Active polymers containing *Lactobacillus curvatus* CRL705 bacteriocins: Effectiveness assessment in Wieners

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Highlights

Films used as *L. curvatus* CRL705 bacteriocins carriers showed antimicrobial activity

Anti-*Listeria* activity was observed in active inoculated wieners packets (45 days)

Inoculated *Lactobacillus* was slightly inhibited during two weeks of wieners storage

PH decrease and gas formation were observed in *Lactobacillus* inoculated packets

Wieners fat content reduced packaging effectiveness against lactic acid bacteria

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Abstract

Bacteriocins from lactic acid bacteria have potential as natural food preservatives. In this study two active (synthetic and gluten) films were obtained by the incorporation of lactocin 705 and lactocin AL705, bacteriocins produced by *Lactobacillus curvatus* CRL705 with antimicrobial activity against spoilage lactic acid bacteria and *Listeria.* Antimicrobial films effectiveness was determined in wieners inoculated with *Lactobacillus plantarum* CRL691 and *Listeria innocua* 7 (10^4 CFU/g) stored at 5ºC during 45 days. Active and control (absence of bacteriocins) packages were prepared and bacterial counts in selective media were carried out. Visual inspection and pH measurement of wieners were also performed. Typical growth of both inoculated microorganisms was observed in control packages which reached 10^6-10^7 CFU/g at the end of storage period. In the active packages, *L. innocua* 7 was effectively inhibited (2.5 log cycles reduction at day 45), while *L. plantarum* CRL691 was only slightly inhibited (0.5 log cycles) up to the second week of storage, then counts around 10^6-10^7 CFU/g were reached. Changes in pH values from 6.3 to 5.8 were produced and gas formation was observed in active and control packages. The low inhibitory effectiveness against lactic acid bacteria is in correlation with the low activity observed for lactocin 705 in the presence of fat; wieners fat content (20-30%) may adversely affect antimicrobial activity. This study supports the feasibility of using polymers activated with *L. curvatus* CRL705 bacteriocins to control *Listeria* on the surface of wieners and highlights the importance of evaluating antimicrobial packaging systems for each particular food application.

Keywords: antimicrobial food packaging; bacteriocins; anti-*Listeria*; wieners; lactic acid bacteria.

1. Introduction

Although food biotechnology has recently made important progresses, food industry and particularly meat industry is still under scrutiny by consumers due to sanitary episodes generated by meat and meat based products (Bremer et al., 2005; CDC, 2007). Modern life conditions related to or as consequence of globalization, contribute to the major incidence of food diseases outbreaks. The major challenges for food safety are the emergent pathogens, among which *L. monocytogenes* is included (Vignolo et al., 2008; Vignolo et al., 2012). During food chain distribution, food needs to be protected from physical, chemical and microbiological spoilage. The shelf life of food is controlled by (i) the product characteristics including formulation and processing parameters (intrinsic factors), (ii) the package properties and (iii) the environment to which the products are exposed during distribution and storage (extrinsic factors). Among intrinsic factors, pH, water activity, enzymes, microorganisms, and concentration of reactive compounds are included. Many of these factors can be controlled by selection of raw materials and ingredients, as well as the choice of processing parameters. However, extrinsic factors namely temperature, relative humidity, light, total and partial pressure of different gases as well as mechanical stresses including consumer handling may affect the rates of deteriorative reactions occurring during the shelf life of food. The properties of package can exert a significant effect on many of the extrinsic factors and thus indirectly on the rates of deteriorative reactions (Robertson, 2006). Interaction of the packaged food with its packaging and the external environment may also change intrinsic food factors, package headspace acting as a buffer between food and packaging material. Due to these

interactions, moisture content (i.e., water activity), dissolved O_2 and CO_2 contents, and preservative concentration can be modified to affect the microbiota and its growth rate (Lee, 2010).

Muscle tissue from healthy animals is free of bacterial or viral pathogens. As with spoilage organisms, pathogens are deposited on meat surfaces during processing and handling of meat carcass. Potential sources of pathogen contamination comprise animal-associated pathogens transferred to meat from the hide, skin, or feathers and the intestinal tract of the animal during carcass processing; human-associated pathogens transferred from personnel during handling of product, processing equipment and tools, which if inadequately cleaned and sanitized may act not only as vehicles for pathogen but also as sources of contamination (Gill and Gill, 2010). Most perishable foods are vulnerable to microbial spoilage even under chilled conditions. Their shelf life is thus, for the most part, terminated when they become unacceptable due to the growth of undesirable microorganisms (Lee, 2010). Within a certain range of environmental conditions, often only one member from the total microflora is responsible for spoilage (specific spoilage organisms—SSO); for cooked meat products, lactic acid bacteria were found as the prevalent spoilage microorganisms (Mataragas et al., 2006; Audenaert, et al., 2010; Chenoll, et al., 2007). For shelf life studies, after determining the SSO and the conditions under which this group of microorganisms is responsible for food spoilage, the next step is to determine the number of SSO responsible for food deterioration producing lack of acceptability (Dalgaard, 1995; Koutsoumanis and Nychas, 2000). The acceptable limit of microbial growth that determines the shelf life differs with food type and storage conditions. SSO counts of 10^5–10^8 bacteria per g^1 or cm^2 are commonly used as a convenient upper limit of quality and are reached mostly during microorganism growth exponential phase (Lee, 2010). Combined intrinsic

