Histamine-producing *Lactobacillus parabuchneri* strains isolated from grated cheese can form biofilms on stainless steel

Maria Diaz, Beatriz del Rio, Esther Sanchez-Llana, Victor Ladero, Begoña Redruello, María Fernández, M. Cruz Martin* and Miguel A. Alvarez

Instituto de Productos Lácteos de Asturias, IPLA-CSIC, Paseo Rio Linares s/n, 33300 Villaviciosa, Spain.

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*Corresponding author
Mailing address: Instituto de Productos Lácteos de Asturias (IPLA-CSIC), Paseo Rio Linares s/n, 33300 Villaviciosa, Spain.
Phone: +34 985 89 21 31
Fax: +34 985 89 22 33
E-mail: mcm@ipla.csic.es

Abstract

The consumption of food containing large amounts of histamine can lead to histamine poisoning. Cheese is one of the most frequently involved foods. Histamine, one of the biogenic amines (BAs) exhibiting the highest safety risk, accumulates in food contaminated by microorganisms with histidine decarboxylase activity. The origin of these microorganisms may be very diverse with contamination likely occurring during post-ripening processing, but the microorganisms involved during this manufacturing step have never been identified. The present work reports the isolation of 21 histamine-producing *Lactobacillus parabuchneri* strains from a histamine-containing grated cheese. PCR revealed that every isolate carried the histidine decarboxylase gene (*hdcA*). Eight lineages were identified based on the results of genome PFGE restriction analysis plus endonuclease restriction profile analysis of the carried plasmids. Members of all lineages were able to form biofilms on polystyrene and stainless steel surfaces. *L. parabuchneri* is therefore an undesirable species in the dairy industry; the biofilms it can produce on food processing equipment represent a reservoir of histamine-producing bacteria and thus a source of contamination of post-ripening-processed cheeses.

Keywords

Biogenic amines, histamine, cheese, *Lactobacillus parabuchneri*, *Lactobacillus buchneri*, biofilms.

1. Introduction

Biogenic amines (BAs) are low molecular weight organic bases with biological activity. They sometimes appear in foods and beverages as the result of the activity of microorganisms that produce different amino acid decarboxylases. For example, histidine decarboxylase catalyses the conversion of histidine to histamine. Although this compound has a number of physiological roles in humans, it is one of the most hazardous of all BAs (EFSA, 2011; Linares et al., 2016), and the ingestion of large amounts can trigger neurological, gastrointestinal and respiratory problems, especially in sensitive people with low detoxification systems (Bodmer et al., 1999; Halasz et al., 1994; Ladero et al., 2010; Silla Santos, 1996).

Although commonly associated with fish and fish products, histamine can also be found in high concentrations in fermented foods. In particular, cheeses have been involved in many cases of histamine poisoning (Silla Santos, 1996). Indeed, concentrations of histamine in cheese can reach over 1000 mg kg^{-1}, far exceeding those recommended for foodstuffs (Fernandez et al., 2007).

The accumulation of BAs in fermented dairy products has mostly been attributed to the presence of certain lactic acid bacteria (LAB) (Lonvaud-Funel, 2001). However, while several species of LAB have been identified as histamine producers, few have actually been isolated from cheese (Burdychova and Komprda, 2007; Carafa et al., 2015; Diaz et al., 2015; Ladero et al., 2008; Linares et al., 2011). The gene coding for histidine decarboxylase in LAB, *hdcA*, lies in the HDC cluster, in which it is usually preceded by *hdcP*, which encodes a histidine/histamine antiporter (Lucas et al., 2005; Martin et al., 2005). It is usually followed by *hdcB,* which codes for a protein that catalyses the maturation of histidine decarboxylase (Trip et al., 2011).

Histamine-producing microorganisms may be naturally associated with milk, form part of starter cultures, or may contaminate cheese during its manufacture (Burdychova and Komprda, 2007; Ladero et al., 2009). Post-ripening processing such as cutting, slicing, grating etc. certainly has a direct impact on the presence of BA-producers and BAs in cheeses (Ladero et al., 2009). Processed cheeses, which are becoming increasingly popular, are subjected to more manipulation than those sold as a whole, and the contact with equipment surfaces increases the risk of

contamination by BA-producing bacteria (Linares et al., 2012). However, it is not well known which microorganisms are responsible for the observed accumulation of histamine and whether these organisms are able to form biofilms on equipment surfaces. Biofilms are defined as sessile communities of cells adhered to one another and/or to a substratum or interface, embedded in a self-produced exopolysaccharide matrix (Donlan and Costerton, 2002). Such an ability would undoubtedly increase the likelihood of contamination (Abdallah et al., 2014; Winkelstroter et al., 2014).

The aims of the present work were to isolate, identify and type the histamine-producing bacteria in a grated cheese with a high histamine content, as well as to test their ability to develop biofilms on polystyrene and stainless steel surfaces.

2. Material and Methods

2.1. Bacterial strains and culture conditions

Lactobacillus buchneri B301 (Martin et al., 2005) was used as a histamine-producing positive control. This strain was first identified using biochemical methods (Joosten and Northolt, 1989), but sequencing of its *16S rRNA* gene (Accession Nº LN877766), *rpoA* (Accession Nº LT547858) and *pheS* (Accession Nº LT547856) revealed it to be 100% identical to that of *L. parabuchneri*. It is therefore referred here as *L. parabuchneri* B301.

All *Lactobacillus* strains used in this study were routinely cultured in MRS broth (Oxoid, Basingstoke, Hampshire, UK). When necessary, the medium was supplemented with 10 mM histidine (Sigma-Aldrich, Madrid, Spain). Unless otherwise stated, strains were incubated at 37ºC under anaerobic conditions (10% H_2, 10% CO_2 and 80% N_2) in a Mac 1000 anaerobic workstation (Don Whitley Scientific, Shipley, UK), a large chamber that controls automatically the temperature and gas concentration.

