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Antimicrobial effectiveness of LAE incorporated into EVOH copolymers to extend the shelf-life of chicken stock and surimi sticks

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Antimicrobial effectiveness of LAE incorporated into EVOH copolymers to extend the shelf-life of chicken stock and surimi sticks

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ABSTRACT

This study was designated to determine the antimicrobial effect of ethyl-N$^{\alpha}$-dodecanoyl-L-arginate hydrochloride (LAE) incorporated into ethylene vinyl alcohol copolymer (EVOH) films on chicken stock and ready-to-eat surimi sticks. Firstly, the effect of LAE against *L. monocytogenes* and *E. coli* was studied by using flow cytometry and scanning electron microscopy. Next, film-forming solutions of ethylene vinyl alcohol copolymers EVOH29 and EVOH44 (29% and 44% molar percentage of ethylene, respectively) containing 0, 5 and 10% w/w of LAE were cast into films. Several experiments were conducted to determine the antimicrobial activity of the films *in vitro* and also *in vivo* with the above-mentioned food products. The outcome of the tests showed a high impact on the viability of bacteria treated with LAE, with dramatic damage to the membrane. The films were able to inhibit the microbiota of the food products studied for 10 days under storage at 4 °C, showing a significant antibacterial effect against *L. monocytogenes* and *E. coli*. These films show great potential as systems for sustained release of active molecules to improve the safety and quality of packaged food products.

Keywords: Ethyl-N$^{\alpha}$-dodecanoyl-L-arginate hydrochloride, ethylene vinyl alcohol copolymers, pathogenic bacteria, surimi sticks, chicken stock.

1. INTRODUCTION

The strong demand for healthy, natural foods rich in proteins and low in fat has increased and they have become popular all over the world. However, food products without or with a low amount of additives undergo faster deterioration as a result of physical, chemical and biological processes. Exposure to these processes tends to modify the nutritional, physiological and/or sensory properties of foods, reducing shelf-life and safety and increasing the risk of food-borne diseases. Microbiological food spoilage can be minimized and food safety ensured by means of effective control of bacterial growth. The short shelf-life of minimally processed and preservative-free refrigerated products creates a need for research into innovative food preservation technologies capable of preserving without compromising product quality.

Technological innovations such as antimicrobial packaging inhibit or retard the proliferation of microorganisms in foods, thus extending the shelf-life and increasing the safety of the product (Sung et al. 2013). Compared with direct addition of active agents to food, antimicrobial active packaging technologies could stabilize labile additives or avoid interactions and chemical reactions with other additives or ingredients during food processing, thus reducing the amount of active agent required, satisfying consumer demands (Kilcast and Subramaniam 2000).

In previous work, antimicrobial packaging films were produced. For this purpose, two different ethylene-vinyl alcohol copolymers (EVOH29 and EVOH44) were used as matrices to incorporate the antimicrobial agent ethyl-N^{α}-dodecanoyl-L-arginate hydrochloride (LAE). The films that were developed presented a strong antimicrobial effect with a wide range of action, without modifying film functional properties (Muriel-Galet et al. 2012; Muriel-Galet et al. 2013). LAE is a cationic surfactant derived from lauric acid, L-arginine, and ethanol. This new molecule has been considered as Generally Recognized as Safe (GRAS) and permitted as a food preservative at a concentration up to 200 ppm by the Food and Drug Administration (FDA) (Bakal and Diaz 2005). Moreover, when LAE is ingested it is hydrolysed in the human body and transformed into natural components (Bakal and Diaz 2005; Hawkins et al. 2009; Muriel Galet et al. 2012). Recent studies have used LAE as an antimicrobial agent to inhibit microbial growth in foods, but limited information is available concerning its use in the design of active food packaging by means of its incorporation into polymer films (Guo et al. 2014; Muriel Galet et al. 2012; Higueras et al. 2013; Theinsathid et al. 2012).

This work considers issues associated with food spoilage and the safety of refrigerated products such as chicken stock and ready-to-eat surimi sticks after package opening. The stipulated

period of time given by the manufacturer for safe surimi and chicken stock consumption after the package is opened is two days when stored under refrigerated conditions. Therefore, the aim of this study is to use the newly developed antimicrobial active films to improve the microbiological safety of these products after package opening.

2. MATERIALS AND METHODS

2.1. Materials

Ethylene vinyl alcohol (EVOH) copolymers possessing 29 and 44% ethylene molar content were kindly provided by The Nippon Synthetic Chemical Company (Osaka, Japan); 1-propanol was purchased from Sigma (Madrid, Spain). Ethyl-N^{α}-dodecanoyl-L-arginate hydrochloride ($C_{20}H_{41}N_4O_3Cl$) was provided by Vedeqsa Grupo LAMIRSA (Terrassa, Barcelona, Spain). Water was obtained from a Milli-Q Plus purification system (Millipore, Molsheim, France).

2.2. Culture preparation

Gram-positive bacteria *Listeria monocytogenes* CECT 934 (ATTCC 19114) and Gram-negative bacteria bacterium *Escherichia coli* CECT 434 (ATCC 25922) were selected because of their importance in food-borne illness. 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 Tryptone Soy Agar (TSA) (Scharlab, Barcelona, Spain) slants at 4 °C and transferred monthly. Prior to the experiments, 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.

2.3. Antimicrobial capacity of LAE

2.3.1 Flow cytometry

The minimum inhibitory concentration (MIC) – considered as the lowest concentration needed to inhibit visible growth of the microorganism – of LAE was tested against *L. monocytogenes* and *E. coli* in a previous work (Muriel-Galet et al. 2012). Dilutions of LAE in peptone water were added to 10 mL of TSB in order to reach a final concentration corresponding to the MIC value, 7 ppm and 20 ppm for *L. monocytogenes* and *E. coli*, respectively. Briefly, 100 µL of each microorganism in exponential phase was inoculated in each test tube and incubated for 24 h at 37 °C. After this time, the samples were centrifuged and resuspended twice in saline solution (0.8% NaCl). The cells were stained with the cell-permeant double-stranded DNA fluorochrome SYTO-13 (Molecular Probes Europe BV, Netherlands) and with propidium iodide

solution (PI, Calbiochem). SYTO-13 stains the nucleic acids in all cells, while PI stains the nucleic acids in cells with a damaged membrane. The studies were conducted in the Central Service for Experimental Research at the University of Valencia (Spain), performed with a BD FACSVerse™ (Becton Dickinson, USA) flow cytometer. Each cell was characterized by four optical parameters: side scatter (SSC), forward scatter (FSC), green fluorescence for SYTO-13 (excitation light 497 nm and emission light 520 nm), and red fluorescence for PI (excitation light 493 nm and emission light 630 nm). The software BD FACS Suite v. 1.0.3.2924 (Becton Dickinson, USA) was used to collect the data.

An untreated control of both pathogens was also analysed.

2.3.2 Scanning electron microscopy

The minimum bactericidal concentration (MBC) – defined as the concentration at which no growth is observed after plating onto agar – values described in previous works, 12 ppm for *L. monocytogenes* and 32 ppm for *E. coli*, were placed in contact with 10 mL of TSB and 100 µL of *L. monocytogenes* and *E. coli* in exponential phase and were inoculated in each test tube and incubated for 24 h at 37 °C. Untreated controls of both pathogens were prepared. The samples were centrifuged and resuspended twice in saline solution (0.8% NaCl). Each suspension was filtered through a 0.2-mm Nuclepore Track-Etch Membrane (Whatman, UK), and the membranes were dehydrated in 30%, 50%, 70%, 90% and 100% graded alcohol series. A small amount of platinum was sputtered on the samples to avoid charging in the microscope. SEM observation of *L. monocytogenes* and *E. coli* was performed in the Central Service for Experimental Research at the University of Valencia (Spain) with an electron microscope (HITACHI S 4100) working at 10 kV.

2.4. Film preparation

Films containing 5% and 10% of LAE in ethylene vinyl alcohol copolymers with different mol % ethylene contents (EVOH29 and EVOH44) were prepared by casting as described in a previous work (Muriel-Galet et al. 2012). Films without antimicrobial agent were prepared as controls.