factors are used to preserve food safety and ensure organoleptic quality, i.e. suitable food shelf life can be obtained by incorporating low levels of additives, mild dehydration and heat processes (Leistner and Gould, 2002). Several technologies can be combined in order to improve food safety and extend shelf life of foods (Rybka-Rodgers, 2001). During the last years, a number of biopreservation technologies has been developed by the inclusion of antimicrobial extracts, lysozyme, bacteria and/or bacteriocins among others, into polymer matrices (Marcos et al., 2008; Gialamas et al., 2010; Ramos et al., 2012; López de Dicastillo et al., 2013; Arcan and Yemenicioğlu, 2013). Nevertheless for bacteriocins biopreservation hurdles, it was found that antimicrobial effect could be affected by food components (Zapico et al., 1999; Aasen et al., 2003; Bhatti et al., 2004). Lactocin 705 and lactocin AL705, are bacteriocins produced by *Lactobacillus curvatus* CRL705. Lactocin 705 has antagonist effect against Lactic acid bacteria (LAB) and *Brochothix thermosphacta*, while AL705 is active against *Listeria* species (Castellano and Vignolo, 2006). Both bacteriocins retained its antimicrobial activity when included in polymer matrices such as LDPE (Blanco Massani et al., 2008, 2012) and gluten (Blanco Massani et al., in press article). In the present study, active LDPE and gluten films obtained by *L. curvatus* CRL705 bacteriocins incorporation were evaluated for antimicrobial effectiveness in contaminated Wieners.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Lactobacillus curvatus CRL705 (producer of the bacteriocins lactocin 705 and lactocin AL705) and *Lactobacillus plantarum* CRL691 (which is sensitive to the activity of lactocin 705) from CERELA culture collection, were grown in MRS broth (Britania,

Argentina) for 16 h at 30ºC. *Listeria innocua* 7 (sensitive to the activity of lactocin AL705) obtained from the Unité de Recherches Laitières et Génétique Appliquée, INRA (France) was grown in trypticase soy broth (Britania) with 5 mg/cm^3 of yeast extract added (Britania, Argentina) for 16 h at 30 ºC. All strains were maintained and stored at −20 ºC in 0.15 g/cm^3 of glycerol until use.

2.2. Wieners elaboration

Wieners were manufactured in a meat processing pilot plant, according to standard procedure (C.A.A.). Beef and pork meat cuts were minced (Themis 32 mincer) and processed together with fat in a vertical cutter (Robot Coupe). Ice (0.11 g/cm^3), sodium phosphate (3 mg/cm^3), sodium erithorbate (0.5 mg/cm^3), sodium chloride (0.017 g/cm^3) and sodium nitrite (0. 15 mg/cm^3) were added and mixed to obtain a homogeneous mass. Finally, starch and water (0.11 g/cm^3) were added to form an emulsion which was filled (Hidraulic filler, RISCO IV 20) into artificial casings (2 cm diameter). Wieners (5 cm, 14 g) were cooked in an oven (Lavaflux) at 80°C for 15 min, cooled in an ice bath to a core temperature below 40°C and refrigerated at 3°C until manual peeled. After that re-pasteurization of vacuum-packed wieners was performed (10 min, 80°C).

2.3. Active solution preparation and quantification

A powder containing lactocin 705 and lactocin AL705 from *L. curvatus* CRL705 was obtained by ammonium sulfate precipitation as earlier reported (Blanco Massani et al., 2008). For activity determination the active powder was resuspended in water and the agar well diffusion assay against *L. plantarum* CRL691 (lactocin 705 sensitive organism) and *L. innocua* 7 (lactocin AL705 sensitive organism) was performed (Blanco Massani et al., 2012). Antimicrobial activity was expressed as AU/cm^3.

