The Use of Plant Bioactives to Control and Eradicate Multi-Species Biofilms containing *Listeria monocytogenes*

Alexa Jollimore[1,*], Rafael Garduno[1,2], Vasantha Rupasinghe[1]

[1] Department of Plant, Food, and Environmental Sciences, Faculty of Agriculture, Dalhousie University, Truro, Nova Scotia, Canada

[2] Microbiology Research Section, Canadian Food Inspection Agency, Dartmouth, Nova Scotia, Canada

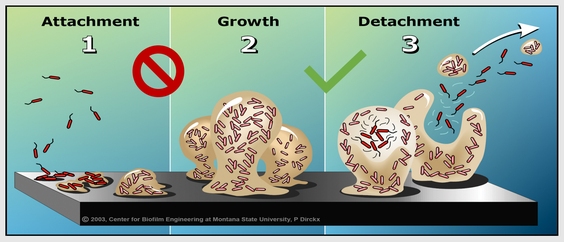
* alexa.jollimore@dal.ca

Abstract

- *Listeria monocytogenes* causes the foodborne illness listeriosis that is associated with a high mortality rate.
- Biofilms are bacterial protective modes of living, enabling microorganism to survive adverse environmental conditions.
- Biofilms enhance *L. monocytogenes* persistence.
- Interest in substances that remove biofilms (including plant secondary metabolites) is increasing.
- Biofilm biomass can be indirectly measured by a crystal violet (CV) binding assay.
- Using the CV assay we assessed biofilm formation by pasteurized milk microbiota and the ability of plant secondary metabolites to remove preformed biofilms.
- The most efficient plant secondary metabolites against biofilms were tea tree and coriander essential oils, thymol, carvacrol, and eugenol.
- However, when tested in a real-life scenario with industrial milk dispensing pumps they were not effective as biofilm removers.
- Future investigation on how to incorporate plant secondary metabolites into cleaning protocols for the industrial milk dispensers is needed.

Introduction

- *Listeria monocytogenes* is among the most serious food safety challenges for the food industry as it is able to survive and replicate at low temperatures [1].
- Therefore ready-to-eat foods from dairy, meat and fish have been categorized as high-risk products for implication of *L. monocytogenes* associated foodborne outbreaks [1].
- Main route of contamination occurs in food processing facilities, where *L. monocytogenes* has attached to food-contact surfaces, such as stainless steel, polystyrene and glass and can persist for several years [1].
- All strains of *L. monocytogenes* are able to persist in the form of biofilms, which allow increased resistance to sanitization practices commonly used in food processing facilities [2].
- As food products have their own microflora, biofilms found in food processing facilities are multi-species which makes the biofilm removal harder than mono-species biofilms [3].
- Multi-species biofilms encourage the attachment and replication of *L. monocytogenes*, also resulting in increased resistances to cleaning practices [4].
- Many plant secondary metabolites have antibiofilm properties that may lead to use of these compounds in food processing facilities to remove problematic biofilms [5] (Figure 1).



https://nodawaynews.com/why-your-immune-system-and-most-prescription-drugs-cant-kill-mold/biofilm-formation-in-three-steps/

Figure 1: Plant secondary metabolites prevent ( ) attachment and growth of biofilms and encourages ( ) detachment and dispersal of preformed biofilms

Methods

Enumeration of *L. monocytogenes* in free standing pumps stored at 4°C

- Commercial milk dispensing units provided by SureShot, were used to pump 2% M.F pasteurized milk inoculated with 1 CFU/ml of *L. monocytogenes* (Figure 2).
- Once milk was incorporated into the entire pump, a 100ml sample was obtained from the pumps twice daily for the duration of the experiment.
- On day 0, 3, 6 and 9, the first sample of the day was diluted and plated onto selective agar for enumeration of *L. monocytogenes* (Results shown in Figure 4).



Figure 2: Images of free standing pumps as well as their dispensing units

Growth kinetics of milk microbiota in 2% M.F pasteurized milk stored at 4°C

- 200 µl of 2% M.F pasteurized milk, 180 µl of 2% M.F pasteurized milk plus 20 µl of Cosmic Serum™(positive control), or 200 µl of boiled 2% M.F pasteurized milk (negative control) was added to a well in a polystyrene 96-well plate .
- At each time point, two wells from each condition were emptied washed with phosphate buffered saline (PBS).
- Crystal violet assay was performed as previously described [6] to quantify biomass (Figure 3).
- Absorbance was measured in a CLARIOstar™ microplate reader (results shown in Figure 5).

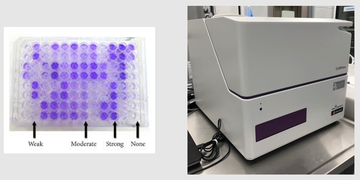


Figure 3: An example of the crystal violet assay performed in a 96 well microtiter plate and the CLARIOstar ™ plate reader

Plant secondary metabolites to remove biofilm containing *L. monocytogenes* in 96-well plates

- Mature biofilms were allowed to form for 74 h in a 96-well plate using milk inoculated with 100 CFU/ml of *L. monocytogenes* .
- Milk was then removed from wells and replaced with selected plant secondary metabolites at either a high or low concentration and with exposure times of 1 hour or 5 hours.
- Crystal violet assay was performed to quantify remaining biomass (results shown in Figure 6).

