Project/Proposal Title: Fate of *Listeria* on apples at ozone and controlled atmosphere storage

Report Type: Final

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Project Duration: 3 Year

Total Project Request for Year 1 Funding: $118,779
Total Project Request for Year 2 Funding: $121,797
Total Project Request for Year 3 Funding: $125,404

WTFRC Collaborative Costs:

Item	2018	2019	2020
Salaries	4,141	4,224	4,308
Benefits	1,367	1,394	1,422
Wages	4,500	4,703	5,267
Benefits	1,485	1,552	1,738
RCA Room Rental	8,316	8,316	8,316
Travel	500	500	500
Total	20,309	20,689	21,551

Budget 1
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Item	2018	2019	2020
Salaries	37,124	38,609	40,154
Benefits	12,412	12,909	13,424
Wages	15,340	15,953	16,592
Benefits	1,094	1,137	1,183
Equipment	0	0	0
Supplies	25,500	25,500	25,500
Travel	2,000	2,000	2,000
Miscellaneous	5,000	5,000	5,000
Plot Fees	0	0	0
Total	98,470	101,108	103,853

OBJECTIVES

1. Assess the fate of *Listeria* on apple surfaces stored under RA and CA with continuous low doses of ozone.
2. Examine the survival of natural microorganisms on apple surfaces stored under RA and CA with continuous low doses of ozone.
3. Evaluate the impacts of ozone in the storage environment on final fruit quality.

SIGNIFICANT FINDINGS

1. A higher die-off rate of *Listeria innocua* on fresh apples was evident within the initial 6 weeks, irrespective of the apple varieties tested (Granny Smith, Red Delicious, and Cosmic Crisp).

   After 6 weeks of cold storage under either commercial RA or CA conditions, Granny Smith and Red Delicious apples exhibited a 1.5-2.0 log reduction in *L. innocua*, while Cosmic Crisp apples showed a slightly higher reduction of 2.8-2.9 logs.
2. Following the initial 6 weeks of RA or CA storage, the die-off rate of *Listeria* on apples diminished. After 36 weeks of CA storage, there was a 2.2-3.0 log reduction on Granny Smith and Red delicious apples and 3.4 log reduction on Cosmic Crisp apples.
3. The die-off rate of *Listeria* on apples of the selected varieties under CA storage with ozone gas was comparable to that observed during RA/CA storage over the initial 6 weeks of storage. During this period, the concentration of ozone gas steadily increased, eventually reaching the targeted concentration.
4. The introduction of ozone gas promotes the die-off of *Listeria* during the 6-24 weeks of storage, irrespective of 1-MCP pretreatment and the apple varieties tested (Granny Smith, Red Delicious, and Cosmic Crisp).
5. The application of ozone gas during a 36-week storage period resulted in an additional reduction of 2.5~3.0 log CFU/apple on Granny Smith and Red Delicious apples compared to CA storage alone.
6. In Cosmic Crisp apple storage study, gaseous ozone was only applied during the initial 24 weeks, followed by standard CA conditions for an additional 12 weeks. This approach achieved a comparable anti-*Listeria* efficacy to Granny Smith and Red Delicious apples treated with 36 weeks of ozone gas application. These findings suggest that the duration of ozone gas application can be shortened to 24 weeks in future industry application.
7. Ozone gas at 50-87 ppb exhibited comparable antimicrobial efficacy across all tested apple varieties. This suggests that a lower concentration within this range could be considered for practical applications in apple industry.
8. The initial indigenous yeast/mold counts of uninoculated apples across the tested apple varieties ranged from 4.5-5.0 $\log_{10}$ CFU/apple. These counts remained stable during the initial 12 weeks of RA regardless of apple varieties. By 24 weeks of storage and beyond, the yeast/mold counts on apples under RA were higher for Granny Smith and Cosmic Crisp apples or similar for Red Delicious apples compared to those during CA storage. The application of low doses of ozone gas decreased yeast/mold counts on apples.
9. Continuous low-dose ozone gas application for 9 months or 6 months at 50-87 ppb did not cause adverse effects on fruit quality or the occurrence of internal and external disorders for all tested apple varieties. However, Granny Smith apples under CA storage could develop ozone burn-like symptoms.
10. *E. faecium* NRRL B-2354 proves a suitable surrogate of *L. monocytogenes* for apple cold storage study.

E. faecium NRRL B-2354 displays survival profiles comparable to *L. innocua* during 36 weeks of RA and CA storage of Cosmic Crisp apples. However, it demonstrates greater resistance to gaseous ozone treatments, regardless of ozone concentration and MCP treatment, in comparison to *L. innocua* on Cosmic Crisp apples.

11. Detailed findings related to Granny Smith apples and Red Delicious apples are available in the published papers by Shen et al (2021) and Sheng et al (2022).

METHODS

<u>Objective 1. Assess the fate of *Listeria* on apple surfaces stored under RA and CA with continuous low doses of ozone.</u>

1. Strain, inoculum preparation, inoculation, and establishment on the apple surface

E. faecium NRRL B-2354 was acquired from the USDA-ARS culture collection located in Peoria, Illinois. For *L. innocua* storage study, a 3-strain *L. innocua* cocktail was utilized, including *L. innocua* NRRL B-33197 (USDA-ARS culture collection), *L. innocua* isolates from the Avocado facility and the Apple facility-Bidart (acquired from Dr. Trevor Suslow, University of California). This cocktail was prepared by mixing equal numbers of each respective strain into a suspension. Unwaxed and unbruised apples of the selected varieties at commercial maturity were individually and separately inoculated to establish 1×10^6 CFU/apple of 3-strain *Listeria* cocktail or *E. faecium* NRRL B-2354 inoculum through dipping inoculation. The inoculated apples were then held at room temperature for 24 h prior to various storage storages.

