Ensuring alfalfa sprout safety *– Silver bullet or finding Waldo?*

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Development of validated seed disinfection strategies for the organic production of sprouted vegetables

Organic Agriculture Centre of Canada

Contributing partners

- AAFC Growing Forward 2
 AgriInnovation Program
- Eatmore Sprouts & Greens Ltd.
- Jonathan Sprouts, Inc.
- Mumm's Sprouting Seeds

Activity researchers

- Siyun Wang, UBC
- Pascal Delaquis, AAFC
- Susan Bach, AAFC
- Kevin Allen, UBC

http://www.anneshealthykitchen.com/alfalfa-sprouts-fresh-cheese-and-tomato-tartine/





With the current healthy eating trend, sprouts are gaining their popularity

Compared to sound seeds, sprouts are low in anti-nutritive compounds, high in oligo- and monosaccharides, free fatty acids, oligopeptides, amino acids, vitamins and phytochemicals





http://n

From 2000 to 2016, US: •23 Outbreaks •788 Illness •79 Hospitalizations •1 Death

(CDC, 2016)

List of Selected Outbreak Investigations, by Year

2017	2016	2015	2014	2013	2012	2011	2010	2009		
 <u>Shell Eggs</u> – <i>Salmonella</i> Oranienburg 										
 <u>Beef Products</u> – <i>E. coli</i> O157:H7 										
 <u>Frozen Strawberries</u> – Hepatitis A 										
• <u>Fro</u>	 <u>Frozen Scallops</u> – Hepatitis A 									
 <u>Alfalfa Sprouts</u> – Salmonella Reading and Salmonella Abony 										
 Flour – E. coli O121 and O26 										
 Frozen Vegetables – Listeria monocytogenes 										
 <u>Raw Milk</u> – Listeria monocytogenes 										
• <u>Pis</u> t	tachios - S	almonella	Montevideo	D						
• <u>Alfa</u>	alfa Sprout	<u>s</u> – <i>E. coli</i> C	0157							
 <u>Alfalfa Sprouts</u> – <i>Salmonella</i> Muenchen and <i>Salmonella</i> Kentucky 										
• <u>Or</u>	ganic Shake	e & Meal Pr	roducts - Sa	almonella V	'irchow					

• <u>Packaged Salads</u> – *Listeria monocytogenes*

The largest hemolytic-uremic syndrome (HUS) outbreak in Europe



Bacterial pathogens

Verotoxigenic E. coli (e.g., E. coli O157:H7)

- Hemorrhagic colitis
- Infectious dose as low as 10 cells
- 50% infections are non-O157

<u>Salmonella enterica</u>

- Non-typhoidal Salmonella
- Abdominal pain, diarrhea, vomiting
- Infectious dose of ~1,000 cells

<u>Listeria monocytogenes</u>

- Relatively rare
- Up to 50% mortality rate
- Attacks CNS, spontaneous abortion
- Can grow at 4°C







(Public Health Agency of Canada)

Why are sprouts so susceptible to pathogen contaminations?



https://www.cdc.gov/salmonella/reading-08-16/



CFIA standards



Canadian Food Inspection Agency

Minimum 3 log reduction

Preventative step

- 2,000 ppm NaClO for 15–20 minutes
- •6–10% H₂O₂for 10 minutes

US Standards/Guidance

- The seeds used to grow sprouts be treated using <u>a</u> <u>scientifically valid method</u> to reduce microorganisms of public health significance
 - Primary consideration given to reduction of <u>Salmonella and E.</u> <u>coli O157:H7</u>
- A <u>3-log reduction</u> is the minimum level of reduction of pathogens the EPA will consider to register an antimicrobial treatment that includes a public health claim on seeds
- Recommend <u>4 or 5-log or higher reduction</u>
- The final FSMA rule includes new requirements to help prevent the contamination of sprouts

Current Seed Treatments

Chemical interventions

- Organic acids
- Elect of zer water

Elected Ozone Biological intervention Conhages

Physical interventions

- Thermal inactivation
- Irradiation
- High pressure

No clear approach to product safety







Today, we will discuss about

- Effects of conventional & organic sanitation treatments on three different types of seeds
 - Alfalfa, mung & radish
 - Salmonella, E. coli & L. monocytogenes
 - Germination rate & yield
- Post sanitation recovery of Salmonella on sprouting alfalfa seeds
 - Cindy Dai, MSc student
- The efficacy of a novel bacteriophage for control of Salmonella on alfalfa sprout seeds
 - Karen Fong, PhD candidate

Experimental Procedures





<image>

Sanitation

- 1) Mild heat + H_2O_2 + Acetic acid @ Room Temp
 - 2) Chlorine (2,000 ppm)
 - 3) H₂O₂ (8%)