2.5. Effectiveness of antimicrobial EVOH films with LAE against *L. monocytogenes* and *E. coli in vitro*

The antimicrobial activity of the EVOH29 and EVOH44 films with 5 and 10% of LAE were tested *in vitro* against *L. monocytogenes* and *E. coli* using an agar diffusion method. For this purpose, 100 µL of a bacteria suspension containing approximately 10^7 CFU/mL was spread over the TSA surface. Disks of the antimicrobial films (40 mm in diameter) were attached to the Petri dish in direct contact with the microorganism. Plates were incubated at 37 °C for 24 h and

the diameter of the resulting inhibition zone in the bacterial lawn was measured. Control films (EVOH29 and EVOH44) without LAE were also tested. The experiment was carried out in triplicate.

2.6. Food samples: chicken stock and surimi sticks

Chicken stock was produced according to the traditional method using a WMF Perfect pressure cooker (Germany). The ingredients employed were chicken, carrots, onion, leek, water and salt, and they were purchased from a local supermarket. After cooking, the chicken stock was cooled at room temperature for 20 min and then kept at 4 °C.

Surimi sticks were also purchased from a local supermarket in Valencia, Spain. The product contained 2.5 g of total fat, 12.9 g of total carbohydrate and 8.4 g of total protein, and was labelled as free of preservatives. The samples were transported to the laboratory under refrigerated conditions and kept at 4 °C until the experimental assays.

2.7. Antimicrobial activity of EVOH29 and EVOH44 films with LAE against microbiota of chicken stock and surimi sticks

Sterilized tubes were prepared with 10 mL of chicken stock produced under sterile conditions; and 0.25 g of film containing 5% or 10% of LAE was immersed. Liquid food samples in contact with EVOH29 and EVOH44 without LAE and samples without film were used as controls. The tubes were stored for 10 days at 4 °C. Analyses were performed on the 2^{nd}, 6^{th} and 10^{th} days of storage. Serial dilutions in peptone water (Scharlab, Barcelona, Spain) were plated on specific media (Scharlab, Barcelona, Spain) with the following culture conditions: a) Violet Red Bile Glucose agar for total enterobacteria, incubated at 37 °C for 24 h; b) Man, Rogosa and Sharpe agar for lactic acid bacteria, incubated at 25 °C for 5 days; c) Nutrient Agar for total aerobic bacteria, incubated at 37 °C for 48 h; d) Nutrient Agar for total aerobic psychrotrophic bacteria, incubated at 10 °C for 10 days; f) King B agar for *Pseudomonas*, incubated at 30 °C for 48 h. The counts were performed in triplicate and expressed as log CFU/mL

Individual pieces (ca. 25 g) of surimi stick were wrapped with EVOH29 films and EVOH44 films containing 5% or 10% of LAE. Samples without film and samples wrapped with EVOH29 and EVOH44 films without LAE were prepared as controls. The wraps covered and touched all surfaces of the surimi. The samples were stored at 4 °C for 10 days. The antimicrobial activity of the films was determined on the 2^{nd}, 6^{th} and 10^{th} days after wrapping the samples. At appropriate times, 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 were plated on specific media with the culture conditions described above.

2.8. Antimicrobial efficacy of EVOH29 and EVOH44 films with LAE in chicken stock and on surimi sticks inoculated with *L. monocytogenes* and *E. coli*

First, 100 µL of cell culture of each microorganism in stationary phase, with an optical density of 0.9 at 600 nm, was diluted in 10 mL of TSB and incubated at 37 °C until exponential phase was reached, corresponding to an optical density of 0.2 at 600 nm (10^5 CFU/mL) measured with an Agilent 8453 UV-visible spectrophotometer (Barcelona, Spain) using TSB as blank. Then 100 µL of pathogenic bacteria in exponential phase was inoculated under sterile conditions into 10 mL of chicken stock contained in sterilized tubes, after which 0.25 g of each antimicrobial film sample was immersed. Liquid food samples without film and samples with film without incorporating LAE were used as controls. The tubes were stored for 10 days at 4 °C. Analyses were performed on the 2^{nd}, 6^{th} and 10^{th} days of storage. Serial dilutions were made and plated on selective media: Palcam Listeria Selective Agar for *L. monocytogenes* and Brilliant Green Agar for *E. coli* (Scharlab, Barcelona, Spain).

Surimi sticks were surface inoculated with 100 µL of each pathogenic microorganism in exponential phase (10^5 CFU/mL). The inoculums were dispersed separately on the surface with a sterile pipette and wrapped as described in the previous section. Surimi stick samples wrapped in film without LAE and samples wrapped in aluminium foil were used as controls. Testing of bacterial growth in surimi sticks was performed on the 2^{nd}, 6^{th} and 10^{th} days after wrapping the samples. Serial dilutions were carried out and plated on the same selective media.

Colonies were counted after incubation at 37 °C for 24 h. Counts were performed in triplicate and expressed as log CFU/mL.

2.9. Analysis of LAE availability

A test to ensure the absence of LAE after gastrointestinal digestion was conducted at the end of the sample storage time. For this purpose, samples of chicken stock and surimi sticks stored in contact with EVOH29 and EVOH44 containing 10% of LAE were analysed. A two-step procedure was carried out. First, a gastrointestinal digestion study was performed, and then the qualitative analysis of LAE was carried out using High Performance Liquid Chromatography (HPLC) coupled with UV-Vis detection.

Gastrointestinal digestion was carried out by dissolving 10 g of each sample (chicken stock and surimi sticks) in water to a final volume of 80 g. The pH was adjusted to 2 and fresh pepsin solution (10% pepsin in 0.1 M HCl) was added. The pepsin-HCl digestion was carried out for 2 h (to simulate gastric digestion), incubated in a shaking water bath at 37 °C. After the pepsin digestion, the pH was adjusted to 7.4 and the pancreatin bile extract solution mixture (0.2 g of pancreatin and 1.25 g of bile extract in 50 mL of 0.1 M $NaHCO_3$) was added and incubated in a

shaking water bath at 37 ºC for 2 h. The experiment was performed in triplicate for each sample. Samples exposed to EVOH films without LAE and samples with a known amount of LAE (200 ppm) directly added to the digestion solution were also measured.

After gastrointestinal digestion, the samples were filtered and LAE was determined by HPLC coupled with a UV-Vis absorbance detector. An Agilent 1200 series HPLC system equipped with a C18 reversed-phase column (150 × 3.9 mm, 5 µm) and a Model G1513A DAD was used and the absorbance data were recorded at 205 nm. The mobile phase was acetonitrile:water 50:50 (v/v) containing 0.1% trifluoroacetic acid, the flow rate 1 ml/min, the temperature 23 °C and the injection volume was 20 µL. A preliminary calibration was performed by injecting known amounts of LAE.

2.10. 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

3.1. Antimicrobial capacity of LAE

3.1.1 Flow cytometry analysis

Flow cytometry analyses the viability of cells. With suitable fluorochromes it is possible to distinguish between a viable and a non-viable cell population to assess the antimicrobial effect of LAE. In previous studies conducted in our laboratory, the minimum inhibitory concentration (MIC) of LAE against *L. monocytogenes* and *E. coli* (Muriel-Galet et al. 2012) was determined. In the present study, flow cytometry was used to confirm the MIC values. The results obtained for *L. monocytogenes* and *E. coli* are shown in Fig 1. In both cases the samples were successfully stained. The positive control of *L. monocytogenes* presented 83.29% of total cells, representing 70% of live cells stained with SYTO-13 and 3.29% of dead cells stained with PI. SYTO-13 penetrates all types of cellular membranes and binds nucleic acids of the cell, whereas PI only penetrates the bacterial membrane when the membrane is damaged (Rodriguez et al. 2004). The percentage of cell death in the control is due to different phases of cellular cycle. After being treated with 7 ppm of LAE, damaged *L. monocytogenes* cells became visible, with a dead population of 23.35% (Fig 1B). With regard to *E. coli*, the untreated control presented 81.90% of live cells stained with SYTO-13 and 4.28% of dead cells stained with PI. After being treated with 20 ppm of LAE the damaged cells became visible, with a dead population of 17.79% (Fig 1D). This study demonstrated that the percentage of damaged cells after exposure to an LAE concentration corresponding to the MIC was in accordance with inhibition growth

results obtained in a previous work (Muriel-Galet et al. 2012). From these results and supporting previous results it can be stated that LAE is slightly more active against Gram-positive bacteria than against Gram-negative bacteria.