2. Cold storage treatments in a commercial packing facility

Apples of selected varieties, inoculated with ~1×10^6 CFU/apple of *L. innocua* or *E. faecium* NRRL B-2354, were randomly separated and assigned into six groups and subjected to three different storages: refrigerated air (RA, 1 °C/ 33 °F), controlled atmosphere (1 °C/ 33 °F, 2 % O_2, 1 % CO_2) treated with (CAMCP) or without 1-methycyclopropene (CA), CA with a low dose gaseous ozone and MCP-1 treatment (CAMCPLowPO_3), CA with high dose gaseous ozone with (CAMCPHigh O_3) or without MCP-1 treatment (CAHighO_3) for up to 36 weeks.

In Cosmic Crisp apple storage study, gaseous ozone application was limited to the initial 24 weeks of storage, followed by standard CA for an additional 12 weeks. Apples under different storage conditions were sampled at 0, 3-, 6-, 12-, 18-, 24-, 30-week, and 36-week of storage, when the counts of *L. innocua* or *E. faecium* NRRL B-2354 survived on apples were enumerated.

3. Microbial analysis

On each sampling day, apples under the respective storage condition were sampled and transferred to sterile Whirl-Pak bags containing 10 ml of 0.1% buffered peptone water. The apples were then gently rubbed to release attached microorganisms, then the resulting microbial suspension was subjected to serial diluted. Appropriate dilutions were plated on agar plates. Plates were incubated at 35˚C (95˚F) for 48h and enumerated manually. Enrichments were done when bacterial levels were under the detection limit of 10 CFU/apple following our previous publication (Sheng et al., 2018).

<u>Objective 2. Examine the fate of natural microorganisms on apple fruit surfaces when stored in refrigerated air or controlled atmosphere in the presence or absence of ozone.</u>

1. Cold storage treatments in a commercial packing facility

Non-waxed, uninoculated apples of the selected cultivars were subjected to different storage conditions (RA, CA, CAMCP, CAMCPLowO_3, CAMCPHingO_3, CAHingO_3) as described previously. Apples were sampled at 0-, 6-, 12-, 24, and 36 weeks of storage for total plate count and yeast and mold enumeration.

2. Survival microorganism analysis

On each sampling day, apples were sampled and transferred to a sterile Whirl-Pak bag with 10 ml of 0.1% buffered peptone water bag, rubbed to release attached microorganisms, followed by serial dilution. The appropriate dilution was plated onto TSAYE plates for total plate count (TPC) and potato dextrose agar (PDA) plates for yeasts and molds, respectively per our established methods (Shen et al., 2019; Sheng et al., 2018; Sheng et al., 2020). TPC colonies were counted manually after incubation at 35 °C (95˚F) for 48h and PDA plates were counted after incubation at room temperature for 5 days.

Objective 3: Examine the effect of ozone in the storage environment on final fruit quality.

1. Fruit quality analysis

Fruit maturity and quality measurements such as firmness, total soluble solids (TSS), and titratable acidity (TA) were performed at harvest, after 6-month and 9-month storage per our established methods (Sheng et al., 2018). Briefly, fruit firmness was assessed with a fruit texture analyzer using a 1 cm diameter probe on a peeled area of ~3 cm^2 on both the sun and shade side of the apples. Total soluble solids were evaluated using Atago PR-32 digital Brix refractometer. The titratable acidity of fruit juice was measured with a potentiometric titrator. Measurements of each parameter were repeated four times independently with a sample size of 10 apples per replication per storage regimen.

2. Disorder analysis

The incidence of disorders was assessed after cold storage followed by one day at room temperature for external disorders and 7 days at room temperature for both internal and external disorders. The absence or presence of the following external disorders was visually inspected and recorded: ozone burn, superficial scald, lenticel decay, visible decay, sunburn, russet, and CO_2 damage. Apples were sliced 3 times to determine the presence of any internal disorders including watercore, internal browning, or cavities.

RESULTS AND DISCUSSION

1. Survival of *L. innocua* on apples of selected varieties under storage conditions with or without low-dose ozone gas.

The survival of *L. innocua*, initially inoculated at levels of 6.0-6.5 Log10 CFU/apple, on Granny Smith, Red Delicious, and Cosmic Crisp apples were investigated under refrigerated air (RA), controlled atmosphere (CA), and CA conditions with varying doses of O3 gas (51-87 ppb).