151

152 **2.4. Active films preparation and antimicrobial activity determination**

153 Two types of active films were prepared. Synthetic: Multilayer films kindly provided by
154 Cryovac (Sealed Air, Argentina) and commercially used as bottom and top of wiener
155 packages, were contacted (1 h, 30°C) with the active solution containing *L. curvatus*
156 CRL705 bacteriocins (1 mg/cm^3, 267 AU/cm^3 and 2133 AU/cm^3 for lactocin 705 and
157 lactocin AL705, respectively) (Blanco Massani et al., 2012). Agro-protein polymer: Wheat
158 gluten (0.779 g of protein, 0.133 g starch and 0.01 g lipids, per gram of gluten on dry
159 weight base) kindly supplied by Molinos Juan Semino S.A. (Carcarañá, Santa Fe) was
160 stirred with sodium sulfite (Merck, Germany), glycerol (Cicarelli, Argentina) and ethanol
161 96% (Merck, Germany) using a mechanical stirrer (Heidolph RZR 2041). After a
162 homogeneous solution was attained, water and the *L. curvatus* CRL705 bacteriocins (1
163 mg/cm^3, 267 AU/cm^3 and 2133 AU/cm^3 for lactocin 705 and lactocin AL705, respectively)
164 solution were added, and the pH was adjusted to 5.0 with acetic acid (Sintorgan,
165 Argentina). The film forming solution was spread onto a continuous Teflon® tape and
166 dried in a warm tunnel with forced air at 50ºC for 4 h (Blanco Massani et al., in press
167 article). Negative controls consisted on either synthetic or wheat gluten films in which
168 active bacteriocins solution was replaced by water. Films were sterilized by UV exposition
169 during 10 min and aseptically stored until use. Antimicrobial activity of the activated and
170 control (without bacteriocins) films was determined by directly placing on the semisolid
171 agar plates seeded with the sensitive organisms (MRS agar plates seeded with *L. plantarum*
172 CRL691 for lactocin 705 and trypticase soy agar + yeast extract seeded with *L. innocua* 7
173 for lactocin AL705 activity determinations). Antimicrobial activity was evidenced as an
174 inhibition zone of the indicator organisms beneath and around the films.

175

176 **2.5. Active packaging preparation and wieners inoculation**

177 For synthetic packaging, each pair of bacteriocins treated Cryovac films (bottom and
178 top of wiener package) (96 cm^2) was thermo-sealed in a sterile cabinet (Biosafety
179 cabinet Labcono, purifier class II), whereas active gluten film was included as a pad (48
180 cm^2) inside packaging made with untreated Cryovac films (96 cm^2). Control packaging
181 (without bacteriocins) were also prepared. All sets were aseptically stored at 5ºC until
182 use.

183 Wieners were separately inoculated under sterile conditions by immersion (30 s) in a
184 solution containing *L. innocua* 7 (10^4 CFU/g) and *L. plantarum* CRL691 (10^4 CFU/g).
185 After drying, three wieners (42 g) were placed into each active and control packaging
186 previously prepared. In parallel, control (without bacteriocins) uninoculated wieners
187 packages were included. All packages were thermo-sealed under vacuum (90%) (Erlich
188 Best Vacuum) and stored at 5ºC for 45 days.

189

190 **2.6. Microbiological determinations**

191 Immediately after inoculation and at 4, 13, 19, 29, 34 and 45 days of storage at 5°C, two
192 synthetic packages with each inoculated microorganism were aseptically opened and
193 microbiological evaluation was performed in 10 g obtained by transversely cutting each
194 wiener. The sample was minced with 90 cm^3 of sterile saline solution (NaCl 8.5
195 mg/cm^3) in a Stomacher (Seward Laboratory Blender, Stomacher 400) for 2 min.
196 Appropriate dilutions from the homogenate were prepared with sterile saline solution
197 and counts of *L. innocua* 7 and *L. plantarum* CRL691 were performed in MOX with
198 sodium moxalactame and MRS in anaerobic conditions, respectively. For the gluten
199 containing packages the same experiment was performed immediately after inoculation
200 and at 4, 19, 34 and 45 days of storage at 5°C. For non-inoculated packages total

aerobic counts were performed at time 0 and at the end of storage (45^{th} day, 5ºC) on Plate Count Agar (Difco). For all the samples duplicate plates were incubated for 48 h at 35ºC. Results were expressed as log CFU/g. A DMFit manual Version 2.0. Program (Baranyi and Roberts, 1994) was used to model inoculated microorganisms growth.

2.7. Residual antimicrobial activity, visual inspection and pH determination

Residual antimicrobial activity of the wieners contacted films and the supernatant liquid from wiener packages (exudate) were determined in semisolid agar against the sensitive microorganisms. Positive bacteriocin activity was evidenced as a zone of inhibition on the indicator organism lawn. Homogenate pH measurements (Hanna Instruments microprocessor pHmeter, HI1332B) and visual inspection of the packages were also performed.

2.8. Statistical analysis

Three independent experiments were performed in duplicate. Data points are represented by the mean, with the standard error indicated by error bars. All data were subjected to analysis of variance (ANOVA), and the Tukey test was applied at the 0.05 level of significance. Statistical analyses were performed using Minitab Statistic Program, release 12 (Pennsylvania, USA).