2.2. Isolation of histamine-producing bacteria from cheese samples

One gram of grated Emmental cheese was homogenized for 2 min in 9 mL of LAPTg broth (1% glucose (w/v), 1.5% peptone (w/v), 1% tryptone (w/v), 1% yeast extract

(w/v), 0.1% tween 80 (v/v)) (Raibaud et al., 1961) using a Lab-Blender 400 Stomacher (Seward Ltd., London, UK). After incubation for 30 min at room temperature, serial dilutions of homogenized suspension were spread on LAPTg agar plates supplemented with 5 mM histidine, 0.005% (w/v) pyridoxal phosphate, and 50 µg/mL cycloheximide (Sigma-Aldrich), in order to obtain isolated colonies. Plates were incubated for 48 h at 37ºC under anaerobic conditions. Isolated colonies present in the agar plates were picked and then each whole colony inoculated into microtitre plates containing liquid decarboxylase medium (MDA) (Bover-Cid and Holzapfel, 1999) supplemented with 10 mM histidine for 72 h at 37ºC. The isolates that caused alkalization of the medium, which is indicated by its change from yellow to a purple colour, were selected for further analysis.

2.3. Quantification of histamine by ultra-high performance liquid chromatography

To test the capacity of each isolate to produce histamine, all isolates were grown in MRS broth supplemented with 10 mM histamine for 24 h at 37ºC without aeration. The cells were then removed by centrifugation at 8000 *g* for 10 min, and 100 µL of the supernatant derivatised with diethyl ethoxymethylenemalonate (DEEMM) (Sigma-Aldrich) to quantify its histamine content by ultra-high performance liquid chromatography (UHPLC) using an H-Class AcquityUPLC system (Waters, Milford, MA, USA) as previously described Redruello et al. (2013). Separation was performed at 35ºC in a Waters AcquityUPLC BEHC18 1.7 µm column (2.1 x 100 mm). Data were acquired and analysed using Empower 2 software (Waters).

To determine the amount of histamine present in the cheese, 1 g samples were processed to extract the BAs as previously described by Herrero-Fresno et al. (2012). One hundred microlitres were then derivatised and analysed as described above.

2.4. DNA manipulation procedures

For each strain, total DNA was isolated from 2 mL of bacterial cultures grown in MRS supplemented with 1% (w/v) glycine (USB Corporation, Cleveland, OH, USA), using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer's instructions. Plasmid DNA was isolated as previously described by

Anderson and McKay (1983). Restriction endonuclease analysis of plasmids was performed with *Eco*RI (Takara Bio Inc., Shuzo Co., Ltd., Japan) using a 0.7% agarose gel in TAE [40 mM Tris/acetate (pH 8.0), 1 mM EDTA] buffer. A GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA) was used as a molecular marker.

The PCR products were purified using the ATP Gel/PCR Extraction Kit (ATPTM Biotech Inc., Taipei City, Taiwan) and sequenced by Macrogen Inc. (Seoul, Republic of Korea).

2.5. Histamine-producing isolates: identification at the species level by 16S rRNA, rpoA *and* pheS *genes sequencing*

Histamine-producing isolates were identified at the species level by amplification of part of the *16S rRNA, rpoA* and *pheS* genes using 1 ng of total DNA as a template, the universal primer pairs 27F/1492R (5'-AGAGTTTGATYMTGGCTCAG-3'/5'-TACGGYTACCTTGTTACGACTT-3') (Lane, 1991), pheS-21-F/pheS-22-R (5'-CAYCCNGCHCGYGAYATGC-3'/5'-CCWARVCCRAARGCAAARCC-3') (Naser et al., 2005) and rpoA-21-F/rpoA-23-R (5'-ATGATYGARTTTGAAAAACC-3'/5'-ACHGTRTTRATDCCDGCRCG-3') (Naser et al., 2005) or rpoA-21-F /rpoA-22-R (5'-ATGATYGARTTTGAAAAACC-3'/5'-ACYTTVATCATNTCWGVYTC-3') (Naser et al., 2007) respectively and 5PRIME Taq DNA Polymerase (5 PRIME GmbH, Hilden, Germany) using an iCycler thermocycler (Bio-Rad, Spain). The thermal programmes consisted of 5 min at 95ºC, followed by 35 amplification cycles of 30 s at 95ºC, 30 s at 50ºC, and 90 s at 68ºC, plus a final extension step of 10 min at 68ºC for primers 27F/1492R and 5min at 95 ºC followed by 3 cycles of 1 min at 95 ºC, 2 min 15 s at 46 or 42 ºC and 1 min 15 s at 72 ºC followed by 30 cycles of 35 s at 95 ºC, 1 min 15 s at 46 or 42 ºC, 1 min 15 s at 72 ºC and a final step of 7 min at 72 ºC (Naser et al., 2005) for primers pheS-21-F/pheS-22-R,rpoA-21-F/rpoA-23-R and rpoA-21-F /rpoA-22-R The resulting sequences were aligned and compared with the eubacterial *16S rRNA, rpoA* and *pheS* gene sequences available in the GenBank and EMBL databases using BLAST software (Altschul et al., 1997).

2.6. PCR amplification of the hdcA gene

The presence of *hdcA* in the histamine-producing isolates was checked by PCR using primers hdc3 (GATGGTATTGTTTCKTATGA) and hdc4 (CAAACACCAGCATCTTC) (Coton and Coton, 2005). PCR was performed as described in *2.5.* but using DreamTaq DNA Polymerase (Fermentas, Vilnius, Lithuania) and employing the conditions described by Coton and Coton (2005). *L. parabuchneri* B301 was used as a histamine-producing positive control.