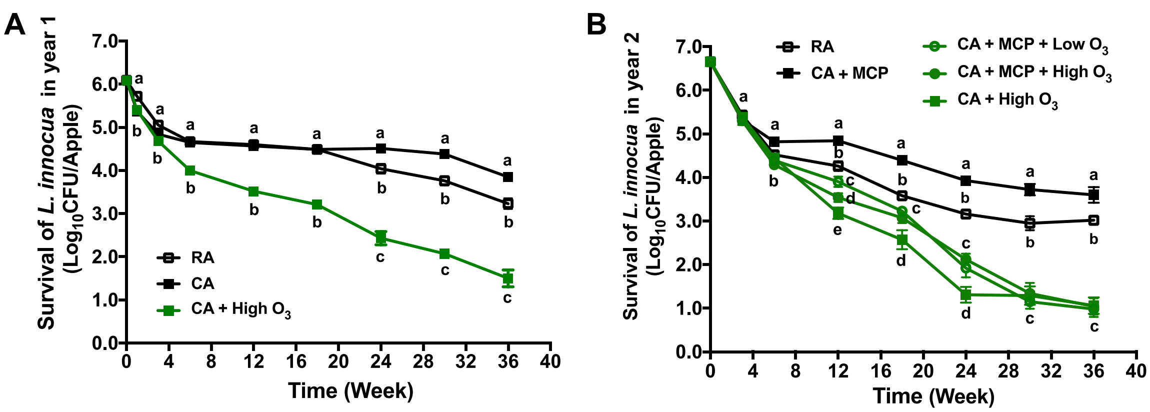


Figure 1. Survival of *Listeria* on Granny Smith apple under commercial cold storages. RA: refrigerated atmosphere (33°F); CA: controlled atmosphere (33°F, 2% O2, 1% CO2). Year1, CA + High O_3: CA with 87 ± 38.8 ppb ozone gas; Year 2, CA + Low O_3; Ozone gas concentration at 51 ± 5 ppb; CA + High O_3: Ozone gas concentration at 68 ± 7 ppb. Mean ± SEM; n=40. [a-d]Means within a column within the same sampling point with no common letter differ significantly ($P < 0.05$).

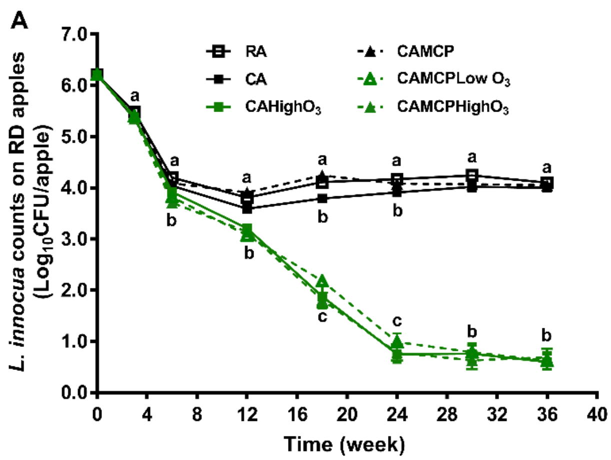
1.1 Survival of *L. innocua* on Granny Smith apples

Fates of *L. innocua* inoculated on Granny Smith apples at a level of 6.0-6.5 log_{10} CFU/apple level under RA, CA, and CA with different doses of O_3 gas (51-87 ppb) were studied during two cropping seasons. Over the 3-week cold storage, *L. innocua* was reduced by 1.0-1.5 log_{10} CFU/apple on Granny Smith apples stored in RA, CA, and CA plus different doses of O_3 with a die-off rate of 0.35-0.45 Log_{10} CFU/apple/week (Figure 1).

There were a 2.9-3.5 log reduction of *L. innocua* on Granny Smith apples over 36 weeks of cold storage under a commercial RA environment, and a 2.2-2.7 log reduction under CA storage conditions (Figure 1) (Sheng et al., 2022). Continuous low dose ozone gas application in CA storage generated additional ~2-log reduction of *L. innocua* on Granny Smith apples. The test range of ozone gas doses (51-87ppb) demonstrated similar bactericidal effects against *Listeria* (Figure 1B). However, MCP-1 application in CA room slightly decreased antimicrobial efficacy of ozone gas.The population of *Listeria* in CA+MCP+ High O3 group at 12-24 weeks of storage was significantly higher than that in the CA+ High O3 group (Figure 1B) (Sheng et al., 2022).

1.2 Survival of *L. innocua* on Red Delicious apples

Studies on Granny Smith apples revealed that continuous low dose ozone gas application in CA cold storage effectively eliminates or controls *L. innocua*, resulting ~ 5 Log_{10} CFU/apple reduction. We further evaluated efficacy of low dose ozone gas against *L. innocua*, on Red Delicious apples. Results revealed a reduction of 0.7-0.9 log_{10} CFU/apple on Red delicious apples stored in RA, CA, and CA plus different doses of O_3 with a die-off rate of 0.24-0.29 log_{10} CFU/apple/week over 3 weeks of cold storage (Figure 2) (Shen et al., 2021), which is smaller than that observed on Granny Smith apples (Sheng et al., 2022). There was ~2.2 Log_{10} CFU/apple reduction of *L. innocua* on Red Delicious apples over 36 weeks of cold storage under a commercial RA and CA storage environment. This reduction was smaller, especially in RA storage, compared to that in Granny Smith apples (Sheng et al., 2022). In comparison to Granny Smith apples, low dose ozone gas at similar dose (60-80 ppb for Red Delicious apples vs 51-87 ppb for GSA) was more effective against *L. innocua* on Red Delicious apples (Figure 1-2) (Shen et al., 2021; Sheng et al., 2022). An additional 3.3-3.4 Log_{10} CFU/apple reduction was observed compared to RA or CA storage. MCP-1 treatment prior to storage had no effects on *L. innocua* survival on Red Delicious apples ($P > 0.05$) (Figure 2), while MCP-1 application in CA room slightly decreased anti-*Listeria* efficacy of ozone gas on Granny Smith apples (Sheng et al., 2022).



