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Title: Evaluation of Microbial Safety of Dairies using Bacterial Proteomic Profiling via MALDI Approach

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **NO**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **YES, all done**

3. **Filming location:** Will the filming need to take place in multiple locations? **NO**

4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **NO**

Current Protocol Length
Number of Steps: 23
Number of Shots: 44

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

1.1. **Paweł Pomastowski:** We developed a protocol to prepare and isolate microbial communities from various dairy products, such as milk, cream, butter, and cheese. Once isolated, these microorganisms can be rapidly and accurately identified using MALDI-TOF MS.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.3*

What are the current experimental challenges?

1.2. **Ewelina Sibińska:** Optimizing culture conditions remains a key challenge, along with database gaps and the demanding extraction of Gram-positive and spore-forming bacteria.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

What advantage does your protocol offer compared to other techniques?

1.3. **Michał Złoch:** By combining culturomics with tailored sample preparation, MALDI enables more precise and faster microbiome analysis at lower cost, producing results comparable to the reference 16S rRNA method.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested B-roll: 3.10.1*

How will your findings advance research in your field?

1.4. **Michał Złoch:** This protocol enables routine, high-throughput monitoring of microorganisms in dairy products, improving detection speed and accuracy and thereby strengthening food quality and safety systems.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.2.1*

What questions will future research focus on?

1.5. **Paweł Pomastowski:** Expanding MALDI databases and automating workflows in industry labs will enhance microbial identification, increase throughput, reduce errors, and speed microbiota monitoring.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Isolation of Dairy-Related Bacteria

Demonstrator: Sibińska Ewelina

2.1. To begin, obtain a representative sample of the dairy product and mix thoroughly to ensure uniformity **[1]**. Aseptically transfer 1 milliliter or 1 gram of solid samples into a 15-milliliter conical centrifuge tube containing 9 milliliters of sterile liquid medium **[2]**.

2.1.1. WIDE: Talent shows the dairy sample and mixes it.

2.1.2. Talent using a pipette or sterile scoop to transfer the sample into a 15 milliliter conical centrifuge tube containing the sterile solution.

2.2. Vortex the tube vigorously for 30 to 60 seconds to homogenize the sample **[1]**.

2.2.1. Talent vortexing the centrifuge tube containing the sample and sterile solution.

2.3. Next, prepare a series of ten-fold dilutions by transferring 1 milliliter of the homogenized suspension into successive tubes, each containing 9 milliliters of sterile solution **[1]**. Mix each dilution thoroughly by vortexing or shaking **[2]**.

2.3.1. Talent pipetting 1 milliliter from the homogenized tube into a new tube containing 9 milliliters of sterile solution.

2.3.2. Talent mixing each dilution thoroughly by vortexing.

2.4. Then take 0.1 milliliter of the original sample or one of the prepared dilutions and transfer it onto the surface of a prepared Petri dish containing the appropriate agar medium **[1]**. Using a sterile spreader, evenly distribute the inoculum over the surface of the agar plate **[2]**.

2.4.1. Talent pipetting 0.1 milliliter of the sample or dilution and dispensing it onto the agar plate surface.

2.4.2. Talent using a sterile spreader to uniformly spread the inoculum over the agar surface.

2.5. For aerobic incubation, inoculate APT *(A-P-T)*, M-17 *(M-Seventeen)*, CBL *(C-B-L)*, MPCA *(M-P-C-A)*, and TSA *(T-S-A)* agar plates with the prepared samples **[1]**. Incubate the plates under aerobic conditions at 37 degrees Celsius **[2]** and 30 degrees Celsius for 24 hours **[3]**. **NOTE**: VO adjusted for the extra shot

2.5.1. Shot of the inoculated APT, M-17, CBL, MPCA, and TSA agar plates.

2.5.2. Talent placing the inoculated plates into incubators set at 37 degrees Celsius.

Added shot 2.5.2A: Talent placing the inoculated plates into incubators set at 30 degrees Celsius.