3. Results and Discussion

All control packages (without bacteriocins) either synthetic or those containing gluten pads (Figs. 1 and 2, respectively), showed the typical growth of both *L. plantarum* CRL691 and *L. innocua* 7 inoculated which reached maximum level of 10^7 CFU/g at

day 45 of storage at 5ºC. Total aerobic counts in non-inoculated wieners at time 0 were below the detection limit (30 CFU/g) either for synthetic or gluten containing control packages~~Total aerobic counts in non-inoculated wieners at time 0 were below the detection limit (30 CFU/g) either for synthetic or gluten containing control packages~~, reaching values of 1 x 10^2 (synthetic packages) and 4 x 10^2 CFU/g (gluten containing packages) at day 45 of storage. Food shelf life is defined as the time during which all of the primary characteristics make the food acceptable for consumption. Thus, the shelf life refers to the time period that food stays on both the retailer´s and consumer´s shelf before it becomes unacceptable (Robertson, 2006). Counts of LAB have often been used as a quality criteria for shelf life determination of chill stored cooked meats and fresh vegetables packaged under vacuum, low O_2, or high CO_2 modified atmospheres (Lee, 2010). Vacuum-packaging and meat moisture inside the bags enable excellent contact between the meat surface and bacteriocins (Ming et al., 1997). In this study, the presence of lactocin 705 incorporated in synthetic packages produced a slight decrease in *L. plantarum* CRL691 counts in wieners over two weeks of storage at 5ºC (0.5-log CFU/g cycles lower than the control, Fig. 1a), and a slight delay in the microorganism growth (μ_{max}= 0.008 and μ_{max}= 0.007 h^{-1}, respectively for the control and active packages). Nevertheless, from the 19th day to the end of storage (45 days), the same *L. plantarum* CRL691 counts ($P \geq 0.05$) were observed for the control and active packets (around 7.3±0.5 log CFU/g). When the growth of *L. plantarun* CRL691 was evaluated in the packages containing the active gluten pad, even though different growth patterns were observed, a lack of inhibition at the end of storage was also found (Fig. 2a). A mildly extended lag phase was observed in the presence of lactocin 705 (193 h and 85 h for active and control packaging, respectively), specific growth rates for gluten active packages being higher than those for synthetic packages (μ_{max}= 0.017 and μ_{max}= 0.012

h^{-1} for active and control, respectively). This result might suggest that gluten film components could have been used by *L. plantarum* CRL691 as nutrients source.

On the other hand, a bacteriostatic effect against *L. innocua* 7 was observed in both synthetic and gluten activated packages until the fourth week of storage, then exhibiting a slight decrease in *Listeria* counts ($P<0.05$, Fig. 1b and 2b) with death rates of -0.0003 h^{-1} for synthetic and -0.0002 h^{-1} for gluten containing active packages. At the end of storage at 5ºC (45 days), *L. innocua* 7 counts were 2.5-log cycles lower (1.7×10^4 and 1.5×10^4, respectively for the active synthetic and gluten containing packets) than each respective control ($7.4\ 10^6$ for synthetic and $2.2\ 10^6$, gluten containing packages, Fig. 1b and 2b). These results are in agreement with those reported using various packaging materials (PE, PE/PA, LDPE, celullosic inserts) containing bacteriocins (lacticin 3147, nisin, enterocin 416K1, bacteriocin produced by *L. curvatus* 32Y) assayed in different food systems such as sliced cheese and ham, pork steak, ground beef, frankfurters and fresh cheeses (Scannell et al., 2000; Mauriello et al., 2004; Iseppi et al., 2008). In cooked meat products, post-processing contamination represents a major safety concern; product handling, processing surfaces, equipments and tools are often involved in this type of contamination (Korkeala and Björkroth, 1997). *Listeria* inhibition in the wiener samples depends on two opposite phenomena: the growth rate, which is principally related to food characteristics and storage temperature, and the killing rate of the antibacterial compounds (bacteriocins) as well as its diffusion rate out of the coating (Iseppi et al., 2008). It is essential that preservatives applied have low diffusivity in their host film to remain at the surface of the food, since diffusion into the food core results in a preservative concentration reduction at the surface (Scannell et al., 2000). Anti-*Listeria* activity was observed in ham wrapped with enterocins alginate films due to a balanced ratio between the release rate of bacteriocins and the growth rate of *L.*

276 *monocytogenes* (Marcos et al., 2007). On the contrary, results from Iseppi et al. (2008)
277 showed a decrease in anti-*Listeria* activity as a function of time when inoculated
278 frankfurters samples were packed with an enterocin-doped LDPE film, suggesting that
279 the diffusion out of the coating was fast for the bacteriocin contained within the first
280 layers of the coating, while enterocin release from deeper layers was slower than
281 *Listeria* growth rate. In our study, *L. innocua* 7 inhibition in both active packages would
282 indicate that the release rate of bacteriocin is higher than bacterium growth rate, anti-
283 listerial lactocin AL705 reaching a concentration equal or greater than the MIC
284 throughout the experiment (Blanco Massani et al., 2008). Some bacteriocins have
285 shown the same effect (bactericidal or bacteriolytic) over the target cells either in
286 culture media or in foods systems (Sabia et al., 2004; Ercolini et al., 2006; Iseppi et. al.,
287 2008). Nevertheless, even when lactocin 705 and AL705 bactericidal effect on *L.*
288 *plantarum* CRL691 and *Listeria* 7 in laboratory media was demonstrated (Vignolo et
289 al., 1996; Cuozzo et al., 2003; Castellano et al., 2004), a bacteriostatic effect was
290 observed in meat and meat products (Vignolo et al., 1996; Castellano and Vignolo,
291 2006). Inactivation of peptide antimicrobial compounds by endogen meat enzymes or
292 fat particles may be responsible for the decreased antimicrobial activity in food systems
293 (Castellano et al., 2008). Moreover, even when bactericidal effect of lactocin 705
294 extract (256 AU/cm^3) against *L. plantarum* CRL691 was earlier reported (Cuozzo et al.
295 2003), in our work active films with 267 AU/cm^3 lactocin 705 added were not able to
296 inhibit *L. plantarum* CRL691 in inoculated wienners. Lactocin 705 inactivation by
297 contact with fatty substances during and after its adsorption on the synthetic film was
298 previously reported (Blanco Massani et al., 2012, 2013). Here, the presence of fat (20-
299 30%) in wieners could have negatively affected lactocin 705 antimicrobial activity,
300 decreasing its inhibitory ability against *L. plantarum* CRL691. This fact shows the

