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Inhibition of food-borne pathogenic bacteria by antagonistic bacteria

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Az iskolavezető jóváhagyása A témavezető jóváhagyása

1. Background and objectives of the work

Food-borne pathogenic microorganisms are the cause of many human diseases nowadays. The WHO estimates that there are around 600 million foodborne illnesses each year, all of which could be prevented (WHO, 2020).

Food production must strive to create safe products with good quality. In addition to food safety, consumer demand is also a key driver, which has an impact on industry. As a result, over the last decade, more and more products have appeared on the shelves of supermarkets to meet this demand.

The use of biocontrol can be an alternative to well-established preservation methods such as heat treatment, freezing and water reduction, especially for foods where the aim is to preserve their freshness.

One of the best sources of antagonistic microbes, which play an important role in the biocontrol strategy, may be the food itself or its processing environment. Potential biocontrol micro-organisms may be present in or on raw materials or raw materials in contact with food commodities, and their direct or indirect use in the food industry may create new opportunities for the production of safe and shelf stable food products that meet consumer needs.

In order to protect the safety of food processing and food production, as well as the health of consumers, the aim of my PhD thesis was to isolate bacteria from the food environment and the raw materials used in the food industry that have inhibitory effects on various pathogenic bacteria of food origin, and then to study the biocontrol properties of the isolated bacteria in order to establish their future applicability, including industrial applications, by determining their inhibitory mechanisms of action as precisely as possible.

To achieve my goals, I have taken the following steps in my work:

- isolation of bacteria from food processing environments and raw food materials;
- characterisation of isolates by biochemical tests,
- screening the isolated bacteria for inhibitory activity by contact inhibition testsagainst four pathogenic bacteria of food safety concern (*Salmonella* Hartford, *E.coli*, *L. monocytogenes,* and *Y. enterocolitica*).
    - Identification of inhibitory isolates by rapid tests and molecular biology assays, and typing of strains by DNA-based techniques;

- identifying the mechanisms behind inhibition: protease activity assay; siderophore production testing; prodigiosin production testing; chitinase production testing.
- further testing of the strains with the strongest inhibitory effect:
    - cell-free supernatants under different treatments,
    - together with culture studies.
- Inhibitory activity against foodborne *Salmonella enterocolitica*;
- testing by other methods to detect the inhibitory effect
    - cellophane test;
    - determining the effect of a supernatant concentrated by lyophilisation
- analysis of the inhibitory component(s) by chromatographic methods.

2. Material and method

2.1 Pathogenic bacteria used in the work

I used the following pathogenic bacteria for my studies: *Listeria monocytogenes* (CCM 4699), *Salmonella* Hartford (NCAIM B1310), *Yersinia enterocolitica* (HNCMB 98002), *Escherichia coli* (NCAIM B01909).

2.2 Isolation and maintenance of bacteria

The surface samples from which the bacteria used in this work were isolated were taken using two different methods. For smooth, even surfaces, I used Contact slides containing TSA, while for hard-to-reach, non-even surfaces, I used a sampling swab (TSB in broth).

The samples were collected from the following locations:

- Abattoire
- Egg processing plant
- Dairy
- Vegetable processing plant

The samples were incubated at 25° C for 24 hours and then further inoculated from the TSB broth to TSA. Pure cultures of bacteria with different morphologies were stored on TSA slant agar sealed with sterile paraffin oil at 4° C.

I also cultured the isolates on WL agar to group the isolated bacteria and to filter out those with the same morphology. Finally, after grouping colonies with similar morphology, I selected one isolate from each group for further testing.

2.3 Contact inhibition assay by agar spot method

I used this method to screen the isolates with inhibitory effects. Suspensions were prepared in sterile water from one-day-old cultures of the pathogenic bacteria described in section 2.1, and 1 ml (approximately 10^4-10^5 CFU/ml) of suspensions were pipetted onto TSA plates, spread evenly and the excess removed. The surface of the plates was dried and 10 µl (approximately 10^6 cells) of the cell suspensions prepared from the isolates were pipetted onto the agar plates. I incubated the plates at 5, 10, 15, 20, 25, 30, 37 and 42 °C for 6 days to determine the optimal temperature for inhibition and the time required for the inhibition effect to be exerted. Inhibition

was indicated by pathogen clearing zones around the macrocolonies of the tested isolates. Plates were evaluated after incubation for one, two, three and six days.

In my later studies, I repeated the method against four Salmonella isolates from eggs, but this time testing only the isolates that had previously shown inhibitory activity against S. Hartford.

I also carried out the contact inhibition test on different pH media (pH 4, 5, 6, 7, 8, 9). The plates were incubated at 20° C and 30° C for 24 hours. Isolates, showing inhibitory effects in the preliminary testing, the "screening", were included in this study.

2.4 Biochemical and physiological tests

The following physiological parameters were tested: the effect of temperature (5, 10, 15, 20, 25, 30, 37 and 42° C) and pH (3, 4, 5, 6, 7, 8, and 9) on the growth of isolates was investigated, while for the biochemical tests I performed KOH test, catalase test and oxidase test.

