Isolation of antimicrobial-producing bacteria from artisanal cheeses and characterization of potentially novel antimicrobial agents produced

Gabriella Gephart

Bachelor of Science in Food Science and Technology

Research Advisor: Dr. Ahmed Yousef

Research Mentor: Dr. Ahmed Abdelhamid

Abstract

Synthetic preservatives are traditionally used in food processing to enhance the safety and quality of a product. Recently, consumers have been demanding natural alternatives to these synthetic preservatives. Few bacteria produce antimicrobial peptides that can be used as food preservatives. This study aimed to isolate beneficial antimicrobial-producing bacteria from artisanal cheeses, with the long-term goal of characterizing promising antimicrobial agents produced by these bacteria. Screening for antimicrobial activity was done using a bioassay via a cellulose grid; this was followed by a quantitative bioassay using a microdilution approach. Isolates identities were determined using 16S rRNA sequencing and whole genome sequencing was used to evaluate genomes for biosynthetic gene clusters associated with antimicrobial production. Eight isolates were found to have strong antimicrobial activity on the bioassays, and biosynthetic gene clusters associated with antimicrobial production were found within their genomes. These clusters could be encoding for potentially novel antimicrobial peptides. These strains should continue to be studied to confirm novelty and determine structure and use of their peptides in food.

Introduction

Preservatives are often used in foods to enhance the quality and safety of a product. Traditional synthetic preservatives include nitrates, nitrites, sulfites, sorbates, benzoates, and many others. These preservatives have a largely negative consumer perception. Nitrates have been associated with an increased risk of cancer, which has led consumers to avoid buying products that contain it (Chazelas et al, 2022).

In recent years the demand for natural preservatives has greatly increased due to consumers becoming more health conscious. Few bacteria produce antimicrobial peptides that have the potential to be used as natural food preservatives. These peptides would help to satisfy consumer

demand for natural preservatives while ensuring product quality and safety (Gerst and Yousef 2018). The most well-known antimicrobial peptide used for food preservation is nisin (Hansen and Sandine, 1994). Nisin is produced by *Lactococcus lactis*, a common starter culture used in cheese making. Nisin is FDA approved and is effective against Gram-positive bacteria. There is not yet an FDA approved antimicrobial peptide effective against Gram-negative bacteria; therefore, there is a demand for this class of peptides in the food industry. This study focuses on isolating antimicrobial-producing bacteria from artisanal cheese. Artisanal cheeses were selected due to their diverse and unique microbiota, hence, the high likelihood for novel antimicrobial peptides, produced by these microbiota, that would be safe for human consumption.

Objective

The objective was to isolate and identify antimicrobial-producing bacterial strains from artisanal cheese and characterize their novel antimicrobial agents.

Methods

Sample collection and isolation

Artisanal cheeses were collected from small producers in Columbus, Ohio. A representative sample was taken from each cheese and was homogenized with warm sodium citrate to release cheese microbiota. Serial dilutions were prepared using sterile saline and 0.1mL of each dilution was spread-plated onto deMan, Rogosa, Sharpe (MRS) agar. Plates were incubated at 30°C for 24-72 hours until colony growth was sufficient. Colonies were streaked for isolation onto MRS agar and incubated at 30°C for 24-72 hours. A stock of each culture was prepared in 25% glycerol and stored at -80°C freezer for future use.

Bioassay using cellulose grid

Cellulose grid filters were placed on MRS plates, one plate for *Escherichia coli* and another plate for *Listeria innocua*. A colony, from each isolated strains, was transferred onto the grid using a loop. The grid plates were incubated for 48 hours at 30°C. Grids were removed from plates and discarded using sterile forceps. Tryptic soy agar (TSA) semi-solid (soft) overlays were prepared. Aliquots (10µl each) of *Escherichia coli* overnight culture was mixed with 10mL of soft TSA and poured onto its respective plate. 10ul of *Listeria innocua* overnight culture was mixed with 10mL of soft TSA and poured onto its respective plate. The overlay plates were allowed to solidify and were incubated for 24 hours at 37°C. The plates were then observed for clearing of indicator microorganisms in the spots where colonies on the grid were located.

