

# Characterisation of *Pseudomonas lundensis* CP-P-5 as a potential antagonist of foodborne pathogenic bacteria

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## ABSTRACT

Previously isolated *Pseudomonas lundensis* CP-P-5 had antagonistic activity against *Salmonella* Hartford, *Yersinia enterocolitica*, and *Escherichia coli*. In this study, determination of its antagonistic mechanism and potential field of application in food industry was aimed. Using cellophane-test and microcultures of the test strain's cell-free supernatant mixed with the pathogens, our results showed that cells of *P. lundensis* CP-P-5 and its concentrated cell-free supernatants were effective against the foodborne bacteria, and the supernatants contained more than one compound responsible for inhibitory activity. Searching for the antagonistic compound, NaOH, protease, and heat treatments were done to the supernatants, and proteolytic activity and siderophore production were also tested using the antagonistic strain. Our results support the potential applicability of *P. lundensis* CP-P-5 as a bioprotective agent against foodborne pathogens in food processing environments.

## KEYWORDS

bioprotective agent, foodborne pathogens, biocontrol, *P. lundensis*, *S. Hartford*, *Y. enterocolitica*, *E. coli*

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## 1. INTRODUCTION

Food safety has a worldwide importance as different foodborne pathogenic microorganisms threaten our health when consuming contaminated or poorly preserved food products. In the European Union, the reported number of human cases, hospitalisations, and deaths due to zoonoses in 2018 were 359,692, 41,203, and 572, respectively (EFSA, 2019), but the real numbers are likely to be much higher.

Relatively new physical technologies (e.g. ionising radiation, high hydrostatic pressure – HHP, or pulsed electric field - PEF) can be used for ensuring safety of foods. Biocontrol can also be an alternate preservation method, and it can be part of a hurdle technology (Farkas, 2001) or sometimes it could replace the traditional preservation techniques.

*Pseudomonas* species are considered effective biocontrol agents of soilborne (Weller, 2007) as well as foodborne pathogenic bacteria (Alegre et al., 2013). Scientific publications demonstrate that isolates of the genus *Pseudomonas* – mainly fluorescent pseudomonads – have antagonistic ability against different foodborne pathogens (Alegre et al., 2013; Olanya et al., 2014; Belák & Maráz, 2015; Oliveira et al., 2015). Secondary metabolites of fluorescent *Pseudomonas* species, such as phenazines (PHZ), 2,4-diacetylphloroglucinol (DAPG), pyoluteorin (PLT), pyoverdine (pseudobactin), pyrrolnitrin (PRN), cyclic lipopeptides (CLPs), indoleacetate, and volatile organic compounds (VOCs), such as hydrogen cyanide (HCN), play important role in their biocontrol activity. These metabolites have antifungal, antibacterial, antiviral, antitumor, and anti-nematicidal properties (Haas et al., 1991; Mishra & Arora, 2018).

In our previous study, bacteria with antagonistic activity were isolated from various food processing environments (Baráti-Deák et al., 2020). One of the most effective isolates against the tested pathogens proved to be *Pseudomonas lundensis* CP-P-5. In this study this bacterium was characterised and tested to determine its antagonistic mechanism and potential field of application in the food industry.

## 2. MATERIALS AND METHODS

### 2.1. Bacterial strains tested and their maintenance

Pathogenic bacteria used for testing the inhibitory effect of *P. lundensis* CP-P-5 (antagonistic bacterial strain) were the followings: *Salmonella* Hartford (NCAIM B1310, National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary), *Yersinia enterocolitica* (HNCMB 98002, Hungarian National Collection of Medical Bacteria, Budapest, Hungary), and *Escherichia coli* (NCAIM B01909, National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary). All strains were maintained on TSA (Biokar Diagnostics, Cedex, France) slants at 4 °C.

### 2.2. Contact inhibition test using agar plates with different pH values

These tests were done with all pathogens, using spot method described in the paper of Baráti-Deák et al. (2020). To obtain agar with different pH values (pH 4, 5, 6, 7, 8, and 9) TSA plates were made using different buffers as described in our previous study (Baráti-Deák et al., 2020). The incubation temperatures were 20 and 30 °C, respectively, and the incubation time was 24 h at both temperatures. The tests were done in duplicates.