2.7. Macrorestriction of genomic DNA and analysis by pulsed-field gel electrophoresis

Twenty one histamine-producing *L. parabuchneri* isolates and *L. parabuchneri* B301 were analysed by PFGE following the protocol described by Herrero-Fresno et al. (2012) with the exception that the agarose plugs were treated with *Not*I endonuclease (Takara Bio Inc) and the pulse ramps used were 0.1-2 s for 12 h and 2-20 s for 6 h. A low range PFG marker (New England Biolabs, Ipswich, MA, USA) was used as a molecular size marker.

GeneTools software (SynGene, Cambridge, UK) was used to analyse the PFGE patterns. An UPGMA (unweighted pair-group method with arithmetic mean) dendrogram was produced by profile comparison based on the molecular weight (MW) alignment and a position tolerance of 1%.

2.8. Biofilm formation on polystyrene

The ability of each strain to form a biofilm on polystyrene was tested using overnight cultures (MRS broth) diluted to approximately 10^6 cfu/mL. Eight wells of a round-bottomed polystyrene 96-well microtitre plate (Nunc MicroWell Plates with a Nunclon Delta Surface: Thermo Fisher Scientific) were inoculated with 200 µL of these dilutions (performed in triplicate). The negative control consisted on eight wells filled with sterile medium. The plates were then incubated at 37ºC. Biofilm-biomass quantification was performed using the crystal violet staining method (CV assay) described by Kubota et al. (2008) with modifications. After 20, 24, 28, 32, 48 and 72

h of incubation, the supernatant was removed and all wells were rinsed twice with 225 µL of PBS buffer to remove non-adherent cells. The plates were then air-dried for 30 min at room temperature in a CRUMAir 9005-FL laminar flow cabinet (CRUMA, Barcelona, Spain). Any biofilm present was then stained with 250 µL of 0.5% (w/v) crystal violet in distilled water (dH_2O) for 30 min at room temperature. The non-bound dye was removed and rinsed three times with 300 µL of dH_2O. Finally, the bound dye was extracted using 250 µL of acetone/ethanol (80/20) and absorbance was measured at 595 nm using a Benchmark Plus microplate spectrophotometer (BioRad, Hercules, CA, USA).

Biofilm production ability was expressed using cut-off values (Extremina et al., 2011). The mean±SD of the optical density (OD) of three replicates was calculated for each strain. The cut-off value between biofilm-producers and non-producers was defined as the mean of the negative controls (ODnc) plus three SDs (ODc). The strains were then classified into the following categories: ODc<OD≤2×ODc=weak biofilm producer; 2×ODc<OD≤4xODc=moderate biofilm producer; OD>4×ODc=strong biofilm producer.

2.9. Biofilm formation on stainless steel

The test surfaces used in this assay were 1 cm^2 stainless steel (type AISI 304) coupons. These were washed with soap and dH_2O, rinsed with dH_2O, and immersed in acetone for 30 min to remove any grease or fingerprints. They were then rinsed once again with dH_2O, autoclaved (Bayoumi et al., 2012), and immersed singly in tubes containing MRS broth inoculated with 10^6 cfu/mL of the assayed strain (performed in triplicate). Each coupon was then incubated at 37ºC for 24, 36 or 48 h before removal using sterile forceps. Non-adherent cells were removed by rinsing the coupon three times in PBS buffer. They were then re-immersed in 10 mL PBS buffer, and the adherent cells detached from the coupon by sonication in an ultrasonic bath (Ultrasons-H, Selecta, Spain) for 15 min. The bacterial suspension produced was serially diluted in PBS, and 100 µL of 10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions were plated on MRS and incubated for 48 h (Kruszewski et al., 2013). Three replicates were performed for each strain, using independent bacterial cultures. Bacterial counts were expressed as $\log_{10}$ cfu/cm^2 (mean±SD of three replicates).

ANOVA with *post-hoc* Bonferroni correction was used to analyse all data. Significance was set at $p<0.05$. All statistical calculations were undertaken using SPSS v.15.0 software (SPSS Inc., 2006).

To test the tolerance of the cells to sonication, a bacterial suspension was serially diluted in PBS. This suspension was sonicated for 15 min and 10-fold dilutions plated. These were incubated for 48 h and the cells enumerated. No differences were seen between pre- and post-sonication cell counts.

2.10 Scanning electron microscopy (SEM)

To observe the biofilms developed on the stainless steel coupons, the method of Kubota et al. (2008) was followed with some modifications. Briefly, the coupons were rinsed twice for 15 min in PBS and then fixed in 2.5% glutaraldehyde (Sigma-Aldrich) in PBS for 16 h at room temperature. The fixed bacteria were then dehydrated using a graded series of acetone solutions (50 to 100% [v/v]), and the coupons dried with CO_2 using a CPD-030 critical point dryer (Bal-Tec AG, Balzers, Liechtenstein). They were then coated with gold (SCD 004 Sputtering Coater, Balzers, Liechtenstein) and observed using a JSM-6610LV scanning electron microscope (JEOL USA, Inc, Peabody, MA, USA).

3. Results

3.1. Isolation of histamine-producing bacteria

A sample of grated Emmental cheese, which contained 155.48 mg kg^{-1} of histamine, was used as starting material for screening for histamine-producing bacteria. As described in section 2.2, the sample was homogenized and the colonies were isolated on solid LAPTg supplemented with histidine, pyridoxal phosphate, which is the coenzyme for histidine decarboxylases in Gram-negative bacteria, and cycloheximide to inhibit yeast and mould growth. All the colonies from dilution 10^{-7} (the dilution with perfectly colonies isolated), a total of 94 colonies were then picked off, placed on new LAPTg plates, and then subjected to functional selection in microtitre plates containing MDA decarboxylase medium with 50 mM histidine. On the basis of a colour change in the medium, 25 isolates were selected as putative

histamine-producing bacteria. UPLC analysis of the cell-free supernatants confirmed the production of histamine by 21 of the 25 isolates. From the 94 isolates initially selected, 21 were histamine-producers, which represent a population of $3.78x10^9$ cfu of histamine-producing bacteria per g of cheese.