2.6. For anaerobic incubation, place the inoculated MRS agar plates in an anaerobic chamber and incubate at 37 degrees Celsius and 30 degrees Celsius for 24 to 72 hours **[1]**. Remove the plates from the incubator **[2]** and examine them daily to monitor microbial growth **[3-TXT]**. **NOTE**: VO adjusted for the extra shot

2.6.1. Talent placing the inoculated plates into an anaerobic chamber or jar, then setting the temperature.

2.6.2. Talent opening the chamber and taking out the MRS plates.

Added shot 2.6.2A: Talent visually inspecting the MRS plates for microbial growth. **TXT: Select colonies from the incubated plates for purification**

2.7. Using a 1 microliter microbial loop, streak the selected colonies onto the same agar medium to subculture them **[1]**. Incubate under the same conditions used previously **[3]**.

2.7.1. Talent using a 1 microliter microbial loop to streak the colonies onto fresh agar plates.

2.7.2. Talent placing the subcultured plates back into the appropriate incubator.

3. Bacterial Samples Preparation for MS Analysis

3.1. For mass spectrometry analysis, begin by preparing the MALDI matrix solution **[1]**.

3.1.1. Talent organizing required materials for MALDI matrix solution preparation on a lab bench.

3.2. Prepare the solvent mixture using 50% acetonitrile, 47.5% HPLC-grade water, and 2.5% trifluoroacetic acid **[1]**. Dissolve the matrix compound into the solvent mixture to a final concentration of approximately 10 milligrams per milliliter **[2]**.

3.2.1. Talent adding the solvents in the specified proportions inside a labeled container.

3.2.2. Talent adding the matrix to the prepared solvent and stirring.

3.3. For MS identification of bacteria using the Direct Colony Transfer method, use a sterile loop to transfer 1 microliter of a grown bacterial colony onto a spot on the MALDI target

plate **[1-TXT]**. Spread the bacterial material to create a thin, even layer on the plate **[2]**. Once dried, add 1 microliter of HCCA *(H-C-C-A)* matrix solution on top and allow it to air dry at room temperature **[3]**.

3.3.1. Talent picking up a small colony using a sterile loop and placing it on the MALDI plate.

3.3.2. Talent spreading the bacterial sample into a thin, even layer on the MALDI plate.

3.3.3. Talent pipetting 1 microliter of HCCA matrix solution onto the dried sample spot and letting it air dry.

3.4. For the On Target Formic Acid Extraction method, use a disposable microbial loop or sterile toothpick to pick a small amount of bacterial biomass from a single colony **[1]**. Smear the material directly onto a spot on the MALDI target plate to create a thin, even layer **[2]**. Add 1 microliter of 70 percent formic acid solution over the smeared area **[3]**.

3.4.1. Talent collecting bacterial biomass from a colony using a sterile toothpick.

3.4.2. Talent smearing the biomass evenly onto the MALDI target plate.

3.4.3. Talent pipetting 1 microliter of 70 percent formic acid onto the smear.

3.5. Once the spot has dried, overlay it with 1 microliter of HCCA matrix solution and allow it to air dry at room temperature **[1]**.

3.5.1. Talent pipetting 1 microliter of HCCA matrix solution onto the dried formic acid-treated spot.

3.6. For the In-Tube Protein Extraction method, transfer 1 to 3 complete colonies from an agar culture plate into a 1.5-milliliter microcentrifuge tube containing 300 microliters of sterile water **[1]**. Add 900 microliters of absolute ethanol to achieve a final ethanol concentration of approximately 75% **[2]**. After mixing the content briefly, centrifuge the tube for 2 minutes at 15,000 *g* at room temperature **[3]**.