Plant secondary metabolites to remove milk microbiota biofilm in free standing pumps

- Mature milk microbiota biofilms were formed in free standing pumps.
- Milk was replaced with selected plant secondary metabolites in high concentration for 1 hour exposure.
- Crystal violet assay was performed to quantify remaining biomass (results shown in Figure 7).

Results

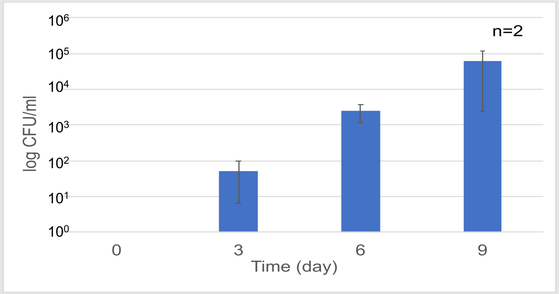


Figure 4: Enumeration of *L. monocytogenes* in free standing pumps stored at 4°C containing 2% M.F pasteurized milk over time

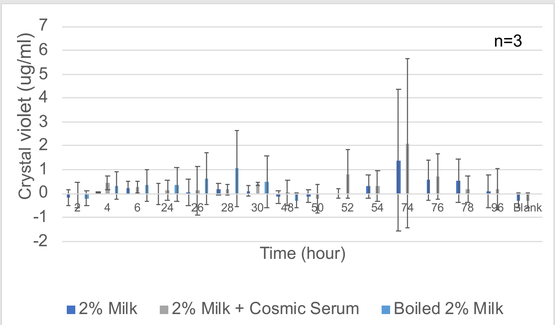


Figure 5: Growth kinetics of 2% M.F pasteurized milk, with the addition of Cosmic Serum™ and boiled milk over time using the crystal violet assay. The crystal violet method can indirectly quantify biofilm biomass.

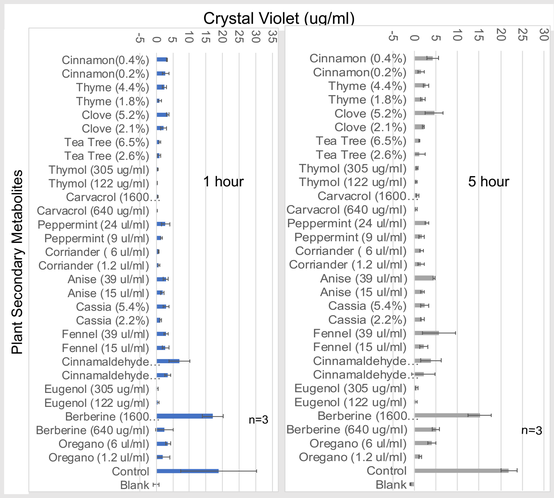


Figure 6: Measurement of the removal of biofilm form from 2% M.F pasteurized milk and *L. monocytogenes* in 96 well microtiter plates after 1 hour and 5 hour exposure to secondary plant metabolites using the crystal violet assay. The crystal violet method can indirectly quantify biofilm biomass

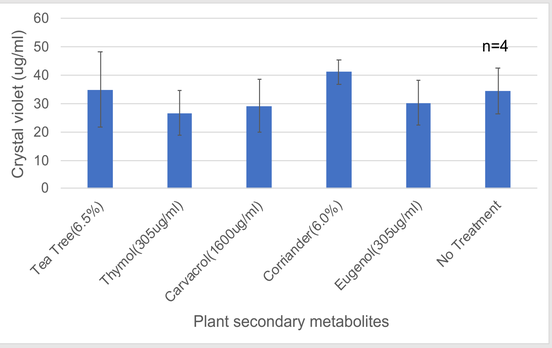


Figure 7: Measurement of biofilm removal formed from milk microbiota in free standing pumps after 1 hour exposure to secondary plant metabolites using the crystal violet assay. The crystal violet method can indirectly quantify biofilm biomass.

Conclusion

- *L. monocytogenes* is able to colonize and replicate within the free standing pumps used to dispense 2% M.F pasteurized milk.
- Optimal biofilm formation of 2% M.F pasteurized milk stored at 4°C occurs in a polystyrene 96-well plate at 74 hours.
- The presence of *L. monocytogenes* in 2% M.F pasteurized milk increase amount of biomass in a 96 well plate.
- Tea tree essential oil, coriander essential oil, carvacrol, eugenol, and thymol were able to remove pre-formed biofilms containing *L. monocytogenes* in polystyrene 96-well plates.
- However, these plant secondary metabolites were unable to remove biofilms in free-standing pumps.

Future Directions

- Test combinations of plant secondary metabolites within free standing pumps.
- Use mechanical force in association with plant secondary metabolites, such as the pumps circulating during the 1 hour exposure.
- Utilize plant secondary metabolites as a pretreatment to decrease the amount of cell attachment to the surfaces within the free standing pumps.
- Investigate the usage of higher concentrations of plant secondary metabolites in the free standing pumps.

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