Article

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A New Strategy to Select *Pediococcus pentosaceus* Strains for the Application of Protective Cultures in Foods and Care Products

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Abstract: *Pediococcus pentosaceus* is a lactic acid bacterium used *inter alia* for fermentation of milk, meat, vegetables, fruits, and even beer. The resulting food products are tasty and safe. Furthermore, health-promoting effects such as anti-inflammation, anti-cancer, and detoxification have been attributed. Beside refining foods during the fermentation process, *P. pentosaceus* might be added to foods as a protective culture to improve food safety and extension of shelf-life, leaving the organoleptic properties of the food untouched. We have addressed the search for novel protective *P. pediococcus* strains by investigation of 38 isolates from milk samples and teat canal biofilms from dairy farms located in the vicinity of Muenster, Germany. *P. pentosaceus* strains were unequivocally identified by DNA sequencing of the gene encoding 16S rRNA. Binary matrices obtained from random amplification of polymorphic DNA experiments showed that all isolates differed and thus represent subspecies. The antibacterial profiles of 13 selected isolates showed that seven food pathogens and food spoilage bacteria could efficiently combat, although to various extends, gram-negative bacteria such as *Pseudomonas aeruginosa* or *Salmonella enterica*, and gram-positive bacteria such as *Staphylococci* and *Listeria monocytogenes*. Interestingly, acid production was dependent on the presence of the challenged pathogen but did not significantly correlate with the extend of inhibition. The genomes of three top-ranked isolates were sequenced. We found that the genome size and the harbored plasmids differed significantly, confirming the subtle genomic plasticity. One to two genes encoding bacteriocins could be detected in each of the three genomes. Our approach for protective culture screening demonstrates that it is worth to examine many isolates from a specific species to select the best performing strain for application in food and care products.

Keywords: *Pediococcus pentosaceus*; protective cultures; antibacterial; genome; lactic acid bacteria; food safety; bacteriocins

1. Introduction

A whole variety of microorganisms is used since thousands of years to produce fermented beverages and food, which are very tasty and resilient to food spoilage [1–4]. In the early 20th century, Illia Iljitsch Metschnikoff postulated that the yogurt-producing lactic acid bacteria (LAB) *Lactobacillus bulgaricus* and *Streptococcus thermophilus* exert health-promoting effects and help humans live longer by acting as intestinal residents [5]. Since then, LAB and bifidobacteria have been thoroughly investigated regarding their biology and their probiotic efficacy to uncover and prove beneficial activities [4–6]. The result is a huge market that offers to the customer probiotic foods, nutraceuticals, and care products for human and animal health[4,7]. Furthermore, traditional spontaneous (wild) fermentation of food has undergone a renaissance in the past years as a food trend that has been, among other things, strongly acclaimed by chefs at Michelin-starred restaurants to present dishes with novel flavor profiles [8,9].

From a taxonomic point of view, LAB are grouped in the order *Lactobacillales* comprising the families *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae*, and *Streptococcaceae* [10]. The

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latter two are the most relevant families, of which *Lactobacillaceae* stand out [11]. They were recently re-classified based on a molecular phylogenetic approach at whole genome level. The authors included additionally the *Leuconocaceae* family giving a total of 261 described species that comprise 25 genera[11].

The ten species of the genus Pediococcus receive increasingly attention as food fermenting and protecting cultures [12,13]. One most studied member is *Pediococcus pentosaceus*, which became originally famous for spoiling beer and at the same time is appreciated for its buttery and sour taste in special beer types [14,15]. Strains of *P. pentosaceus* have become attractive for the fermentation of vegetables, sausages, vegetables, fruits, and wine [16]. Numerous health-promoting effects have been associated including microphage stimulation, anti-inflammation, anti-cancer, antioxidant, detoxification, and cholesterol-lowering [16]. As other LAB, *Pediococci* contribute to food safety as they combat pathogenic bacteria by producing bacteriocins or other antibacterial compounds such as lactic acid, small peptides, hydrogen peroxide, or diacetyl [12,17,18].

Here, we present the isolation of *P. pentosaceus* strains from one specific natural environment, the udder of milk cattle to study their potential application as protective culture in foods. We show that *P. pentosaceus* isolates exhibit a high biodiversity at the subspecies level. We show that each isolate exerts antibacterial activities to various extends against representative pathogens. We provide genomic data from the best performing isolates to provide a molecular view into the genome plasticity. The implication of our approach for the discovery of protective cultures in foods to enhance food safety is discussed.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

P. pentosaceus were cultivated on modified DeMan-Rogosa Sharp agar plates (MRS) [19]. If appropriate, MRS plates were supplemented with cysteine (mMRS) to promote growth and with bromophenol blue (mMRS-bpb) [20] to better distinguish colony morphologies by blueish coloration. Strains were freshly propagated in MRS broth for 48 hours at 30°C. Seven bacterial species were employed as indicators and surrogates for foodborne pathogens and spoilage organisms. These strains, except *Listeria monocytogenes*, were cultivated in Lysogeny broth (LB) after Lennox at 37°C for 24 hours. Brain Heart Infusion (BHI) Bouillon was utilized for the cultivation of *L. monocytogenes*.

Table 1. List of strains.

species	origin	comment
Bacillus subtilis	laboratory strain collection	surrogate food spoilage
Citrobacter koseri	laboratory strain collection	fish-borne pathogen
Listeria monocytogenes	DSM 20600	food pathogen
Pseudomonas aeruginosa	ATCC 15442	food spoilage & pathogen
Salmonella enterica	LT-2	food pathogen
Staphylococcus haemolyticus	laboratory strain collection	surrogate food pathogen
Staphylococcus warneri	laboratory strain collection	surrogate food pathogen

[1] re-classified from originally S. typhimurium [21] to *S. enterica ssp. enterica ser. Typhimurium* [22].