2.5 Molecular typing by RAPD-PCR

To test the clonal similarity of the inhibitory isolates, molecular typing was performed by RAPD-PCR using the following primers: OPE 18 (Belák, 2009), M13 (Vassart et al., 1987), D8635 (Van Looveren et al., 1999). The PCR products were separated and detected on a 1.5% agarose gel by gel electrophoresis using a DNA Molecular Weight Marker VI ladder. Samples were compared using GelCompar. II (Applied Maths NV, Belgium) software.

2.6 Identification of antagonistic isolates

The isolates with antagonistic activity were first identified by miniaturized identification tests. For Gram-negative isolates I used API 20 NE and API 20 E kits, while for Gram-positive isolates I used the BBL Crystal test.

Second, the isolates were identified to genus or species level by direct Sanger sequencing of 16S rDNA PCR products generated with primer pairs 27f-1492r (Maiwald, 2004). The ramplicons were purified using a PCR product purification kit PCR-Advanced™ Clean Up System and sequencing was performed by Eurofins BIOMI Kft. (Gödöllő). Nucleotide sequencing was performed using the reverse primer. The resulting sequences were processed using MEGA6 (Tamura et al., 2013) and evaluated using the EzTaxon

(http://www.ezbiocloud.net/eztaxon) and Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) databases.

2.7 Co-culture inhibition in TSB broth and milk

In the contact inhibition studies, two strains with inhibitory activity against *Salmonella* Hartford, *Pseudomonas lundensis* CP-P-5 and *Serratia marcescens* CSM-RMT-1, were tested by co-culturing with the same *Salmonella* and with an *S. enterica* strain isolated from egg juice in TSB broth at the following pathogen: inhibitory microorganismconcentration ratios: 1:1, 1:10, 1:100 and 1:1000, with the lowest concentration being 10^1 cells/ml. Cultures were incubated for 6 days at 25° C under static conditions. On the first, second, third and sixth days, inoculation was performed by spreading the cultures on TSA and selective Harlequin agar (Lab M Limited) for the respective pathogen in two parallel sprays.

Co-cultivation in a food matrix was also performed for strain CSM-RMT-1, using UHT milk with 2.8% fat content as the culture medium instead of TSB broth. The method for this study was identical to that described above.

2.8 Investigation of the inhibitory effect of cell-free supernatants

To investigate the inhibitory effect of cell-free supernatants, I examined the production of extracellular inhibitory metabolites using microcultures. The inhibitory effect of one-, three- and six-day-old cell-free supernatants produced from TSB broth cultures of strains showing inhibitory activity in the screening was investigated using Multiskan Ascent with a microtitre plate culture method. The inoculated microtiter plates were incubated at 25 °C and the absorbance values measured at 595 nm were automatically recorded every 30 min during 24 h of culture by the instrument used for measurement. Three parallel measurements were performed during the assays. Growth curves were plotted from the time-dependent absorbance values to show the effect of cell-free supernatants on pathogen growth.

2.8.1 Investigation of the inhibitory effect of cell-free supernatants under different culture conditions

The study was performed with two strains that inhibited *Salmonella* Hartford (*Ps. lundensis* CP-P-5 and *Ps. lundensis* CE-EJ-2) to determine the effect of altered culture conditions on the production of the antagonist compound(s). Cultivation was carried out as follows: static and shaken (180 rpm, 25 °C) incubation conditions in TSB broth in TGE broth. The inhibitory effect of the extracted cell-free supernatants was tested.

2.8.2 Examination of the effect of different treatments on cell-free supernatant inhibition

Ps. lundensis strain CP-P-5, I applied the treatments listed below and examined the effect of the treated supernatants on the growth of Salmonella Hartford as described above: protease treatment at 37 °C for 90 min (proteinase K 200 μg/mL, protease from Streptomyces griseus 200 μg/mL, trypsin from bovine pancreas 100 μg/mL, α-chymotrypsin from bovine pancreas 100 μg/mL), 0.2 N NaOH treatment, heat treatment (boiling at 95 °C for 5 min and 30 min and autoclaving at 121 °C for 15 min at about 1.2 bar). Following the treatments, the inhibitory effects were assayed as previously described in a microtitre plate using a Multiscan Ascent against the previously inhibited pathogen. Treatments were also performed with lyophilized supernatants for several strains.

2.8.3 Analysis of cell-free supernatants concentrated by lyophilisation

Cell-free supernatants of the selected inhibitory strains were lyophilized to 10x concentrations. For this purpose, supernatants were prepared from cultures prepared under static conditions in TSB broth as described above after one, three and six days of culture. The supernatants were then lyophilized after freezing at -80 °C.

2.9 Cellophan test

The study was based on the study described by Giolitti and Bertani (1953) with minor modifications. Sterile cellophane discs of about 4 cm diameter were placed on TSA plates and 10 μl of an aqueous suspension of the antagonist isolate (about 10^6 cells) were dropped onto the centre of the cellophane. After incubation for 24 h at 25 °C, the cellophane was removed with sterile forceps and colonies were formed on the surface. The TSA plates were then inoculated with the pathogenic microorganisms tested according to the method used for screening and incubated at temperatures favourable for the appearance of inhibitory effects at 15, 20, 25 and 30 °C for six days. Results were read after one, two and six days.In an attempt to determine the inhibitory component,

As an attempt to determine the inhibitory component, I also determined the UV spectra of the extracts of agar pieces containing the inhibitory component. The 1 cm^3 pieces of agar cut from the cellophane culture method were placed in a test tube containing 2 ml of organic solvent or water. After two hours of extraction at 25 °C, 1 ml of sample from each tube was analysed with a Secord Plus 2000 UV spectrophotometer, recording spectra from 200 nm to 800 nm, with a measurement at every 2 nm.