B

Reduction of *L. innocua* during storage (log_{10}CFU/apples)

		CA		CA+1-MCP		
Week	RA	0	High O_3	0	Low O_3	High O_3
0	0.00	0.00	0.00	0.00	0.00	0.00
3	0.73	0.86	0.83	0.80	0.81	0.86
6	2.01	2.17	2.29	2.12	2.39	2.50
12	2.40	2.62	3.00	2.30	3.14	3.07
18	2.10	2.42	4.33	1.97	4.03	4.40
24	2.04	2.30	5.46	2.13	5.22	5.43
30	1.97	2.19	5.45	2.14	5.42	5.58
36	2.15	2.21	5.61	2.18	5.59	5.52

Figure 2. Survival of *L. innocua* on Red Delicious apples during 36-week of commercial cold storage. A. *L. innocua* count on apples over cold storage period. Mean ± SEM, n = 32-40. Different letters (a-c) at each sampling point indicated significant differences ($P < 0.05$); B. Reduction of *L. innocua* on apples under different storages. Mean averaged from 32-40 apples. RA: refrigerated atmosphere; CA: controlled atmosphere; MCP: apples were treated with 1-methycyclopropene before subjecting to cold storage; CAHighO3: CA storage with continuous gaseous O_3 application at 78.7 ± 13.2 ppb; CAMCPLowO3: CA storage with continuous gaseous O_3 application at 60.2 ± 5.7 ppb, where apples were treated with 1-methycyclopropene treatment before subjecting to storage.

1.3 Survival of *L. innocua* on Cosmic Crisp apples

Data collected from Fuji (Sheng et al., 2018), Red Delicious (Sheng et al., 2022), and Granny Smith (Sheng et al., 2022) apples revealed that the most substantial reduction in *L. innocua* counts occurred within a 24-week storage period. Therefore, in this study, a low-dose continuous gaseous ozone was applied only during the initial 24 weeks of CA storage, followed by an additional 12 weeks of CA storage. The survival *L. innocua* on Cosmic Crisp apples were evaluated throughout the 36-week storage under different conditions.

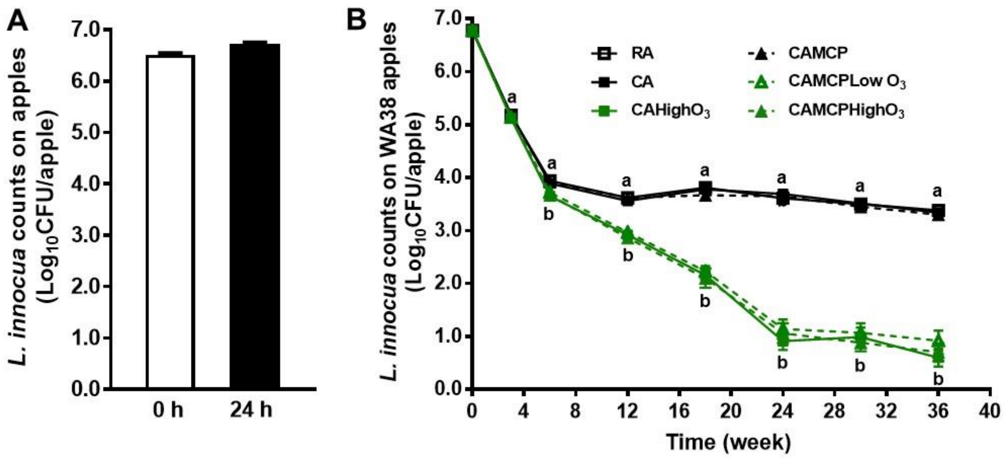


Figure 3. Fates of *L. innocua* on Cosmic Crisp apples during 36 weeks of cold storage under different storage regimes. A. The initial bacterial population on apples; B. Survival of *L. innocua*; RA: refrigerated atmosphere; CA: controlled atmosphere; MCP: apples were treated with 1-methycyclopropene prior to cold storage; CAHighO$_3$: CA storage with continuous gaseous O$_3$ application at 78.2 ± 12.2 ppb; CAMCPHighO$_3$: CA storage with continuous gaseous O$_3$ application at 78.2 ± 12.2 ppb, where apples were treated with 1-methycyclopropene prior to cold storage; CAMCPLowO$_3$: CA storage with continuous gaseous O$_3$ application at 55.5 ± 8.8 ppb, where apples were treated with 1-methycyclopropene prior to cold storage. Different letters (a-b) at each sampling point indicated significant differences ($P < 0.05$).

As results observed in Granny Smith and Red Delicious apples, the greater die-off rate of *L. innocua* on Cosmic Crisp apples was observed during the initial 6 weeks of storage (Figure 3). During the initial 3 weeks, before reaching the target ozone concentration, the populations of *L. innocua* on Cosmic Crisp apples exhibited a reduction of 1.6-1.7 log10 CFU/apple across all storage conditions (Figure 3). This reduction surpassed that observed on Red Delicious apples (Shen et al., 2021). Over the entire 36-week storage period, regardless of atmospheric conditions or 1-MCP treatment, there was a substantial 3.4-3.5 log10 CFU/apple reduction in *L. innocua* on Cosmic Crisp apples (Figure 3), surpassing reductions seen in Granny Smith and Red Delicious apples (Shen et al., 2021; Sheng et al., 2022).