2.2. Species Identification Through 16S rRNA Gene Sequencing

Genomic DNA extraction was performed by resuspending a single colony in 50 µl of 0.05 M NaOH. The mixture was incubated at 100°C for 1 minute. The lysate was precipitated by centrifugation. The supernatant with the genomic DNA was 1:10 diluted in sterile DNAse-free demineralized water. Polymerase chain reactions (PCR) were carried out in a volume of 25 µl, consisting of 12.5 µl oneTaq Polymerase Hot Start mastermix (New England Bio Labs, England),

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0.5 µl (10 pmol/µl) F08 primer (5′-AGAGTTTGATCATGGCTCAG-3′), 0.5 µl (10 pmol/µl) 535R primer (5′-TATTACCGCGGCTGCTGGCA-3′) , 9 µl DNase and nuclease-free water, and 2.5 µl (0.1 to 10 ng) DNA template. The resulting amplicon encompassed bases 8 to 535 of the gene encoding 16s rDNA representing the variable regions V1-V3 of the 16S rDNA gene. PCRs were carried out with an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds. PCR amplicons were sequenced at Microsynth (Switzerland). The Basic Logical Alignment Tool (BLAST) service provided by the National Center for Biotechnology Information (NCBI, USA) was used for species identification.

2.3. Random-Amplified-Polymorphic-DNA Analysis (RAPD)

RAPD-PCR fingerprinting was carried out by applying the M13 minisatellite core primer (5'-GAGGGTGGCGGTTCT-3') [23]. The PCR reaction mixture contained equal amounts of 10 ng genomic DNA, 2 µl primer (10 pmol) and 10 µl of a 2x concentrated OneTaq polymerase master mix (New England Bio Labs, England). The thermal cycling protocol involved an initial denaturation step at 94°C for 7 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 42°C for 30 seconds, and extension at 68°C for 4 minutes. A final extension cycle was performed at 68°C for 10 minutes (modified after Sirichoat et al. [24]). PCR products were subjected to electrophoresis on a 2% agarose gel (AppliChem, Germany).

2.4. Hydrogen Peroxide Production

Hydrogen peroxide production was conducted following the method described by Tomás et al. [25]. Fresh cultures of each isolate were plated on mMRS agar plates supplemented with 1 mM 3,3',5,5'-Tetramethylbenzidin (TMB) and 2 U/ml of type II horseradish peroxidase (Carl Roth, Germany). Agar plates were incubated anaerobically for 48 hours at 30°C. Subsequently, the Petri dish lid was removed, exposing the cultures to fresh air for 10 minutes. Hydrogen production was visible when the yellowisch colonies turned black. Data were reproduced in triplicate.

2.5. In Vivo Detection of Antimicrobial Activity

Antimicrobial activities of P. pentosaceus isolates against foodborne pathogens and food spoilage organisms were evaluated with lawn-on-spot assays modified as described [26,27]. Isolates were inoculated from a liquid overnight culture by positioning 2 µl into the center of an MRS agar plate. Agar plates were incubated under anaerobic conditions at 30°C for 48 hours. The freshly grown colonies were overlaid with 10 ml of LB-soft agar (8%) or BHI-soft agar (8%) in the case of L. monocytogenes. Before pouring, indicator strains were applied by adding 0,2 ml of a cell suspension of $1{,}0 \times 10^7$ CFU/ml. Following a 20- to 30-hour co-cultivation at 37°C, the diameter of the zone of growth inhibition, from which the colony diameter was subtracted, was measured in millimeter. Acid secretion of the P. pediococcus isolates was determined with pH stripes. In this way, it was possible to measure changes of pH by comparing the pH in the inhibition zone with the pH of the indicator strain grown on the same media in the absence of a P. pediococcus isolate.

2.5. Whole Genome Sequencing

Genomic DNA was extracted using the Wizard HMW DNA Extraction Kit (Promega, USA) and quantified using a DeNovix QFC Fluorometer (DeNovix, United States). Sequencing libraries were prepared with the Rapid Sequencing Kit (SQK-RAD004) (ONT, GB). After that, samples of 30 µl were subjected to a Flongle Flow Cell R9.4.1 (FLO-FLG001) (ONT, GB) on a MinION Mk1B device (ONT, GB) with MinKNOW 22.08.9 [28].

2.6. Data Analyses and Statistics

Data analyses comprised subspecies identification, antimicrobial activity in vivo, and the relationship between antimicrobial compounds and acid secretion. To do so, the statistical and computing package R (v4.2.1) and RStudio (v2023.03.0+386) with packages tidyverse (v2.0.0), readxl

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(v1.4.2), ggplot2 (v3.4.2), vegan (2.6-4) and ggdendroplot (v0.1.0) [29–34]. Subspecies identification was based on the analysis of the data derived from RAPD-PCR DNA band patterns. Therefore, the unweighted pair group method with arithmetic mean (UPGMA) was used by using the hclust algorithm together with the Dice dissimilarity coefficient matrix [35,36] from the R base package. Phylogenetic trees were generated using a threshold of ≤ 5% dissimilarity to distinguish clonal isolates [37,35,38–40]. Strains were analyzed for in vivo antimicrobial activity via heatmapping and cluster analysis, by normalizing inhibition zone diameters across strains by z-score calculation. Hierarchical clustering of strains and indicators was performed using Euclidean distances and complete linkage in the hclust function, visualized through heatmap and dendrograms in ggplot2 and ggdendroplot. The above-mentioned statistical methods were also used to evaluate the data obtained from acidification experiments. Pearson correlation and scatterplot visualisation performed on the raw replicate values assessed the contributions of antimicrobial compounds and acid secretion at a 95% confidence level.