3.1.2 Scanning electron microscopy (SEM)

In order to determine the morphological changes resulting in the membrane structure of *L. monocytogenes* and *E. coli*, cells were observed by SEM after being exposed to the minimum bactericidal concentration (MBC) of LAE, 12 ppm and 32 ppm, respectively. Untreated *L. monocytogenes* and *E. coli* cells displayed a smooth, intact surface as observed in the micrographs. The outer membrane that surrounds the cell wall of Gram-negative bacteria can also be observed (Fig 2C) The membrane of the cells that were in contact with the corresponding MBC of LAE was seriously damaged and they had an irregular rough surface, compromising cell integrity (Fig 2B and 2D).

Micrographs showed that LAE produced important changes in the cell envelope, indicating that membranes are the main target, producing disruption and instability of the plasma membrane, and presenting more activity against Gram-positive bacteria than against Gram-negative bacteria according to results obtained by other authors (Rodriguez et al. 2004). LAE interacts with lipids from the bacterial membranes, generating changes in membrane potential and bacterial structure in Gram-negative bacteria (Rodriguez et al. 2004). Cell lysis was not observed.

3.2. Effectiveness of antimicrobial EVOH films with LAE against *L. monocytogenes* and *E. coli in vitro*

The antimicrobial activity of EVOH29 and EVOH44 films with 5 and 10% of LAE was studied by the agar diffusion method. Table 1 shows the results obtained for the films against the two microorganisms tested. Growth inhibition appeared as a clear halo around the film, with the inhibition zone of *L. monocytogenes* proving to be greater than the one produced in *E. coli*. It was also observed that for *L. monocytogenes* the inhibition zone increased with the amount of LAE incorporated into the film, with values of 46.30 mm and 50.00 mm for EVOH29 incorporating 5 and 10% of LAE, respectively. This difference decreased for EVOH44 films incorporating 5 and 10% of LAE, which indicates that the amount of antimicrobial released from this copolymer is not enough to cause differences in growth inhibition zone related to the concentrations tested.

No differences were observed in the inhibition zones produced by EVOH29 and EVOH44 films incorporating 5 and 10% of LAE against *E. coli*. Probably, the different amounts released in these samples were not enough to produce a differential effect. In the previous section it was demonstrated that the MIC of LAE is higher against Gram-negative bacteria than against Gram-

positive bacteria, because the outer membrane of the cell wall provides a greater defence system and less susceptibility to the action of the antimicrobial compound (Muriel-Galet et al. 2012).

The inhibition zone formed around EVOH29 or EVOH44 with 5 and 10% of LAE can be explained by the high relative humidity (90% RH) produced in the headspace of the system because of the presence of agar, triggering the release of the antimicrobial compound. In general, the highest inhibition zone values were observed with EVOH29: the more hydrophilic nature of that copolymer compared with EVOH44, conferred by a higher content of –OH groups, favours greater plasticization of the polymer matrix by environmental moisture, facilitating the release of LAE to the agar medium.

3.3. Antimicrobial activity of EVOH films incorporating LAE applied to chicken stock and surimi sticks

After confirming the antimicrobial effectiveness of the EVOH29 and EVOH44 films with 5% and 10% of LAE a new study was carried out with the objective of testing the microbiota of chicken stock and surimi sticks against two pathogenic bacteria: *L. monocytogenes* and *E. coli*. All the samples were stored at 4 °C, simulating commercial conditions.

3.3.1 Chicken stock and surimi stick microbiota counts

Chicken stock samples in contact with the films that had been developed were subjected to microbiological analysis for 10 days; samples were analysed on the 2^{nd}, 6^{th} and 10^{th} days. In all the samples analysed, no growth was observed for enterobacteria, acid lactic bacteria, psychrotrophic bacteria, yeast or moulds. It must be pointed out that no differences were found between the controls, EVOH29 or EVOH44 without LAE and samples without film (data not shown).

Pseudomonas growth was observed after the 2^{nd} day. Values of 1.78 ± 0.01, 2.18 ± 0.063 and 3.34 ± 0.056 log were obtained for the control tubes on days 2, 6 and 10, respectively. Total inhibition was found with EVOH29 and EVOH44 incorporating 5% and 10% of LAE during the entire storage time.

A similar effect on total aerobic count was obtained. Values of 2.05 ± 0.070, 6.25 ± 0.15 and 7.15 ± 0.20 log for days 2, 6 and 10, respectively, were observed in the control samples, and total inhibition was observed with the EVOH29 and EVOH44 samples containing 5% and 10% of LAE during the entire storage time.

Levels of *Pseudomonas* and total aerobic count can be used as indicators of microbiological quality (Kilcast and Subramaniam 2000). *Pseudomonas* is a predominant spoilage bacteria in food stored aerobically under refrigeration conditions. No studies were found in the literature

regarding the level of spoilage bacteria that can confidently be associated with changes in organoleptic properties of chicken stock.

Surimi sticks were individually wrapped with EVOH29 and EVOH44 films with 5% and 10% of LAE. Samples without film and samples wrapped with films without LAE were prepared as controls. The samples were subjected to microbiological analysis on the 2nd, 6th and 10th days of refrigerated storage. Again, no differences were found between unwrapped samples and samples wrapped in film without LAE (data not shown). In all the control samples analysed, no growth was observed for enterobacteria, yeast and moulds during the whole storage time. However, common bacteria for seafood, such as lactic acid bacteria, appeared in the control samples, with values of 3.24 ± 0.21 on the 2nd day of storage, maintaining this concentration until the end of the assay. With regard to the total aerobic count, growth was observed after the 2nd day. Values of 2.38 ± 0.13, 3.45 ± 0.21 and 3.52 ± 0.21 log were obtained for the control tubes on days 2, 6 and 10, respectively. Psychrotrophic bacteria, the major group of microorganisms responsible for aerobic spoilage of fish stored at refrigeration temperatures (Sallam and Ibrahim 2007), proliferated in the control samples on the 2nd day of storage, with values of 2.53 ± 0.34, increasing to 6.04 ± 0.12 on the 10th day. Finally, *Pseudomonas* proliferation in the surimi samples was detected in the control samples on the 6th day of storage, with values of 3.45 ± 0.63, maintaining this value until the end of the storage time.

Total inhibition throughout the storage time was observed for all the microorganisms tested when the product was exposed to EVOH29 and EVOH44 containing 5% and 10% of LAE.

The use of heat treatment can lead to deterioration of food and consumer perception of product quality owing to flavour alterations. One way to prolong the microbiological shelf-life without modifying organoleptic properties is to improve traditional packaging by the use of the newly developed films as an inner coating of the package wall (Appendini and Hotchkiss 2002 ; Han 2013). Chicken stock and surimi sticks do not usually present a high number of microorganisms because they are subjected to a high temperature during processing. Moreover, in the case of commercial UHT chicken stock high levels of additives are added to the product, which is very different from traditional home-made stock. The microbiological shelf-life after package opening is 48 h when stored under refrigeration temperatures. To control undesirable microorganisms in food products after the package has been opened, LAE can be incorporated into the polymer matrix to increase food safety.

