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SEARCHING FOR ANTAGONISTIC ACTIVITY OF BACTERIAL ISOLATES DERIVED FROM FOOD PROCESSING ENVIRONMENTS ON SOME FOOD-BORNE PATHOGENIC BACTERIA

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Bacterial strains with inhibitory effect on *Salmonella* Hartford, *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Escherichia coli*, respectively, were isolated. Out of the 64 bacteria originated from food processing environments, 20 could inhibit at least one of the tested pathogens, and it was proved that growth decline of the pathogenic bacteria was more remarkable by co-culturing than by using cell-free supernatants of the isolates. Seven different genera (*Pseudomonas*, *Bacillus*, *Paenibacillus*, *Macrococcus*, *Staphylococcus*, *Serratia*, and *Rothia*) reduced the pathogens' growth during the time period of analysis, and the strongest inhibitory effect was observed after 24 h between 15 and 30 °C. Sensitivity of the tested human pathogenic bacteria against the inhibitory strains was distinct, as *Y. enterocolitica* could be inhibited by numerous isolates, while *S.* Hartford proved to be the most resistant. Our results reveal that the isolated bacteria or their excreted metabolites could hinder pathogen growth when used in sufficient quantities.

Keywords: food-borne pathogens, food processing environment, biocontrol

Biological control – as an alternative method – has a great importance in sustainable food production (Bale et al., 2008) as well as in in-farm application (Gálvez et al., 2010). It appears to be a good solution to eliminate foodborne pathogenic bacteria mainly by using the native microbiota of the product of interest (Oliveira et al., 2015). However, biocontrol can also be applied as an alternative cleaning and/or disinfection practice (Vandini et al., 2014; Gray et al., 2018) as well. As the need for effective biocontrol is significant, different strategies such as application of bacteriophages and endolysins (Bai et al., 2016), competitiveness enhancement techniques, protective cultures and antimicrobial metabolites (McIntyre et al., 2011; Hossain et al., 2017) were evaluated to enhance safety of food products.

Published research on biocontrol of foodborne pathogens by antagonistic bacteria or competitive exclusion is limited, however, the number of studies focusing on the antagonistic activity of different food derived microorganisms is increasing because of their potential usage as alternatives for preservation, especially in combination with other techniques (such as freezing, refrigeration, etc.) (Farkas, 2001). In a study of Alegre and co-workers (2012), it was found that an *Enterobacteriaceae* species isolated from apple was able to control *E. coli* O157:H7, *Salmonella* spp., and *Listeria innocua* on minimally processed apples and peaches. It was also Alegre and co-workers (2013), who demonstrated the antagonistic activity of a *Pseudomonas graminis* strain against *Salmonella* spp. and *Listeria monocytogenes*

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on minimally processed apple. Leverentz and co-workers (2006) isolated microorganisms (among them a *Gluconobacter asaii* strain) from apple with inhibitory effect against *L. monocytogenes* and *Salmonella* Poona. Moreover, in the scientific literature, studies focusing on antimicrobials produced by lactic acid bacteria and other biocontrol microbes can also be found (Gálvez et al., 2010; Castellano et al., 2017).

The aim of this study was to isolate bacteria with biocontrol activity against various foodborne pathogens in food processing environments, so that our results may reflect interactions between microbial populations in the food and food raw material environments that were – to the best of our knowledge – less examined.

1. Materials and methods

1.1. Isolation of potential antagonistic bacteria

Samples for isolation were taken from food processing environments of swine abattoir, egg, milk, and vegetable processing plants. Sample collection from smooth flat surfaces was done with contact slides with TSA (Tryptone-soy agar, Biokar), while for rugged or not easily accessible places swab sampling rods with TSB were used. All samples were incubated at 25 °C for 24 h. Morphologically different isolates were collected directly from the contact slides, while in case of TSB, serial dilutions were made, and after streaking (100 µl) on TSA (Biokar), the plates were incubated at 25 °C for 24 h. Morphologically different colonies were collected, and the prepared pure cultures were maintained on TSA slants at 4 °C.

1.2. Grouping bacterial isolates by morphological characteristics

To group and reduce the number of isolates by excluding the similar ones, their colony morphologies were studied and compared using WL (Wallerstein Laboratory) Nutrient agar plates (Atlas, 1995). WL and TSA plates were inoculated individually with each isolate being in exponential phase and incubated for 24–48 h at 25 °C. Isolates with similar morphological characteristics both on WL and TSA plates were grouped, and only one isolate from each group was selected for further examinations.