2.7. Bioinformatics Pipeline and Genome Assembly

The bioinformatics pipeline, managed by Snakemake [41], began with high-accuracy base-calling of raw ONT sequencing data via Guppy (v6.3.8) [42]. Porechop was used for adapter trimming [43], and Filtlong filtered out reads shorter than 1 kbp and the lowest 10% by quality [44]. Trycycler was then used for assembly, combining Flye, Raven, and Miniasm+MiniPolish to generate a consensus genome [45–49], with the help of MASH and MUSCLE [50–52], and final polishing by Medaka [53].

2.8. Genome Analyses

Gene annotations, chromosomal organization und circular maps were obtained using the bioinformatic tools Bakta, Prokka, and FastANI as implemented into the Proksee server [54–57]. A Venn-Diagram showing the core genome, the pan genomes and singleton genes was computed with EDGAR 3.0 on the basis of the gene annotation list created with Bakta [54,58]. Genes encoding bacteriocins were screened by uploading fasta files of chromosomes and plasmid DNA sequences into the BAGEL4 server [59].

3. Results

3.1. Isolation of Pediococcus Pentosaceus Strains from the Cattle Udder

In the course of a screening for novel food protective strains, we isolated several hundred microbial strains from the udder of milk cows from 15 farms located in Münsterland, Germany. Among those, we found 38 strains of *P. pentosaceus*, as demonstrated by complete DNA sequence identity of a 483 base pair fragment of the 16S rRNA-encoding gene (data not shown). One isolate, that produced the antibacterial compound hydrogen peroxide (data not shown), was excluded since such strains can alter the taste of food products by oxidation processes, an undesired characteristic for the use of protective cultures in foods [60,61].

3.2. Identification of Subspecies

We then raised the question about the genetic diversity of the obtained strains. Therefore, chromosomal DNA samples of all 37 *P. pentosaceus* isolates were subjected to RAPD-PCR. Representative band patterns of 21 isolates from farms 4 and 13 are shown in Figure 1.

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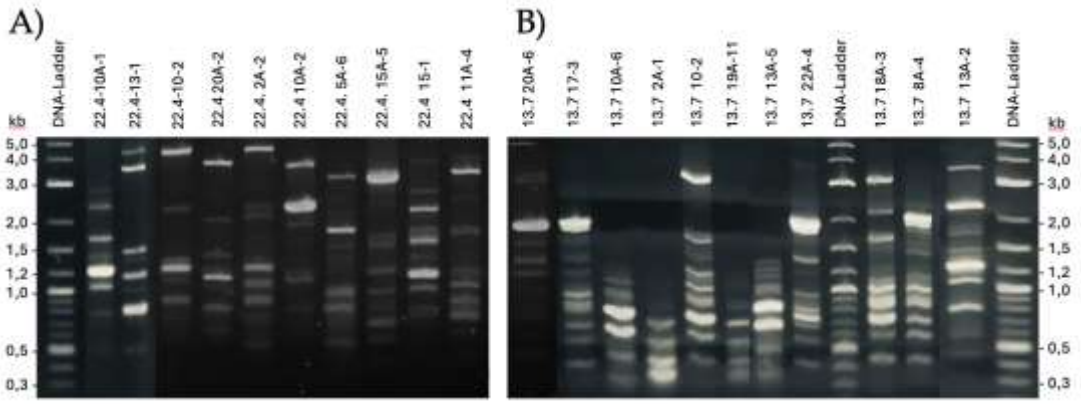

Figure 1. RAPD analyses. Shown are 2% agarose gels showing gene fragment pattern from RAPD analyses. Isolates from farm 4 are shown on the left, those from farm 13 are shown on the right.

A matrix of the fragment size distribution was generated and used for UPGMA cluster analyses to obtain phylogenetic trees of isolates within each farm (Figure 2). Across the four dairy farms, from which *P. pentosaceus* strains were obtained, the relative similarity ranged from 20% to 85%.