3.3.2 Efficacy of EVOH films with LAE against L. monocytogenes and E. coli inoculated into chicken stock and surimi sticks

Tubes containing films in contact with 10 mL of chicken stock inoculated with *L. monocytogenes* and *E. coli* and stored for 10 days at 4 °C were tested for bacterial growth. The analysis performed after 2 days of storage showed total inhibition against both pathogens in all the samples exposed to EVOH29 and EVOH44 films containing 10% of LAE. EVOH29 and EVOH44 films with 5% of LAE produced reductions of 5.37 and 5.48 log, respectively, compared with the control. As has been demonstrated in previous studies, in spite of releasing the same amount of LAE to water as food simulant, EVOH29 releases it faster than EVOH44 because it is more plasticized by water (Muriel-Galet et al. 2013). In the remaining days, a bactericidal effect was observed in all the samples regardless of the copolymer used (EVOH29 or EVOH44). This maximum in microbial growth reduction is probably reached as a result of the release of LAE and the amount of LAE accumulated in the chicken stock during the storage time. The control sample of *L. monocytogenes* reached values of 8.31 ± 0.01 log after 10 days at 4 °C. The pH between 6 and 6.5, the nutrient characteristics of chicken stock and the classification of psychrotrophic pathogens contribute to the growth of *L. monocytogenes* at refrigeration temperature. In contrast, the growth of *E. coli*, a mesophilic pathogen, was reduced significantly at refrigeration temperatures: the samples maintained bacterial counts around 6 ± 0.02 log throughout the experiment.

Surimi sticks were surface inoculated with *L. monocytogenes* and *E. coli* and wrapped in EVOH29 and EVOH44 films with 5% and 10% of LAE as described above. Table 2 shows the antimicrobial effect of these films against *L. monocytogenes* compared with control samples. After the 2nd day of storage, a significant antimicrobial effect was shown by the EVOH29 and EVOH44 films with LAE. At the end of the storage period, after 10 days, all the samples produced significant inhibition compared with the control. Again, sustained release of LAE throughout the storage time and accumulation in the food sample gave rise to microbial reduction compared with the control.

Again, no differences were found between the controls in both assays (data not shown)

Table 3 shows the inhibitory effect of the films against *E. coli* inoculated into surimi sticks. On the 2nd day of storage, the release of LAE produced a reduction of 1 log in the samples wrapped in EVOH29 and EVOH44 with 10% of LAE. On the 6th day of storage, a significant decrease appeared in all the samples: the EVOH29 5% and 10% LAE samples had reductions of 1.36 log and 2.37 log, respectively, and the EVOH44 5% and 10% LAE samples had reductions of 1.11 log and 2.15 log respectively, results that were maintained until the end of the experiment.

It is worth pointing out that the effectiveness of the antimicrobial films varied depending on the food matrix, being more effective in liquid food. This is related to the amount of LAE released

to the media and the release kinetics. These parameters depend on several factors, such as the water activity of the food and consequently the level of plasticization achieved by the films; and also the compatibility of the antimicrobial agent with the film and the food media. It must also be taken into account that in solid foods the morphological structure will also be affected by migration of the antimicrobial agent from the surface to the centre of the matrix.

L. monocytogenes and *E. coli* are common pathogens present in food products as a result of cross-contamination. *L. monocytogenes* is considered one of the most important bacteria of food-borne disease. The manifestations of disease include septicaemia, meningitis (or meningoencephalitis) and encephalitis, usually proceeded by symptoms similar to flu. In pregnant women it can cause spontaneous abortion. It has also been associated with gastrointestinal manifestations with fever. Although the morbidity of listeriosis is relatively low, the rate of mortality from systemic disease can be very high, with values close to 30%. Symptoms of infection by *E. coli* are severe abdominal pain, severe diarrhoea, often bloody, and sometimes nausea, vomiting and mild fever. The individuals susceptible to these pathogenic bacteria include "YOPIs" (the young, the old, the pregnant and the immunocompromised) (Adams and Moss 2008).

Ultra-High-Temperature (UHT) processing normally involves a certain loss of heat-labile vitamins, which mostly have to be restored artificially after thermal treatment; degradation of organoleptic quality and loss of texture can also be produced (Fellows 2009). In addition, the shelf-life of the products after opening is not more than 48 hours. Thus the chances of recontamination with these food-borne bacteria in the processing and packaging plant and after package opening and subsequent manipulation and storage at home are very high.

The results obtained in this study show that EVOH films incorporating LAE can contribute effectively to the development of active food packaging technologies. As an example, these films can be incorporated in a multilayer structure as the inner layer or coating in contact with the food. Thus active packaging could preserve all the food nutrients, without the incorporation of additives or the use of high thermal treatments. Moreover, it could increase the safety of the product after package opening and storage at home.

3.4. *In vitro* availability analysis of LAE

An *in vitro* digestion and subsequent HPLC analysis was carried out to evaluate LAE solubilised in the gastrointestinal tract. LAE was not detected in any of the digestion solutions (LOQ_{LAE} = 0.05mg/mL). (Bakal and Diaz 2005) demonstrated cleavage of the ethyl ester from LAE to form N-lauroyl-L-arginine (LAS) (LOQ_{LAS} = 0.02 mg/mL), but this compound was also not detected in the chromatogram.

As already mentioned, LAE degrades to LAS and hydrolysis of it results in the production of arginine, which converts to ornithine and urea, following acid catabolism via the urea and citric acid cycles, resulting in carbon dioxide and urea, which are excreted in the expired air and in urine respectively (Hawkins et al. 2009). The results demonstrate that these films have a great potential for the food safety industry without compromising human health.

CONCLUSION

The results indicate that LAE is a powerful antimicrobial with a high activity against *L. monocytogenes* and *E. coli*. In this study, LAE was included at different concentrations in films based on EVOH29 and EVOH44. The results demonstrate that these films can be applied to reduce native microflora and the growth of *L. monocytogenes* and *E. coli* in different food matrices (chicken stock and surimi sticks), increasing the safety of the products after package opening. Furthermore, it is interesting to note that after simulated gastrointestinal digestion LAE was not detected in any of the samples, demonstrating the safety of the antimicrobial agent. The application of these films as the internal surface of the package wall for direct contact with a fluid or solid product has potential to improve the microbial stability of food products.

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Legends to figures:

Fig 1. Dual-parameter SYTO-13/PI fluorescence histograms obtained by staining *L. monocytogenes* a) control samples; b) cells after MIC treatment; and *E. coli* c) control samples; d) cells after MIC treatment.

Fig 2. Scanning electron micrographs of pathogen cells: a) *L. monocytogenes* control sample and b) *L. monocytogenes* treated with 12 ppm of LAE, c) *E. coli* control sample and d) *E. coli* treated with 32 ppm of LAE.