The continuous application of low-dose ozone gas during the initial 24 weeks of CA cold storage caused an additional 2.4-2.8 $\log_{10}$ CFU/apple reduction when the ozone gas was discontinued. The anti-*Listeria* efficacy of 24 weeks of gaseous ozone application was similar to that seen in Granny Smith and Red Delicious apples treated with ozone gas for 36 weeks (Shen et al., 2021; Sheng et al., 2022). In alignment with Red Delicious apples (Shen et al., 2021), pre-storage1-methylcyclopropene (1-MCP) treatment had a minor effect on *L. innocua* survival on Cosmic Crisp apples ($P > 0.05$) (Figure 3).

2. Fates of resident microbiota on apples of the selected varieties under RA, CA, and CA with low-dose ozone gas.

Resident bacteria, mold and yeast cause postharvest decay of apples (Janisiewicz & Korsten, 2002). Therefore, the impacts of ozone gas application on the counts of resident bacteria, mold and yeast were examined on Granny Smith, Red Delicious, and Cosmic Crisp apples using uninoculated apples under the above stated various storage conditions, mirroring those applied to the inoculated apples.

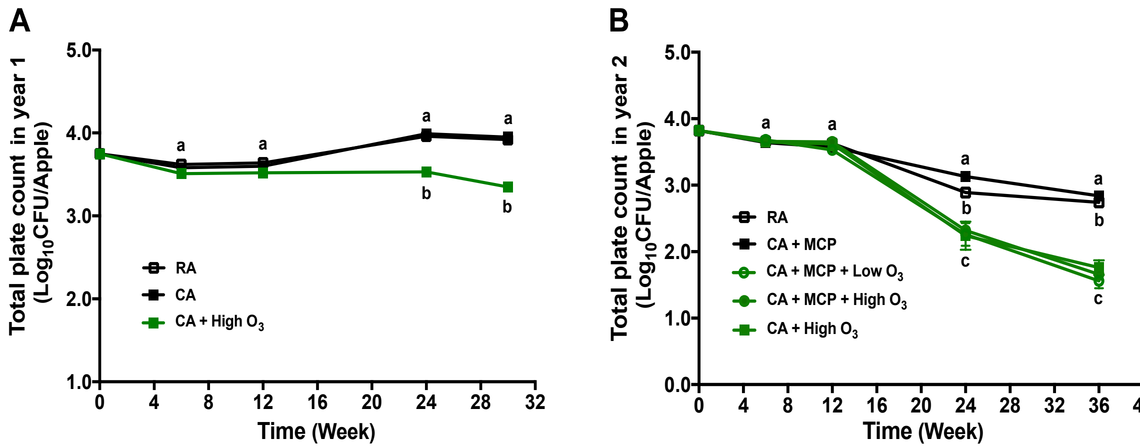


Figure 4. **Survival of resident bacteria on Granny Smith apple under commercial cold storages.** RA: refrigerated atmosphere (33°F); CA: controlled atmosphere (33°F, 2% O2, 1% CO2). A: Year1; B. Year 2; In year 1, CA + High O_3: CA with 87 ± 38.8 ppb ozone gas; In year 2, CA + Low O_3; Ozone gas concentration at 51 ± 5 ppb; CA + High O_3: Ozone gas concentration at 68 ± 7 ppb. Mean ± SEM; n = 40. Different letters (a-c) at each sampling point indicated significant differences ($P < 0.05$).

The initial total plate count for background bacteria in Granny Smith apples was 3.5-4.0 $\log_{10}$ CFU/apple, maintaining relative stable over 36-week storage at RA or CA (Figure 4) (Sheng et al., 2022). Continuous low dose ozone gas application in CA room significantly decreased resident bacteria in Granny Smith apples after 24 weeks of storage, with a more pronounced reduction observed in the year 2 study (Figure 4B) (Sheng et al., 2022).

Regarding indigenous yeast and mold (Y/M) counts, the initial level in un-inoculated Granny Smith apples were 4.5-5.0 $\log_{10}$ CFU/apple, remaining relatively stable during the first 12 weeks of storage in RA and CA conditions before gradually increased (Figure 5) (Sheng et al., 2022). By the 24th week of storage and beyond, the Y/M count in Granny Smith apples stored under RA was significantly higher than that in the CA room (Figure 5) (Sheng et al., 2022). The Y/M count in Granny Smith apples under CA storage with different doses of ozone gas decreased during the initial 24 weeks of storage, followed by a gradual increase (Figure 5) (Sheng et al., 2022).

