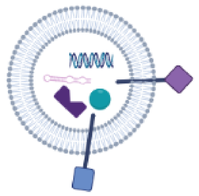
Isolation and Analysis of Extracellular Vesicles from Lactic Acid Bacteria

L. Brooke Busby, Isabel Myers, Klea Hoxha, Gabriela C. Pérez-Alvarado, and Brian M. Lee
Department of Chemistry, Coastal Carolina University, Conway, SC 29528

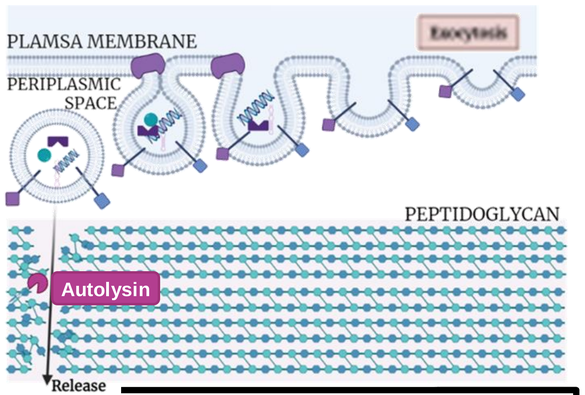
Introduction



Membrane-derived extracellular vesicles (EVs) are produced by both eukaryotic and prokaryotic cells and mediate intercellular communication. In Gram-negative bacteria, EVs can be derived from the outer membrane, but in Gram-positive bacteria it was thought that the thick layer of peptide-glycan would prohibit their release.

More recent studies have shown that EVs can be produced by Gram-positive bacteria. These EVs range from 20 to 200 nm and may contain proteins, lipids, nucleic acids (DNA and RNA) and other small molecules. EVs may interact with cell surface receptors to initiate intracellular signaling cascades or be absorbed through endocytosis to release regulatory factors within the host cells.[1-4]

Mechanism of EV Production and Release



Function of EVs:

- ❑ Detoxification of environmental stress
- ❑ Cellular and host communication
- ❑ Elimination of competitors
- ❑ Nutrition sensing
- ❑ Virulence

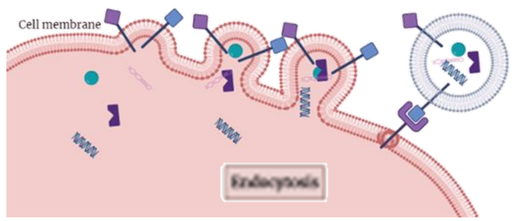
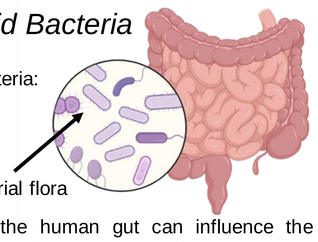


Figure 1: The process of production, autolysis and release of extracellular vesicles, which can then be taken up by surrounding cells (*Adapted from Briaud and Ronan*).[5-6]

Lactic Acid Bacteria

(non-pathogenic) Probiotic Bacteria:

- ❑ *Streptococcus thermophilus*
- ❑ *Lactobacillus acidophilus*
- ❑ *Lactobacillus bulgaricus*



The microbiome found within the human gut can influence the immune response as well as brain functions with effects on mood, cognition, and mental health. EVs produced by the bacterial flora inhabiting the human gut may be found in both blood and urine. Of interests are probiotic bacteria that produce effector molecules with anti-allergy, anti-inflammation, and cancer-inhibiting effects.[6-7]

Results

S. thermophilus Growth Cultures

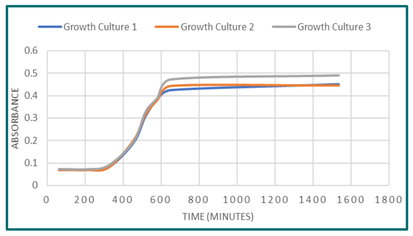


Figure 2: Growth Curve of cultures grown in M17 media with 0.5% lactose at 37 °C. The average doubling time during exponential growth phase is 90.8 min.