301 impact of the food matrix composition on the effectiveness of post-process
302 technologies, highlighting the importance of validation procedures for each particular
303 application (Gálvez et al., 2007).

304 Residual antimicrobial activity in activated packaging at the end of storage and
305 in wieners exudates was evaluated. Results showed a lack of lactocin 705 and AL705
306 activity on synthetic multilayer films and wieners exudate after 45 days of storage at
307 5°C, (Fig. 3). On the contrary, although gluten pads and wieners exudate did not exhibit
308 residual activity for lactocin 705, a residual antilisterial activity due to lactocin AL705
309 was observed during 15 days at 5°C (Fig. 4 b). This result would indicate that this
310 bacteriocin is present in higher concentration in the gluten pads than in the synthetic
311 multilayer films. As was recently reported by Blanco Massani et al. (2013), only the
312 adsorbed lactocin AL705 was shown to exert antimicrobial activity, after synthetic films
313 activation and its saturation concentration (200 AU/cm^3) was lower than the bacteriocin
314 present in the activation solution (2133 AU/cm^3), whereas in the gluten pads, inner
315 lactocin AL705 concentration was that of the added (2133 AU/cm^3), this resulting in a
316 higher gluten residual activity of lactocin AL705 after wieners contact. On the other
317 hand, reduced antimicrobial activity of bacteriocins was earlier reported when recovered
318 from complex matrixes such as food systems (Raju et al., 2003; Aasen et al., 2003).
319 Lack of lactocin 705 activity in films and wieners exudates found in our work is in line
320 with interferences produced by fat as earlier discussed.

321 Changes of wieners pH in packages inoculated whit *L. plantarum* CRL691
322 showed a decrease from 6.3 to 5.9 (day 19th), a final value in the range of 5.7-5.8 being
323 reached towards the end of the experiment (Fig. 5a and b). In wiener packages
324 inoculated with *L. innocua* 7 and those uninoculated, pH values stayed around 6.3
325 throughout the experiment. The pH decrease in the presence of *L. plantarum* CRL691 is

in agreement with its high acidogenic ability as was reported by Fadda et al. (2010). When visual inspection of wiener packages during storage at 5°C was carried out, the appearance of small bubbles from day 19 onwards was registered either in inoculated or uninoculated samples (data not shown). Gas production in meat products is a consequence of heterofermentative metabolism of the naturally present meat borne *Lactobacillus* and *Leuconostocs* species (Korkeala, & Björkroth, 1997; Mataragas et al., 2006; Chenoll et al., 2007). Even when *L. plantarum* CRL691 is a facultative heterofermenter strain, gas production may not be ascribed to its metabolism. Since vacuum packaging thermo-sealing of wieners was performed under non-sterile conditions, contamination with gas-producer organisms could have been occurred.

4. Conclusions

The use of natural substances as biologically derived antimicrobials appears as an important requirement in the active food packaging methodology for the microbial control. Here, assayed as wieners packages, high anti-listerial efficacy for synthetic and gluten containing packaging activated with lactocin AL705, from *L. curvatus* CRL705, was obtained. However, no inhibition of *L. plantarum* CRL691 by lactocin 705 was exerted due to the high fat content of wieners. These results show the importance of particular food characteristics in the design of active packaging.

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Figure Legends

Figure 1. *L. plantarum* (a) and *L. innocua* 7 (b) growth during 45 days at 5 ºC in the active (▼) and control (●) synthetic packages. Lines between points mark tendencies.

Figure 2. *L. plantarum* (a) and *L, innocua* 7 (b) growth during 45 days at 5 ºC in the active (▼) and control (●) gluten containing packages. Lines between points mark tendencies.

Figure 3. Residual antimicrobial activity of lactocin 705 (a) and AL705 (b) in the active synthetic films before (1) and after (2) contact with wieners (15 days at 5 ºC). Wells in the plates correspond to residual analysis in wieners exudates.