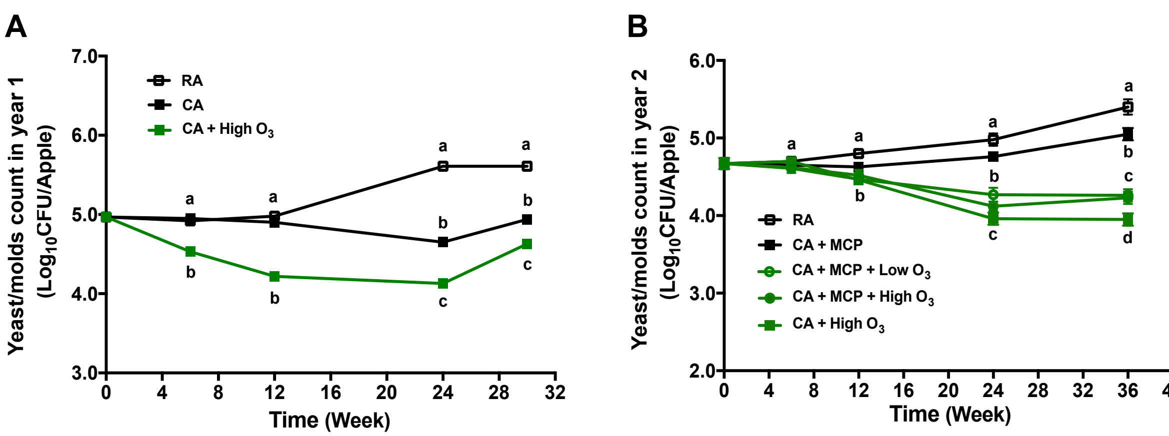


Figure 5. The yeast/mold counts of Granny Smith apple under commercial cold storages. RA: refrigerated atmosphere (33°F); CA: controlled atmosphere (33°F, 2% O2, 1% CO2). A. Year1; B. Year2. In year 1, CA + High O_3: CA with 87 ± 38.8 ppb ozone gas; In year 2, CA + Low O_3; Ozone gas concentration at 51 ± 5 ppb; CA + High O_3: Ozone gas concentration at 68 ± 7 ppb. Mean ± SEM; n = 40. Different letters (a-d) at each sampling point indicated significant differences ($P < 0.05$).

Red Delicious apples had similar initial bacterial count (~ 3.8 Log_{10} CFU/apple). It increased by one log_{10} CFU/apples after 12-week storage at RA or CA at 1°C/33°F and maintained this high level throughout 36-week storage (Figure 6A) (Shen et al., 2021). Ozone gas application at different doses in CA storage decreased resident bacteria in Red Delicious apples by 1.2-1.3 Log_{10} CFU/apple after 36 weeks of storage (Figure 6A) (Shen et al., 2021).

The initial level of indigenous Y/M counts of uninoculated Red Delicious apples was 4.7 log_{10} CFU/apple, which was similar to that of uninoculated Granny Smith apples. The Y/M count gradually increased in apples under RA and CA storages (Figure 6B). By 36-week of storage, the Y/M counts of Red Delicious apples stored under RA or CA room were increased by 1.1-1.3 Log_{10} CFU/apple (Figure 6B) (Shen et al., 2021). On the other hand, the Y/M count on Red Delicious apples gradually decreased under CA with 60-80ppb ozone gas; there was ~ 0 .7 Log_{10} CFU/apple reduction of Y/M at the end of cold storage (Figure 6B) (Shen et al., 2021).

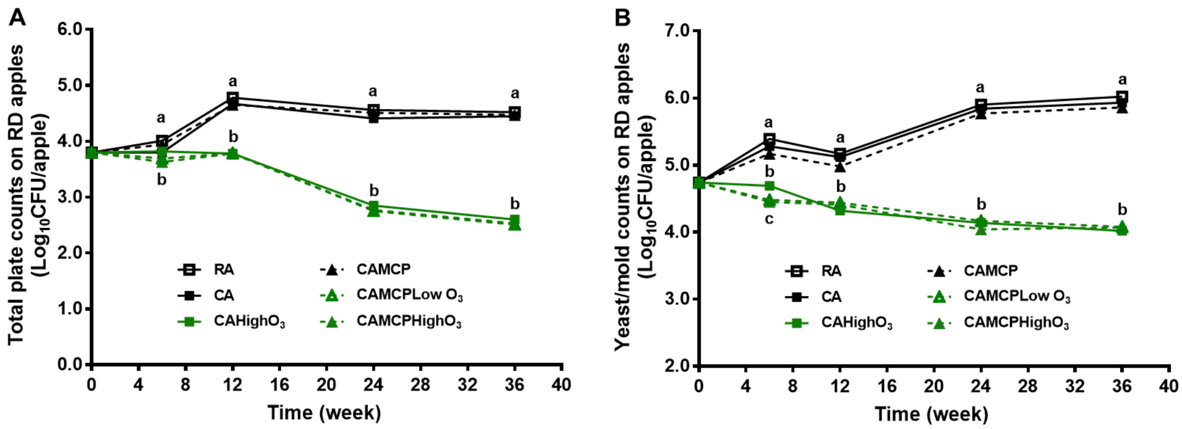


Figure 6. Resident bacteria and natural decay microorganisms on Red Delicious apples during 36 weeks of commercial cold storage. A. Total plate counts on apple over storage; B. Yeast and mold counts on apples during storage; Mean ± SEM, n = 40. Different letters (a-c) at each sampling point indicated significant differences ($P < 0.05$). RA: refrigerated atmosphere; CA: controlled atmosphere; MCP: apples treated with 1-methycyclopropene prior to cold storage; CAHighO_3: CA storage with continuous gaseous O_3 application at 78.7 ± 13.2 ppb; CAMCPHighO_3: CA storage with continuous gaseous O_3 application at 78.7 ± 13.2 ppb, where apples were treated with MCP-1 prior to different storages; CAMCPLowO_3: CA storage with continuous gaseous O_3 application at 60.2 ± 5.7 ppb, where apples were treated with 1-MCP prior to cold storage.