3.2. Molecular identification of histamine-producing isolates.

The 21 histamine-producing isolates were identified as *L. parabuchneri* by *16S rRNA* gene sequencing (Accession Nº LN877763) and database comparison (100% identical). Since *16S rRNA* gene from *L. parabuchneri* and *L. buchneri* is very similar, *rpoA* and *pheS* genes from one representative isolate of each lineage were amplified and sequenced. The sequences of all the isolates were identical (Accession Nº LT547859 and LT547857 respectively) and were compared with databases. The sequence of both genes showed 99-100% identity with deposited *L.parabuchneri* strains, confirming the previous identification. They were thus named as *L. parabuchneri* IPLA11118 to IPLA11138.

3.3. Verification of the presence of hdcA in the genome of the histamine-producing L. parabuchneri *isolates*

The presence of *hdcA* was sought in all the *L. parabuchneri* isolates by PCR using the primers hdc3 and hdc4 (Coton and Coton, 2005) and the genomic DNA of each isolate as a template. An approximately 440 bp amplicon was produced by all isolates. These amplicons were sequenced (Accession Nº LN877764) and compared with those in international databases using BLAST software, and found to be 100% identical to the *hdcA* gene of *L. parabuchneri* B301 (formerly *L. buchneri* B301) (Accession Nº AJ749838) and of two other strains of *L. buchneri* (Accession Nº AY550914 and DQ132890). The *hdcA* gene of *L. parabuchneri* DSM 5987 was also PCR amplified using the primers hdc3 and hdc4, sequenced (Accession Nº LN877765), and also found to be 100% identical.

3.4. PFGE-macrorestriction analysis of genomic DNA of L. parabuchneri *isolates and restriction endonuclease analysis of the plasmid DNA*

To further characterise the 21 histamine-producing *L. parabuchneri* isolates, their genomic DNA was subjected to PFGE analysis, and their plasmid DNA characterised by endonuclease restriction analysis. The strain *L. parabuchneri* B301 was also included in this PFGE analysis.

*Not*I-macrorestriction PFGE analysis (Fig. 1) differentiated the isolates into four *Not*I profiles (N1-N4). The strain *L. parabuchneri* B301 showed a different profile to the present isolates. All except four isolates were assignable to the N1 profile (Table 1). A dendrogram (Fig. 1) was produced from the PFGE profiles using profile-based cluster analysis based on the molecular size of the fragments. Visual examination of the dendrogram showed that most of the *L. parabuchneri* profiles grouped into the same cluster with over 77% similarity. The N3 profile was grouped separately and showed 62% similarity with the other profiles.

Plasmid DNA of the 21 *L. parabuchneri* isolates was extracted and characterized by *Eco*RI restriction analysis and seven different profiles obtained (P1-P7) (Fig. 2). Eleven of the isolates fell into the P1 profile, 2 into the P2 profile, 3 into P3, 1 into P4, 2 into P5, 1 into P6, and 1 isolate without plasmids into P7.

Taken together, these results allowed eight lineages to be distinguished - L1 (N1, P1), L2 (N1, P2), L3 (N1, P3), L4 (N1, P5), L5 (N1, P7), L6 (N2, P6), L7 (N3, P3) and L8 (N4, P3). Lineage L1, represented by 11 isolates, was the most common; lineages L2, L4 and L7 were represented by two isolates each, and lineages L3, L5 L6 and L8 by one isolate each (Table 1).

3.5. Biofilm formation on polystyrene

One representative *L. parabuchneri* isolate of each lineage was selected and assessed for biofilm formation on polystyrene. For all the assayed strains, biofilm biomass was maximal at 48 h of incubation at 37ºC (Fig. 3).

The mean±SD of the absorbance of the negative control was used to calculate the biofilm production strength cut-off values as described in Section 2.8. Bar charts for the OD (mean±SD) at the maximum adhesion times (i.e., 48 h) showed three of the eight strains tested to be strong biofilms producers on polystyrene. The five remaining strains were weak biofilm producers.

3.6. Biofilm formation on stainless steel

The ability of one representative *L. parabuchneri* isolate of each lineage to adhere to stainless steel was tested. For all the assayed strains, maximum adhesion was obtained after 36 h of incubation at 37ºC (Fig. 4). This value was always $>10^3$ cfu/cm^2, except for *L. parabuchneri* IPLA 11138. Significant differences in the number of adhered cells were only seen between *L. parabuchneri* IPLA 11138 and the strains *L. parabuchneri* 11131 and *L. parabuchneri* 11132.

3.7. Imaging of biofilm formation on stainless steel.

Scanning electron microscopy (SEM) photomicrographs of the biofilm formed by *L. parabuchneri* IPLA11129 (the strongest biofilm producer) on the stainless-steel coupons were taken after 36 h of incubation. Figure 5 shows clusters of cells clearly forming a biofilm.

4. Discussion

It is well known that food processing increases the probability of contamination by microbes (Reij and Den Aantrekker, 2004). It could, therefore, also facilitate contamination by histamine-producing microorganisms, potentially leading to high histamine concentrations in final products. Work performed at our laboratory has shown that the post-ripening processing of cheese facilitates such outcomes (Ladero et al., 2009), but no histamine-producing microorganisms have ever actually been isolated from processed cheeses. The present work allowed isolating, identifying and characterizing histamine-producing bacteria from a grated Emmental cheese; a required first step in preventing the problems they can cause.