1.3. Screening for antagonistic activity of the bacterial isolates by spot method

The screening for antagonistic activity was done against the following four bacterial pathogens: *Listeria monocytogenes* (CCM 4699), *Salmonella* Hartford (NCAIM B1310), *Yersinia enterocolitica* (HNCMB 98002), and *Escherichia coli* (NCAIM B01909).

Pathogenic bacteria were cultured on TSA plates at 37 °C for 24 h, except *Y. enterocolitica*, which was incubated at 25 °C. Suspensions of the pathogens were prepared from one-day-old cultures in sterile distilled water. Using Biosan DEN-1B densitometer, the density of the suspensions was adjusted to 2.5 McFarland (approx. 10^8–10^9 CFU ml^{-1}). After preparing a ten-fold dilution, 0.1 ml with a final concentration of 10^6 CFU ml^{-1} was massively inoculated onto TSA plates. After drying the surface of the plate, 10 µl of cell suspension made of the isolates (containing approx. 10^6 cells) was dropped onto the agar surface. The plates were incubated at 5, 10, 15, 20, 25, 30, 37, and 42 °C for 6 days to determine the optimal temperature and time for inhibition. Growth inhibition was detected by measuring the clearing zones around the macrocolonies of the tested isolates after one, two, three, and six days of incubation. These experiments were done in duplicates.

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1.4. Inhibitory effect of cell-free supernatants of potential antagonistic strains

Production of extracellular inhibitory substances was examined using cell-free supernatants of the antagonistic isolates by micro-culturing. Inhibitory effect of one-, three-, and six-day-old cell-free supernatants generated from cultures of the isolates in TSB was tested by Multiskan Ascent (Thermo Fisher Scientific). Cultures of the selected isolates were grown at 25 °C, and after separating the cells from the culture medium by centrifugation (14 000 r.p.m., 15 min), the supernatants were removed and filtered through 0.2 µm pore size membrane filters. The wells of the microplates were filled with 300 µl of liquid consisting of 75 µl of four-fold strength TSB, 75 µl of cell suspension of the pathogen (approx. 10^6 CFU ml^{-1}), and 150 µl cell-free supernatant of the test strain. Inoculated microplates were incubated at 25 °C and the absorbance values at 595 nm were recorded automatically every 30 min during the 24 h of cultivation. Growth curves were generated from the absorbance values versus time data, using the average of triplicates.

1.5. Characterisation of antagonistic bacteria

For characterisation of the isolated bacteria, analyses of optimal growth temperature and pH, KOH test for determination of cell wall properties, and catalase and oxidase tests were done.

To determine the optimal growth temperature of the isolates, TSA plates were surface inoculated with a loopful of overnight cultures and incubated at different temperatures (5, 10, 15, 20, 25, 30, 37, and 42 °C). Formation of colonies was checked after 24 h, and the results were recorded.

For studying the optimal pH range of growth for the isolates, TSA plates containing different buffers (phosphate-citrate buffer for pH 3, 4, 5, 6, 7, Sorensen's phosphate buffer for pH 8, and glycine-NaOH buffer for pH 9 (Ruzin, 1999)) were used. The growth rates were checked after 24 h. Both above mentioned experiments were done in duplicates.

The isolates were further characterised with KOH, catalase, and oxidase tests by conventional methods (Ryu, 1940; Lemberg & Foulkes, 1948; Kovacs, 1956; Powers, 1995).

1.6. Molecular typing and identification of the antagonists

Molecular typing of the antagonistic isolates was done by RAPD-PCR using OPE 18 (Belák, 2009), M13 (Vassart et al., 1987), and D8635 (Van Looveren et al., 1999) primers. Identification was performed with miniaturised identification kits and by sequence analysis of 16S rRNA encoding rDNA genes. For Gram-negative isolates API 20 NE and API 20 E kits (bioMérieux) were used, while for Gram-positive ones BBL Crystal tests (Becton Dickinson) were applied. The isolates were also identified at genus or species level by direct sequencing of the 16S rDNA PCR products generated by 27f–1492r primer pairs (Maiwald, 2004). The sequences were analysed using the databases of EzTaxon and NetBlast.