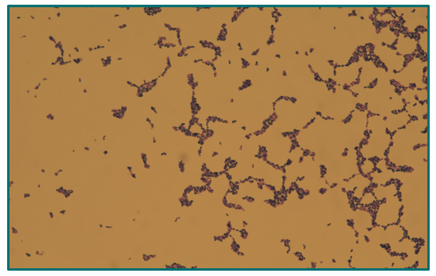
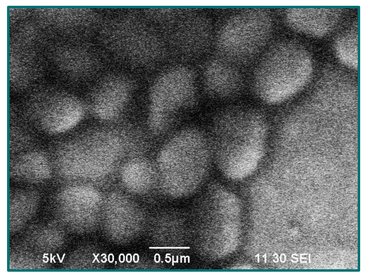


Figure 3: Gram stain of *S. thermophilus* colony grown on M17 agar with 0.5% lactose. Magnification at 1000X.

Scanning Electron Microscopy (SEM)



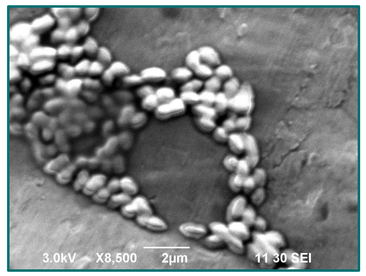


Figure 4: *S. thermophilus* cells fixed by formaldehyde, placed on aluminum foil circles (7 mm) and dehydrated with ethanol. Cells were harvested from culture at 1.0 OD_{600}.

A. 0.5 µm resolution
B. 2 µm resolution

Process Diagram for the Isolation of Extracellular Vesicles

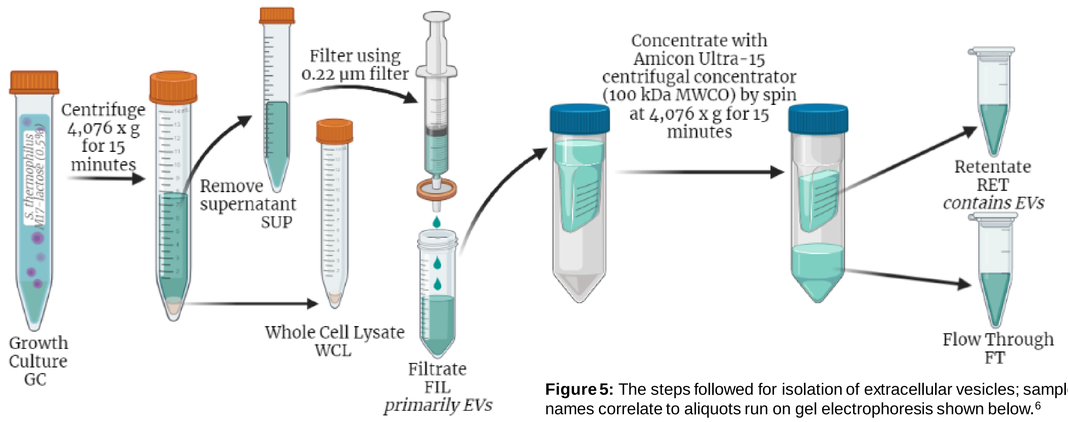


Figure 5: The steps followed for isolation of extracellular vesicles; sample names correlate to aliquots run on gel electrophoresis shown below.[6]

Polyacrylamide Gel Electrophoretic Analysis of Aliquots

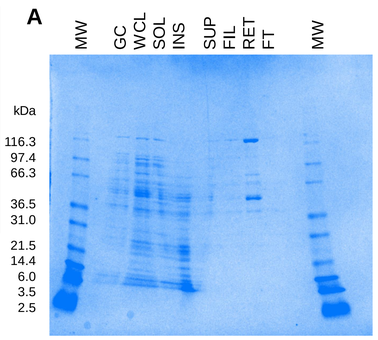
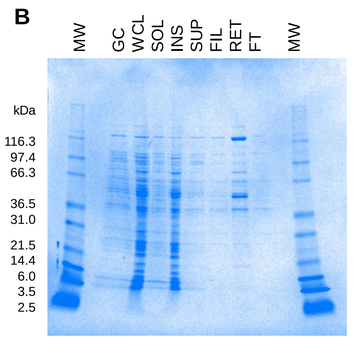


Figure 6:
4-20% Tris-Glycine SDS-PAGE
A. Exponential Growth Phase 0.548 OD_{600} at harvest.