The 21 histamine-producing isolates obtained from the cheese sample were all identified as *L. parabuchneri* based on *rpoA, pheS and16S rRNA* genes sequencing. *L. parabuchneri* is one of the obligate heterofermentative lactobacilli (OHL) most commonly isolated from cheese (Coton et al., 2008). Indeed, it was the first histamine-producing bacterium isolated from it (although not from post-ripening processed cheese) (Sumner et al., 1985) although it was wrongly classified as *L. buchneri* (Fröhlich-Wyder et al., 2013). Histamine-producing *L. parabuchneri* strains have recently been isolated from two traditional, non-post-ripening-processed

cheeses of different origin and characteristics (Carafa et al., 2015; Diaz et al., 2015). Together, these data, plus the present results, suggest *L. parabuchneri* to be the main bacterium responsible for the accumulation of histamine in cheese.

In the present work, PCR confirmed the presence of the *hdcA* gene in all the *L. parabuchneri* isolates. The sequence of the amplicon produced showed 100% similarity to those of the *hdcA* genes of several *L. buchneri* strains held in public databases (Accession Nos AJ749838, AY550914 and DQ132890); this suggests these latter strains may be incorrectly identified. Surprisingly, these databases hold no sequence for the *hdcA* of *L. parabuchneri*, even though this species has been described as a histamine producer. The decision was therefore taken to sequence the *hdcA* gene of the collection strain *L. parabuchneri* DSM 5987, and it was found to be 100% identical to those of the present isolates. The *rpoA, pheS* and *16S rRNA* genes of *L. buchneri* B301 was therefore also sequenced, which confirmed the strain as belonging to *L. parabuchneri* (note: Fröhlich-Wyder et al. [2013] reported a similar misidentification). Besides the obvious taxonomic interest of this finding, the proper classification of these species is very important, since, while *L. buchneri* has a Qualified Presumption of Safety status, awarded by the European Food Safety Authority, *L. parabuchneri* does not (EFSA Panel on Biological Hazards, 2015).

The 21 *L. parabuchneri* isolates were classified into eight lineages, with four different *Not*I PFGE profiles and seven different *Eco*RI plasmid profiles. The existence of eight lineages shows that the isolates do not belong to a clonal group, and indicates that the presence of *L. parabuchneri* was not due to some unfortunate but random contamination event; rather, this species would appear to be perfectly adapted to the grated cheese manufacturing environment.

It is important to remember that processing equipment may have areas that are difficult to clean and which are therefore susceptible to biofilm formation (Winkelstroter et al., 2014). The CV assay, which is routinely used to measure the biomass of biofilms, showed that the eight lineages detected in the present work were able to form biofilms on polystyrene surfaces, with three being strong biofilm producers. Further, the average number of cells adhered to the stainless steel coupons was $>10^3$ cfu/cm^2 for all lineages but one. Cells adhering to steel processing equipment could be an important contamination source. Few studies have been undertaken on biofilm formation by food spoilage lactobacilli (Fernández Ramírez et al., 2015; Kubota et al., 2008; Somers et al., 2001); to our knowledge this is the first

time that the ability of BA-producing food microorganisms to form biofilms has been described.

In conclusion, the results of this work highlight the importance of controlling the formation of biofilms involving BA-producing bacteria in the food processing industry.

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6. Figure legends

Figure 1. UPGMA-based dendrogram resulting from cluster analysis of the *L. parabuchneri* isolates' PFGE fingerprints.

Figure 2. *Eco*RI restriction profiles of the plasmids carried by the *L. parabuchneri* isolates. M: GeneRuler 1 kb DNA Ladder.

Figure 3. Biofilm-forming ability on polystyrene of the histamine-producing *L. parabuchneri* isolates (one representative of each of the eight lineages). Data represent means±SD (error bars) of three experiments. Values marked with the same letter do not differ significantly ($p>0.05$ according to the Bonferroni *post hoc* test). (___) Cut-off line (ODc); (__ __ __) 2xODc; (.....) 4xODc.

Figure 4. Adherence to stainless steel coupons by histamine-producing *L. parabuchneri* isolates (one representative of each of the eight lineages). Data are expressed as $\log_{10}$ cfu/cm^2 and represent the mean±SD (error bars) of three

446 experiments. Values marked with the same letter do not differ significantly ($p>0.05$
447 according to the Bonferroni *post hoc* test).
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449 Figure 5. (A): Scanning electron photomicrograph of a biofilm formed by *L.*
450 *parabuchneri* IPLA11129 on a steel coupon; (B): Biofilm-forming *L. parabuchneri*
451 IPLA11129 cells.
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L4 (2)	N1	P5	IPLA11125, IPLA11137
L5 (1)	N1	P0	IPLA11122
L6 (1)	N2	P6	IPLA11131
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L8 (1)	N4	P4	IPLA11138