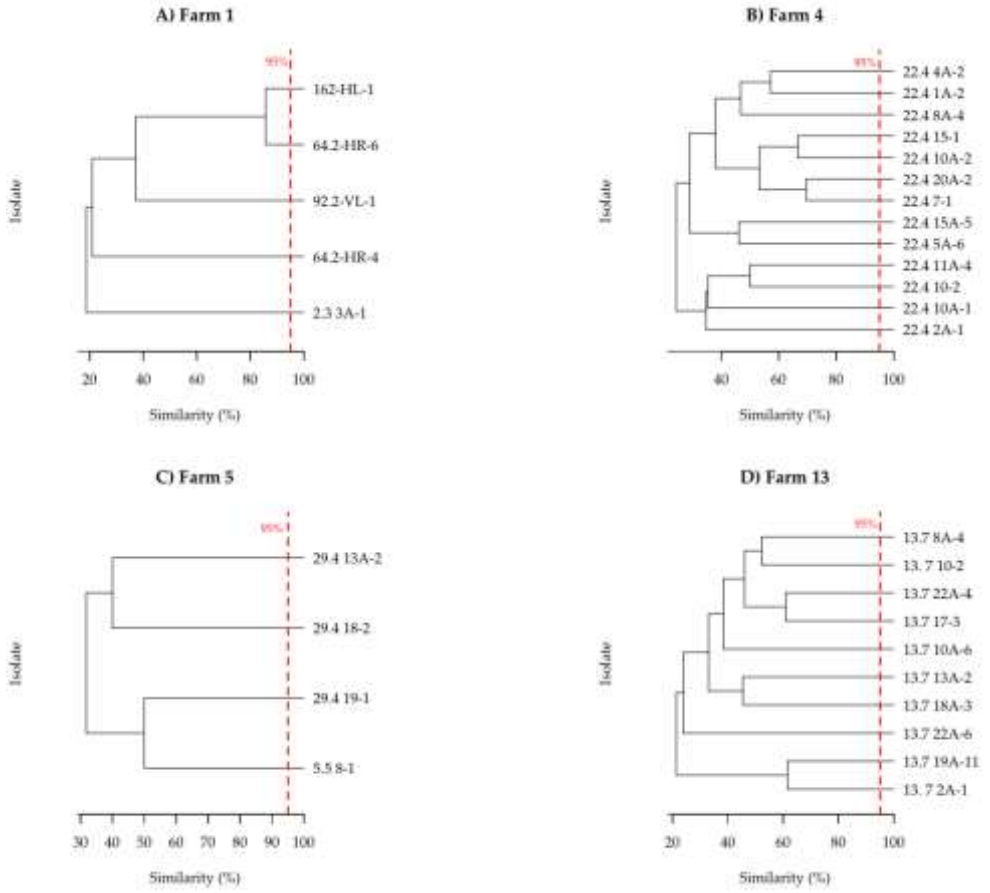

Figure 2. Depiction of the phylogenetic relationships within isolates from one farm. Relative phylogenetic distances are indicated by scale. The red line depicts the 95% similarity criterium to define a subspecies. A) farm 1 (n=5); B) farm 4 (n=16); C) farm 5 (n=5); D) farm 13 (n=11).

While the spatial proximity of isolates within the phylogenetic tree suggested a higher degree of genetic similarity and potential relatedness among individuals, none of the isolates within a farm

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exhibited a phylogenetic dissimilarity of less than 5%, the threshold to discriminate subspecies. Hence, all isolates were classified as a distinct subspecies within one farm.

3.3. Antimicrobial Profiles

To uncover the most important prerequisite for a protective culture, the antimicrobial potential, we took thirteen diverse isolates for further investigations. They were tested against a set of indicator strains comprising food pathogenic and spoilage bacteria and representative surrogates. A heatmap that was derived from inhibition zone sizes of conducted spot-on-lawn assays is presented in Figure 3. The data were normalized to ensure comparability. Based on the calculated z-values, indicator strains could be clustered according to their antibacterial profiles. Most susceptible were *Pseudomonas aeruginosa* and *Salmonella enterica* situated in the first subcluster on the left with inhibition zones of 34 and 56 millimeter.

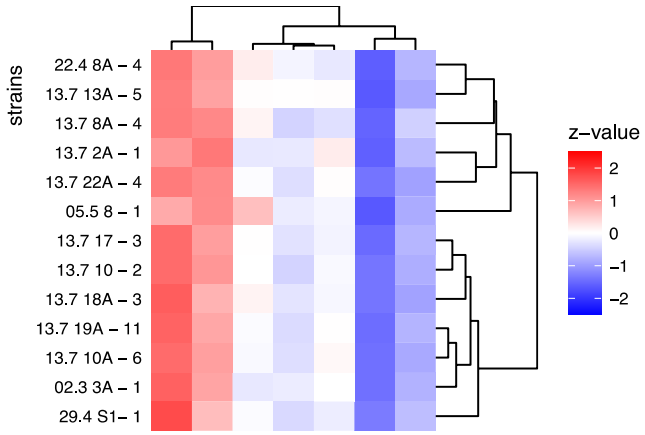

Figure 3. Heatmap of antibacterial activities. The color scale shows the normalized z-values. Indicators are ordered by falling susceptibility from left to right. The clustering on the right shows the subspecies of *P. pentosaceus* according to the similarity of their antibacterial profiles. Experiments were carried out in triplicate.

A second cluster of indicators comprised the gram-negative, enteric bacterium *Citrobacter koseri* and the two gram-positive *staphylococci*, ranging from inhibition 17 to 24 millimeter. While *C. koseri* was generally more sensitive to *P. pentosaceus* isolates, three of the thirteen isolates were more effective against the *Staphylococcus* strains. The antimicrobial effect of the third cluster, consisting of the gram-positives *L. monocytogenes* and *B. subtilis*, was below average. It is noteworthy that *L. monocytogenes* was generally susceptible to most isolates, especially to isolate 13.7.8A-4. The zones of inhibition for *L. monocytogenes* ranged from eight to 17 millimeter. The only indicator that showed very weak or in three cases even zero antibacterial activity was the food spoilage surrogate *B. subtilis*.

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3.4. Acidification

After the antimicrobial profiles were uncovered, it was crucial to know which isolates have a low capacity to produce acids, in order to avoid unwanted changes in the taste of food products. To monitor this, we examined the extent of acidification of *P. pentosaceus* isolates exposed to all indicator strains (Figure 4).