### 2.3. Testing the inhibitory ability of *P. lundensis* CP-P-5 by cellophane test

The analysis was made according to [Giollitti and Bertani \(1952\)](#) with some minor modifications. Small cellophane (Hewa, Sarantis Hungary Ltd., Budaörs, Hungary) disks with approximately 4 cm diameter were cut and placed in a glass petri dish using filter paper to separate them. The filter papers were previously soaked in distilled water, and the cellophane disks were carefully put on the wet paper. After autoclaving, the cellophanes were placed onto TSA plates together with the filter paper using sterile forceps, which, therefore, was able to minimise the chance of air bubbles appearing under the cellophane. The filter paper was then removed and 10 µl of the antagonistic strain (approx.  $10^8$ – $10^9$  CFU mL<sup>-1</sup>) was dropped onto the cellophane disks. After 24 h of incubation at 25 °C, the cellophane – which contained the colonies on its surface – was removed using sterile forceps. The inoculation of these TSA plates with the pathogenic bacteria – after removing the cellophane – was the same as described at the screening method in our previous study ([Baráti-Deák et al., 2020](#)).

### 2.4. Inhibitory effect of cell-free supernatants of *P. lundensis* CP-P-5 generated by different culturing conditions

Inhibitory assays using cell-free supernatants of static and shake-flask (180 r.p.m.) TSB (Biokar Diagnostics, Cedex, France) and peptone-glucose-yeast extract (PGY, Biokar Diagnostics, Cedex, France) cultures were made as described in [Baráti-Deák et al. \(2020\)](#) to determine the optimal conditions for production of the antagonistic compound(s). The incubation temperature was 25 °C, and samples were taken on the first, the third, and the sixth days of incubation. The flasks were duplicated.

### 2.5. Production of concentrated cell-free supernatant by lyophilisation

To get more concentrated supernatants of *P. lundensis* CP-P-5, the one-, three-, and six-day-old cell-free supernatants generated from cultures in TSB were frozen at –80 °C, then lyophilised (ScanVac CoolSafe™, Labogene, Allerød, Denmark) and soaked in one-tenth amount of TSB. Therefore, a ten times more concentrated supernatant was used for further tests. The inhibition assays were repeated with this concentrated supernatant using Multiscan Ascent photometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) as described by [Baráti-Deák et al. \(2020\)](#).

### 2.6. Protease, NaOH, and heat treatments of the cell-free supernatants

24-h-old cell-free supernatants of static TSB cultures and the concentrated supernatants were treated with the followings to obtain more information of the antagonistic compound: (i) protease treatment by one of the following: proteinase K (Sigma-Aldrich, St. Louis, MO, USA) 200 µg mL<sup>-1</sup>, protease from *Streptomyces griseus* (Sigma-Aldrich, St. Louis, MO, USA) 200 µg mL<sup>-1</sup>, trypsin from bovine pancreas (Sigma-Aldrich, St. Louis, MO, USA) 100 µg mL<sup>-1</sup>, and α-chymotrypsin from bovine pancreas (Sigma-Aldrich, St. Louis, MO, USA) 100 µg mL<sup>-1</sup>, at 37 °C for 90 min, (ii) 0.2 N NaOH treatment, and (iii) heat treatment at 95 °C for 5 min and 30 min, or at 121 °C for 15 min. The treated supernatants were used against *Salmonella* Hartford NCAIM B1310 observing its growth using Multiscan Ascent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with the method described previously ([Baráti-Deák et al., 2020](#)).



## 2.7. Analysis of siderophore production

Siderophore production was tested using CAS agar plates according to [Schwyn and Neilands \(1986\)](#) and [Verma et al. \(2012\)](#) with the modification of the base medium. The CAS plates were made using 900 mL Kings B (Merck, Darmstadt, Germany) mixed with the dye containing 60.5 mg chromeazurol-S (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 50 mL water and mixed with 10 mL iron (III) Solution (1 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  and 10 mM HCl). During stirring, the solution was added to 72.9 mg HDTMA (hexadecyltrimethylammonium bromide, Sigma-Aldrich, St. Louis, MO, USA), which was previously dissolved in 40 mL water. The base medium and the CAS dye were autoclaved separately and mixed before usage. The CAS agar plates were inoculated with the overnight culture of *P. lundensis* CP-P-5 and incubated for 24 h at 25 °C.

## 2.8. Testing of proteolytic activity of *P. lundensis* CP-P-5

Protease activity of the antagonistic strain was tested using skim milk agar (SMA) (yeast 2.5 g L<sup>-1</sup>, peptone 1 g L<sup>-1</sup>, glucose 5 g L<sup>-1</sup>, milk powder 10 g L<sup>-1</sup>, and agar 15 g L<sup>-1</sup>). A loopful of overnight culture was put in 0.5 mL sterile water and mixed vigorously. Ten microlitres of the suspension was dropped onto the surface of SMA. The plates were then incubated at 20, 25, or 30 °C. The appearing clearing zones were measured and checked after one, two, and five days of incubation. The test was done in triplicates.