For Cosmic Crisp apples, the initial resident microflora and indigenous yeast/mold count in the receiving apples were 3.75 log_{10} CFU/apple and 4.87 log_{10} CFU/apple, respectively (Figure 7). These levels fall within the range observed for Granny Smith and Red Delicious apple (Shen et al., 2021; Sheng et al., 2022). The resident bacteria on Cosmic Crisp apples showed an increase of 0.6-0.7 log_{10} CFU/apple under both RA and CA storage within the first 12 weeks of storage (Figure 7A). Total plate counts on Cosmic Crisp with low dose ozone treatment were initially reduced by 1.2-1.3 log_{10} CFU/apple at 24 weeks of storage, followed by subsequent increase, although still lower than that at harvest (Figure 7A).

The Y/M counts of Cosmic Crisp apples exhibited an increase of 0.5-0.6 log_{10} CFU/apple after 36 weeks of both RA and CA storages (Figure 7B). However, Yeast/mold counts decreased by 0.2-0.4 log_{10} CFU/apple on Cosmic Crisp apples treated gaseous ozone for 24 weeks (Figure 7B).

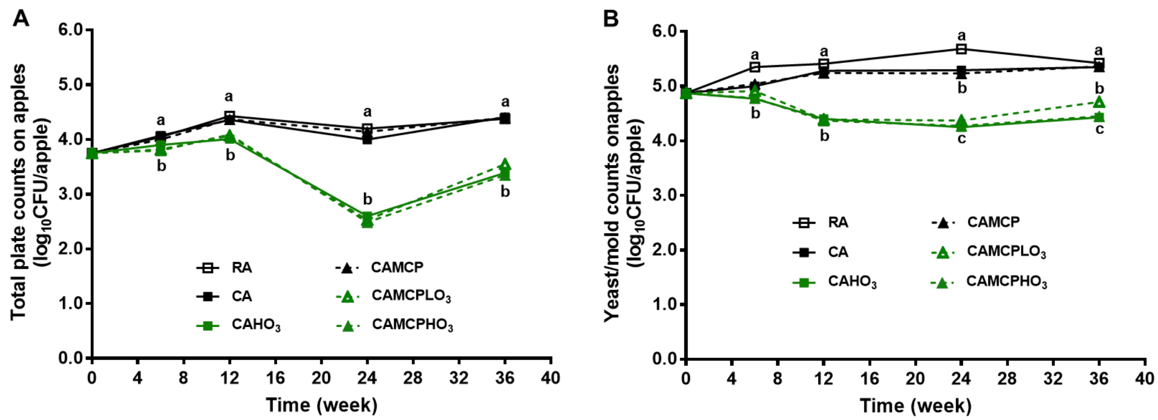


Figure 7. Resident bacteria and natural decay microorganisms on Cosmic Crisp apples during 36 weeks of commercial cold storage. A. Total plate counts on apple over storage; B. Yeast and mold counts on apples during storage; Mean ± SEM, n = 32. [a-b] Different letters (a-c) at each sampling point indicated significant differences ($P < 0.05$). RA: refrigerated atmosphere; CA: controlled atmosphere; MCP: apples were treated with 1-methycyclopropene before cold storage; CAHighO_3: CA storage with continuous gaseous O_3 application at 78.2 ± 12.2 ppb during the initial 24 weeks of storage; CAMCPHighO_3: CA storage with continuous gaseous O_3 application at 78.2 ± 12.2 ppb during the initial 24 weeks of storage, where apples were treated with MCP-1 before different storages; CAMCPLowO_3: CA storage with continuous gaseous O_3 application at 55.5 ± 8.8 ppb during the initial 24 weeks of storage, where apples were treated with 1-MCP before cold storage.

3. Effects of continuous low-dose ozone application in cold storage environment on final fruit quality.

Fruit quality is a crucial factor influencing consumer preference. CA storage is known to help maintain fruit quality for extended periods (Tasdelen & Bayindirli, 1998) and retard apple decay during prolonged storage (Xuan & Streif, 2005). Improper cold storage can resulted in a significant decline in fruit quality (Davis & Blair, 1936). The aforementioned data suggest the potential efficacy of low-dose ozone gas in *Listeria* control; however, uncontrolled or excessive ozone gas administration may lead to ozone burn and quality deterioration, resulting in substantial financial losses. Therefore, apple fruit quality parameters were evaluated both at harvest and after storage.

For Granny Smith apples, both years of study revealed that 6 months of RA storage resulted in a significant reduction of firmness and TA (Sheng et al., 2022). CA storage alone significantly mitigated the loss of firmness and TA. The incorporation of ozone gas in CA storage had a minor impact on Granny Smith apple quality, while the 1-MCP treatment further reduced the loss of firmness and TA in CA storages, independent of ozone gas (Sheng et al., 2022). In terms of physiological disorders, CA storage with or without ozone reduced the incidence of superficial scald and lenticel decay in year 1(Sheng et al., 2022). In year 2, although CA+HO_3 storage alone failed to inhibit superficial scald, 1-MCP pre-storage treatment significantly reduced the incidence of superficial scald on GSA in CA storage with or without ozone (Sheng et al., 2022). However, in the year 2 studies, apples under CA storage showed ozone burn-like symptoms, and apples treated with 1-MCP, and ozone gas showed a higher ozone burn-like symptom (Sheng et al., 2022). To confirm the observed symptoms, we conducted another quality evaluation in year 3, and no ozone burn-like symptoms were observed on any apples regardless of treatment. (Sheng et al., 2022). 1-MCP treatment of Granny Smith apples in CA with ozone resulted in ozone burn-like symptoms in year 2 but not in year 3, indicating the potential role of preharvest properties of apples on their reactions to ozone treatment. CA storage, regardless of ozone gas and 1-MCP treatment, significantly reduced the incidence of internal browning compared to RA storage in all three years (Sheng et al., 2022).