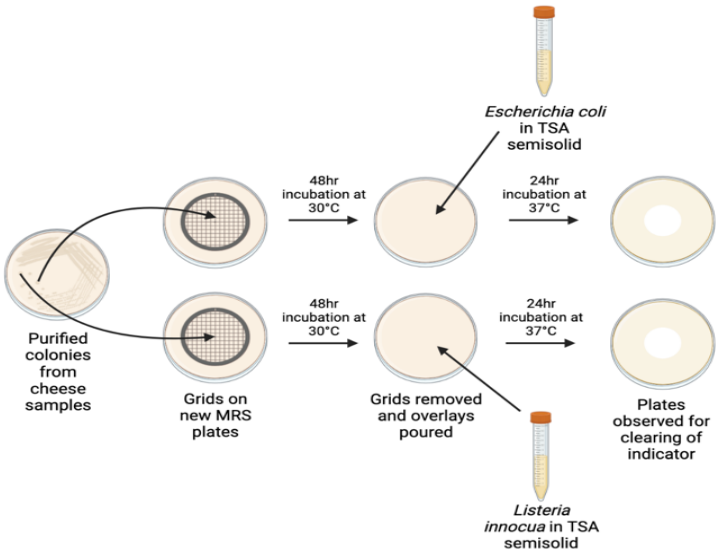

Figure 1: bioassay using cellulose grid method. Created in Biorender.com.

Quantitative bioassay

Isolates were grown in MRS broth, supplemented with 1% yeast extract (YE), with and without 125 rpm of shaking for 24 hours at 30°C. The liquid cultures were then centrifuged and the supernatant was passed through a 0.46-µm filter to create a cell-free supernatant. A series of two-fold dilutions was done in 96-well microtiter plates using the cell free supernatants and MRS-YE broth as a diluent. Each isolate underwent identical dilutions for each indicator microorganism. *E. coli* and *L. innocua* were diluted 1:100 in sterile saline and 25-µl aliquot of each diluted indicator

organism was added to its respective wells. The positive control was 25µl of indicator and 125µl of media, and the negative control was 125µl of media. The plates were incubated for 14 hours and OD_{600} readings were taken on a plate reader following incubation. Antimicrobial activity was calculated using equation 1 after data analysis. D is the highest dilution that inhibited indicator growth.

Bacteriocin activity determined as arbitrary units (AU)/mL = (1000/125) x (1/D) (Equation 1)

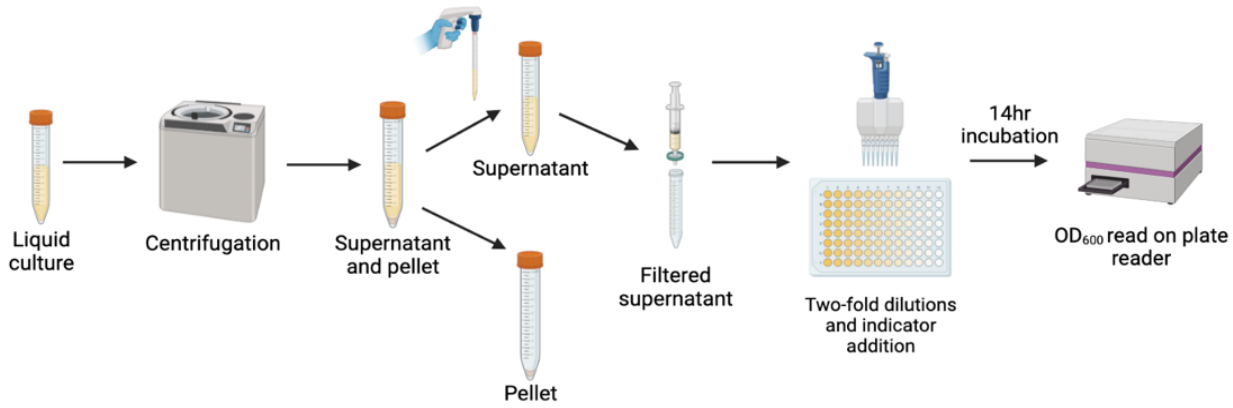

Figure 2: quantitative bioassay method. Created in Biorender.com.

16S rRNA sequencing

Isolates were grown in MRS broth for 24 hours at 30°C. DNA was extracted using the Qiagen DNA extraction kit. Following DNA extraction, PCR was performed using 16S forward and reverse primers. The PCR product underwent gel electrophoresis, and the bands were cut and purified using Qiagen's gel purification kit. Samples were prepared and Sanger sequenced at the Genomics Shared Resource at The Ohio State University. For isolate identification, genomes were compared against the NCBI nucleotide database using the Basic Local Alignment Search Tool (BLAST).

Whole genome sequencing

Whole genomes were sequencing using the Illumina MiSeq platform and the resulting FASTQ files were analyzed.

Genome mining techniques

Genomes were assessed for quality using FASTQC. Genomes were assembled using SPAdes. Genome-based discovery of biosynthetic gene clusters associated with antimicrobial production was done using antiSMASH and Bagel4 software.