## 2.9. Statistical analysis

Standard deviation of the collected data was calculated using Microsoft Excel's STDEV function.

# 3. RESULTS AND DISCUSSION

Based on previous results, *P. lundensis* CP-P-5 was chosen for further analyses, as this strain showed strong antagonistic effect on three tested pathogens (*S. Hartford*, *Y. enterocolitica*, and *E. coli*) by contact inhibition tests ([Baráti-Deák et al., 2020](#)). Furthermore, it was not able to grow at 37 and 42 °C – human and animal pathogens usually propagate well at these higher temperatures – and the optimum was between 20 and 30 °C, which overlaps the most effective inhibition temperature range of the above-mentioned pathogens.

To observe whether the pH has any effect on the inhibitory property of *P. lundensis* CP-P-5 against the tested foodborne pathogenic bacteria, contact inhibition tests (agar spot method) using agar plates with different pH values were done. The results showed that in case of *S. Hartford* and *E. coli*, the lower pH values (4, 5, and 6) combined with lower temperature (20 °C) had stronger inhibitory effect on the growth of the pathogens. At higher pH values and temperature (30 °C) – which are closer to the optimal for the pathogens – the growth inhibition was only partial. In case of *Y. enterocolitica*, low pH (4, 5, and 6) and temperature (20 °C) proved to be enough to inhibit its growth, as the pathogen was not able to grow in itself on the positive control. At 30 °C and with higher pH (7, 8, and 9), inhibition of *Y. enterocolitica* was only partial as well. These results highlight the importance of unfavourable environmental parameters for growth inhibition of pathogenic bacteria and the additive effect of ecological parameters and biocontrol metabolites.

Before further analysis of the inhibitory compound(s), cellophane test was performed for determining whether the metabolite(s) derived from the antagonistic strain could inhibit the



pathogens without the presence of its cells. It was important, as cell-free supernatants of the antagonistic strains were not as effective in growth inhibition as the cells in co-culturing study (Baráti-Deák et al., 2020). After the incubation time, visible clearing zones of the pathogens were observed on those points of the agar, where the cellophane was previously placed, and the bacterium cells were cultured (Fig. 1). This means that *P. lundensis* CP-P-5 produced the inhibitory compound(s) during its cultivation without the presence of the inhibited pathogen, and the metabolites were able to diffuse into the agar through the cellophane layer. It supports the presumption that the inhibitory compounds are extracellular substances and could be primary metabolites of the producing strain.

As the cell-free supernatants of the isolated antagonistic strains did not show as effective inhibition as it was seen in co-culturing study, different circumstances using TS and PGY broth under static or shaking culturing conditions were tested in case of *P. lundensis* CP-P-5. Our results showed that neither PGY broth nor shaking conditions had any positive effect on the inhibitory compound production of the strain. Furthermore, the previously seen antagonistic activity was weakened as a cause of change in parameters of culturing conditions. The best inhibitory effect was seen using TS broth under static condition.

As our results generated by agar spot method refers to the fact that the inhibitory compounds have better effect if they are present in more concentrated form, lyophilisation of cell-free supernatant (generated from static TSB culture) of *P. lundensis* CP-P-5 was done. The results proved that the ten times concentrated supernatant had stronger antagonistic effect against all three tested pathogens. Moreover, the best inhibitory effect was detected with supernatants obtained from 24 hour-old culture broth of the strain, while the three- and six-day-old lyophilised supernatants were not as effective as the one-day-old.

Neither protease nor NaOH treatments of non-concentrated cell-free supernatants generated in TSB under static conditions affected their slight inhibitory effect on the tested pathogens, however, after heat treatments, there was no inhibition observed. It may refer to the fact that the inhibitory compound is heat sensitive and resistant to the applied proteolytic enzymes. In