Figure 4. Residual antimicrobial activity of lactocin 705 (a) and AL705 (b) in the active gluten (A) and control (C) films after contact with wieners (15 days at 5 ºC)

Figure 5. Changes of pH during storage (45 days at 5ºC) in active (▼) and control (●) synthetic (a) and gluten (b) wiener packages inoculated with *L. plantarum* CRL691 .

Figure

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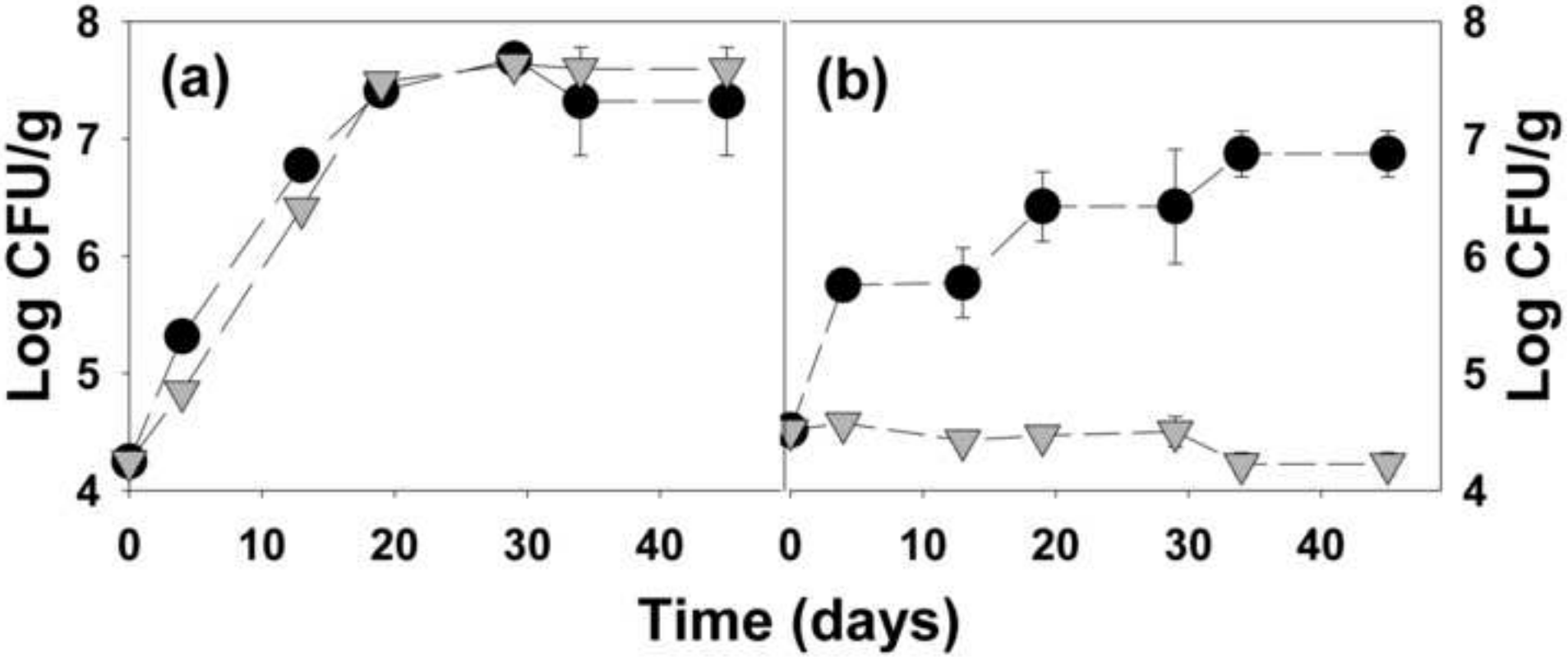

