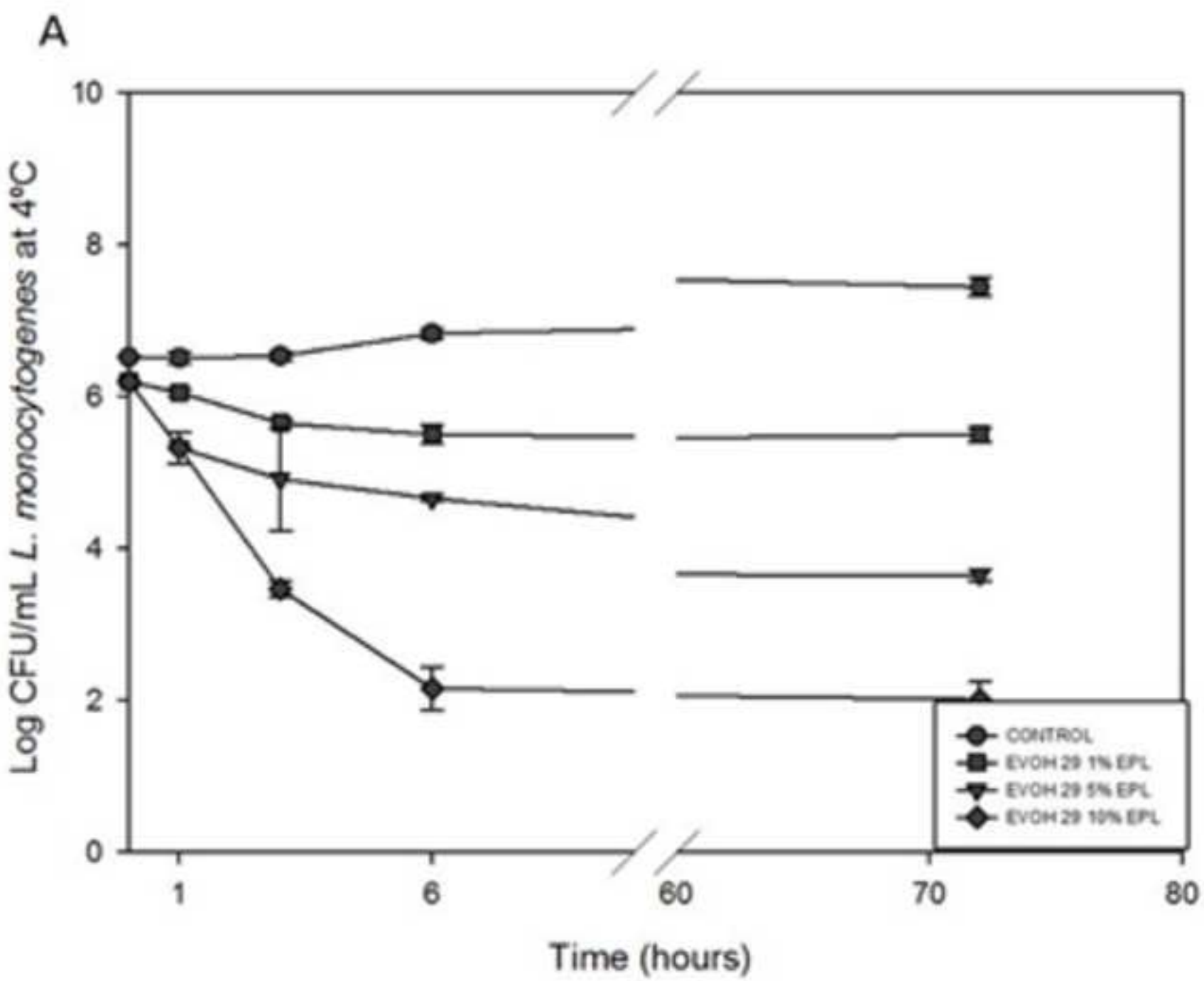