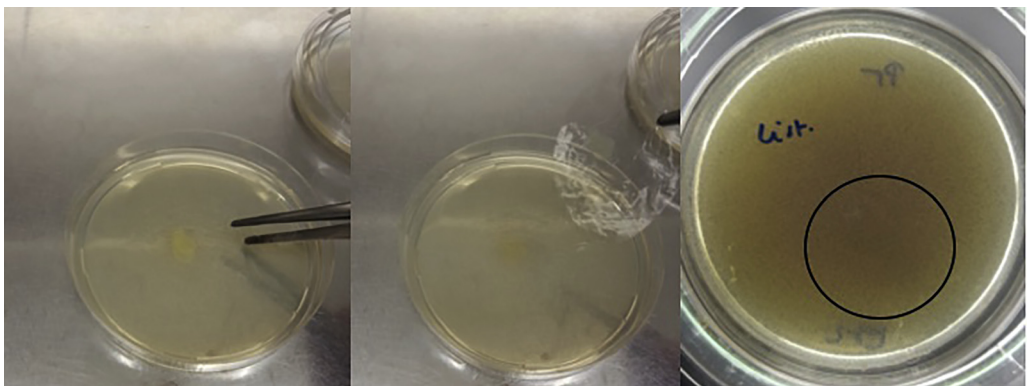


Fig. 1. Cellophane test with *P. lundensis* CP-P-5 strain and its partial inhibition against *L. monocytogenes* (first and second pictures: cellophane removal after 24 h of incubation with *P. lundensis* CP-P-5 on it, third picture: mild clearing zone – where previously the test strain grew on the cellophane – after 24 h of incubation with the pathogen)



contrast with these results, the enzymatic treatments of the lyophilised supernatants showed that protease from *S. griseus* and proteinase K could decrease the antagonistic effect of the concentrated supernatants on *S. Hartford*, while the other treatments (NaOH, heat treatments, trypsin, and  $\alpha$ -chymotrypsin) did not affect them. In case of *E. coli*, the antagonistic effect of supernatants decreased after some of the treatments (the NaOH treatment had the highest impact on the supernatants), while the protease from *S. griseus* and  $\alpha$ -chymotrypsin did not influence their antagonistic activities. In case of *Y. enterocolitica*, none of the treatments had any effect on the growth inhibitory effect of the cell-free supernatants, however, this pathogen was the most sensitive in each examination (Table 1).

Summarising these results, it can be said that in the non-concentrated supernatants, the substrates of the proteolytic enzymes might have been in low concentrations, that is why in these cases there were no changes in the inhibitory pattern. At the same time, in the ten-times concentrated samples, the metabolites were enriched and proved to be present in sufficient concentration for enzymatic break-down. As it can be seen from Table 1, significantly different results were generated by the enzymatic, heat, and NaOH treatments, which could refer to the fact that more than one inhibitory compounds were responsible for the growth decline of the pathogens, and, in addition, various metabolites have impact on the different pathogenic bacteria.

Siderophore production of *P. lundensis* CP-P-5 was tested using CAS agar. The occurring orange clearing zone on the blue agar meant that the strain could produce chelating metabolite (Fig. 2). Proteolytic activity was also confirmed in case of this strain, as at all three tested temperatures clearing zones were observed on SMA plates. The size of the zones increased significantly during the 72 h of incubation, and the best proteolytic activity was observed at 25 °C (Table 2). As in case of *P. lundensis* CP-P-5, the best antagonistic activity was observed at 25 °C, there could be correlation between the inhibitory effect and the ability of protease production of this strain.

Application of biocontrol agents can be the solution for eliminating foodborne pathogens. Leverentz et al. (2006) found four antagonists (one bacterium and three yeast strains), which were effective in preventing the growth or survival of foodborne human pathogens on fresh-cut

Table 1. Effects of enzyme, heat, and alkaline treatments on antagonistic activity of supernatants derived from *P. lundensis* CP-P-5 (number of observations,  $n = 3$ )

Treatment of the cell free supernatant	Supernatant of CP-P-5	10× concentrated supernatant of CP-P-5		
	<i>Salmonella</i> Hartford	<i>Salmonella</i> Hartford	<i>E. coli</i>	<i>Y. enterocolitica</i>
Proteinase K	–	+	+	–
Protease	–	+	–	–
Trypsin	–	–	+	–
$\alpha$ -Chymotrypsin	–	–	–	–
0.2 N NaOH	–	–	+	–
95 °C for 15 min	+	–	+	–
95 °C for 30 min	+	–	–	–
121 °C for 15 min	+	–	–	–

+: The enzyme or treatment reduced the antagonistic effect.

–: The enzyme or treatment did not affect the antagonistic effect.