Results

Bioassay using cellulose grid results

Sixteen isolates from 12 cheeses showed zones of inhibition against *E. coli*, *L. innocua* or both. These zones ranged from 5mm to 35mm in diameter indicating strong antimicrobial activity.

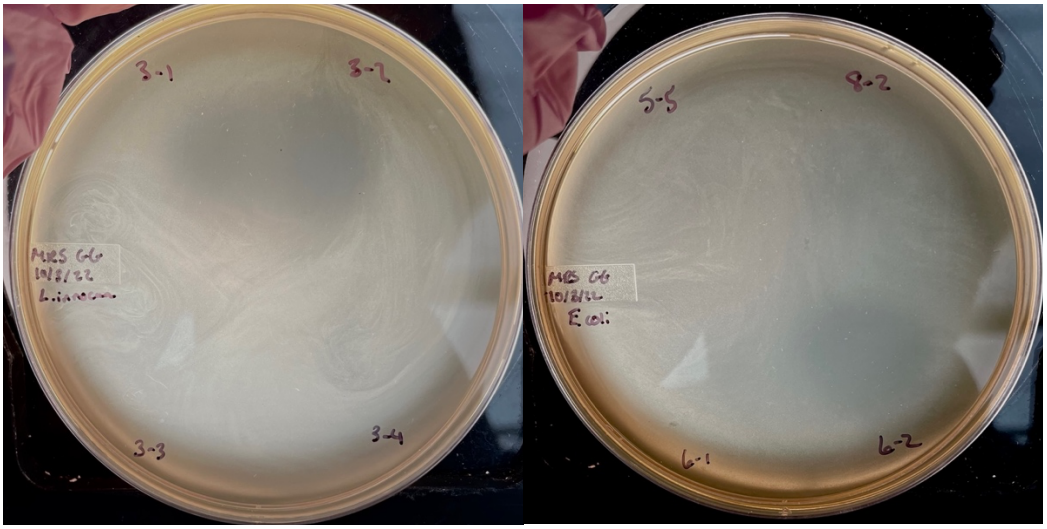

Figure 3: Right, zone of inhibition from isolate 6-2 against *E. coli.* Left, zones of inhibition from isolates 3-1 and 3-2 against *L. innocua*

Quantitative bioassay results

As shown in figure four, most isolates' cell-free supernatants inhibited both indicators. It can be seen that *E. coli* was inhibited more than *L. innocua* by most isolates. As supernatants became more dilute, the inhibition lessened for all isolates.

Isolates 3-1 and 3-2 showed almost no antimicrobial activity when grown without shaking but showed strong antimicrobial activity when grown with shaking. Isolates 3-2, 4-1, 5-4 and 11-2 showed the strongest broad-spectrum antimicrobial activity. Isolates 3-2, 5-4 and 11-2 had the highest antimicrobial activity while grown with shaking while 4-1 had more antimicrobial activity without shaking. These four isolates each produced 256 AU/mL of inhibition against *E. coli* and 64 AU/mL of inhibition against *L. innocua*. pH 4 and 5 buffers, served as negative controls, and both did not inhibit indicator bacteria.