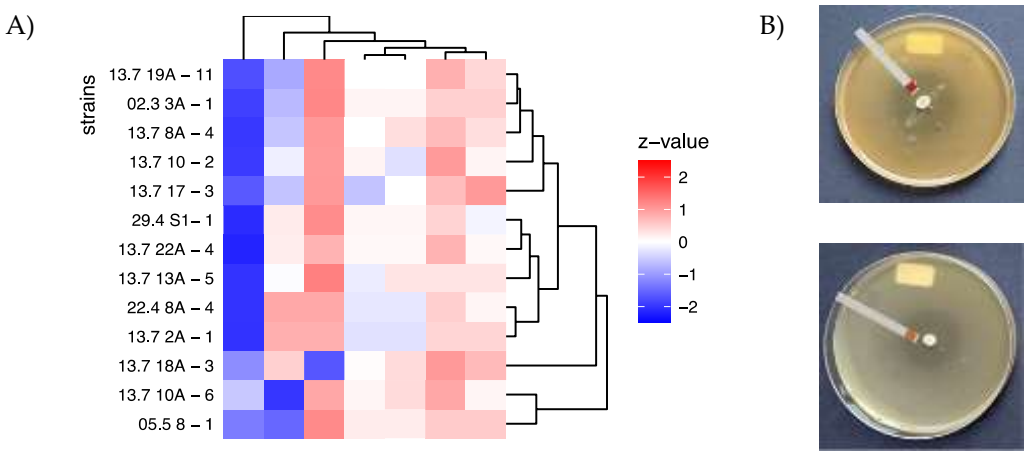

Figure 4. Heatmap of pH drop of *P. pentosaceus* isolates against indicator strains. The columns show the normalized z-values of acidification ranging from blue (strong)) to white (fair) to red (low). Indicator strains clustering is shown on the top, while clustering of the isolates is shown on the right. Data represent the average of triplicates.

Acidification of the medium in response to the indicator strains was considerably variable. Notably, the presence of *S. enterica* induced the strongest acidification by an average decrease in pH from 0.79 (± 0.1). By contrast, the presence of *C. koseri* yielded the mildest acidification with a pH drop ranging around 0.1 (± 0.08) units. All isolates behaved quite similar in acidification with an average of pH reduction of 0.26 (±0.04) units.

To elucidate the influence of acid secretion on the measured antimicrobial activity, a Pearson correlation analysis was performed, and non-linear correlations were visualized through scatterplot analysis. Figure 5A shows that acid secretion had a slight but significant effect on the measured antimicrobial activity for *Listeria monocytogenes*, with a correlation coefficient of -0.33 and a p-value of 0.042. No other indicator organism demonstrated sensitivity solely through acid secretion.

Out of the thirteen isolates, five revealed a significant moderate to strong correlation between acid secretion and measured antimicrobial activity across all indicators. These were the isolates 02.3 3A-1, 13.7 10A-6, 13.7 17-3, 13.7 9A-11, and 13.7 8A-4 (Figure 5B).

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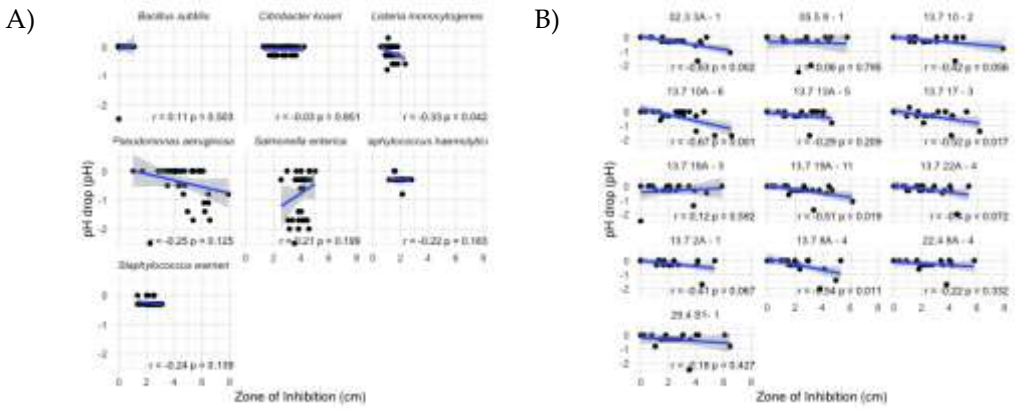

Figure 5. Pearson correlation analysis. Shown is the drop in pH versus the inhibition zone for A) indicators and B) *P. pentosaceus* isolates. r, correlation coefficient; p, p-value significance of <0,05.

Assuming that a protective culture should effectively inhibit a broad spectrum of food pathogens and food spoilage microorganisms without affecting the flavor and texture of the food products, isolates were ranked as shown in Table 2. The criteria included above-average inhibition of the number of indicator species combined with below-average acidification.

Table 2. Ranking of isolates regarding antibacterial activity.

ranking	strain	No. inhibited pathogens above average	sum	mean	p_value
1	13.7 13A - 5	5	2.465265	0.3521807	0.20896
2	05.5 8 - 1	3	2.728224	0.3897463	0.79546
3	13.7 2A - 1	3	2.654947	0.3792781	0.06718
4	13.7 10 - 2	3	2.612546	0.3732209	0.05576
5	22.4 8A - 4	3	2.607411	0.3724873	0.33180
6	13.7 22A - 4	3	2.606640	0.3723771	0.07161
7	13.7 18A - 3	3	2.577932	0.3682761	0.59202
8	29.4 S1- 1	2	2.506960	0.3581371	0.42734

The best performing strain was isolate 13.7 13A-5, which inhibited five out of seven indicator bacteria above average, followed by isolates that inhibited three indicator species above average. Any isolate could be selected from this group, considering the specific properties of the food in question and the most critical species to be controlled.