1A

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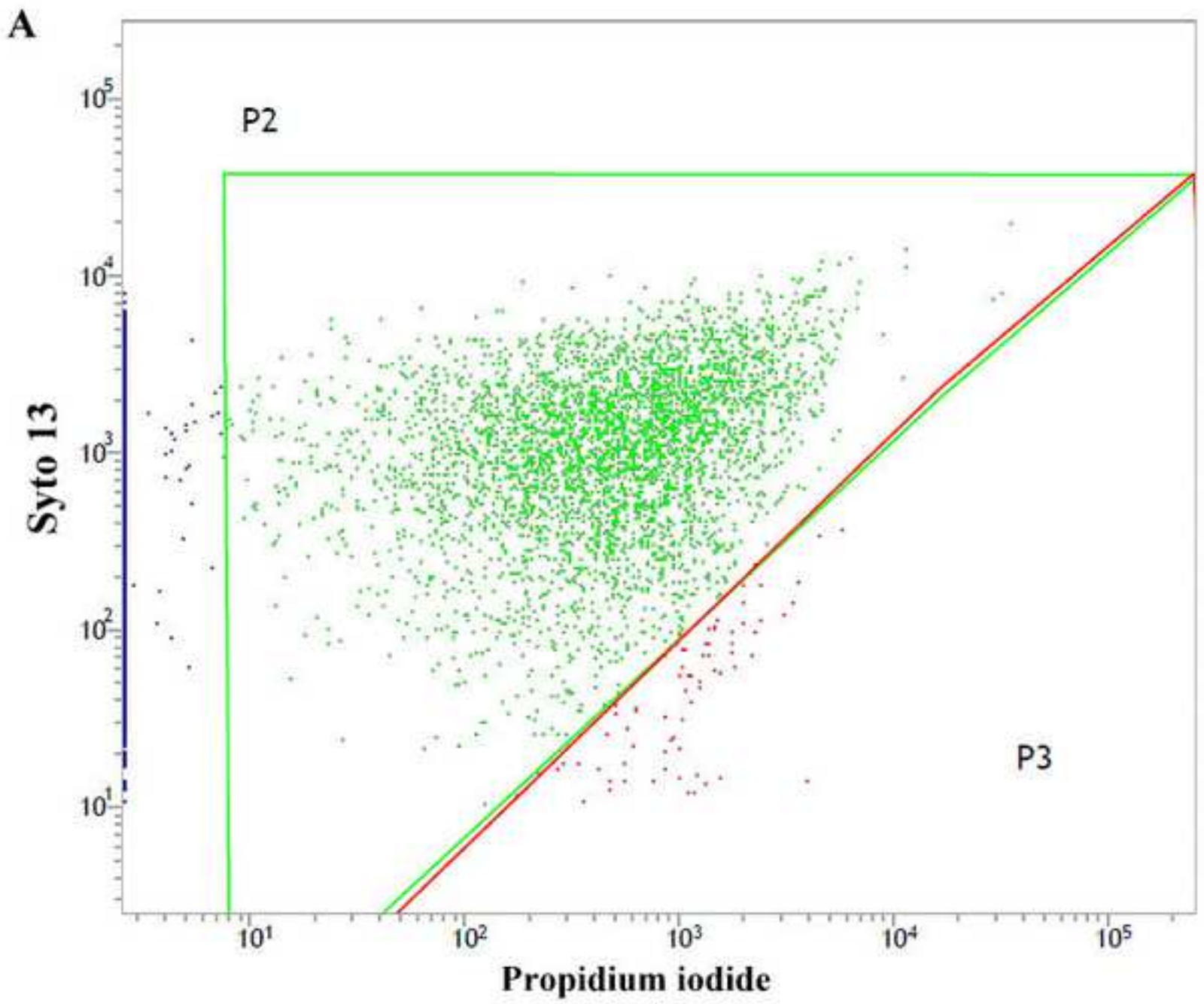

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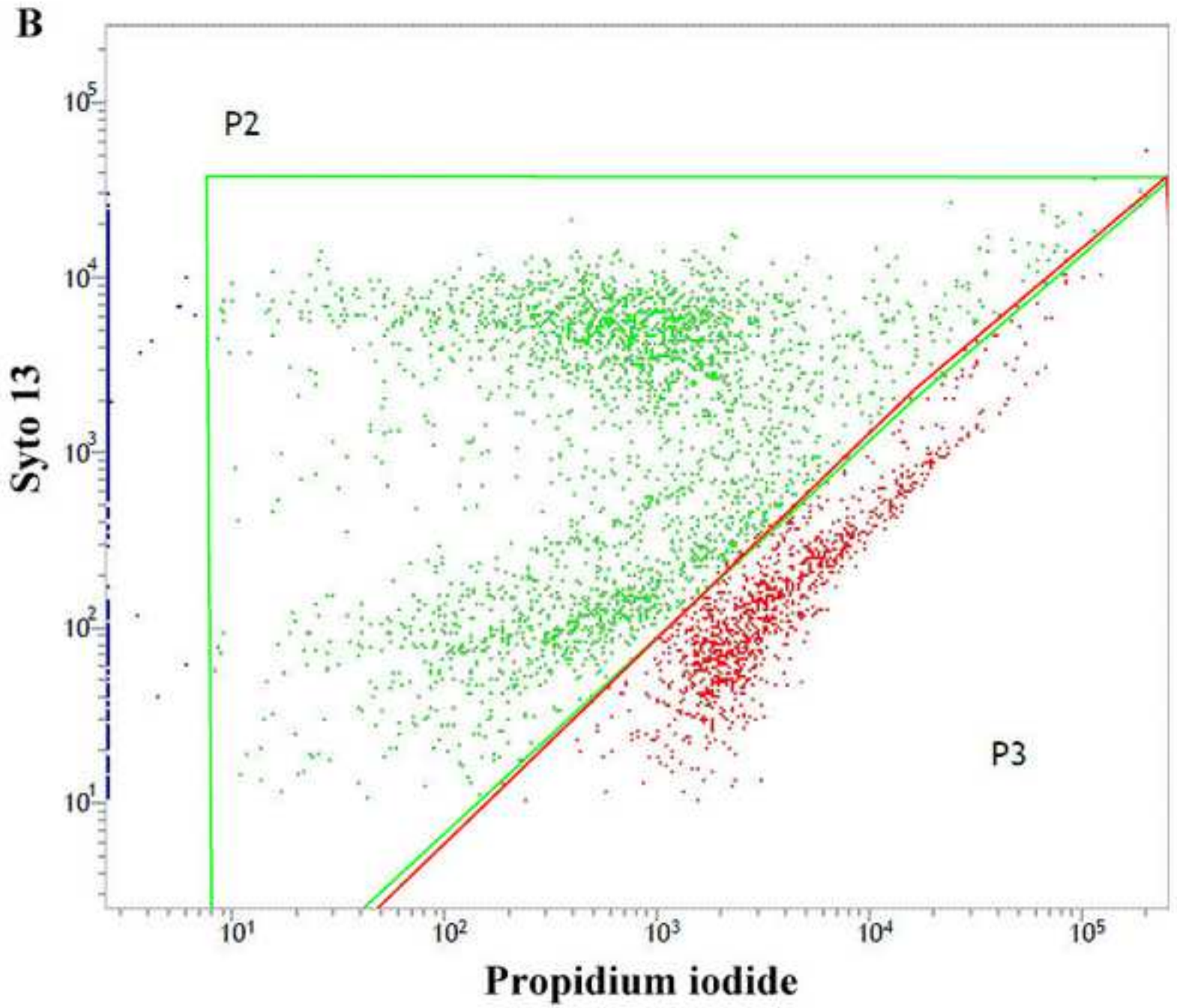

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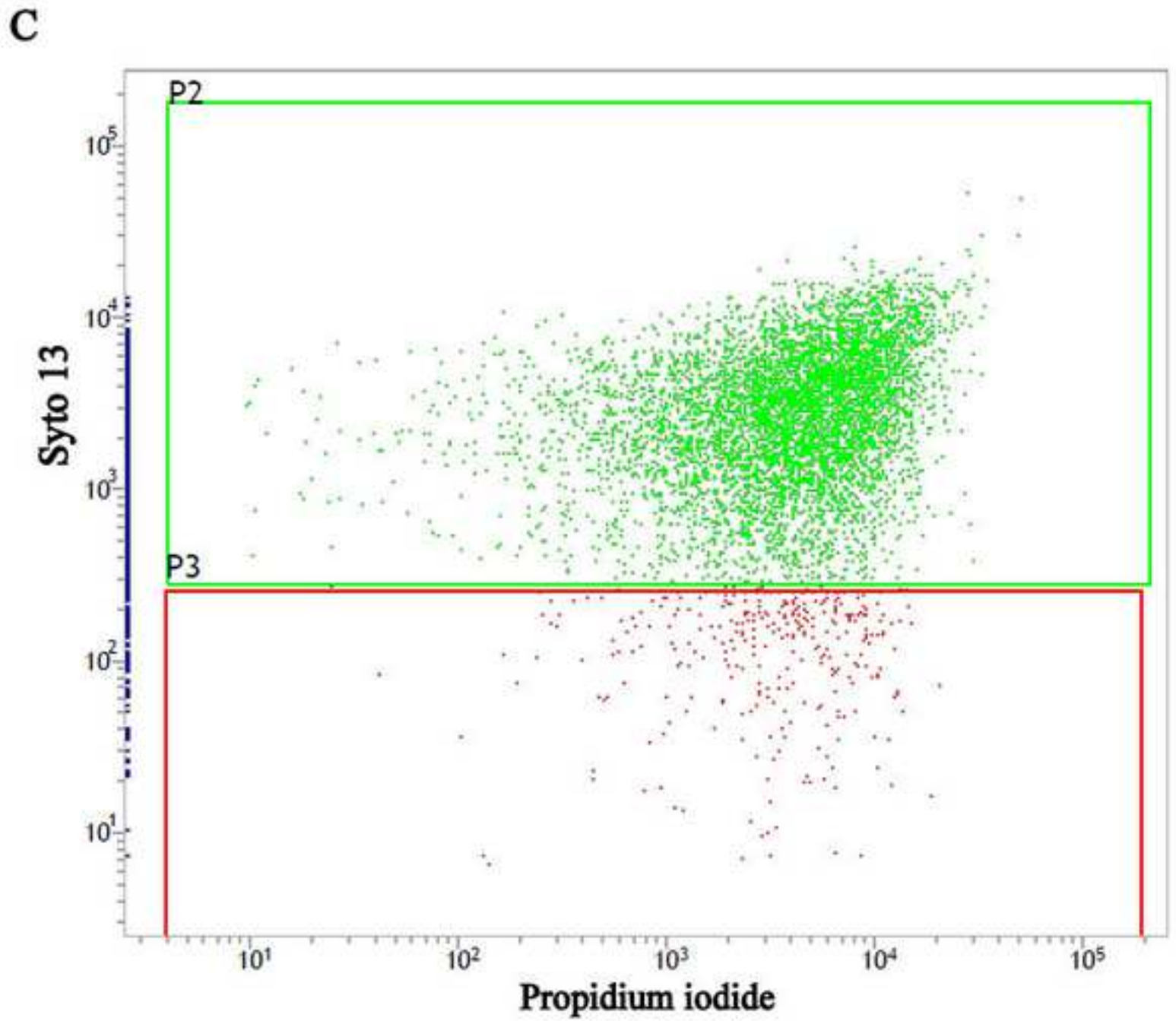