2.10 Antibiotic susceptibility testing

To determine the antibiotic susceptibility of inhibitory strains, the qualitative disc diffusion susceptibility testing method described by Bagul and Sivakumar (2016) was used for the antibiotics chloramphenicol, ceftadizime, imipenem, erythromycin, nalidixic acid, meropenem, norfloxacin, cholistin sulfate, ciprofloxacin, piperacillin and amikacin. Incubation was carried out at the optimum temperature of 25°C for the strains tested and results were read after 24 hours.

2.11 Tests performed for the determination of the inhibitory component

2.11.1 Protease activity assay

The proteolytic activity of the inhibitory strains was assayed using milk powder TGE agar. A cell suspension of 0.5 McFarland standard density (10 µl) of the strains was pipetted onto the surface of the milk powder TGE plates, which were subsequently incubated at 20, 25 and 30 °C. Clearing zones were read after one, two and five days of incubation.

2.11.2 Siderophore production testing

Siderophore production was investigated using the method of Schwyn and Neilands (1987) as described by Verma et al. (2012) using CAS agar plates with modification of the base medium. To prepare CAS plates, I mixed the specially prepared CAS stain to the prepared Kings B agar. The CAS agar plates were then inoculated and incubated at 25 °C for 24 hours. The colour change around the colonies that developed on the blue CAS agar, the orange zone, indicated siderophore activity, which is due to the fact that siderophores are strong chelating compounds that are able to leach iron from the complex, resulting in a change in the colour of the stain in the agar.

2.11.3 Investigation of chitinase production

2.11.3.1 Testing chitinase production using a culture method

Chitinase production and activity were detected according to the method described by Leisner et al. (2008). For this assay, cell suspensions of antagonistic *S. marcescens* strains were droppped onto media containing basic chitin (BMC) and after three days of incubation at 30 °C, the clearing zones were evaluated for the presence of chitinase activity.

2.11.3.2 Detection of the chitinase encoding gene by PCR

The chiA gene of *S. marcescens* strains was detected by PCR as described by Ramaiah et al. (2000).

2.11.4 Prodigiosin production study

2.11.4.1 Preliminary colour test

S. marcescens strains were cultured on Nutrient agar plates and incubated overnight at the following temperatures: 20, 25, 30, 37 and 40 °C. Prodigiosin was extracted with EtOH at room temperature for 24 hours, and after centrifugation, 37% HCl or ammonia was added to the pellet in the tube. The apparent red (acidic environment) or yellow (alkaline environment) colour change is observed in the presence of prodigiosin (Bharmal and Jahagirdar, 2012)

2.11.4.2 PCR analysis of prodigiosin producing genes

To investigate the presence of a gene cluster responsible for prodigiosin production, PCR amplification of the cueR and copA genes was performed as described by Harris et al. (2004).

2.11.5 Fluorescence testing of *Pseudomonas* strains

To test the fluorescence dye-producing ability of *Pseudomonas* strains, the bacteria were inoculated onto Pseudomonas F selective agar and incubated at 25 °C for 24 hours. Fluorescent dye production was then detected under 365 nm UV light.

2.12 Chromatographic tests

Chromatographic analyses were carried out in collaboration with the Plant Protection Institute of the Centre for Agricultural Research. In these studies, those two strains' 10-fold concentrated supernatants were used, which had the most effective inhibitory effect (*Ps. lundensis* CP-P-5 and *S. marcescens* CSM-RMT-1).

2.12.1 HPTLC test

Extracts of lyophilized supernatants were first examined by HPTLC. In the first assay, extraction was performed with 5 ml ethanol followed by 5 ml 50% aqueous ethanol. The extracts were tested by HPTLC-*Bacillus subtilis* assay (Móricz et al., 2016).

2.12.2 Investigation of the inhibitory effect of fractionated supernatants

The HPTLC studies were confounded by the large amount of peptide in the culture medium, so a new, smaller matrix culture medium was needed to grow the inhibitory strains in which the inhibitory components could be produced in sufficient amounts. On this basis, the bacteria were cultured in the following media: *S. marcescens* CSM-RMT-1 in minimal medium, *Ps. lundensis* CP-P-5 in Mineral 0 (liquid mineral medium), Mineral 1 (liquid mineral medium with fructose carbon source) and Mineral 2 (modified mineral medium).

2.12.3 Separation of cell-free supernatants

The following extractions were performed during the study:

- Hex: extraction with 7.5 ml hexane followed by drying of the supernatant and dissolution in 100 µl EtAc,

- EtAc: extraction of the residue with 5 ml EtAc, followed by drying of the supernatant and dissolution in 100 µl EtAc,

- EtOH: extract the residue with 1 ml EtOH and filter the supernatant.

For the next study, supernatants were fractionated on a C18 SPE column (500 mg Merck Lichrolut).

For the third test, the lyophilizate of the supernatants was successively extracted with more and more polar solvents, and the residue was dissolved in water to prepare the different fractions, which I could test for inhibitory effects.