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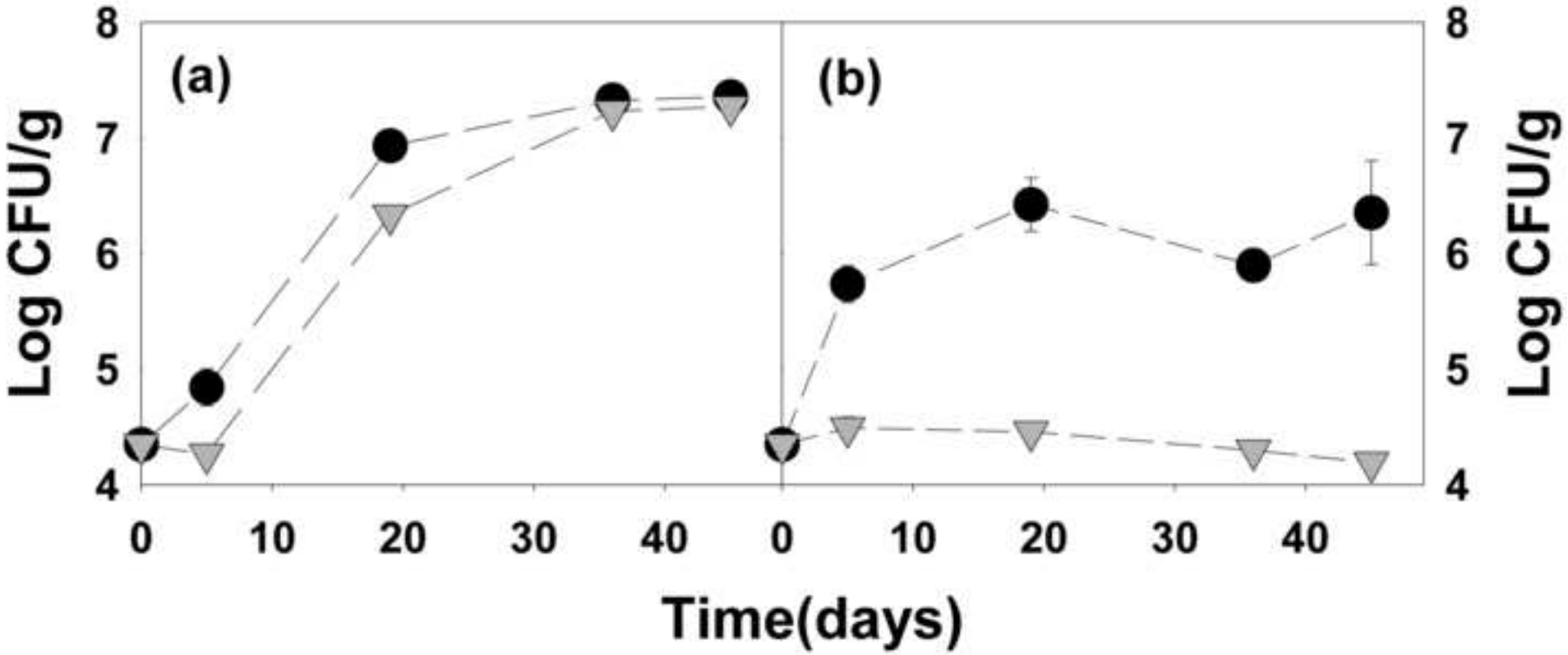

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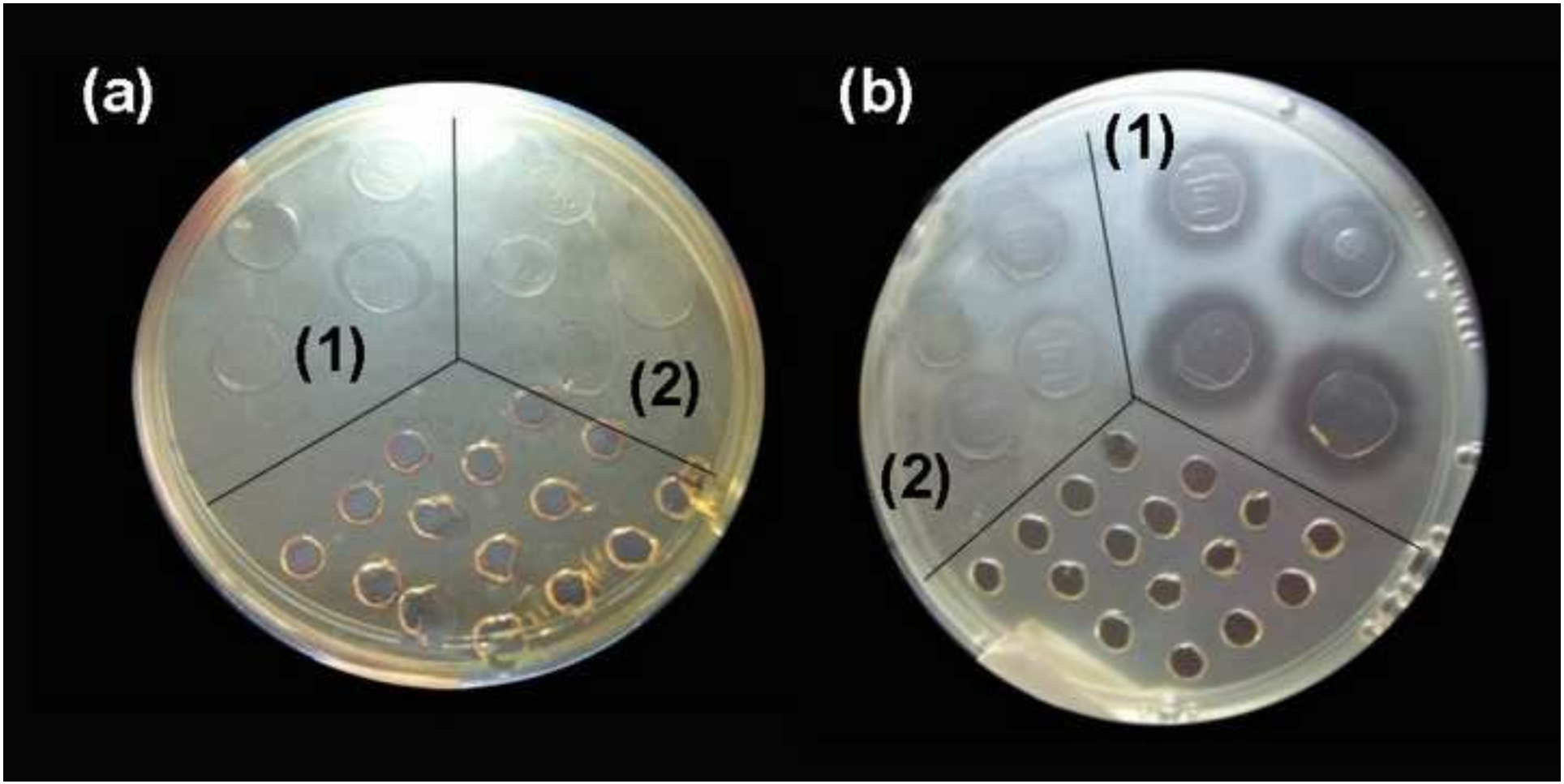