3.6. Genome Analyses

Since, and that was somehow surprising, all isolates were according to our RAPD analyses greater than 5% dissimilar, they were classified as subspecies [39]. We sequenced the genomes of the

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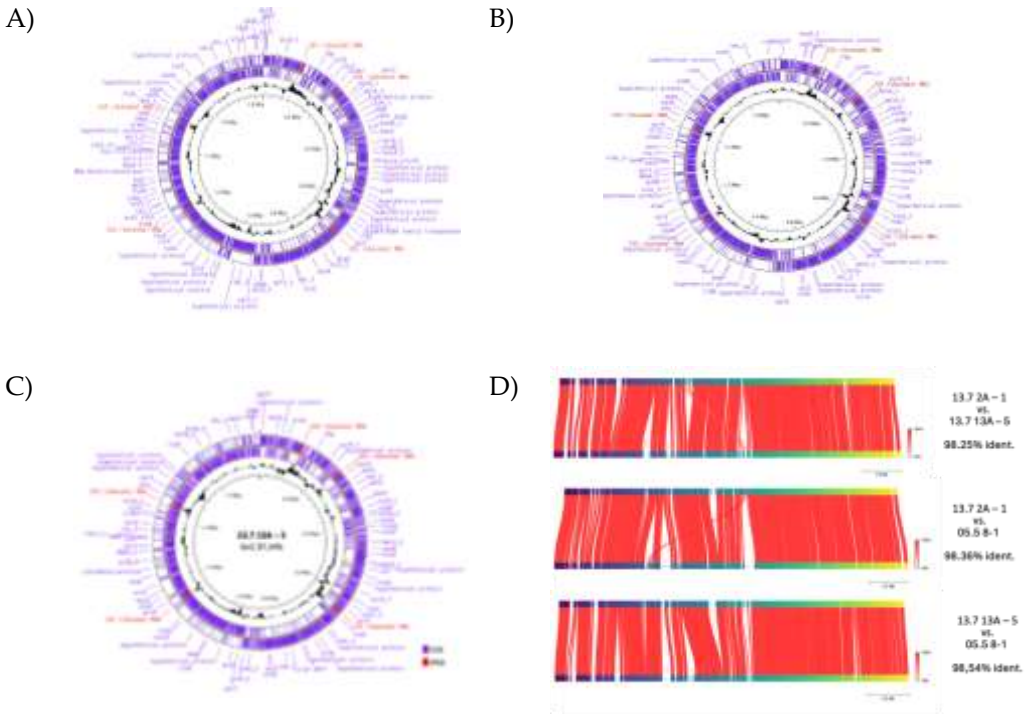
best three performing candidates to proof and substantiate this at the molecular level (Table 2). The chromosomes of the isolates differed already in size, namely 1.771.607 bp, 1.731.150 bp, and 1.835.763 bp for isolates 13.7 13A5, 05.5 8-1, and 13.7 2A-1, respectively. The isolates harbored unequal numbers of three, five, and six plasmids (Table 3). Another measure was comparing the relative locations of homologous genes present in all three genomes. Therefore, we determined the distance from base one of the circular maps, the first nucleotide of the *dnaA* gene, by taking three genes positioned at around three, six, and nine o'clock of the chromosomal map (Table 2; Figure 6A-C).

Table 3. Key data of the genomes.

strain	13.7 13A–5	05.5 8-1	13.7 2A-1
genome (bp)	1771607	1731150	1835763
predicted genes	1,816	1,914	2,332
plasmids (bp)	12,144; 12,513; 29,159	13,332; 20,517; 21,974; 24,254; 68,459	12,153; 16,367; 22,192; 36,437; 42,648; 44,253
G+C content (%)	37.14	37.41	37.11
uvrA[1]	490,671	464,679	459,969
polC[1]	856,007	806,61	867,588
secY[1]	1,320,181	1,287,919	1,381,749

[1] position gene start.

Beside the genome sizes and different cellular plasmid numbers and types, the genomic plasticity became apparent if the distance of homologous genes was reviewed. From the circular genome maps, we could infer that the gene order was overall the same, which can be easily seen by the locations of the five 23S ribosomal DNA gene cluster labeled in red (Fig. 6A-C) and the relative distance in base pairs from chromosome base one to *uvrA*, *polC*, and *secY*, respectively (Table 2).



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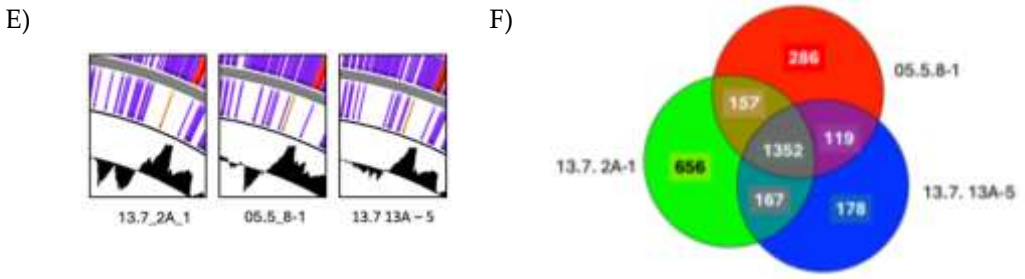

Figure 6. Genomic landscape. (A-C) Shown are the chromosomal maps of the three indicated *P.pentosaceus* genomes. Genes are in blue, the five 23S rRNA gene cluster are labeled red. (D) FastANI diagrams showing shifts of gene segments or insertion or deletions of binary genome comparisons. (E) Sections from each genome map around the first rRNA region (red) show gene insertions visible by the G+C content graph (black). (F) Venn diagram showing the numbers of common genes between the genomes.