B. Lag Growth Phase 1.300 OD_{600} at harvest

Molecular Weight Marker – MW
Growth Culture aliquot – GC
Whole Cell Lysate – WCL
BPER soluble portion – SOL
BPER insoluble portion – INS
Supernatant – SUP
Filtrate – FIL
Retentate – RET
Flow Through – FT



Methods and Materials

Streptococcus thermophilus (ATCC 19258) glycerol stocks were used to isolate individual colonies by streaking on plates M17 agar with 0.5% lactose grown at 37 °C. Selected colonies were used to inoculated overnight starter cultures, which were then diluted 5-fold for 10 mL growth cultures in M17 broth with 0.5% lactose incubated at 37 °C. Incubation was done under anaerobic conditions using an anaerobic jar, oxygen absorber CO_2 generator and oxygen indicator (AnaeroPack, Mitsubishi). At log phase (0.548 OD_{600}) and late log, stationary phase (1.30 OD_{600}), 1 mL aliquots were removed for growth culture (GC), whole-cell lysate (WCL), and soluble/insoluble fractionation with B-PER reagent (Pierce). The WCL aliquot, B-PER aliquot and remaining culture (6 mL) were harvested by centrifugation at 4,076 x *g*. The WCL pellet was resuspend in SDS sample buffer (Novex). The B-PER pellet was treated with B-PER reagent to separate into soluble (SOL) and insoluble (INS) fractions before adding SDS sample buffer to 1X final concentration. The supernatant of the remaining culture was decanted and after removing an aliquot (SUP), was filtered through a 0.22 µm membrane (Millipore) to remove any bacterial cell contaminants (FIL). The filtrate was then concentrated with an Amicon Ultra-15 centrifugal concentrator with a 100 kDa cut-off membrane (Millipore). The retentate and an aliquot of the flow through used for gel after adding SDS sample buffer. SDS-PAGE gels for the exponential growth phase aliquots and stationary phase aliquots were run using 4-20% gradient gels in Tris-Glycine buffer (Invitrogen/Novex) and stained with Coomassie brilliant blue R-250 (BioRad). Gels images were taken with Gel-Doc XR+ gel documentation center with trans-white light conversion screen (BioRad).

The Gram stain image of *S. thermophilus* cells was prepared from one colony selected from the M17 agar streak plate and transferred to a glass slide. Heat-fixed cells were treated with crystal violet then washed with water. Cells were treated with Gram's iodine and then washed with water. The slide was then treated with decolorizing agent and counterstained with safranin. After a final wash with water, the sample was blotted dry and covered with a plastic coverslip. The micrograph image was taken at 1000X under oil emersion with an Olympus BX51 fluorescence microscope.

The SEM sample was prepared from a growth culture of *S. thermophilus* with a 750 µL aliquot removed at 1.0 OD600 and fixed with 5% formaldehyde for 10 minutes at room temperature. Fixed cells were harvested at 4,000 x *g* and resuspended in ultrapure water (MilliQ) after washing once with water. Serial dilutions in water were prepared at 10^2, 10^4 and 10^6 dilutions. For each sample, a 10 µL aliquot was placed on an aluminum foil circle (7 mm) and airdried overnight. Samples were dehydrated with ethanol. The SEM micrograph was taken with a Jeol JSM-6490LV scanning electron microscope. Images were taken under vacuum at 3 kV and 5 kV electron beam strengths.

Conclusion

A difference can be observed between the protein content of samples taken throughout the process of isolating EVs. Distinct bands are observed around 50 kDa and 120 kDa in the retentate aliquot after concentration with a 100 kDa cut-off membrane. These bands can be attributed to either large or multimeric proteins secreted from the bacterial cells or proteins packaged within the membrane-derived extracellular vesicles.

Future Studies

Future studies will include the isolation of EVs from other lactic acid bacteria, such as *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*. Our aim is to identify the proteins found in EVs and compare the EV content from different lactic acid bacteria. Further methods development will include using a mini bead mill homogenizer to improve lysis of Gram-positive bacterial cells and using gel filtration (size-exclusion) chromatography to separate EVs from large secreted proteins that may remain in the retentate sample. We hope to observe budding of EVs using SEM, which will require improving the sample preparation by fixing the bacterial cells on poly-L-lysine coated glass slides and using sputtering with gold or gold-palladium for increased contrast. We are working on techniques for RNA preparation of sample aliquots to run by native PAGE to visualize RNA molecules found in EVs at various growth stages. Our aim is to identify small RNA transcripts in bacterial EVs that regulate host cell activities that affect the immune response and mental health functions.

References

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