2. Results and discussion

2.1. Isolation of bacteria and in vitro test for their inhibitory effect

Altogether 78 bacteria were isolated from four different food processing environments: 20 from a swine abattoir, six from vegetable processing environment, 18 from surface samples of an egg

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processing plant, and 34 from a diary product plant. After examining the colony morphology of the isolates on WL and TSA plates, 64 bacteria – 13 from the abattoir, 6 from vegetable processing environment, 18 from the egg processing plant, and 27 from the diary product plant – showing different characteristics on the agar plates were selected for inhibitory assay.

The selected isolates were screened for their antagonistic activity using spot method against *L. monocytogenes*, *Salmonella* Hartford, *Y. enterocolitica*, and *E. coli*. The results of the assay showed that out of the investigated 64 isolates 20 could inhibit at least one of the tested pathogens (Table 1). In these cases, the observed clearing zones around the isolates referred to partial or total inhibitory effects. Two of the isolated bacteria could inhibit all four pathogens, further two inhibited three of them, three isolates had negative effect on two of the pathogens' growth, and 13 could inhibit only one of the pathogenic bacteria (Table 1).

Table 1. Results of screening for growth inhibition of the 20 potential antagonistic bacteria

Source	Isolate code	*L. monocytogenes*	*S.* Hartford	*Y. enterocolitica*	*E. coli*
Vegetable processing plant	6/2 Z	(+)	–	–	–
	C2Z	–	–	(+)	–
Swine abattoir	CP-P-2	(+)	–	–	–
	CP-P-5	–	+	(+)	(+)
	CP-P-8	–	–	(+)	–
	CP-S-8	–	–	–	(+)
Egg processing plant	CSE-B-2	–	–	(+)	–
	CE-B-1	–	–	(+)	–
	CE-PT-1	–	–	+	–
	CE-EJ-2	–	+	–	(+)
	CE-EJ-3	+	–	–	(+)
	CE-EJ-4	+	–	–	(+)
	CSE-T-1	–	–	(+)	–
	CSE-T-3	–	–	+	–
	CSE-T-4	+	–	+	(+)
	CE-E-1	–	–	(+)	–
Dairy product plant	CM-CT-2	+	+	+	+
	CM-SMT-1	–	–	+	–
	CSM-RMT-1	+	(+)	+	+
	CSM-RMTII-1	–	–	+	–

+: total inhibition
(+): partial inhibition
–: no inhibition

Regarding the optimal inhibitory temperature, it was observed that at most applied temperatures growth inhibition was only partial, and total inhibition occurred merely at one or two tested temperatures in each case. The most effective inhibition was always detected between 15 °C and 30 °C (Table 1). Inhibitory effect of the isolates could already be detected after one day of incubation at temperatures that supported the growth of the pathogen. However, in some cases, for the sixth day of the test the pathogen overgrew the bacterial strain that could inhibit its growth earlier.

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The most sensitive pathogenic bacterium proved to be *Y. enterocolitica*, as out of the 20 antagonistic isolates 14 had negative effect on its growth. *L. monocytogenes* and *E. coli* were sensitive to seven and eight isolates, respectively, and only four bacteria could inhibit the growth of *S.* Hartford.

2.2. Characterisation, identification, and molecular typing of the antagonistic bacterial strains

On the basis of in vitro inhibition test results, only the 20 above mentioned isolates were selected for further characterisation and identification.

The physiological tests showed that the isolates were mostly neutrophilic and mesophilic with an optimal pH of 7 and temperature of 25 °C. All 20 antagonistic isolates were catalase positive, and eight of them did not show the activity of cytochrome c oxidase. Based on the results of the KOH test, nine isolates were Gram-positive and eleven proved to be Gram-negative.

Identification of the strains was done by miniaturised identification tests and sequence analysis of the 16S rRNA encoding rDNA genes. As can be seen in Table 2, the two methods gave significantly different results. Identification of *Pseudomonas* isolates could be accepted at genus level in the case of API tests, while all other isolates were misidentified by miniaturised kits, which emphasize the necessity of molecular identification of non-clinical isolates.