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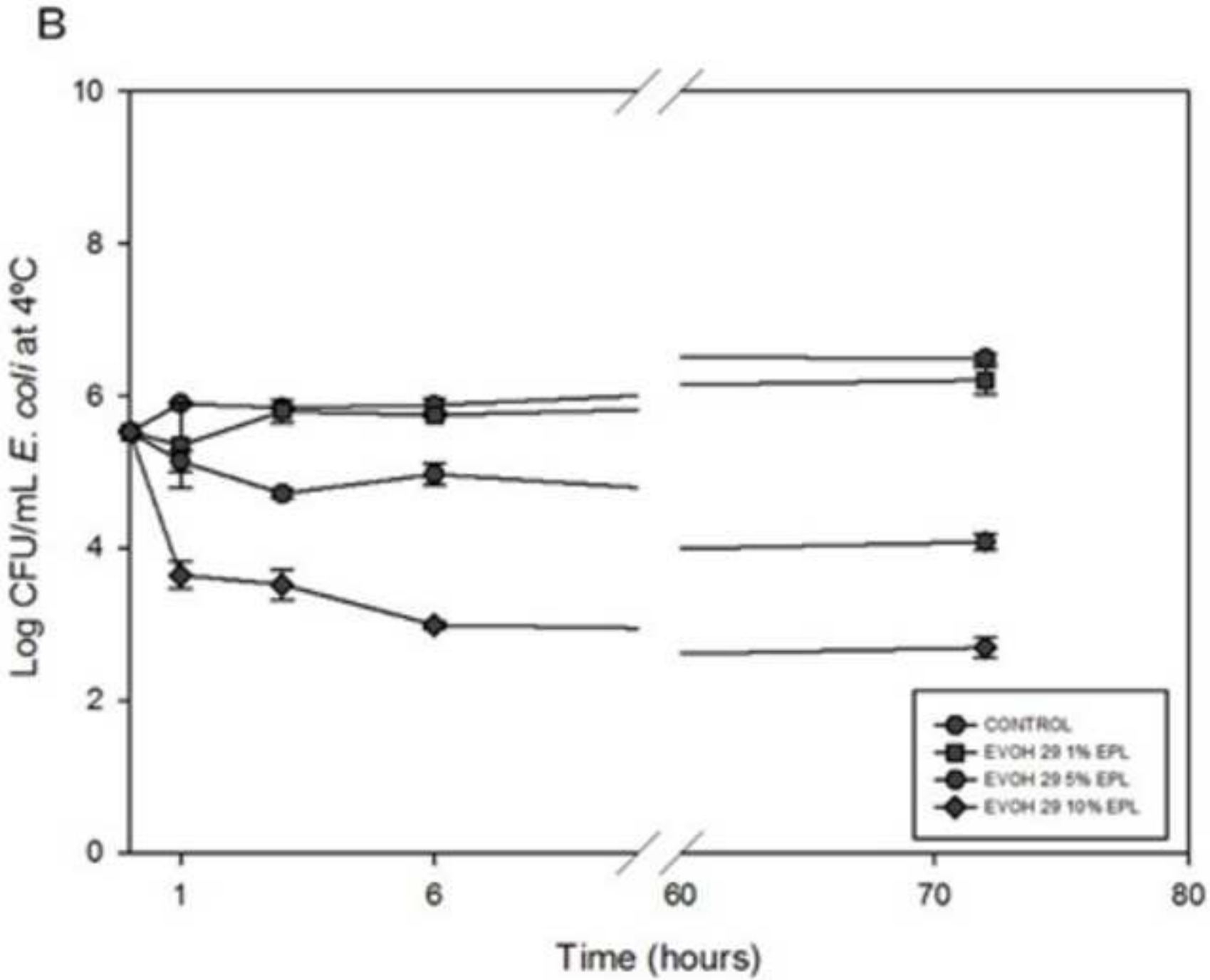