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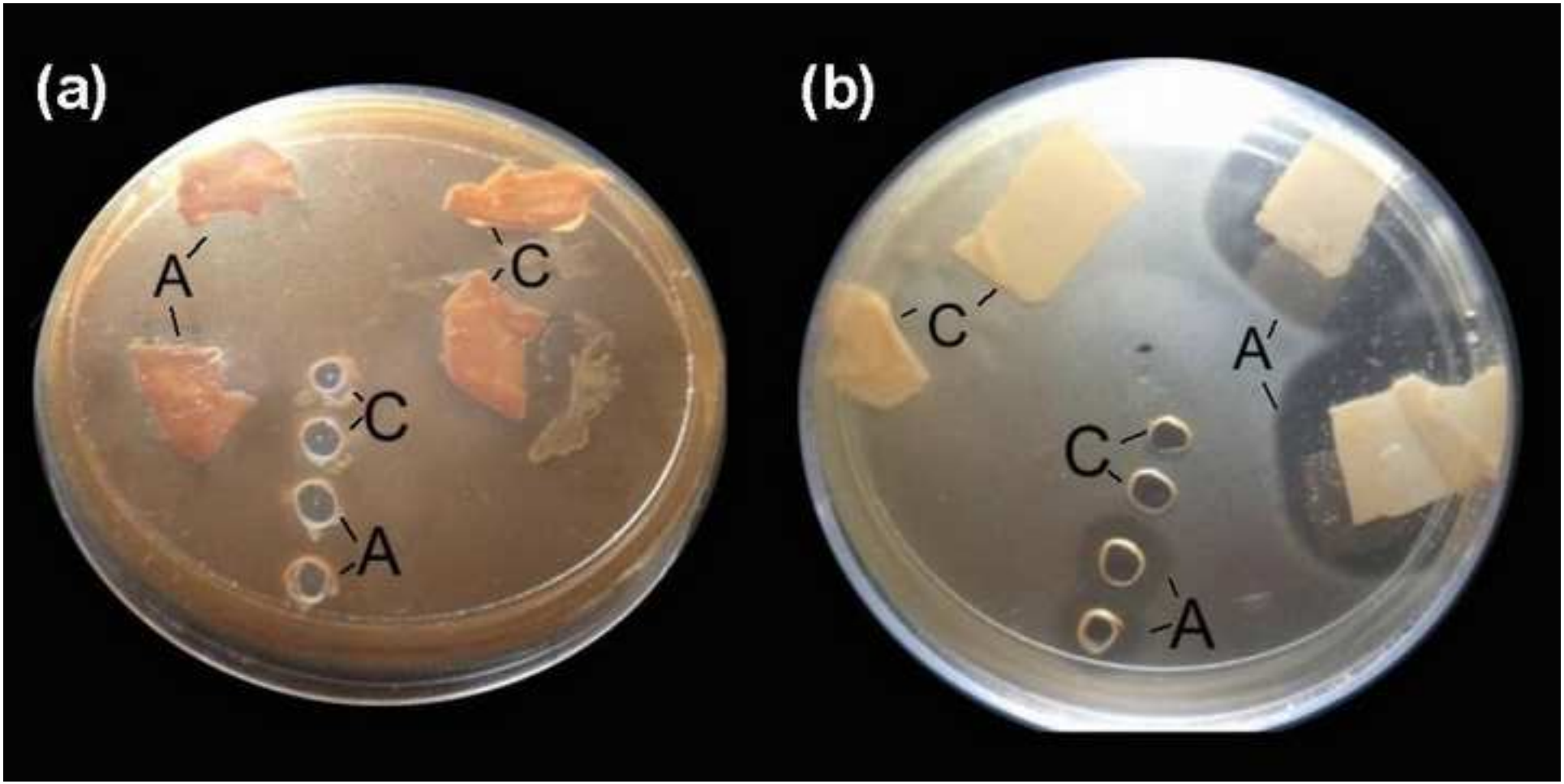

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