Table 2. Results of identification of antagonistic isolates using miniaturised kits (API and BBL Crystal) and 16S rDNA sequencing

Source	Code	API or BBL Crystal	Sequencing of 16S rDNA gene (similarity percentage)
Vegetable processing plant	6/2 Z	*Lactococcus lactis ssp. cremoris*	*Bacillus toyonensis* (100%)
	C2Z	*Enterococcus avium*	*Bacillus weihenstephanensis* (99.91%)
Abattoir	CP-P-2	*Pseudomonas fluorescens*	*Pseudomonas azotoformans* (99.7%)
	CP-P-5	*Pseudomonas putida*	*Pseudomonas lundensis* (99.9%)
	CP-P-8	*Sphingomonas paucimobilis*	*Paenibacillus pabuli* (99.9%)
	CP-S-8	*Pseudomonas fluorescens*	*Pseudomonas granadensis* (100%)
Egg processing plant	CSE-B-2	*Acinetobacter baumanii/ calcoaceticus*	*Pseudomonas rhizosphaerae* (99.05%)
	CE-B-1	*Corynebacterium renale*	*Macrococcus caseolyticus* (99.8%)
	CE-PT-1	*Staphylococcus kloosii*	*Rothia endophytica* (100%)
	CE-EJ-2	*Pseudomonas putida*	*Pseudomonas lundensis* (99.9%)
	CE-EJ-3	*Pseudomonas fluorescens*	*Pseudomonas extremaustralis* (99.81%)
	CE-EJ-4	*Pseudomonas fluorescens*	*Pseudomonas azotoformans* (99.59%)
	CSE-T-1	*Lactococcus lactis ssp. cremoris*	*Staphylococcus vitulinus* (100%)
	CSE-T-3	*Staphylococcus haemolyticus*	*Macrococcus caseolyticus* (99.8%)
	CSE-T-4	*Helcococcus kunzii*	*Bacillus pumilus* (100%)
	CE-E-1	*Satphylococcus haemolyticus*	*Macrococcus caseolyticus* (99.79%)
Dairy product plant	CM-CT-2	*Pseudomonas 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%)

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For the molecular typing of the antagonistic isolates by RAPD-PCR, D8635 primer gave the best patterns. Typing with OPE18 was not successful, as in many cases amplicons were not generated, while typing with M13 – together with D8635 – showed that all our twenty antagonistic isolates were clonally diverse (results are not shown).

Comparing the results of non-staining KOH method and molecular identification, it was observed that the Gram-positive *Paenibacillus pabuli* gave different results, since Gram-negative property was determined in its case. However, this phenomenon was also observed earlier by Tsukatani and co-workers (2011), who also got false positive reaction with *Paenibacillus macerans* using the KOH string test.

Based on the results of molecular identification, inhibitory isolates belonged to seven different genera (*Pseudomonas*, *Bacillus*, *Paenibacillus*, *Macrococcus*, *Staphylococcus*, *Serratia,* and *Rothia*). *Pseudomonas* strains were described earlier as potential biocontrol bacteria of food-borne pathogens (Alegre et al., 2013; Jiang et al. 2019), however, the other genera are not frequently mentioned in the scientific literature as biocontrol agents for these bacteria. Földes and co-workers (2000) isolated a *Bacillus subtilis* strain, which was able to inhibit the growth of *L. monocytogenes* and *Staph. aureus* to a high degree. In a publication of the FDA (2019), a non-pathogenic *Paenibacillus alvei* strain is mentioned as an effective inhibitor of growth for human foodborne bacterial pathogens like *Salmonella*, *Escherichia*, *Listeria*, *Shigella*, *Enterobacter*, and *Staphylococcus*.

Bacteria in the genus *Staphylococcus* are pathogens of man and other mammals. Coagulase-positive strains are considered as the most pathogenic ones, while coagulase-negative staphylococci are common commensals of skin, although some species can cause infections (Foster, 1996). The genus *Macrococcus* is evolutionarily closely related to *Staphylococcus*, however, in contrast to *Staphylococcus*, species of *Macrococcus* are regarded as avirulent bacteria to their animal hosts (Mazhar et al., 2018). Purkayastha and co-workers (2018) proved the plant growth promoting and biocontrol efficacy of a *Serratia marcescens* strain isolated from tea rhizosphere, however, *Rothia endophytica* was first isolated from healthy roots of an aquatic perennial herb (*Dysophylla stellata* (Lour.) Benth.) by Xiong and co-workers (2013). To our best knowledge, out of the seven isolated genera mentioned in our study as inhibitory ones, *Macrococcus*, *Staphylococcus*, *Serratia,* and *Rothia* are not mentioned as potential biocontrol agents of foodborne pathogenic bacteria in the scientific literature until now.