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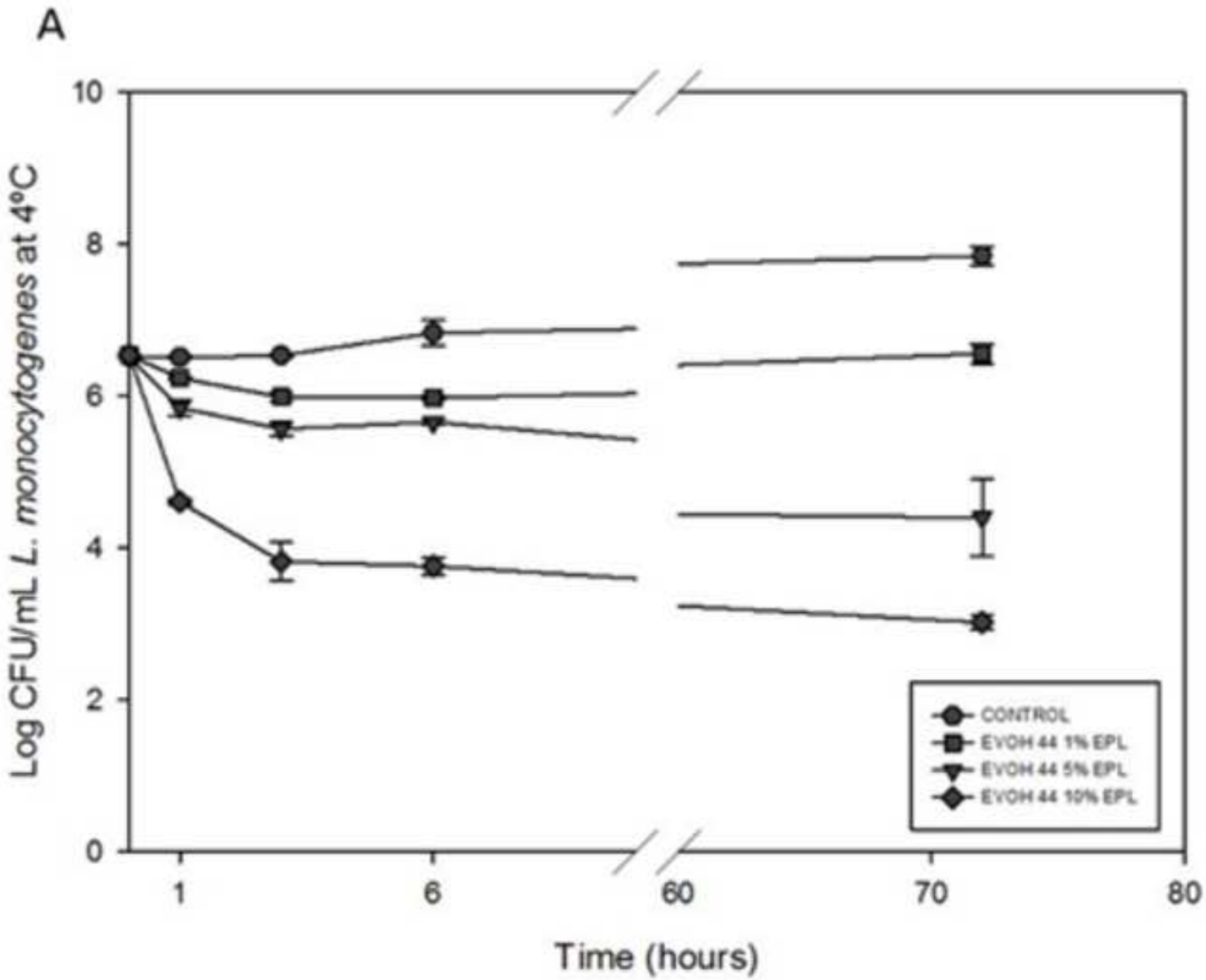

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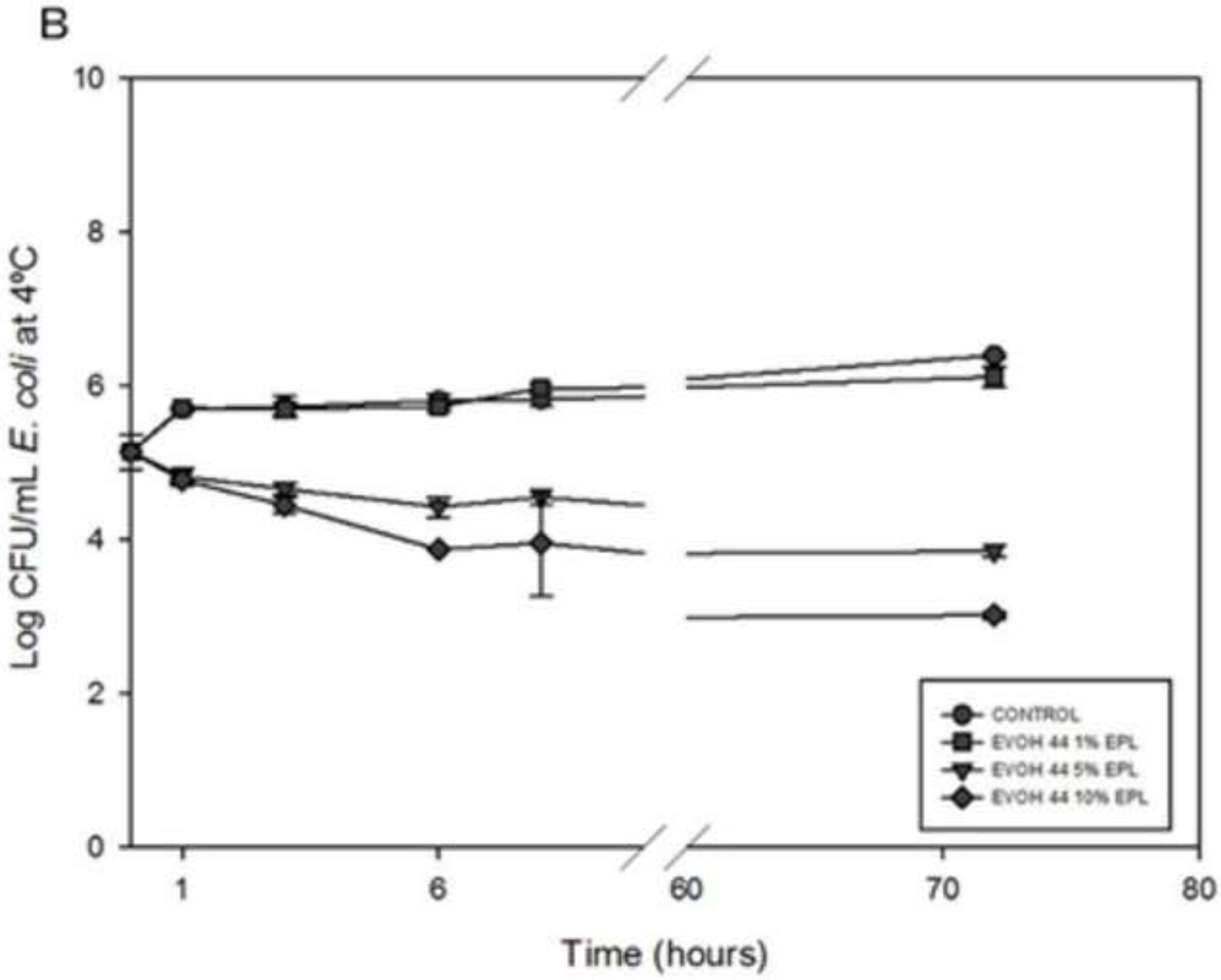