Figure

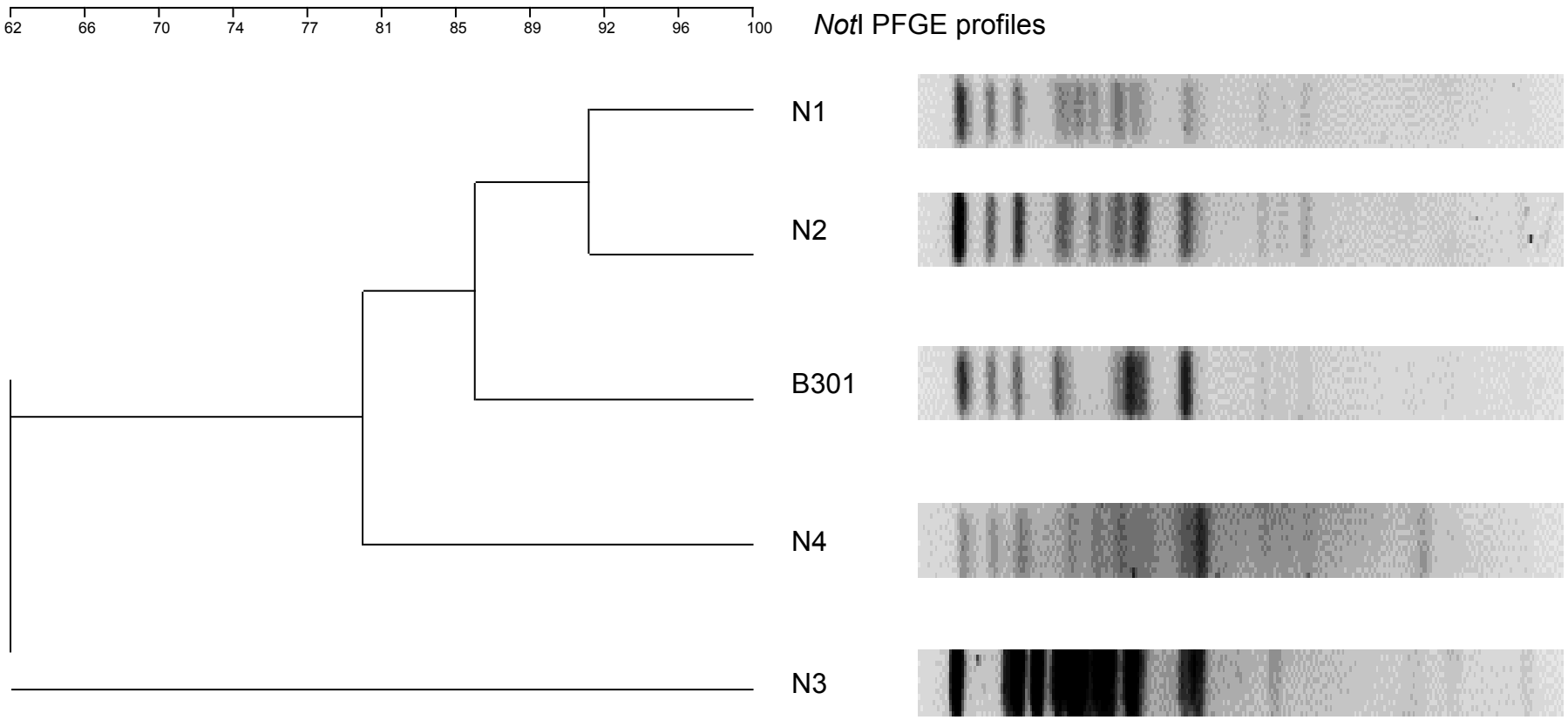

Figure 1

Figure

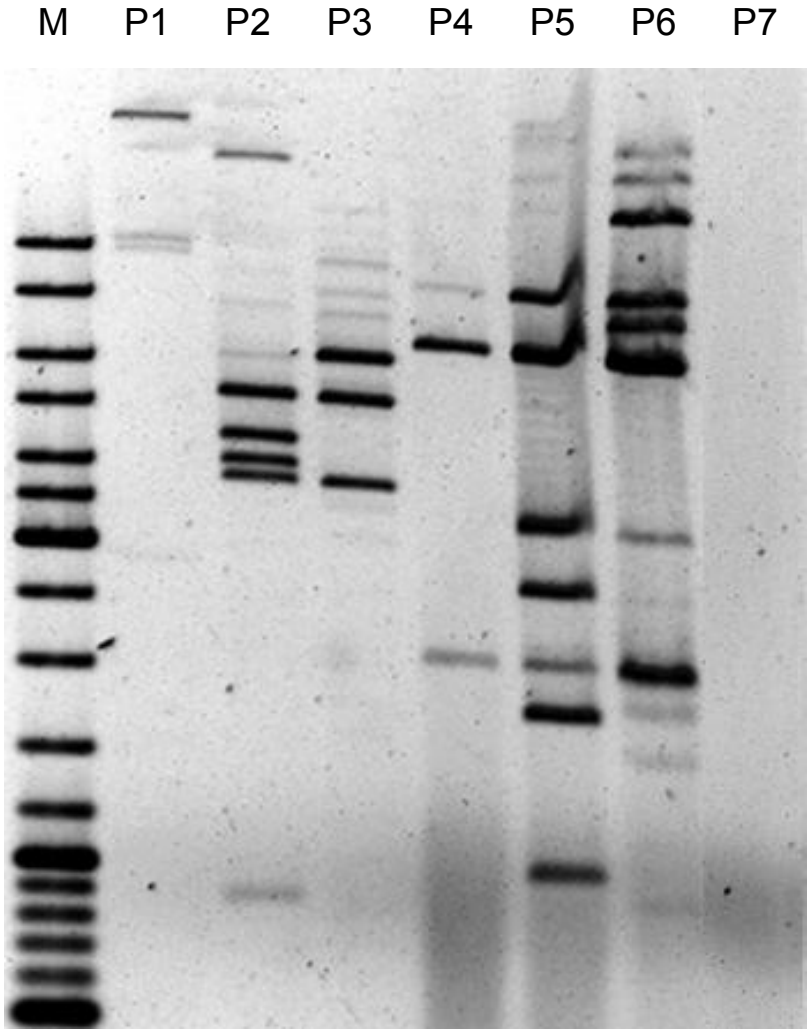