2.3. Effect of cell-free supernatants for inhibitory isolates on pathogenic bacteria

Examination of cell-free supernatants of the inhibitory isolates by micro-culturing using Multiscan Ascent mostly resulted in partial growth inhibition of the pathogens, total inhibition occurred only in the case of *Y. enterocolitica* (Table 3).

By testing the effect of cell-free supernatants, it was observed that while in contact inhibition study the clearing zones appeared after 24 h of incubation, in some cases of cell-free supernatants inhibition was only detected after 48 h. Moreover, there was no connection between the sizes of the clearing zones observed in spot method and the effectiveness of the cell-free supernatants (data are not shown). Additionally, the total and partial inhibitory effect of the isolates at co-culturing study did not correlate with the results of cell-free supernatants.

In co-culturing, the formed clearing zones remained during the whole study, while inhibitory effect of cell-free supernatants could not be detected on any day of the study, especially in cases of total inhibition, where growth decline of the pathogen was observed only once during the studied time period. Moreover, appearance of inhibition was random

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during the six days of incubation. Partial inhibition could be detected on more examination days, however, six-day-long inhibitory effects were not detected.

Table 3. Inhibitory effect of cell-free supernatants on growth of the pathogenic bacteria

Source	Code of the isolate	*L. monocytogenes* CCM4699	*S.* Hartford B1310	*Y. enterocolitica* 98002	*E. coli* B01909
Vegetable processing plant	6/2 Z	+	nd	nd	nd
	C2Z	nd	nd	+	nd
Abattoir	CP-P-2	–	nd	nd	nd
	CP-P-5	nd	(+)	–	(+)
	CP-P-8	nd	nd	+	nd
	CP-S-8	nd	nd	nd	–
Egg processing plant	CSE-B-2	nd	nd	+	nd
	CE-B-1	nd	nd	(+)	nd
	CE-PT-1	nd	nd	+	nd
	CE-EJ-2	nd	(+)	nd	–
	CE-EJ-3	(+)	nd	nd	(+)
	CE-EJ-4	(+)	nd	nd	(+)
	CSE-T-1	nd	nd	+	nd
	CSE-T-3	nd	nd	+	nd
	CSE-T-4	–	nd	+	–
	CE-E-1	nd	nd	(+)	nd
Dairy product plant	CM-CT-2	–	–	(+)	–
	CM-SMT-1	nd	nd	+	nd
	CSM-RMT-1	–	–	(+)	–
	CSM-RM-TII-1	nd	nd	+	nd

+: total inhibition
(+): partial inhibition
–: no detectable inhibition
nd: not determined

Comparing the results of spot method with those of cell-free supernatants, it can be said that in case of co-culturing, 7, 4, 14, and 8 isolates could inhibit the growth of *L. monocytogenes*, *S.* Hardford, *Y. enterocolitica*, and *E. coli*, respectively (Table 1), while in liquid cultures – where the effect of extracellular metabolites was tested – only 4, 2, 13, and 3 isolates had negative impact on their propagation ability, respectively (Table 3). This observation is analogous with that of Pelyuntha and co-workers (2019), who tested the effect of cell-free supernatants from cultures of lactic acid bacteria on *Salmonella* strains, and determined that fewer isolates kept their activity against the pathogens when using their supernatants compared with the agar spot test. They assumed that the limited solubility of the inhibitory metabolites in the agar was behind this result. In our study, micro-culturing in liquid medium was used when testing the effect of extracellular metabolites. Thus, the decreasing number of

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inhibitory strains can refer to the fact that metabolites that are responsible for the inhibition were not present in adequate quantity in the broth to have negative effect on the pathogens, while in the solid medium these compounds could have been enriched. Furthermore, the inhibitory metabolites could have limited solubility in the applied liquid medium, which can result in the same observation. Accordingly, the antagonistic compound(s) should be present in concentrated form in the environment for effective inhibition of the pathogen, however, further studies are required to confirm it.

3. Conclusions

Food processing environments can contain microorganisms that are able to inhibit the propagation of food-borne pathogenic bacteria like *L. monocytogenes*, *S.* Hartford, *Y. enterocolitica*, or *E. coli*. These microbes can negatively influence the growth of the pathogens by antagonism or competitive exclusion. Further experiments will focus on the mechanism of biocontrol in case of inhibitory strains isolated in this study, and the potential application of these bacteria in combination will also be studied.

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