3. Results and discussion

3.1 Isolates collected during the work

Altogether, 78 bacteria were isolated from four different food processing environments. Of these, 20 were collected from a pig slaughterhouse, 6 from a vegetable processing plant, 18 from an egg processing plant and 34 from a dairy processing plant.

After testing the colony morphology of isolates with similar morphology on the surface of both TSA and WL feed agar, only one isolate was selected for further testing, resulting in 64 bacteria tested for inhibitory activity: 13 from the abattoire, 6 from the vegetable processing plant, 18 isolates from the egg processing plant and 27 from the dairy processing plant.

All the isolation sites selected during the work had a high number of bacteria present at the time of sampling, with different morphologies. From the four food processing environments from which isolates were collected, 6 and 18 isolates from the vegetable processing and egg processing plants, respectively, were found to be different in morphology, indicating a significant diversity of bacteria present. Of the 20 and 34 isolates from the abattoire and dairy plants respectively, 7-7 were excluded on the basis of morphological similarities, but still a large number of bacteria with different teleomorphology were present in these sampling sites.

3.2 Selection of isolates inhibiting the growth of foodborne pathogenic bacteria

The antagonistic activity of isolates collected from food raw materials and their processing environment was tested against *L. monocytogenes*, *Salmonella* Hartford, *Y. enterocolitica* and *E. coli* by agar spot method. The results showed that 20 of the 64 isolates tested were able to inhibit at least one of the tested pathogens. In these cases, partial or complete cleararing zones appeared around the isolates. Two of the bacteria isolates (CM-CT-2, CSM-RMT-1) were able to inhibit all four pathogens at least partially, two isolates inhibited three pathogens, three isolates had a negative effect on the growth of two pathogens and 13 isolates were able to inhibit only one pathogen at any of the temperatures tested during the six-day incubation.

In the contact inhibition study, the inhibitory effect was mostly observed between 15 °C and 30 °C, which may be due to the fact that these temperatures may be ideal for the antagonistic bacteria to grow, while for the pathogens they are lower than their optimum. In some cases, the

inhibitory effects of the isolates were detectable after only one day of incubation at temperatures at which the pathogens were able to grow, but in most cases the inhibitory effects were observed after the 2nd to 3rd day of incubation. This suggests that the amount of the metabolites responsible for the inhibition are increasing during the multiplication process.

3.3 Characterisation, typing and identification of inhibitory isolates

Based on the results of the in vitro inhibition study, only the 20 isolates that inhibited the growth of any of the pathogens were further characterized and identified.

In conclusion, the isolates are neutrophilic and mesophilic: their optimum pH is around 7, while their optimum growth temperature is close to 25 °C.

The results of the biochemical tests showed that all 20 antagonist isolates were catalase positive, seven of them showing no cytochrome c oxidase activity. The KOH test showed that nine isolates were Gram-positive and 11 Gram-negative.

Based on the molecular typing results, the best patterns were obtained with the D8635 primer. Typing with OPE18 was not successful as in many cases no amplicons were generated, while typing with M13 and D8635 showed that all twenty antagonist isolates were clonally distinct, and representing different strains.

Strains were identified by miniature identification tests and sequence analysis of 16S rDNA genes encoding 16S rRNA. The two methods gave significantly different results (Table 1).

Table 1: Results of bacterial isolate identification by miniaturized identification tests and 16S rDNA gene sequencing

Source	Code	Miniaturised identification test	16S rDNA sequencing (*similarity percentage*)
Vegetables	6/2 Z	*Lactococcus lactis ssp. cremoris*	*Bacillus toyonensis (100%)*
	C2Z	*Enterococcus avium*	*Bacillus weihenstephanensis (99.91%)*
Meat	CP-P-2	*Ps.fluorescens*	*Pseudomonas azotoformans (99.7%)*
	CP-P-5	*Ps. putida*	*Pseudomonas lundensis (99.9%)*
	CP-P-8	*Sphingomonas paucimobilis*	*Paenibacillus pabuli (99.9%)*
	CP-S-8	*Ps.fluorescens*	*Pseudomonas granadensis (100%)*
Egg	CSE-B-2	*Acinetobacter baumanii/ calcoaceticus*	*Pseudomonas rhizosphaerae (99.05%)*
	CE-B-1	*Corynebacterium renale*	*Macrococcus caseolyticus (99.8%)*
	CE-PT-1	*Staph. kloosii*	*Rothia endophytica (100%)*
	CE-EJ-2	*Ps. putida*	*Pseudomonas lundensis (99.9%)*
	CE-EJ-3	*Ps.fluorescens*	*Pseudomonas extremaustralis (99.81%)*
	CE-EJ-4	*Ps.fluorescens*	*Pseudomonas azotoformans (99.59%)*
	CSE-T-1	*Lactococcus lactis ssp. cremoris*	*Staphylococcus vitulinus (100%)*
	CSE-T-3	*Staph. haemolyticus*	*Macrococcus caseolyticus (99.8%)*
	CSE-T-4	*Helcococcus kunzii*	*Bacillus pumilus (100%)*
	CE-E-1	*Satph. haemolyticus*	*Macrococcus caseolyticus (99.79%)*
Milk	CM-CT-2	*Ps. fluorescens*	*Pseudomonas azotoformans (99.52%)*
	CM-SMT-1	*Streptococcus intermedius*	*Staphylococcus sciuri subsp. sciuri (100%)*
	CSM-RMT-1	*Burkholderia cepacia*	*Serratia marcescens subsp. marcescens (99.5%)*
	CSM-RMTII-1	*Aeromonas hydrophilia/caviae*	*Serratia marcescens subsp. marcescens (100%)*

Half of the antagonistic bacteria (10 strains) were Gram-negative, and the genus *Pseudomonas* was represented with the highest number of strains, as a total of 8 isolates were identified as *Pseudomonas*. This is also in line with previous observations on biocontrol strains, as findings of antagonistic strains from the genus *Pseudomonas* are also very frequent in the literature.