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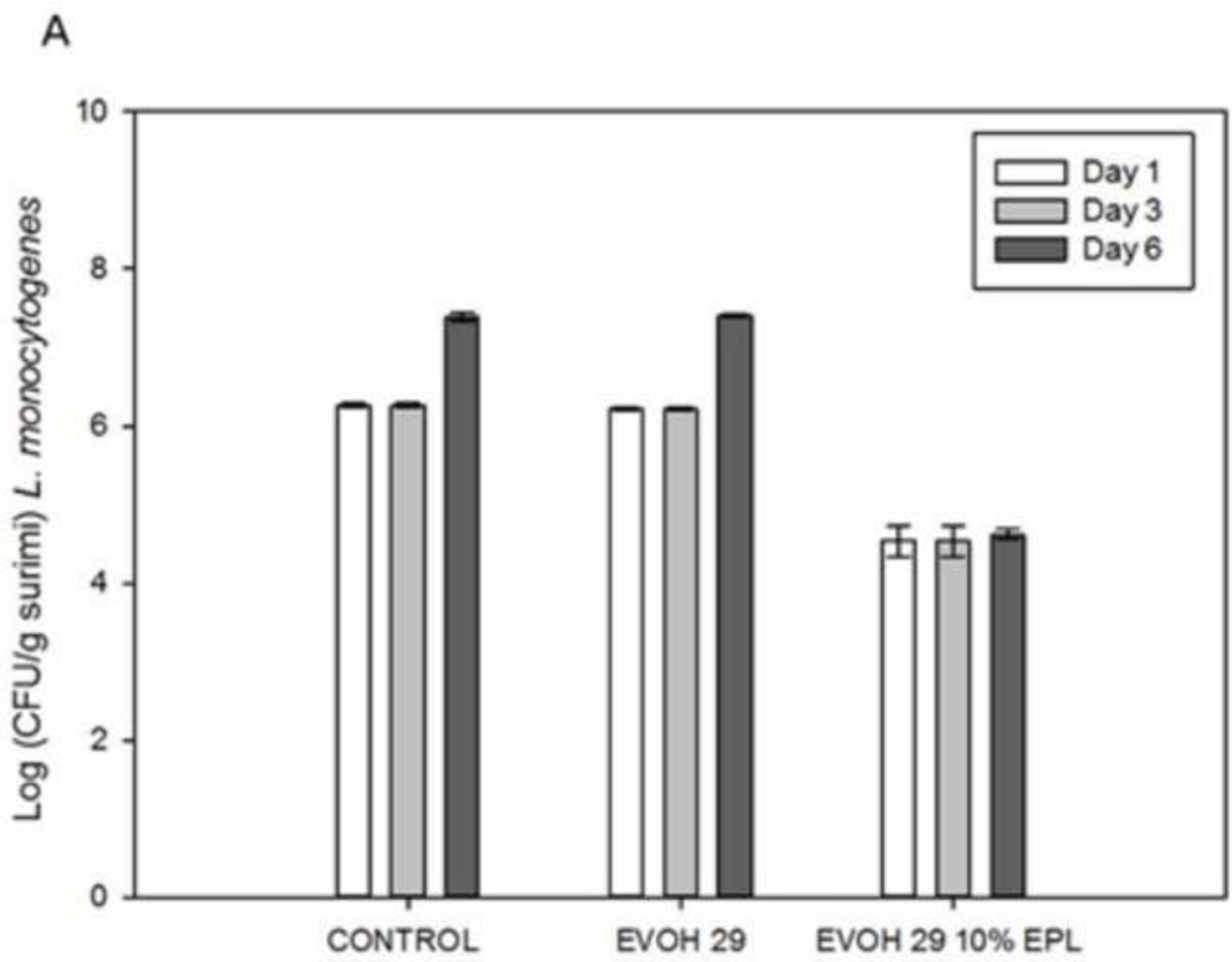