Inoculation

3 major foodborne pathogens: *E. coli* O157:H7 *Salmonella enterica Listeria monocytogenes* ~5 log cfu/g





Germination & Yield Assay

Alfalfa seeds





Using 50C as the mild heat treatment, the germination rate of treated seeds did not differ significantly from that of the control pm Chlorine

)2 at 55°C

icat 55°C

perature

E,

m Chlorine

Alfalfa seeds

0 min in 2% H2O2 nin in 4% H2O2 + min in 2% H2O2 min in 4% H2O2 min in 4% H2O2 min in 4% H2O2 min in 4% H2O2 min in 2% H2O2 + + 0.2% 0.2% acetic acid + 0.1% acetic acid + 0.1% acetic acic 0.2% 0.2% 0.2% acetic acic 0.1% acetic add acetic acic acetic acid acetic acid

min in 2000ppm C

in 4% H2O2

+ 0.2% acetic acid

in 4% H2O2 + 0.2% acetic acid

in 4% H2O2

H202

in 2% H2O2

H202

at 50°

2%

H202

in 4% H2O2

in 4% H2O2

tic acio

tic acio

tic acid

H₂O₂ slightly promotes the yield of sprouts Increasing the water temperature from 50 °C to 55 °C will significantly decrease the yield 2 om Chlorine tic at 55°C at 55°C

Alfalfa seeds

Radish seeds

Germination rate

Yield

The combined treatment of mild heat (60C), H₂O₂ and acetic acid achieved a higher log reduction (> 3-log) of pathogens compared to other treatments evaluated.

Salmonella

Listeria monocytogenes

Salmonella became more resistant to sanitation treatments after a 4-week storage.

Conclusions

- There is no universal "optimal" treatment for different kinds of seeds
- •The combined treatment of mild heat, H₂O₂ and acetic acid was effective in achieving at least 3-log reduction in alfalfa seeds and mung beans

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Salmonella enterica

- Bacteria that can cause food-borne infection called Salmonellosis
- Globally, 80.3 million cases yearly, 155, 000 deaths/yr
- No. 3 cause of bacterial foodborne disease overall in Canada
- Found in domestic and wild animals
- Serotypes: antigenic specificity

Multistate Salmonella Outbreaks linked to Alfalfa sprouts in US

Salmonella enterica includes >2,600 serotypes, but only a few of them are responsible for causing the majority of the illnesses

Salmonella on sprouting seeds

Maximum 10⁸ CFU/g

100,000-fold increase after 48-hour germination

Infective does: Fewer than 1,000 cells

(Matthews, 2006; Gandhi and Matthews, 2003; FDA, 2012)

Research Objective 1

Can Salmonella cells survive and recover from sanitation stress on sprouting alfalfa seeds?

To investigate the ability of *Salmonella enterica* to colonize on sprout seeds

H2: the survival of sanitizer-injured Salmonella on sprouting alfalfa seeds is regulated by

To investigate Salmonella's survival mechanisms leading to sprout contamination

Salmonella strains

Sprout Isolate (Agona)

Human Isolate (Agona)

Irrigation water isolates (Typhimurium, Daytona, Enteritidis)

Seed treatments

Research Objective 2

Can Salmonella cells survive and recover from sanitation stress on sprouting alfalfa seeds?

To investigate the ability of *Salmonella enterica* to colonize on sprout seeds

How does Salmonella survive and recover from sanitation stress on sprouting alfalfa seeds?

To investigate Salmonella's survival mechanisms leading to sprout contamination

Metabolomics

Metabolomics of Tomato Plant Surface

Environmental Metabolomics of the Tomato Plant Surface Provides Insights on Salmonella enterica Colonization

Sanghyun Han,^a* Shirley A. Micallef^{a,b}

Department of Plant Science and Landscape Architecture, University of Maryland, College Park, Maryland, USA^a; Center for Food Safety and Security Systems, University of Maryland, College Park, Maryland, USA^b

			Proportion			
			0%	50%	100%	
	Veter	Heinz-1706 Plum Dandy VF				
Seedling Shoot	1 S	Nyagous Rutgers VFA				
Seedling	展	Heinz-1706				
Root	11	Nyagous				
		Rutgers VFA				
Flowering Dloot	*	Nyagous	5			
Flowening Plant	TAK	Plum Dandy VF				
Shoot	11	Rutgers VFA				
		Heinz-1706				
	300	Heinz-1706	6			
Flowering Plant	1 the	Rutgers VFA				
Shoot	1	Nyagous	5			
Shoot	P N	Plum Dandy VF				
	late	Heinz-1706	;			
Fruit		Plum Dandy VF				
TTUR		Rutgers VFA				
		Nyagous				
		Amino acid	2.53	Fatty acid	d	
		Nucleobase & nucleic acid Organic acid Rhanolics Reveal & Sugar &				
		No group		Unidenti	fied	

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- Maximum populations attained in exudates of fruits and flowering plants were smaller than those in seedlings
- Sugars, sugar alcohols, and organic acids were associated with increased S. enterica growth, while fatty acids, including palmitic and oleic acids, were negatively correlated.