The molecular identification of the bacterial isolates with inhibitory activity revealed that they belong to seven different genera: *Pseudomonas, Serratia, Bacillus, Paenibacillus, Macrococcus, Staphylococcus,* és *Rothia*. *Pseudomonas* törzseket már korábban is leírtak és vizsgáltak, mint potenciális biokontroll baktériumokat, de a többi nemzetséghez tartozó baktériumok ilyen irányú vizsgálatáról a szakirodalomban csak elvétve található leírás.

3.4 Co-culturing studies

Based on the results of the co-culture studies, the tested antagonist strains were able to reduce *Salmonella* cell proliferation, but no significant cell reduction was observed, and the cells of the inhibitory strains would have to be applied in high concentrations compared to the pathogen, which could even induce spoilage processes in food. Based on these observations, I conducted further experiments focusing on the metabolic products of the inhibitory strains.

3.5 Testing the inhibitory effect of cell-free supernatants

The inhibitory effect of cell-free supernatants was not detectable on each day of measurement, and the appearance of inhibition was random over the six-day incubation. Partial inhibition was detectable on several test days, but no inhibitory effect was detected over six days. Thus, comparing the results of the spot method and the cell-free supernatants, 4, 8, 7 and 14 isolates were able to inhibit the growth of *L. monocytogenes*, *S.* Hardford, *Y. enterocolitica* and *E. coli* in the contact inhibition assay (spot method), whereas only 2, 4, 3 and 13 isolates had a negative effect on the ability of the pathogens to multiply in the liquid culture, where the effect of extracellular metabolites was investigated.

The altered culture conditions did not promote the production of the inhibitory component, so I continued to use bacteria grown in TSB broth under static conditions for the studies.

Based on the studies to observe the inhibitory effects, four strains were selected for further experiments. These had the strongest and/or most inhibitory activity against most pathogens in both the contact inhibition assays and the supernatant tests. These strains were:

- *Pseudomonas lundensis* CP-P-5
- *Serratia marcescens subsp. marcescens* CSM-RMT-1
- *Pseudomonas lundensis* CE-EJ-2
- *Pseudomonas azotoformans* CM-CT-2.

3.6 Testing the inhibitory effect of concentrated cell-free supernatants

The results of the cell-free supernatants inhibition **tests** suggested that the inhibitory components were unlikely to concentrate sufficiently in liquid culture media, so I tried to create more concentrated supernatants by lyophilisation. This was supported by the results of the effect of the lyophilized supernatants, as 10-fold concentrates were much more effective in

suppressing pathogen growth. This suggests that extracellular metabolite(s) are responsible for the inhibitory effects and that the most effective supernatants were those of the one- and three-day cultures, except for the CM-CT-2 *Ps. azotoformans* strain, where the inhibitory effect of the one-day supernatant was greater than that of the supernatants from three- and six-day cultures only during the first half of the 24-hour culture period, and later approached or even exceeded the growth curves of the control pathogens.

3.7 Antibiotic susceptibility testing

Although the cell-free supernatants with inhibitory activity do not contain bacterial cells, it is possible that lysed cells may be present in the cell suspension and their genetic material may be incorporated into the filtrate, and these extracellular DNA molecules may be involved in natural recombination processes, with the result that inhibitory strains with potential antibiotic resistance may play a role in the transmission of resistance.

Antibiotic susceptibility testing showed resistance to several antibiotics in all three *Pseudomonas* strains. These antibiotics were primarily β-lactams, but given the size of the zones, it is likely that these strains are also resistant to the two antibiotics that inhibit protein synthesis on the 50S ribosomal subunit.

The EUCAST breakpoint table contains data mainly for bacteria associated with human diseases and does not include, for example, *S. marcescens*, so it is not possible to compare the results of the disc diffusion test for the CSM-RMT-1 strain. However, taking into account the relatively large zone sizes for the antibiotics tested, it can be concluded that the strain is presumably resistant only to erythromycin.

The antagonistic strains have a certain degree of resistance to antibiotics that inhibit cell wall and protein synthesis, but further studies would be needed to determine the rate of cell death, the amount of DNA released and the stability of the cells in the environment during the culture time. It can be assumed, however, that the 24 h incubation period does not yet initiate significant lysis of the cells and that the nucleases present in the environment contribute to the faster degradation of extracellular DNA, but it would be worthwhile to perform experiments that confirm or reject the involvement of the released DNA in horizontal gene transfer.