3.6.1. Talent transferring colonies into a microcentrifuge tube containing sterile water.

3.6.2. Talent adding ethanol into the tube.

3.6.3. Talent placing the tube in a centrifuge and starting a spin at 15,000 g.

3.7. Afterward, carefully discard the supernatant without disturbing the cell pellet **[1-TXT]**.

3.7.1. Talent decanting or pipetting off the supernatant without disrupting the pellet.
TXT: Allow the pellet to dry at RT

3.8. Then, add 20 to 50 microliters of 70 percent formic acid to the dried cell pellet **[1]**. Mix

the contents by pipetting or vortexing until the pellet is fully resuspended **[2]**.

3.8.1. Talent pipetting formic acid into the microcentrifuge tube containing the dried pellet.

3.8.2. Talent mixing the pellet by pipetting or vortexing until it is completely dissolved.

3.9. Now, add an equal volume of acetonitrile to the formic acid suspension and mix thoroughly **[1]**. Centrifuge the tube at 15,000 *g* for 2 minutes **[2]**.

3.9.1. Talent adding acetonitrile to the formic acid mixture and gently mixing the contents.

3.9.2. Talent placing the tube in the centrifuge and starting the spin.

3.10. Transfer 1 microliter of the resulting supernatant to a designated spot on a MALDI target plate and allow it to dry **[1]**. Then, overlay the dried spot with 1 microliter of HCCA matrix solution **[2-TXT]**.

3.10.1. Talent pipetting 1 microliter of the clear supernatant onto the MALDI target plate.

3.10.2. Talent adding 1 microliter of HCCA matrix solution onto the dried sample spot. **TXT: Allow the matrix to dry before MS analysis**

4. Spectra Acquisition and Microbial Identification using the MS Technique

Demonstrator: Złoch Michał

4.1. Pipette 1 microliter of either the Bacterial Test Standard, containing an extract of *Escherichia coli* DH5 alpha, or the Microbiology Calibrator, containing *E. coli* ATCC *(A-T-C-C)* 25922 *(Two-Five-Nine-Two-Two)* protein extract, ribonuclease, and myoglobin **[1].** For spectra acquisition and microbial identification, prepare calibration spots on the MALDI target plate **[2]**.

4.1.2. Talent pipetting 1 microliter of the appropriate calibrant standard onto designated calibration spots on the plate.

4.1.1. Talent setting up a MALDI target plate for calibration. **NOTE**: The shots and VO are inverted

4.2. Load the MALDI target plate with both the prepared sample spots and the calibrant spots into the mass spectrometry instrument **[1]**. Run the calibration analysis in calibration mode to calibrate the system **[2]**.

4.2.1. Talent placing the MALDI plate into the instrument's loading dock.

4.2.2. SCREEN: step-4.2.2.mp4: 00:02-00:14

4.3. Operate the mass spectrometer in linear positive ion mode for general spectra acquisition **[1]**. Then, set the mass detection range of the mass spectrometer to cover from 2,000 to 20,000 mass-to-charge ratio **[2]**.

4.3.1. SCREEN: step-4.3.mp4: 00:00-00:08

4.3.2. SCREEN: step-4.3.mp4: 00:08-00:14

4.4. ~~Measure at least 2 different spots for each bacterial isolate to ensure sample representation **[1]**~~. Acquire spectra in duplicate for each spot to generate at least 4 technical replicates per sample **[1-TXT]**.

~~4.4.1. Talent placing two sample spots per isolate on the MALDI target plate.~~ **NOTE**: VO is moved as on screen text for the deleted shot

4.4.2. SCREEN: step-4.4.2.mp4: 00:04-00:17 **TXT: Measure at least 2 different spots for each bacterial isolate**

4.5. Now, apply the Savitzky-Golay method as a smoothing algorithm. Use the TopHat algorithm for baseline correction to remove background noise. Then, detect peaks using centroid mode **[1]**.

4.5.1. SCREEN: step-4.5.mp4

4.6. Upload the processed mass spectra of unknown bacterial isolates to the microbial identification platform within the MALDI software and run the identification **[2]**. Evaluate the identification scores according to the manufacturer's threshold standards **[2]**.