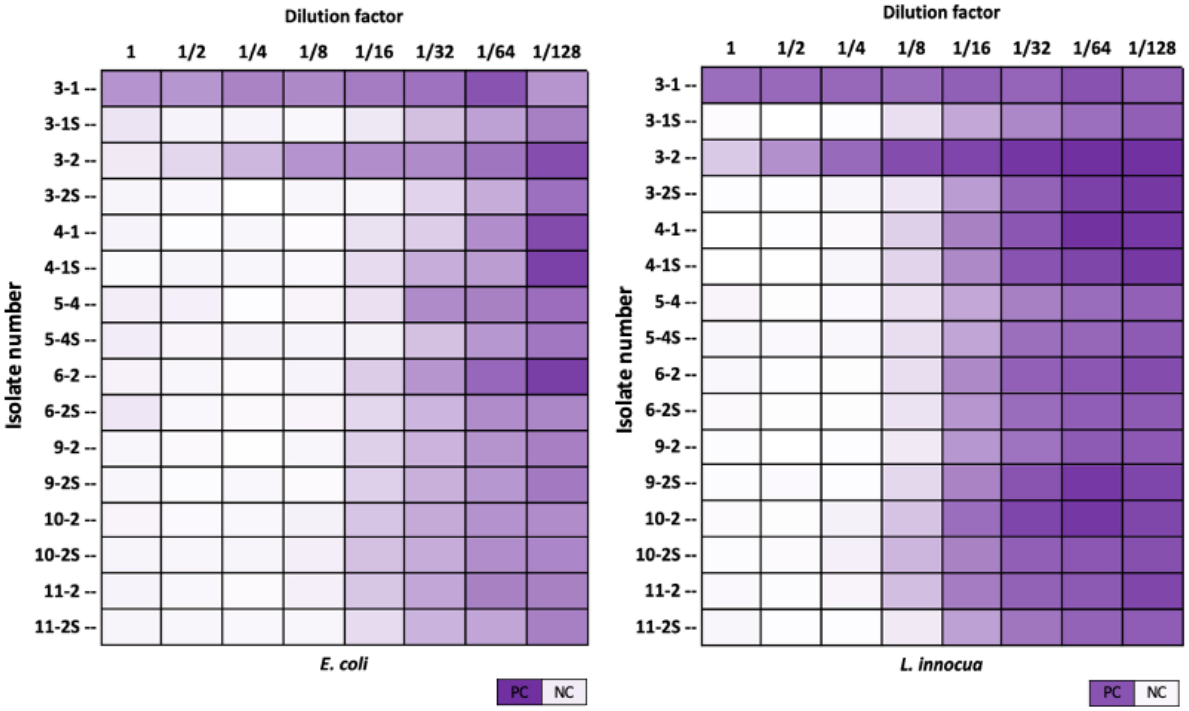

Figure 4: Left, heat map showing cell-free supernatant two-fold dilutions against *E. coli.* Right, heat map showing cell-free supernatant two-fold dilutions against *L. innocua.* An "S" next to the isolate number indicates it was grown with 125rpm of shaking.

Table 1: identities of isolates from 16S rRNA sequencing and cheese sources.

Isolate #	Isolate identity	Strain identifier	Cheese source
3-1	*Lactobacillus paracasei*	OSY-GG31	Amish sharp cheddar
3-2	*Lactobacillus paracasei*	OSY-GG32	Amish sharp cheddar
4-1	*Lactococcus lactis*	OSY-GG41	Stout beer washed
5-4	*Leuconostoc mesenteroides*	OSY-GG54	Bloomy rind
6-2	*Leuconostoc mesenteroides*	OSY-GG62	Cave-aged cheddar
9-2	*Lactococcus lactis*	OSY-GG92	Raw sheep's milk
10-2	*Enterococcus faecium*	OSY-GG102	Raw sheep's milk
11-2	*Leuconostoc pseudomesenteroides*	OSY-GG112	Amish basil mozzarella

Whole genome sequencing results

Whole genome sequencing produced extremely high-quality genomes. All isolates had Phred quality scores of at least 37 for both forward and reverse reads. AntiSMASH software revealed multiple biosynthetic gene clusters across all isolates that encode potentially novel antimicrobial peptides.

Conclusion

Antimicrobial activity was detected in eight isolates. Antimicrobials exhibited up to 256 AU/mL of inhibition against *E. coli* and up to 64 AU/mL of inhibition against *L. innocua.* Genome mining revealed ribosomally-synthesized and post-translationally modified class II bacteriocins produced by the isolates. Strong anti-Gram-negative activity was detected. Natural anti-Gram-negative compounds are in high demand for food preservation. These peptides should continue to be studied to determine their novelty and potential application in food.

References

Eloi Chazelas, Fabrice Pierre, Nathalie Druesne-Pecollo, Younes Esseddik, Fabien Szabo de Edelenyi, Cédric Agaesse, Alexandre De Sa, Rebecca Lutchia, Stéphane Gigandet, Bernard Srour, Charlotte Debras, Inge Huybrechts, Chantal Julia, Emmanuelle Kesse-Guyot, Benjamin Allès, Pilar Galan, Serge Hercberg, Mélanie Deschasaux-Tanguy, Mathilde Touvier, Nitrites and nitrates from food additives and natural sources and cancer risk: results from the NutriNet-Santé cohort, *International Journal of Epidemiology*, 51:1106–1119. https://doi.org/10.1093/ije/dyac046

Gerst M, Yousef A (2018) Modified microassay for the isolation of antimicrobial-producing, spore-forming and nonspore-forming bacteria. *J. Appl. Microbiol*. 124:1401-1410. https://doi.org/10.1111/jam.13724.

Hansen N & Sandine WE (1994) Nisin as a model food preservative, *Critical Reviews in Food Science and Nutrition*, 34: 69-93. https://doi.org/10.1080/10408399409527650