3.8 Inhibitory activity of selected antagonist strains against *Salmonella enterica* isolated from egg powder

Testing of strains CP-P-5, CE-EJ-2, CM-CT-2, and CSM-RMT-1 against *Salmonella* using agar spot methods yielded results similar to those previously observed with *S.* Hartford. All four

newly isolated *Salmonella* were at least partially inhibited by the four antagonist strains at 15 °C and 20 °C, and there was no major difference between the inhibitory effects on *Salmonella* strains (S1, S2, S3, S4). The inhibition zones appeared after 24 h, and by the end of the six-day incubation, the pathogens were unable to overgrow on the antagonists.
Based on the results of this study, the antagonist strains tested were able to inhibit all five salmonellae tested, suggesting that they have a general inhibitory effect, however, neither serotyping nor molecular characterisation of the newly isolated *Salmonella enterica* bacteria was performed, so no conclusions on strains can be drawn.

3.9 Inhibition results determined by the cellophane test

On all incubation days, the inhibitory effect was clearly observed on the agar surface at 20, 25 and 30 °C, but cultivation at 15 °C did not promote the development of the inhibitory effect in this method. Again, *Y. enterocolitica* was found to be the most susceptible pathogenic bacterium, multiplying only at the higher temperatures (25-30 °C) on the first two days of culture.
At the end of the incubation period, visible clearing zones of pathogen colonisation were observed in the agar where I had previously placed the cellophane and where the antagonistic bacteria cells had grown. This suggests that the antagonistic strains produced the inhibitory compounds during their culture without the presence of the pathogen to be inhibited, thus they are not induced compounds. In addition, the metabolites were able to diffuse through the cellophane layer into the agar, this phenomenon supports the hypothesis that the inhibitory compounds are extracellular substances and are the primary metabolites of the producing strains because they were secreted in sufficient quantities during 24 h of culture to achieve the required inhibitory effect.

3.10 Results of characterisation and identifying the inhibitory component

3.10.1 UV spectra analysis of agar pieces extracted from the cellophane test
Spectral analysis of agar slices from the cellophane cultivation method did not give valuable results. There were no peaks in any of the curves that would have indicated the chemical properties of the compounds responsible for the antagonistic effect, so I was unable to characterise the inhibitory compounds using this method.
3.10.2 Proteáz aktivitás vizsgálatának eredményei

With the exception of *Ps. rhizosphaerae* strain CSE-B-2, all antagonistic bacteria had some degree of protease activity and the size of the clearing zone on the milk powder agar increased with the incubation time, i.e. the enzyme activity increased. These results suggest that the inhibitory effect of the isolates may be due to their protease activity, but an increasing inhibitory effect would be expected to accompany the increasing proteolytic activity. However, the results observed in the contact inhibition studies do not suggest this, where the inhibitory effect did not clearly increase with increasing incubation days and temperatures. Typically, stronger inhibitory effects were observed at lower temperatures and during shorter incubations (1-3 days).

3.10.3 Inhibitory effect at different pH values

The results showed that for *S.* Hartford and *E. coli*, lower pH values (4, 5, 6) combined with lower temperatures (20 °C) had a stronger inhibitory effect on the growth of the pathogens tested than was observed with the contact inhibition agar spot method. At higher pH values and temperatures (30 °C), which are more optimal for the pathogens, the inhibitory effect was only partial or even disappeared. In the case of *L. monocytogenes*, lower pH values (5, 6) combined with lower temperature (20 °C) helped to induce the inhibitory effect in *Ps. azotoformans* strain CM-CT-2, whereas the inhibitory effect of *S. marcescens* CSM-RMT-1 was not affected by the altered environmental parameters. In the case of *Y. enterocolitica*, low pH (4, 5, 6) and temperature (20 °C) were sufficient to inhibit growth, as the pathogen was unable to grow on control plates. At 30 °C and higher pH (7, 8, 9), the inhibition of *Y. enterocolitica* was only partial for *Ps. lundensis* CP-P-5, whereas the other isolates were able to inhibit its growth completely.

The results also suggest an even wider range of possible uses for metabolites as biocontrol compounds as an element of hurdle-technology as a gentle preservation process.

3.10.4 inhibitory effect of cell-free supernatants after different treatments

With the supernatants of the strains tested, it was not clear whether a heat-sensitive component or a protein-derived component was involved in the inhibition effect. The results suggest that several substances are responsible for inhibition, which may differ between pathogens.
The variable effects of the treatments on *Ps. lundensis* CP-P-5 and *S. marcescens* CSM-RMT-1 are summarised in Table 2.

Table 2 Effect of different treatments on the inhibitory effect of concentrated cell-free supernatants of *Ps. lundensis* CP-P-5 and *S. marcescens* CSM-RMT-1 strains

Ps. lundensis CP-P-5				
Treatment	*S.* Hartford	*E. coli*	*L. monocytogenes*	*Y. enterocolitica*
proteinase K	+	+		-
protease	+	-		-
trypsin	(+)	+		-
α-chymotrypsin	(+)	(+)		-
0,2 N NaOH	-	+		-
95 °C, 15 min	-	+		-
95 °C, 30 min	-	-		-
121 °C, 15 min	-	+		-
S. marcescens CSM-RMT-1				
Kezelés	*S.* Hartford	*E. coli*	*L. monocytogenes*	*Y. enterocolitica*
proteinase K	(+)	(+)	-	-
protease	(+)	(+)	-	-
trypsin	(+)	(+)	-	-
α-chymotrypsin	(+)	(+)	-	-
0,2 N NaOH	(+)	-	-	-
95 °C, 15 min	(+)	(+)	+	-
95 °C, 30 min	(+)	(+)	+	-
121 °C, 15 min	(+)	(+)	(+)	-

+	influenced the inhibitory effect
(+)	slightly influenced the inhibitory effect
-	did not influenced the inhibitory effect
	not examined

3.10.5 Results of tests with antagonistic *S. marcescens* strains

One of the strains with the best inhibitory activity was CSM-RMT-1, which was identified as a *Serratia marcescens* strain based on its 16S rDNA sequence. The bacterium was able to produce the enzyme chitinase, which was confirmed by the results of the chiA-specific PCR assay. This strain showed inhibitory activity against all four food pathogens tested in the inhibition assays as well as in the concentrated supernatant assays.