It is a quite common event in evolution that the genetic plasticity between subspecies is caused by horizontal gene transfer and transposable elements giving insertions of genes from other species that can be identified by regions of higher or lower G+C-content, that differ from the chromosomal G+C content of the investigated genome. This was observed as shown by the black areas in Figure 6E. Two areas of distinct lower G+C content occur in the genome on the left, one area in the middle genome, and none in the genome on the right, while the patterns are very similar further downstream. A Venn-diagram was computed showing the core set of 1352 genes present in all three genomes (Figure 6F). Strain 13.7.2A-1 had the largest genome with six inclusive plasmids. It possesses an astonishing number of 656 genes that are not present in the other two. Hence, the genome data substantiate the RAPD experiments und demonstrate the genomic plasticity of the three here in depth examined *pediococcal* isolates.

3.7. Bacteriocin Encoding Genes

The chromosomal and plasmidal DNA sequences were screened for the presence of bacteriocin-encoding genes using the BAGEL4-server, which, to our knowledge, emphasizes the most comprehensive bacteriocin database. As shown in Figure 7, gene regions were detected within the chromosomes of 13.7.13A-5 and 13.7.2A-1 that harbor a putative gene for the bacteriocin penocin A (light green). Directly upstream on the left is a gene associated that might confer immunity to penocin A (red). Two open reading frames are situated downstream. They encode a putative two-component system with a histidine kinase and a response regulator gene. The four genes upstream encode a putative PEP:dependent phosphotransferase system (PTS) for the consumption of ß-glucosides and a gene encoding a IIA^{Glc} homolog of the PTS, which serves in many bacteria in glucose transport and down-regulation of second choice carbon sources when glucose is available [6].

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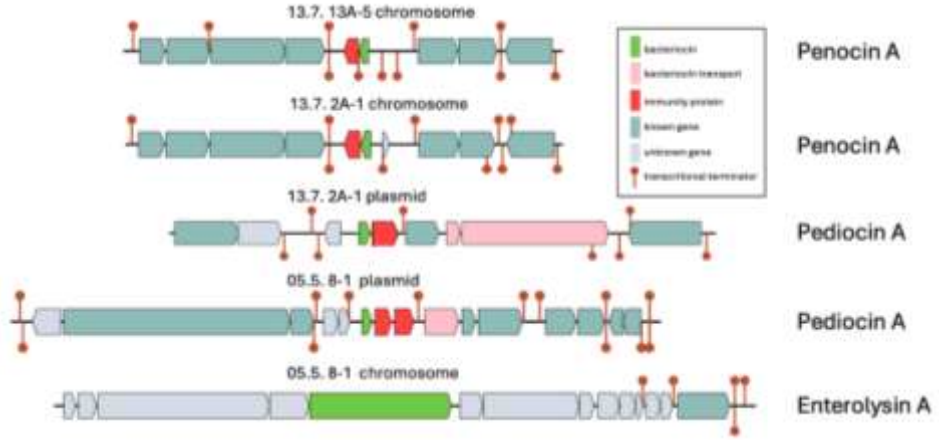
A)



B)

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13.7.13A-5c*   MTEIKVLNDKELKNVVGGKYYGNGVHCGKKTCYVDWGQATASIGKIIVNGWTQHGPWAHR--   Penocin A
13.7.2A-1c*    MTEIKVLNDKELKNVVGGKYYGNGVHCGKKTCYVDWGQATASIGKIIVNGWTQHGPWAHR--   Penocin A
13.7.2A-1p**   MKKIEKLTEKEMANIIGGKYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC   Pediocin A
05.5.8-1p**    MKKIEKLTEKEMANIIGGKYYGNGLYCGKHSCSVDWGKATTCIINNGAMAWATGGHQGTHKC   Pediocin A
               *.:*: *.:**: *::*********: ***::* ****:**:.* : . .*:  *  . :
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Figure 7. A) Genetic regions of genes involved in bacteriocin metabolism. B) Multiple alignment of bacteriocin protein sequences. The conserved sequence motifs "YGNGV(L) including the two functional cysteines are shown in red. *chromosomal; **plasmidial. The consensus sequence below shows identical (*) and conserved (:, .) amino acid positions.

A second bacteriocin gene for pediocin A was found on a plasmid of isolate 13.7.2A-1, showing that this isolate has two bacteriocins at its disposal. Isolate 05.5. 8-1, however, harbors a chromosomal gene locus for enterolysin A, a class III bacteriocin, and on one plasmid a locus with putative genes for pediocin A, an immunity protein, and genes that could encode an exporter for the secretion of the bacteriocin. The gene products of the plasmid gene loci were almost identical regarding pediocins A and the immunity proteins sharing 95% and 98% amino acid identities, respectively. The surrounding genes, including some for bacteriocin export (pink), were quite dissimilar. When all four bacteriocin proteins were aligned, they exhibited an overall protein sequence identity of 45% harboring the conserved motif "YGNGV(L)" and two conserved cysteins that form disulfite-bridges to determine the three-dimensional structures of these class II bacteriocins.