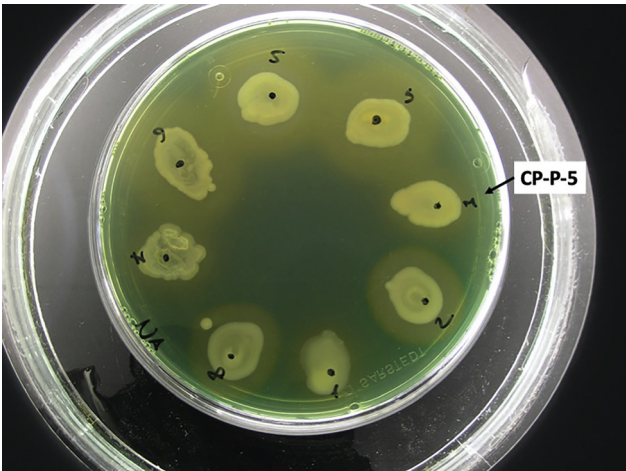


Fig. 2. Siderophore activity of *P. lundensis* CP-P-5 strain on CAS agar after incubation at 25 °C for 24 h

apple tissue. All antagonists were originally isolated from apple surfaces. In another study, biopreservative bacterial strain CPA-6 belonging to the family *Enterobacteriaceae* and isolated from minimally processed (MP) apples was able to control a non-pathogenic strain of *E. coli* O157:H7, *Salmonella*, and *Listeria innocua* on MP apple and peach (Alegre et al., 2012). Alegre et al. (2013) isolated *Pseudomonas graminis* CPA-7 from fruit, which could inhibit the growth of some foodborne pathogenic bacteria on fresh-cut apple and peach. Biocontrol can also be used as an alternative disinfection practice. A surface sanitiser comprising three lactic acid bacteria (*Carnobacterium maltaromaticum*, *C. maltaromaticum*, and *Enterococcus mundtii*) together with nisin for the control of *L. monocytogenes* was patented (Twele et al., 2011). Furthermore, Schöbitz et al. (2014) formulated and designed a biocontroller for application in the food processing environment and evaluated its inhibitory capacity against biofilms of *L. monocytogenes* grown in floor gutters in a salmon processing plant.

Many *Pseudomonas* species can be biocontrol and growth-promoting agents, as they can grow rapidly *in vitro* and generate biomass, they are able to produce a wide spectrum of bioactive metabolites (i.e. antibiotics, siderophores, volatiles, and growth-promoting substances), they may compete aggressively with other microorganisms, and they can adapt to environmental stresses (Weller, 2007). In previous studies, *Pseudomonas* strains with antagonistic effect on

Table 2. Proteolytic activity of *P. lundensis* CP-P-5 using skim milk agar for the detection of clearing zones (diameter, mm)\*

Incubation temperature	Incubation time		
	24 h	48 h	120 h
20 °C	3.33 ± 0.236	6.17 ± 0.236	17.67 ± 0.236
25 °C	4 ± 0	7.33 ± 0.236	15.17 ± 0.236
30 °C	1.83 ± 0.417	6 ± 0.707	10 ± 0

\*: Values are means ± SD, based on three repetitions.



foodborne pathogenic bacteria were isolated (Alegre et al., 2013; Olanya et al., 2014; Belák & Maráz, 2015; Oliveira et al., 2015) and most of them were fluorescent pseudomonads. *P. lundensis* CP-P-5 can also produce fluorescent pigments on King B agar (data are not shown). As this strain was isolated from a surface of an abattoir, the application of *P. lundensis* CP-P-5 as a biocontrol strain can have a potential in the food industry. Till now we could not find any scientific publication in which antagonistic *P. lundensis* had been previously described.

## 4. CONCLUSIONS

*P. lundensis* CP-P-5 isolated from an abattoir was previously selected from potential antagonistic isolates, as it had the most promising inhibitory effect against the tested foodborne pathogenic bacteria. This strain was able to inhibit the growth of *S. Hartford*, *Y. enterocolitica*, and *E. coli*. Inhibitory ability of CP-P-5 was demonstrated in contact inhibition and micro-culturing tests as well. Both approaches showed that *P. lundensis* CP-P-5 can be a good candidate as biocontrol agent, however, as direct application of bacterial cells can lead to spoilage of food products, usage of compounds produced and excreted by the strain can be an alternative solution. Moreover, concentrated extracellular metabolites of *P. lundensis* CP-P-5 may also serve as bio-disinfectants in food producing environments. However, further trials are needed to ensure that this strain is safe to use in food processing environments. To our best knowledge, this is the first study in which a *P. lundensis* strain was described as a potential antagonist of foodborne pathogenic bacteria, however, the characterisation of inhibitory compounds taking part in its antagonistic action has to be continued.

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