3.10.6 Results of studies with *Pseudomonas* strains

When the siderophore activity of eight strains belonging to the genus *Pseudomonas* was tested on CAS agar, all eight had chelating properties and all eight were able to produce fluorescent pigments when tested on King B agar.

Among the isolates belonging to the genus *Pseudomonas*, the strain with the most significant antagonistic effect was *Ps. lundensis* CP-P-5, which was able to inhibit the other tested pathogens except *L. monocytogenes*, as well as S. Hartford and later four *Sa. enterica* isolated from food also showed the strongest inhibitory effect against Based on our tests, this strain is capable of fluorescent pigment production, and in addition to its protease activity, several components may be involved in the formation of the inhibitory effect, which was indicated by the results of enzyme and heat treatment of the supernatants, as well as the results of the inhibitory effect test following chromatographic fractionation.

3.10.7 Results of chromatograph tests and fractions

Seeing the results of the inhibitory effect of the fractions of the supernatants, it can be said that several components are definitely responsible for the development of the inhibitory effect in the case of *Ps. lundensis* CP-P-5, since several of the tested fractions also had an inhibitory effect. Therefore, in the future, it would be worthwhile to further investigate the inhibitory fractions in order to determine the component.

In the case of *S. marcescens* CSM-RMT-1, there was a fraction (S3) which in all cases was able to achieve some degree of inhibitory effect against the tested pathogens, here it is more likely that the inhibitory component remained in the phase prepared from the methanol extraction.

4. Conclusions and recommendations

Foods and their processing environment contain many microorganisms that can have a negative impact on pathogens that cause human disease. It is therefore worthwhile to investigate naturally occurring micro-organisms in the food environment, as they may be used as biocontrol agents (e.g. for surface disinfection, Twele et al., 2011). In my work, 20 of the 78 bacteria isolated from different food raw materials and food contact surfaces were able to inhibit one of the pathogens tested (*S*. Hartford, *E. coli*, *Y. enterocolitica* or *L. monocytogenes*).

In the search for isolates with inhibitory activity, the results of contact inhibition on the agar surface could not be confirmed by studies with cell-free supernatants, with much less inhibition observed. In this study, liquid media were used, so the moderate inhibition may also indicate that the metabolites responsible for inhibition were not present in the media in sufficient quantities to have a negative effect on the pathogens, whereas in the solid media these compounds may have been accumulated. It can also be assumed that the metabolites with inhibitory activity may have had limited solubility in the liquid medium used, which could lead to the same observation. Accordingly, it can be concluded that the antagonistic compounds must be present in concentrated form in the environment to inhibit the pathogen more effectively.

The results obtained when investigating the effect of pH and temperature on the production of inhibitory substances highlight the importance of unfavourable environmental parameters in inhibiting the growth of pathogenic bacteria and the additive effect of environmental factors and biocontrol metabolites.

After examining proteolytic enzyme activity, it can be said that proteases may be involved in pathogen inhibition, but they are not the main inhibitory compounds.

The cellophane assay and the testing of supernatants in concentrated form also support the observation that a more concentrated presence of inhibitory components is necessary for the inhibitory effect to occur. In addition, both tests support the observation that the presence of the pathogenic microorganism is not necessary for the production of the inhibitory substance, thus it is not the presence of the pathogen that induces the production and secretion of the metabolites responsible for the inhibition, but they are continuously produced and released into the extracellular space. Taking into account the culture time, the strongest inhibitory effect in

these studies was generally observed after 24 hours, so it is likely that primary metabolites are responsible for the inhibitory effect. This observation is supported by the clearing zones that appeared in the contact inhibition studies after 24 hours. In addition, the stronger inhibitory effect observed in the concentrated supernatant test on supernatants from one-day-old cultures compared to three- and six-day-old cultures also points to this fact. The inhibitory effect observed in the co-culture tests of the two strains with the most significant and broadest inhibitory effect, *Ps. lundensis* CP-P-5 and *S. marcescens* CSM-RMT-1, also points to the primary metabolite nature of the inhibitory compounds, since in both cases the most significant reduction in pathogen numbers was observed after 24 hours in the presence of the antagonist isolates.

All microorganisms have different environmental and nutrient requirements for optimal reproduction, factors that influence the nature and quantity of the primary metabolites they select. Based on these factors, it is not possible to generalise about the conditions under which the isolates I have collected can optimally produce the inhibitory components, and therefore the isolates need to be examined individually. Based on the results obtained in my work, concentrating on the two strains mentioned above (CP-P-5, CSM-RMT-1), the following can be said about the compounds responsible for the inhibitory effect.