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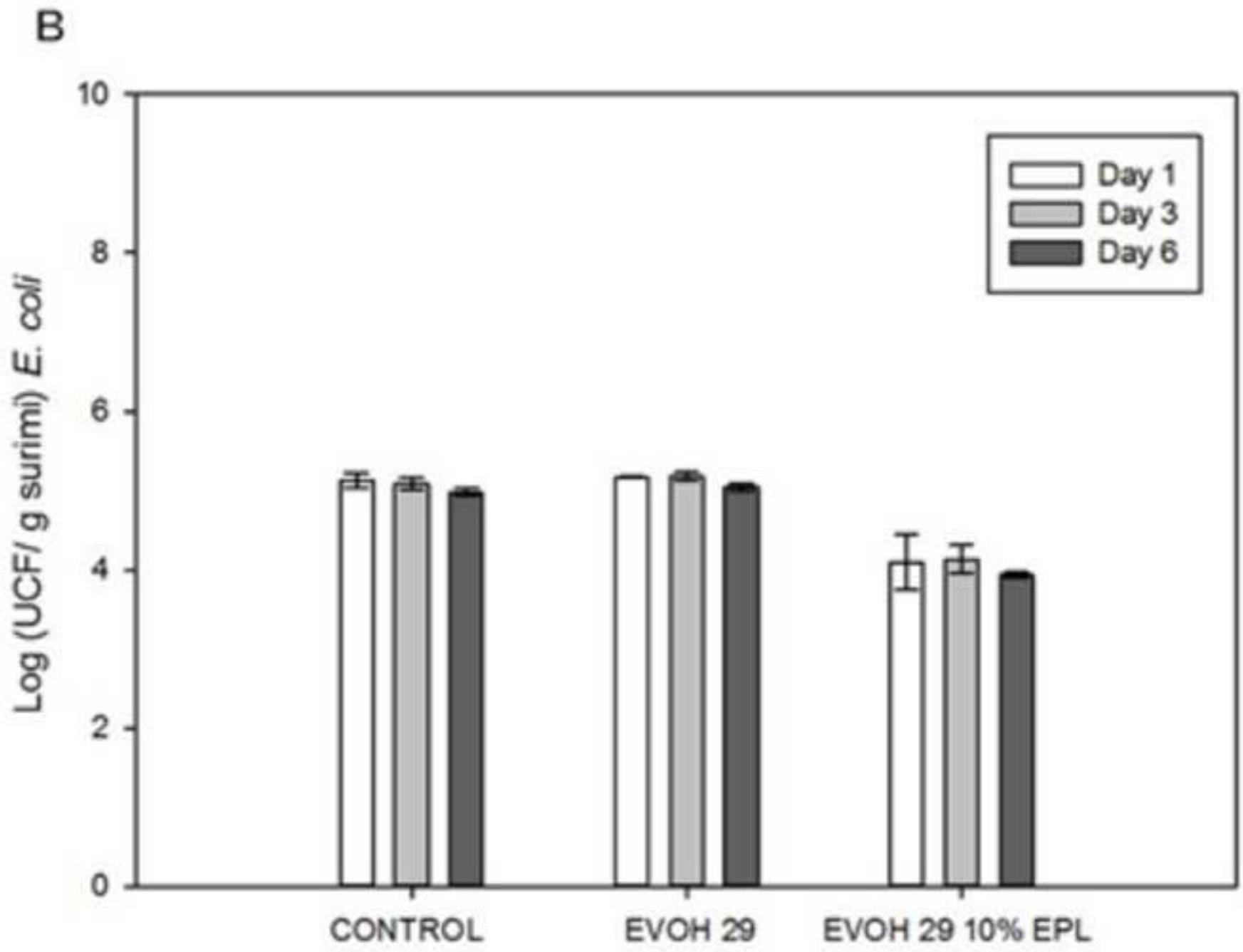