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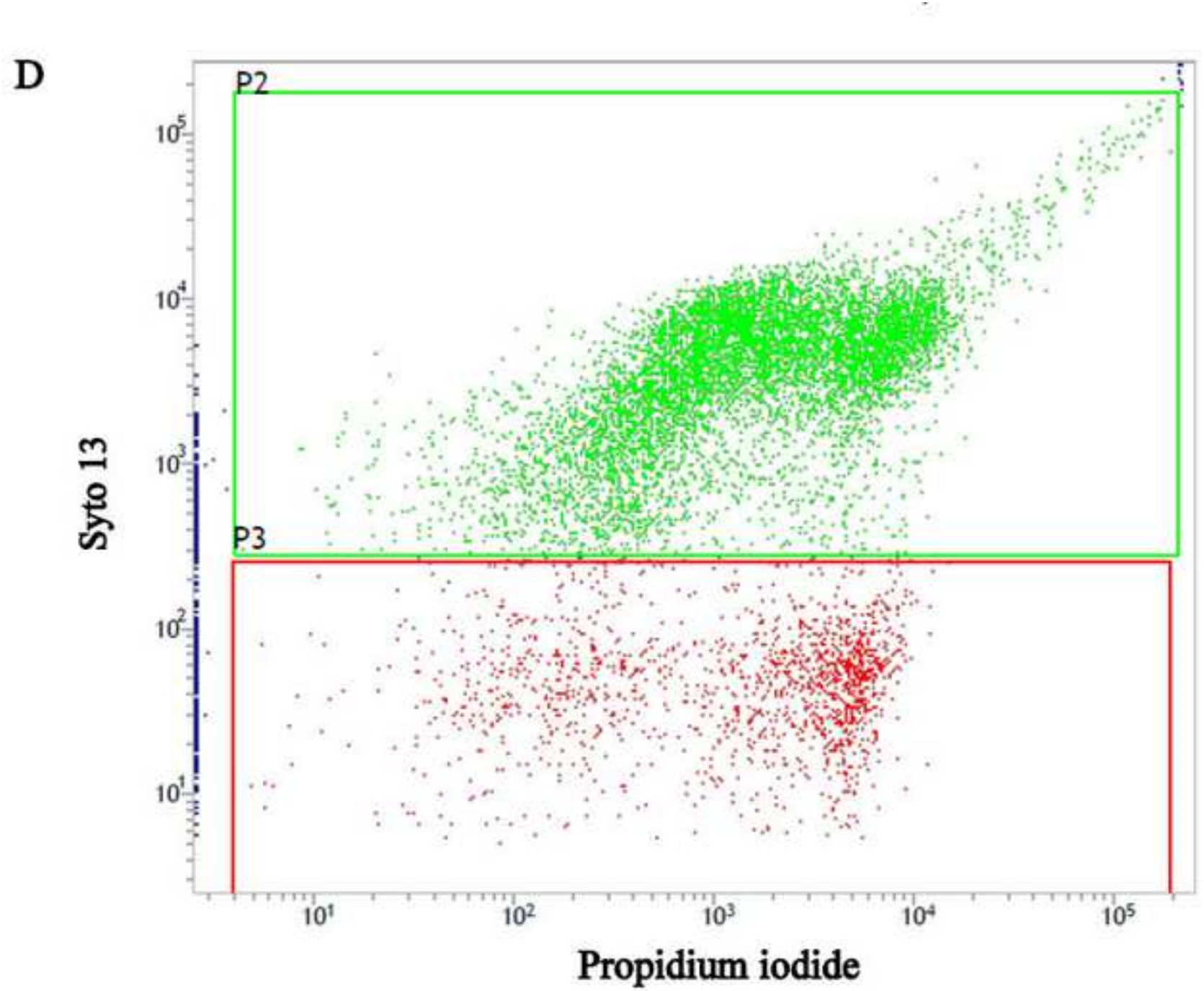

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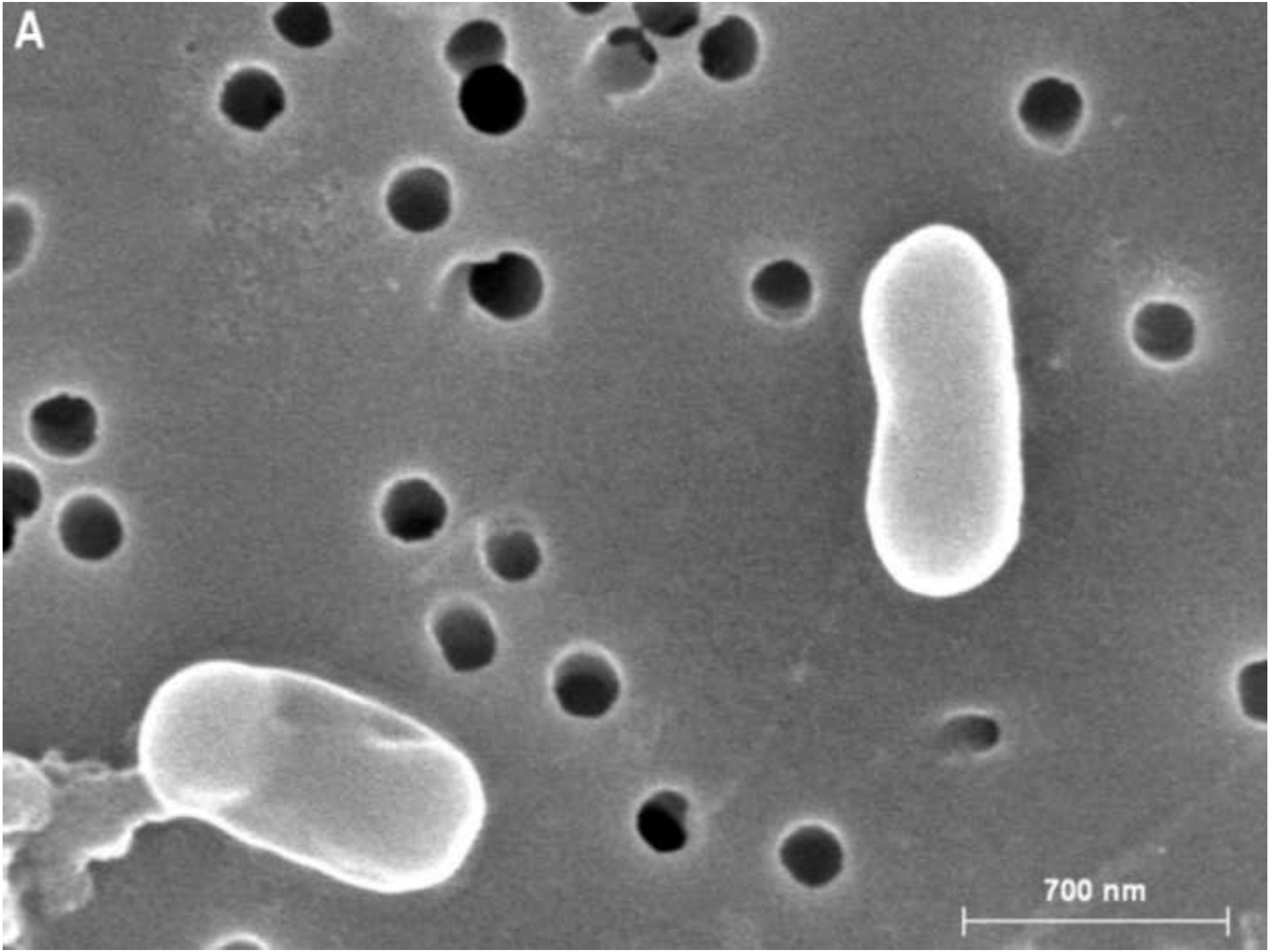