The molecules responsible for the inhibitory effect of the *S. marcescens* CSM-RMT-1 strain tested are extracellular metabolites, since cellophane and supernatant studies have shown that inhibition of pathogens is observed both on the solid agar surface and in the medium after culture of the antagonist strain and after removal of bacterial cells, i.e. the compounds produced and secreted by the antagonist strains have the same effect as the cells themselves. Prodigiosin has no relevant role in inhibition, as CSM-RMT-1 strain is not able to produce this pigment, yet it has good inhibitory activity against the pathogens tested. However, various hydrolytic enzymes that can degrade essential macromolecules of target organisms are likely to be among the inhibitory substances produced by CSM-RMT-1. In addition, this strain has the chiA (chitinase encoding) gene in its genome and is also capable of producing chitinase, an enzyme with antifungal and antibacterial activity, so overall it can be concluded that these enzymes may also be responsible for the antagonistic effect.

After analysis of the fractions obtained from the chromatographic studies, it can be concluded that several components may be responsible for the inhibitory effect of CSM-RMT-1, however, the compound with the most significant inhibitory effect is soluble in methanol, so further

investigation of this would bring us closer to the identification of the metabolite with inhibitory effect.

Based on the results and observations, a concentrated cell-free supernatant of the bacterium could be used as an antagonist of various pathogenic bacteria in the food industry, even as a bio-disinfectant on surfaces in food processing areas. In addition, its antifungal activity through the production of chitinase may also be worth investigating. However, further analyses are needed to verify how diffusible proteins or other extracellular compounds may affect the composition of food or food raw materials.

Many *Pseudomonas* can be used as biocontrol microbes, as they multiply rapidly under *in vitro* conditions, produce biomass and a wide range of bioactive metabolites (e.g. antibiotics, siderophores, volatiles and growth promoters), can also compete aggressively with other microorganisms and can adapt to environmental stresses (Weller, 2007). Previous studies have isolated several strains of Pseudomonas with antagonistic effects on pathogenic bacteria in food (Alegre et al., 2013, Olanya et al., 2014, Belák and Maráz, 2015, Oliveira et al., 2015), most of which were fluorescent *Pseudomonas*. *Ps. lundensis* CP-P-5, which I isolated, was also able to produce fluorescent pigments on King B agar, and siderophore production was also observed on the surface of CAS agar plates.

The results of the float studies suggest that several components may be responsible for the inhibitory effect of this strain. In the different treatments, the substrates of proteolytic enzymes in the non-concentrated supernatants may have been at low concentrations, and therefore there was no change in the inhibitory effect in these cases. However, in the tenfold concentrations of the supernatants, the metabolites were enriched and present in sufficient amounts for enzymatic degradation. Different results were obtained for each pathogen after enzymatic, thermal and NaOH treatment, suggesting that several inhibitory compounds are responsible for the inhibition of pathogens. This is supported by the inhibitory effect of the fractions prepared in the chromatographic studies, where components that are highly soluble in different polar solvents may be the metabolites with antagonistic activity. Similar to the biocontrol strain *S. marcescens, Ps. lundensis* CP-P-5 may also be used in the food industry to inhibit certain pathogenic bacteria. However, it should be mentioned that direct use of bacterial cells may lead to food spoilage, so the use of compounds produced and selected by the strain may be an alternative solution. To the best of my knowledge, this is the first study in which a *Ps. lundensis* strain has been described as a potential antagonist of pathogenic bacteria in food, but the

characterisation of the inhibitory compounds involved in the antagonistic effect would certainly be worthwhile to continue.

5. New scientific results

1. For the first time, bacteria from the genera *Macrococcus* and *Rothia*, which can be classified as potential biocontrol strains against food-borne pathogens, have been isolated and described, belonging to the species *Macrococcus caseolyticus* and *Rothia endophytica*. These species have not yet been reported in the scientific literature as potential biocontrol bacteria for foodborne pathogenic bacteria.

2. I first described a biocontrol strain of *Pseudomonas lundensis* isolated from a slaughterhouse environment that is capable of inhibiting the growth of food-borne pathogenic bacteria. Several extracellular metabolites are involved in this inhibition: compounds that are soluble in organic solvents and may include siderophores and proteases produced by the bacterium. Based on my results, a concentrated cell-free supernatant of *Ps. lundensis* CP-P-5 could be used to control the growth of various pathogenic bacteria in food processing plants.

3. In my work, a non-pigment-producing *Serratia marcescens* biocontrol strain was isolated from a dairy environment for the first time. Based on my results, I found that the compound(s) responsible for the inhibitory effect are extracellular metabolites extractable by methanol and that the production of protease and chitinase by the *S. marcescens* strain CSM-RMT-1 may also contribute to the inhibition. These results suggest that a concentrated cell-free supernatant of the biocontrol effect prodigiosinnegative *S. marcescens* strain could be used against various pathogenic bacteria in the food industry.

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6. Publications

IF journal articles

Baráti-Deák, B., Da Costa A., Giseli C.a, Perjéssy, J., Klupács, A., Zalán, Zs., Mohácsi-Farkas, Cs., Belák, Á., (2023) Inhibition of Foodborne Pathogenic Bacteria by Excreted Metabolites of Serratia marcescens Strains Isolated from a Dairy-Producing Environment. *Microorganisms* 11(2): 403. IF: 4,926

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