4.6.1. SCREEN: step-4.6.mp4: 00:05-00:25

4.6.2. SCREEN: step-4.6.mp4: 00:45-00:54

Results

5. Results

5.1. The mass spectrometry spectra displayed species-specific protein profiles, enabling the identification at the genus or species level **[1].**

5.1.1. LAB MEDIA: Figure 4. *Video editor: Highlight the line "Lactobacillus plantarum DSM 1055 DSM" with the log score value of 2.240 in the species list*.

5.2. The dendrogram revealed high similarity observed among strains of the same species and clear differentiation between species **[1].**

5.2.1. LAB MEDIA: Figure 5.

5.3. A shorter incubation time of 12 hours for *Bacillus licheniformis* produced a higher ID score value of 2.38 **[1]**, while a longer incubation of 18 to 24 hours led to a lower ID score value of 1.85 due to the dominance of spore signals **[2].**

5.3.1. LAB MEDIA: Figure 6. *Video editor: Highlight the lower mass spectrum labeled "Shorter incubation of Bacillus licheniformis (12h)" with ID score 2.38.*

5.3.2. LAB MEDIA: Figure 6. *Video editor: Highlight the upper mass spectrum labeled "Long incubation of Bacillus licheniformis (18–24h)" with ID score 1.85 and the annotation "intensive signal from spores".*

5.4. Among the sample preparation methods, in-tube protein extraction gave the highest ID score value of 2.23 **[1],** followed by on-target formic acid extraction at 1.97 **[2],** and direct colony transfer which resulted in the lowest score of 1.61 **[3].**

5.4.1. LAB MEDIA: Figure 7. *Video editor: Highlight the mass spectrum labeled "In-Tube Protein Extraction" with ID score 2.23 in green.*

5.4.2. LAB MEDIA: Figure 7. *Video editor: Highlight the spectrum labeled "On-Target Formic Acid Extraction" with ID score 1.97 in yellow.*

5.4.3. LAB MEDIA: Figure 7. *Video editor: Highlight the spectrum labeled "Direct Colony Transfer" with ID score 1.61 in red.*

- **conical**

Pronunciation link:
https://www.merriam-webster.com/dictionary/conical
IPA: /ˈkɑn·ɪ·kəl/
Phonetic spelling: **KAH-nih-kəl**

- **centrifuge**

Pronunciation link:
https://www.merriam-webster.com/dictionary/centrifuge
IPA: /ˈsɛn·trəˌfjuʒ/
Phonetic spelling: **SEN-truh-fyoohj**

- **homogenize**

Pronunciation link:
https://www.merriam-webster.com/dictionary/homogenize
IPA: /həˈmɑ·dʒəˌnaɪz/
Phonetic spelling: **huh-MAHJ-uh-nyz**

- **inoculum**

Pronunciation link:
https://www.merriam-webster.com/dictionary/inoculum
IPA: /ɪˈnɑ·kjə·ləm/
Phonetic spelling: **ih-NAHK-yuh-luhm**

- **anaerobic**

Pronunciation link:
https://www.merriam-webster.com/dictionary/anaerobic
IPA: /ˌæn·əˈroʊ·bɪk/
Phonetic spelling: **AN-uh-ROH-bik**

- **spectra**

Pronunciation link:
https://www.merriam-webster.com/dictionary/spectra
IPA: /ˈspɛk·trə/
Phonetic spelling: **SPEK-truh**

- **Savitzky-Golay**

Pronunciation link:
(No confirmed entry in Merriam-Webster)
IPA: /səˈvɪts·ki goʊˈleɪ/
Phonetic spelling: **suh-VITS-kee goh-LAY**

- **centroid**

Pronunciation link:
https://www.merriam-webster.com/dictionary/centroid

IPA: /ˈsɛn·trɔɪd/
Phonetic spelling: **SEN-troyd**