4. Discussion

The incorporation of protective cultures in food is increasing to meet the challenges of food safety and enhance food security [3,62]. Due to the diverse metabolic performance of LAB regarding the inhibition of pathogens, species from the order *lactobacillales* are of particular interest [3,18]. It has been reported that the LAB *P. pentosaceus* can serve in food protection and that isolates from different environments behave different, since they adapt genetically to the respective biotope from which they have been isolated [16]. To uncover novel *P. pentosaceus* strains for food protection, we retrieved from a typical LAB ecosystem, the udder of milk cows. LAB from milk have a long tradition to serve as the prime starter cultures for food fermentation giving taste and furthermore providing food preservation over months. Our data on 37 isolates of *P. pentosaceus* strains revealed that they effectively could combat pathogenic bacteria. However, there were differences as to the extent and to the ability regarding the inhibition of the challenged pathogens.

Pediococci have been isolated from milk, dairy products, fermented vegetables, sausages, or even aquatic products [12,16,63]. The data of the isolates derived from RAPD experiments showed throughout genetic variations suggesting that all isolates represent subspecies. In a previous study, although in less detail, comparable results from *pediococci* were reported for plant-derived LAB from traditional Ethiopian foods such as tef dough and kocho [64]. Quite similar results were reported when *L. plantarum* strains were compared [65]. Rossetti and Giraffa analyzed more than thousand

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LAB strains from raw cheese [23]. They established a databank of RAPD-profiles which can be used to identify an unknown isolate just by the gene fragment pattern of RAPD. However, *P. pentosaceus* strains were not included. The here presented data add to this. It is to our knowledge, the first of a set of *P. pentosaceus* isolates from one environment.

We found that all isolates showed antibacterial activity against a series of food pathogens and surrogates. The isolate *P. pentosaceus* 13.7 13A-5 was the best promising one. It was able to inhibit five out of indicators above average. The second-best performing isolates were a group of six that inhibited three out of seven above averages. The usage of the antimicrobial potential of *P. pentosaceus* in foods has been addressed recently on one *P. pentosaceus* and one *Pediococcus acidilactici* isolate from silage [66]. It was reported that they could combat 74 *Listeria monocytogenes* isolates and 27 different *enterococci* resistant to vancomycin. Furthermore, it was shown that these isolates inhibited many fungal species, most likely by several anti-fungal metabolites produced by this strain [66]. In another study, addition of a mixture of *P. pentosaceus*, *P. acidilactici*, and *L. plantarum* to alfalfa sprouts reduced the presence of *L. monocytogenes* by 4.5 log and of *S. enterica* by 1.0 log [67]. In accordance with our observation that isolates of the same species may have different antimicrobial profiles, Santini *et. al.* published that different isolates of strains of LAB and bifidobacteria exhibited different efficacies against three *Campylobacter* species [68]. Numerous reports focused on the role of purified bacteriocins produced by *Pediococci*. Especially, the presence of pediocin and pediocin-like bacteriocins from *P. pentosaceus* in meat products has been thoroughly examined [69].

The production of acid by a protective culture is an important issue to combat unwanted microorganisms. However, too much acidification may change the organoleptic characteristics of the food product [70,71]. We like to highlight that acid production not only depended on the respective *P. pentosaceus* isolate, but also on the presence of the competing strain, in this case the applied indicator strain. Our approach to measure the acidification capacity of each isolate against each indicator allowed us to select for weak acid producers while maintaining antimicrobial efficacy.

Finally, the genomes of the three best performing isolates were elucidated. The bioinformatic analyses corroborated the genetic plasticity of the *P. pentosaceus* isolates, and thus support the data obtained from RAPD analyses on all isolates. The analysis of the core genome shows this in striking detail. Only 1352 common genes occurred in the three genomes, while 178, 286, and 656, respectively, were singletons [58]. These findings were supported when we counter-checked available *P. pediococcus* genomes in the databases [63]. The number of plasmids of the sequenced isolates was three, five and six ranging from 12 kb to 68 kb. Plasmids carry often operons encoding genes for the metabolization of unusual carbon sources, bacteriocin-encoding genes, and transposable elements [72]. This can be explained by the adaptation to the various environments in which *Pediococci* occur [63]. Genome analysis for bacteriocin genes revealed that the strains harbor chromosomal and/or plasmidial genes for the bacteriocins pediocin A and penocin A together with genes for immunity to and export of the bacteriocin. The respective genes, genetic organization and functions have been characterized in molecular detail . The genetic surroundings, i.e. the presence of a carbohydrate:phosphotransferase system (PTS) for metabolism of the ß-glucosides and the PTS factor IIA^{Glc} that controls the utilization of carbon sources is commonly associated with the pen operon [63,73,74]. The operons found in our sequenced genomes are conventionally present in many isolates of *P. pentosaceus* from diverse environments [75–78].

Although it is appealing to suggest that the action of bacteriocins is a major factor to push back unwanted microorganisms in food products, it should be noted that they might not always be expressed [79,80]. Expression greatly depends on growth conditions and is influenced by pH, temperature, quorum sensing, and the availability of carbon sources, essential amino acids, vitamins and energy [81]. In such cases other compounds such as diacetyl or chemicals like phenyllactate that lower the rH-Value could account for the protective action [3,18]. The answer to this question must be given by a proof of principle for each foodstuff containing an effective protective culture.

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5. Conclusions

We showed that the isolation of *P. pentosaceus* strains from different milk cattle of different dairy farms revealed a high biodiversity. All isolates exhibited inhibitory activity against diverse pathogenic or food spoiling bacteria making them applicable in food products as protective cultures. Hence, the derived data can be used to select the matching isolate for a specific food product that needs a robust food safety along its shelf-life.

Our study reveals that it is worthwhile to set up a screening that includes several isolates of one species for a specific application, in this case for food protection. Our approach might be used as a blueprint for similar purposes such as the choice of the best starter culture as food fermentater and/or as a probiotic health-promoter.

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