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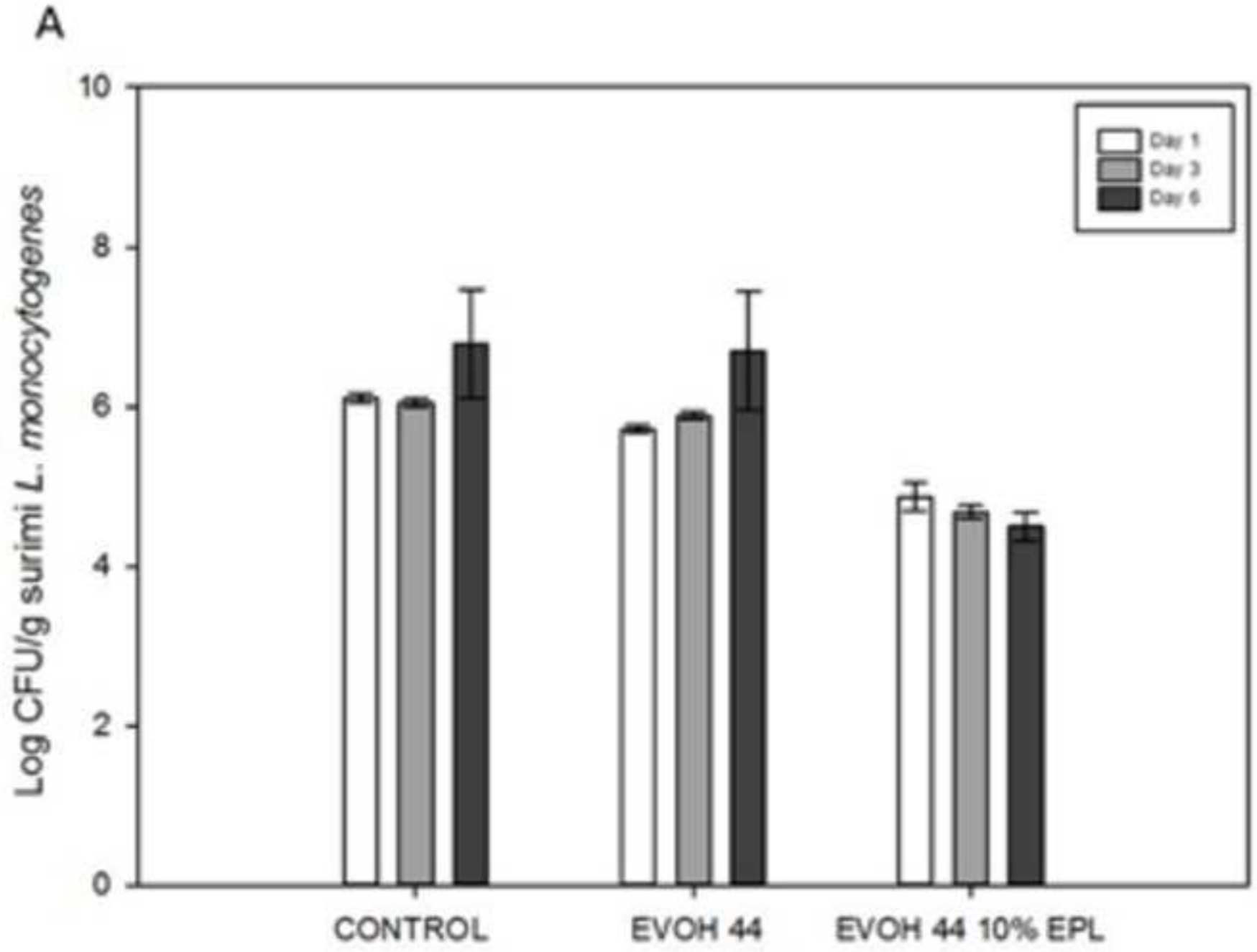