Figure 2

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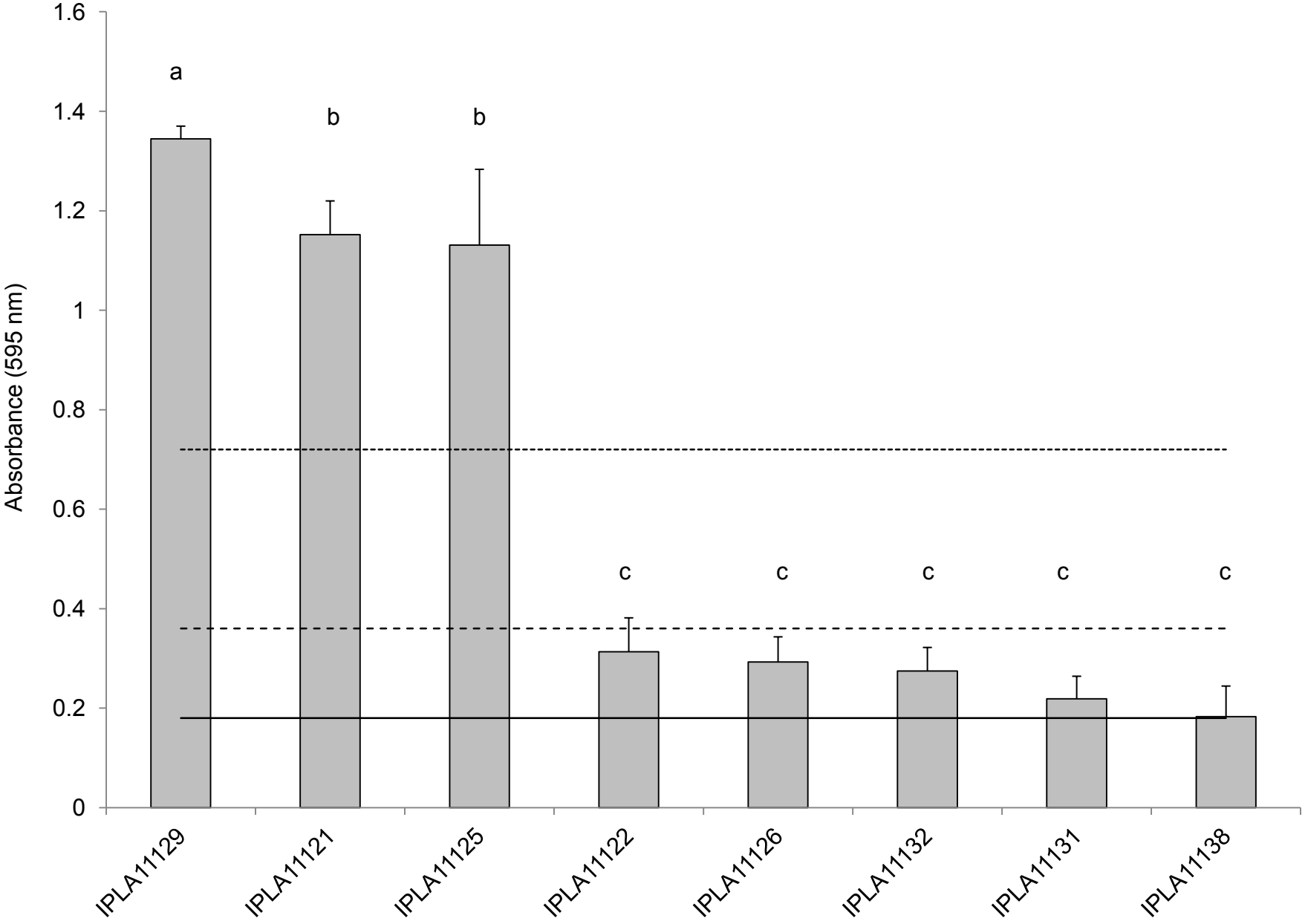