Why metabolomics?

- Seedling surface compounds drive bacterial colonization success
 - Plant exudate profiles may differ after different seed treatments
 - Differences in exudate profiles may help explain some of the variability in *Salmonella* colonization susceptibility seen among serotypes.
- Some bacteria from the natural microbiota are also recovering from sanitation stress during germination

Significance

- Address the importance of pathogens' postsanitation recovery in sprout outbreaks
- Provide insight on mechanisms that may be important for pathogen interactions with sprouts
- Devise more targeted interventions for the production of pathogen-free sprouted vegetables

Conclusions

- The selected 3 seed treatments reduced, but did not eliminate, *Salmonella*
- The selected 5 Salmonella strains can all survive and recover from sanitation stress on sprouting alfalfa seeds
- A maximum level of 10⁶⁻⁸ CFU/g after 20-32 hours of germination
- Survival mechanism is under investigation
- Note:
 - Artificially contaminated (10^{3.5-4.5} CFU/g)
 - No intervention strategy was used during the germination to inhibit pathogen growth

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Phages, the "edible viruses"

- Viruses, predators of bacteria
- Most abundant replicating biological entity on Earth
 - Reduces bacterial population by 50% every 48 h
- Structural characteristics:
 - 0.2-0.4 microns (5 10X smaller than bacteria)
 - Tailed viruses are most common
- Cause lysis (killing) of hosts
 - Very specific to bacterial hosts
 - Harmless to humans, animals & plants
 - Uses host for reproduction

Phages as antimicrobials in produce

- Growing interest in using live phage as antimicrobials in the food industry
- Lytic phages offer several desirable attributes:
 - 1. "Green" technology
 - 2. Designed to kill host cells only
 - Safe for human consumption
 - 3. Highly specific; usually do not cross genus or species barriers
 - 4. Self-replicating and self-limiting
 - 5. Ubiquitously distributed in nature (and also in food)

Phage preparations

Research objective:

To evaluate the efficacy of a novel phage, SI1, for control of *Salmonella* on alfalfa sprout seeds

Irrigation water from Abbottsford, BC

Isolation of Salmonella phage SI1

In vitro testing

- Broad host range phage
- Kills S. Enteritidis & S. Typhimurium, causing highest proportions of outbreaks worldwide
- Stable at a variety of pH & temperatures
- Produces ~83 phage progeny in 25 mins

Salmonella biocontrol on alfalfa sprout seeds

Majority of outbreaks linked to alfalfa seeds

S. Enteritidis FSL S5-483 *S.* Agona FSL S5-517

Previously involved in North American sprouts outbreaks

2 Salmonella biocontrol on alfalfa sprout seeds

2 Salmonella biocontrol on alfalfa sprout seeds

Effect of phage treatment on sprout yield

Table 1 | Effect of phage SI1 treatment on final yield of alfalfa sprouts, measured on day 6 of sprouting. Presence of an asterisk indicates significance (p<0.05; Student's *t*-test).

Conclusions

- Phage SI1 was a novel phage possessing suitable characteristics for *Salmonella* biocontrol
- Assessment of its efficacy to control Salmonella on alfalfa seeds:
 - 2.6 log CFU/g reduction of *S*. Enteritidis on day 1 post-treatment
 - 3.1 log CFU/g reduction of S. Agona on day 1 post-treatment → consistent with Health Canada standards
 - However, no significant effect observed on days 2-6
- Phage treatment did not significantly reduce sprout yield (p>0.05)
- Treatment with phage SI1 could potentially be used for Salmonella decontamination of alfalfa seeds

What's next for phage biocontrol?

- Assessing effect of SI1 on different concentrations of *Salmonella*
- Validating SI1 for control of other *Salmonella* serotypes (*S.* Typhimurium, *S.* Heidelberg) on a variety of sprouts
- Evaluating combinatorial treatment
- Evaluating optimal method of application (soak, rinse, spray)
- Assessing industry & consumer perception of viral phage treatment

Organic Sprouts & Greens Grown in the Comox Valley Year Round!

Carmen Wakeling

Dr. Pascal Delaquis & Susan Bach, AAFC