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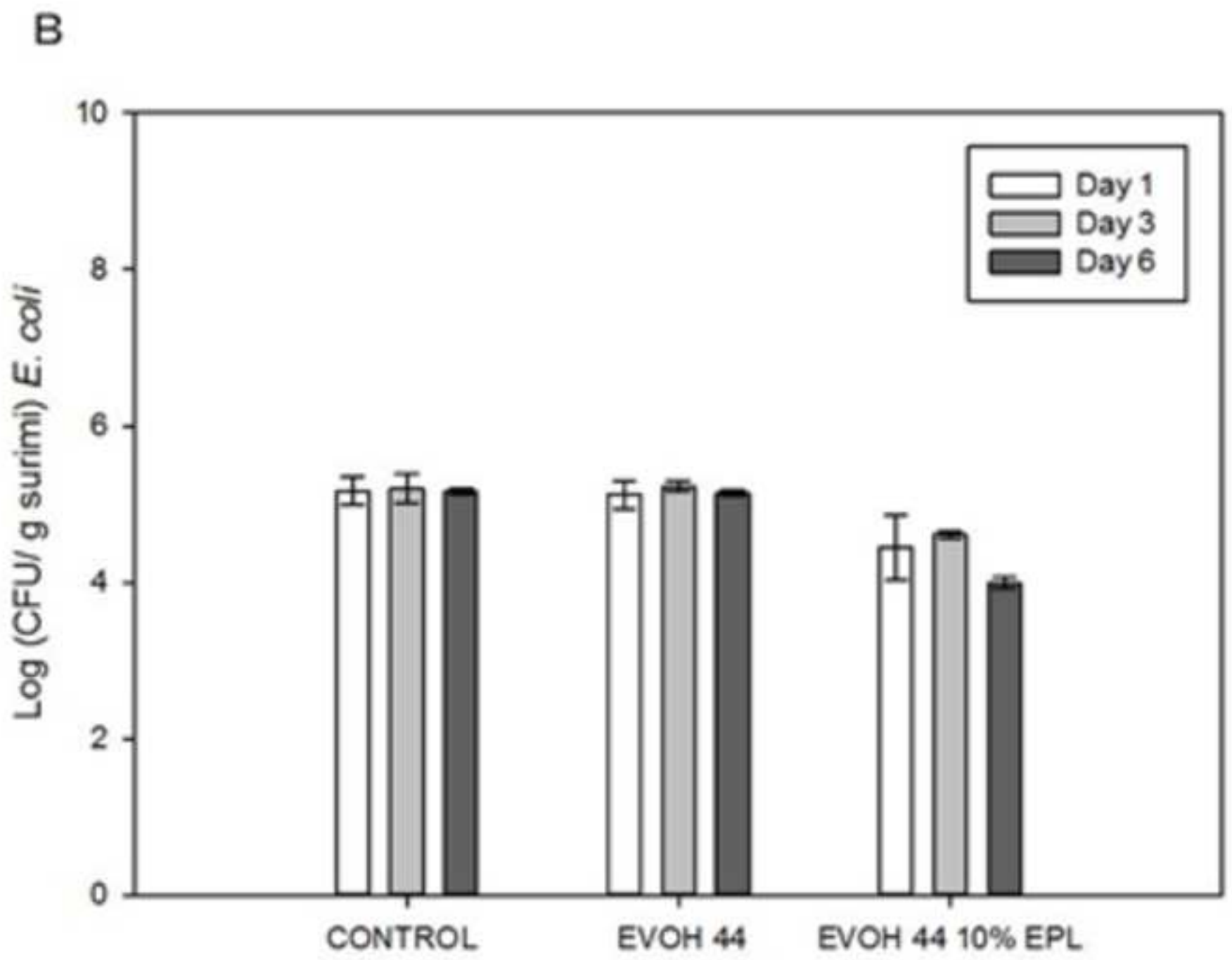

Table
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Table 1. Colour parameters of EVOH 29 and EVOH 44, without and with 1, 5 and 10% of EPL.

EVOH 29	**L***	**a***	**b***	**C***	**hº**
CONTROL	90.27 ± 0.18[a]	–0.86 ± 0.02[a]	1.09 ± 0.04[a]	1.39 ± 0.02[a]	128.22 ± 1.65[a]
1% EPL	89.43 ± 0.65[a]	–0.89 ± 0.02[a]	1.13 ± 0.10[a]	1.44 ± 0.08[a]	128.41 ± 2.64[a]
5% EPL	89.40 ± 0.70[a]	–0.93 ± 0.04[a]	1.13 ± 0.08[a]	1.47 ± 0.04[a]	129.49 ± 3.04[a]
10% EPL	89.84 ± 0.43[a]	–0.97 ±0.02[b]	1.30 ± 0.04[b]	1.62 ± 0.02[b]	129.82 ± 1.53[a]

EVOH 44	**L***	**a***	**b***	**C***	**hº**
CONTROL	89.82 ± 0.97[a]	–0.88 ± 0.05[a]	1.02 ± 0.12[a]	1.35 ± 0.07[a]	129.12 ± 4.34[a]
1% EPL	89.98 ± 0.53[a]	–0.86 ± 0.06[a]	1.07 ± 0.08[a]	1.38 ± 0.08[a]	128.86 ± 2.59[a]
5% EPL	89.80 ± 0.67[a]	–0.89 ± 0.04[a]	1.16 ± 0.08[b]	1.47 ± 0.05[b]	127.42 ± 2.83[a]
10% EPL	90.21 ± 0.66[a]	–0.98 ± 0.06[b]	1.24 ± 0.09[b]	1.57 ± 0.10[b]	127.55 ± 0.95[a]

[a-c] Different letters in the same column indicate significant differences among the values of the color parameter of each EVOH copolymer (Turkey's adjusted analysis of variance, $P<0.05$).