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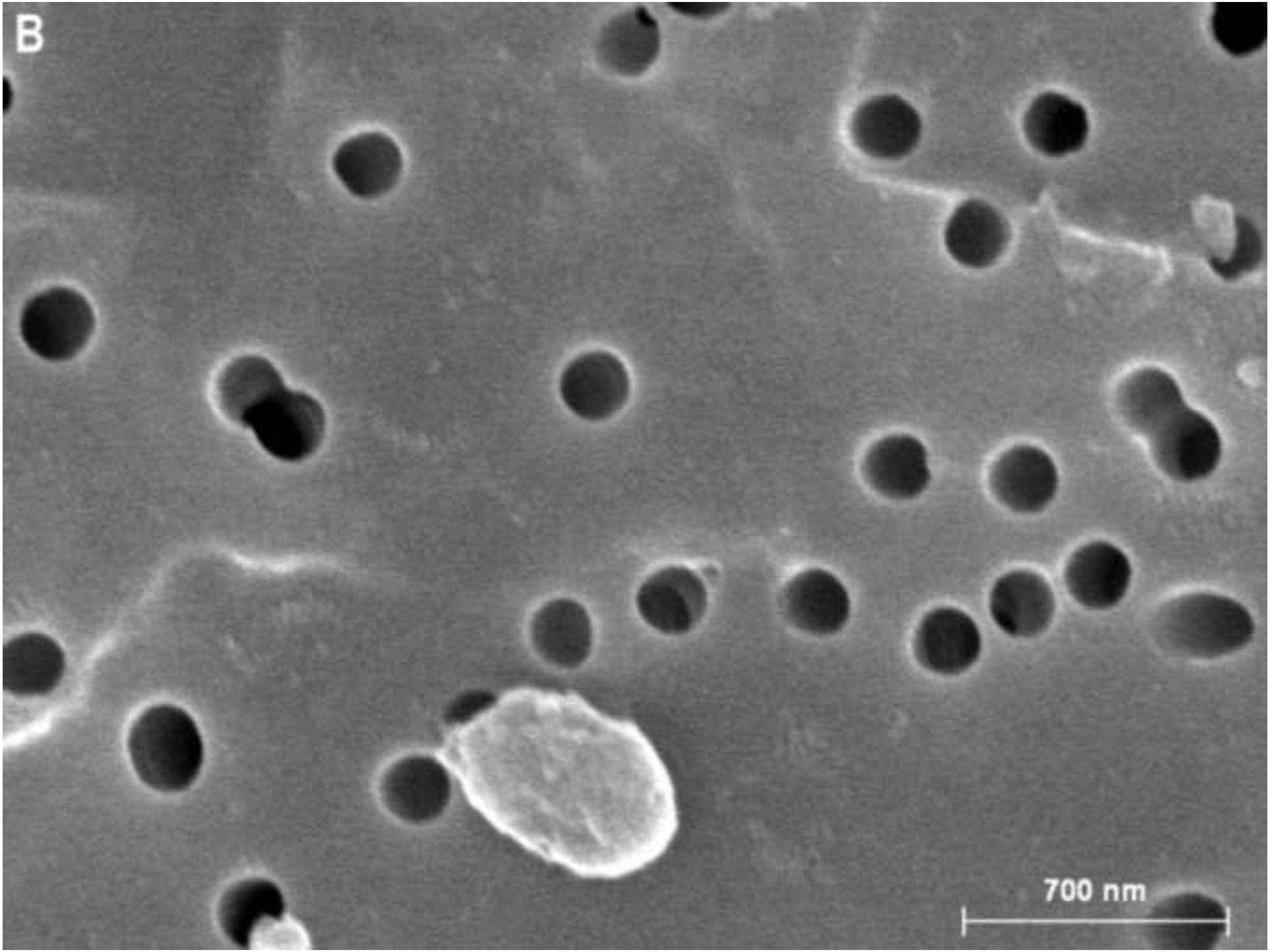

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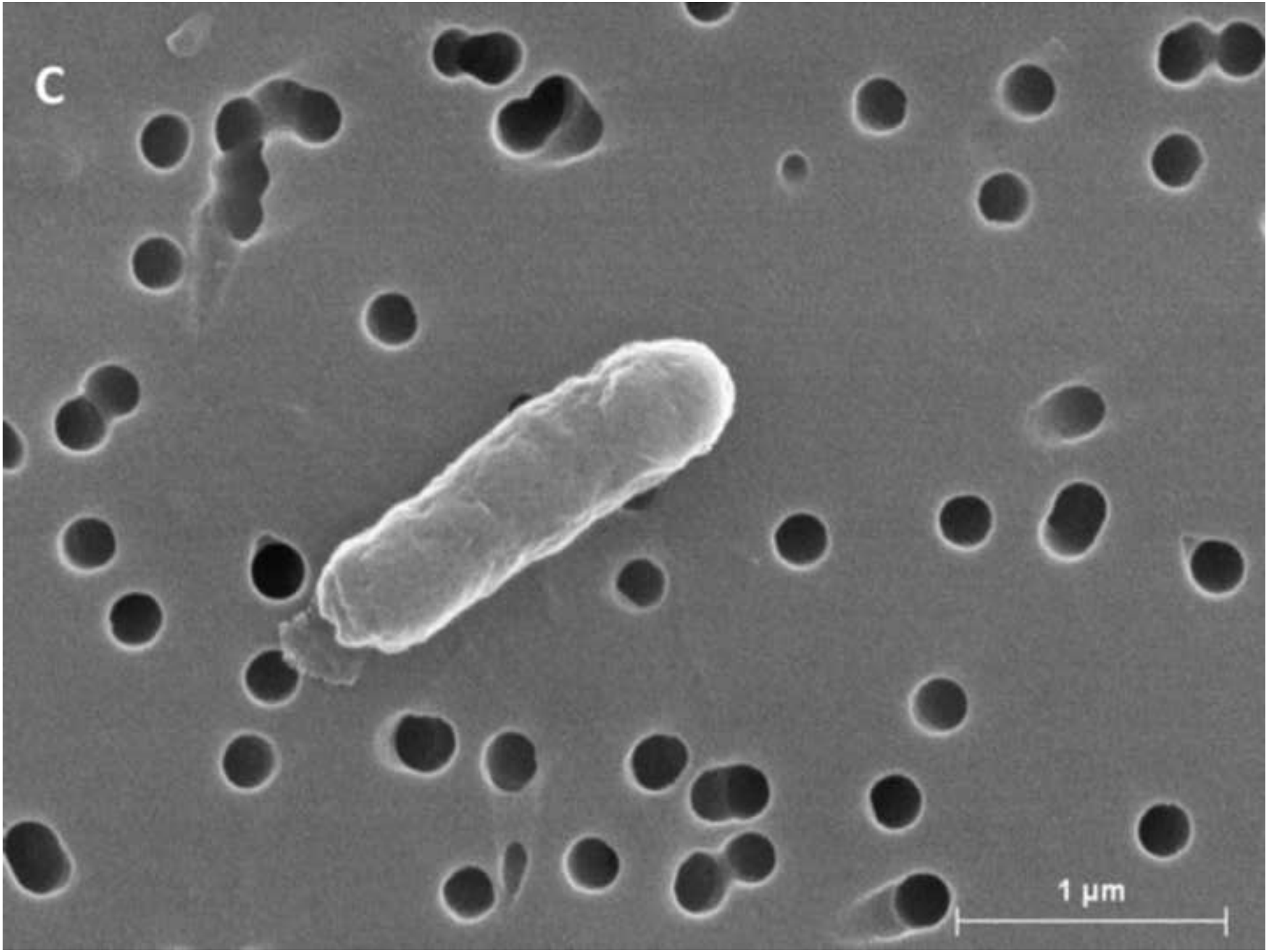