Figure 3

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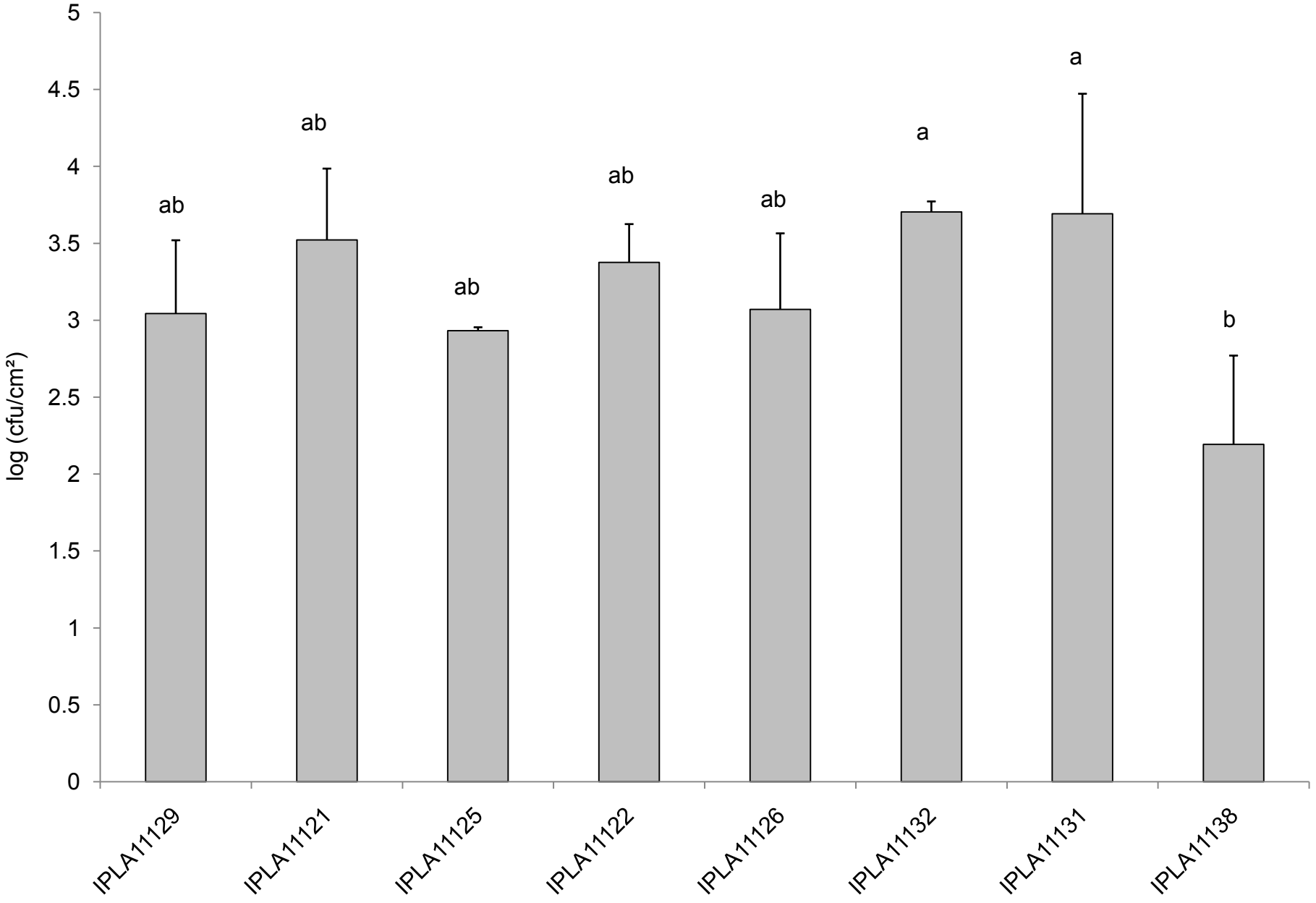
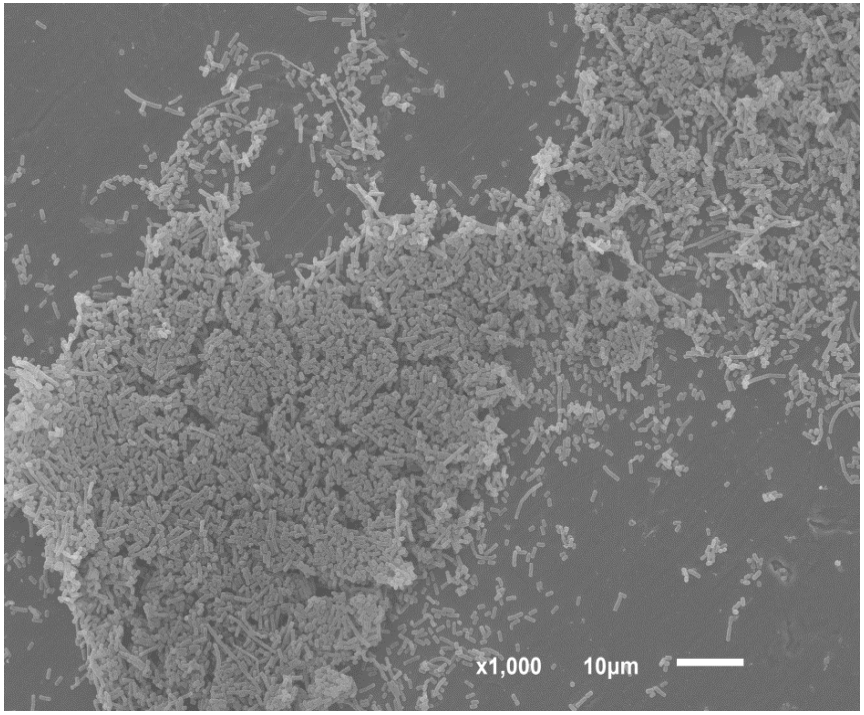


Figure 4

Figure

A



B

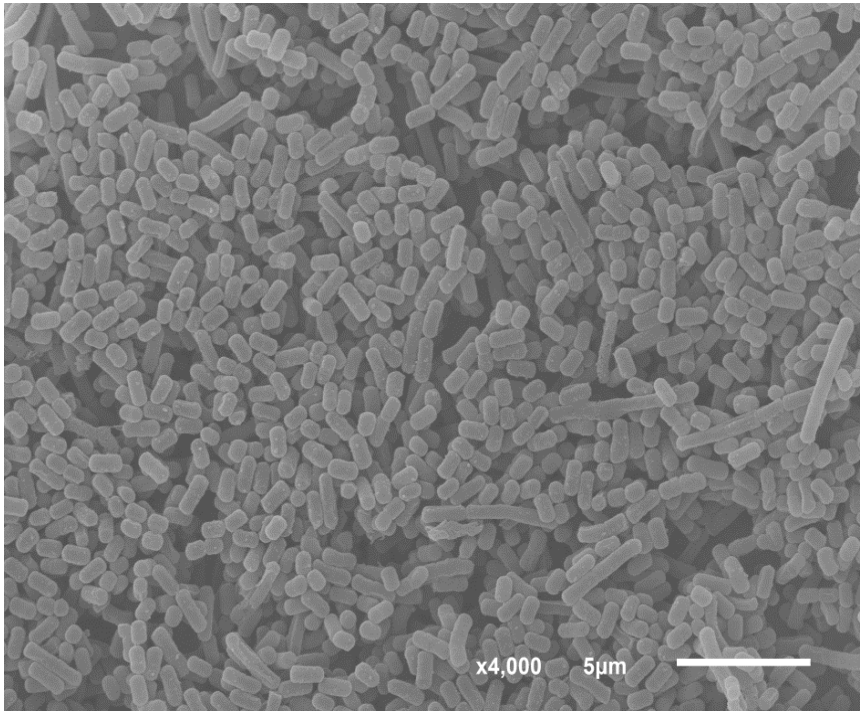


Figure 5