Table
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Table 2. EPL migrated from EVOH films to phosphate buffer pH 7.5 at 37 °C after 24 hours.

EPL in EVOH (%)	From EVOH 29 (µg/mL)	Migration (%)	From EVOH 44 (µg/mL)	Migration (%)
1% EPL	47.43 ± 0.02[a]	18.97	42.87 ± 0.01[a]	17.15
5% EPL	68.19 ± 0.02[b]	5.45	58.82 ± 0.01[b]	4.71
10% EPL	95.90 ± 0.03[c]	3.84	71.46 ± 0.03[c]	2.86

[a-c] Different letters in the same column indicate significant differences among the values of EPL migrated to the liquid medium (Turkey's adjusted analysis of variance, $P<0.05$).

Table
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Table 3. Antimicrobial effectiveness of EVOH 29 films against *L. monocytogenes* and *E. coli* at 37 °C expressed as logarithm of colony forming units per mL (Log (CFU/mL)) and logarithm reduction value (LRV).

	L. monocytogenes		***E. coli***	
	Log (CFU/mL)	**LRV**	**Log (CFU/mL)**	**LRV**
CONTROL	8.49 ± 0.08^{c}		9.08 ± 0.02^{c}	
EVOH 29 1% EPL	4.40 ± 0.12^{b}	4.09	6.34 ± 0.12^{b}	2.74
EVOH 29 5% EPL	2.40 ± 0.09^{a}	6.09	3.50 ± 0.12^{a}	5.58
EVOH 29 10% EPL	Total inhibition	8.49	Total inhibition	8.49

[a-c] Different letters in the same column indicate significant differences in antimicrobial effectiveness of different EVOH 29 films (Turkey's adjusted analysis of variance, $P<0.05$).

Table
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Table 4. Antimicrobial effectiveness of EVOH 44 films against *L. monocytogenes* and *E. coli* at 37 °C expressed as logarithm of colony forming units (Log (CFU/mL)) and log reduction value (LRV).

	L. monocytogenes		***E. coli***	
	Log(CFU/mL)	**LRV**	**Log(CFU/mL)**	**LRV**
CONTROL	8.15 ± 0.06^{c}		8.86 ± 0.07^{c}	
EVOH 44 1% EPL	6.76 ± 0.03^{b}	1.39	8.34 ± 0.15^{c}	0.52
EVOH 44 5% EPL	3.18 ± 0.01^{a}	4.97	4.92 ± 0.09^{b}	3.94
EVOH 44 10% EPL	Total inhibition	8.15	2.22 ± 0.19^{a}	6.64

[a-c] Different letters in the same column indicate significant differences in antimicrobial effectiveness of different EVOH 44 films (Turkey's adjusted analysis of variance, $P<0.05$).

Table
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Table 5. Enumeration of microbial population in surimi: *Lactic acid bacteria*, *Psychrotrophic bacteria*, *Total aerobic count* and *Pseudomonas* expressed as logarithm of colony forming units per g (Log (CFU/g)) and logarithm reduction value (LRV).

	Day 3		**Day 6**	
	Log (CFU/g)	LRV	Log (CFU/g)	LRV
Lactic acid bacteria				
CONTROL	3.45 ± 0.23		3.89 ± 0.15	
EVOH 29 10% EPL	Total inhibition	3.45	Total inhibition	3.89
EVOH 44 10% EPL	Total inhibition	3.45	Total inhibition	3.89
Psychrotrophic bacteria				
CONTROL	3.09 ± 0.14[b]		5.00 ± 0.13[b]	
EVOH 29 10% EPL	1.64 ± 0.61[a]	1.45	2.84 ± 0.11[a]	2.16
EVOH 44 10% EPL	2.02 ± 0.37[a]	1.07	3.29 ± 0.09[a]	1.71
Total aerobic count				
CONTROL	2.34 ± 0.06		3.18 ± 0.02[b]	
EVOH 29 10% EPL	Total inhibition	2.34	Total inhibition	3.18
EVOH 44 10% EPL	Total inhibition	2.34	1.90±0.24[a]	1.28
Pseudomonas				
CONTROL	3.04 ± 0.12[b]		3.63 ± 0.03[b]	
EVOH 29 10% EPL	Total inhibition	3.04	2.20 ± 0.10[a]	1.43
EVOH 44 10% EPL	2.36 ± 0.14[a]	0.68	2.62 ± 0.11[a]	1.01

[a-b] Different letters in the same column for each microorganisms indicate significant differences in antimicrobial effectiveness of different EVOH films (Turkey's adjusted analysis of variance, $P<0.05$).