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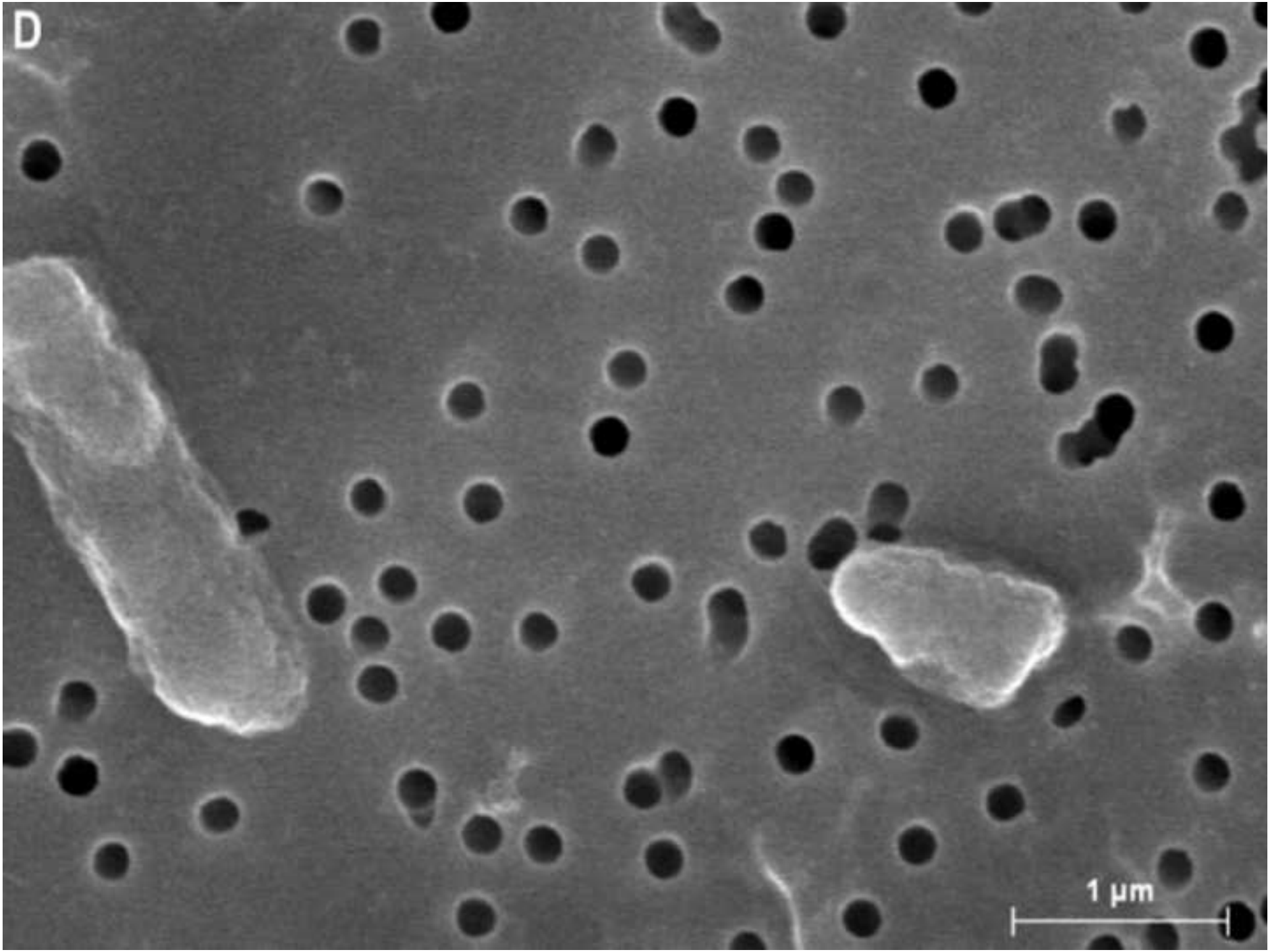

Table
Click here to download Table: Table 1.docx

Table 1. Antimicrobial effectiveness of EVOH29 and EVOH44 films incorporating several amounts of LAE against *L. monocytogenes* and *E. coli*, expressed as mm of growth inhibition zone.

	Inhibition zone (mm)	
	L. monocytogenes	***E. coli***
EVOH29-0%	No inhibition zone	No inhibition zone
EVOH29-5%	46.30	44.50
EVOH29-10%	50.00	44.50
EVOH44-0%	No inhibition zone	No inhibition zone
EVOH44-5%	46.50	42.00
EVOH44-10%	48.00	43.00

Table
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Table 2. Antimicrobial activity of EVOH29 and EVOH44 films with 5 and 10% of LAE in surimi sticks inoculated with *L. monocytogenes* during 10 days of storage at 4 °C expressed as logarithm of colony forming units (Log (CFU/g)) and log reduction value (LRV).

	L. monocytogenes					
	Day 2		**Day 6**		**Day 10**	
	Log(CFU/g surimi)	**LRV**	**Log(CFU/g surimi)**	**LRV**	**Log(CFU/g surimi)**	**LRV**
CONTROL	6.00 ± 0.02c	----	7.09 ± 0.05c		8.29 ± 0.02c	----
EVOH29-5%	5.07 ± 0.03b	0.93	4.67 ± 0.09b	2.42	3.98 ± 0.02a	4.31
EVOH29-10%	3.91 ± 0.15a	2.08	2.93 ± 0.04a	4.13	2.97 ± 0.04a	5.42
EVOH44-5%	6.15 ± 0.02c	----	6.28 ± 0.01c	0.80	6.34 ± 0.01b	1.95
EVOH44-10%	4.23 ± 0.03a	1.77	4.93 ± 0.01b	2.16	5.04 ± 0.02b	3.25

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

Table
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Table 3. Antimicrobial activity of EVOH29 and EVOH44 films with 5 and 10% of LAE in surimi sticks inoculated with *E. coli* during 10 days of storage at 4 °C expressed as logarithm of colony forming units (Log (CFU/g)) and log reduction value (LRV).

	E. coli					
	Day 2		**Day 6**		**Day 10**	
	Log(CFU/g surimi)	**LRV**	**Log(CFU/g surimi)**	**LRV**	**Log(CFU/g surimi)**	**LRV**
CONTROL	6.12 ± 0.04c		6.22 ± 0.02c		6.63±0.06c	
EVOH29-5%	5.96 ± 0.04c	----	4.86 ± 0.04b	1.36	5.33±0.06b	1.30
EVOH29-10%	4.98 ± 0.14b	1.23	3.85 ±0.05a	2.37	4.24±0.02a	2.39
EVOH44-5%	6.29 ± 0.15c	----	5.11 ± 0.03b	1.11	4.85±0.01b	1.78
EVOH44-10%	5.14 ± 0.05b	1.01	4.07 ± 0.05a	2.15	4.31±0.01a	2.32

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