Red Delicious apples are particularly prone to developing watercore compared to other commercially grown apple varieties, posing an increased risk of developing internal disorders such as internal browning during long-term CA storage (Mattheis, 2008). Quality attributes of Red Delicious apple fruits under different storage conditions were further assessed both at harvest and after 6-month or 9-month storage. TSS of Red Delicious apples did not differ among storage treatments and over 9 months storage (Shen et al., 2021). Red Delicious apples subjected to RA storage exhibited significantly lower firmness and TA compared to those with CA with MCP-1 pretreatment at 6-month and 9-month storages (Shen et al., 2021). Ozone gas application significantly improved the firmness and increased TA of apples comparted to RA and CA storage (Shen et al., 2021). Both ozone gas application at 60-80 ppb did not induce ozone burn in Red Delicious apples at both 6-month and 9-month storage (Shen et al., 2021). Neither of ozone application had effects on superficial scale, lenticel decay, Russet, CO_2 damage compared to CA, all of which were significantly better than those observed in RA storage (Shen et al., 2021). Ozone applications further enhanced the visual appearance of apples (Shen et al., 2021).

Cosmic Crisp apples have garnered consumer popularity due to their appealing crisp texture, juiciness, and other desirable traits (Evans et al., 2012). These apples possess remarkable storage capabilities (Evans et al., 2012). The weight and TSS of apple fruits at 9 months of storage were not different from that at harvest, regardless of storage conditions (Table 1). The firmness of Cosmic Crisp apples, after 9 months of CA storage with or without 1-MCP and gaseous ozone treatments, was the same as that measured at harvest. However, the firmness of Cosmic Crisp apples under RA storage was significantly reduced (Table 1). TA of Cosmic Crisp apples after 9-month storage was significantly lower than that of apples at harvest, regardless of storage treatments. Gaseous ozone application had no impact on TA (Table 1). No external disorder or internal disorder was observed in any of Cosmic Crisp apples after 9 months of storage (data not shown).

Table 1. Fruit quality attributes of Cosmic Crisp apples at harvest and after storage

Treatment	**Weight (kg)**		**Firmness (kg)**		**TSS (% Brix)**		**TA (% malic acid)**	
	0-m	9-m	0-m	9-m	0-m	9-m	0-m	9-m
RA	0.26 ± 0.05^{A}	0.25 ± 0.04^{aA}	8.33 ± 0.16^{A}	6.22 ± 0.13^{aB}	NA	14.25 ± 0.25^{a}	0.77 ± 0.03^{A}	0.26 ± 0.02^{aB}
CA		0.26 ± 0.06^{aA}		8.00 ± 0.11^{bA}		15.05 ± 0.15^{a}		0.34 ± 0.02^{bB}
CAMCP		0.24 ± 0.03^{aA}		8.22 ± 0.08^{bA}		14.43 ± 0.17^{a}		0.41 ± 0.00^{bB}
CAMCPLowO_3		0.26 ± 0.04^{aA}		8.24 ± 0.09^{bA}		14.18 ± 0.15^{a}		0.37 ± 0.04^{bB}
CAMCPHighO_3		0.25 ± 0.05^{aA}		8.29 ± 0.10^{bA}		14.38 ± 0.27^{a}		0.41 ± 0.03^{bB}
CAHighO_3		0.26 ± 0.05^{aA}		8.12 ± 0.07^{bA}		14.55 ± 0.19^{a}		0.37 ± 0.09^{abB}

TSS: Total soluble solids; TA: titratable acidity. RA: refrigerated atmosphere; CA: controlled atmosphere; MCP: apples were treated with 1-methycyclopropene before cold storage; CAHighO_3: CA storage with continuous gaseous O_3 application at 78.2 ± 12.2 ppb during the initial 24 weeks of storage; CAMCPHighO_3: CA storage with continuous gaseous O_3 application at 78.2 ± 12.2 ppb during the initial 24 weeks of storage, where apples were treated with MCP-1 before different storages; CAMCPLowO_3: CA storage with continuous gaseous O_3 application at 55.5 ± 8.8 ppb during the initial 24 weeks of storage, where apples were treated with 1-MCP before cold storage. $^{a-d}$ Mean within a column of the selected quality attribute without a common letter differ significantly ($P < 0.05$). $^{A-B}$ Mean the comparison of an individual quality parameter at 0-month (at-harvest) and 9-month storage without common letter differ significantly ($P < 0.05$). Mean ± SEM, n=40.

4. *E. faecium* NRRL B-2354 is a suitable surrogate of *L. monocytogenes* for Cosmic Crisp apple during cold storage study with or without ozone gas treatment

For apples stored under regular atmosphere (RA) and controlled atmosphere (CA) conditions, the survival pattern of *E. faecium* NRRL B-2354 on Cosmic Crisp apples closely mirrored that of *L. innocua* over a 24-week storage period (Figures 3B & 8), resulting in a reduction of 3.0-3.1 log10 CFU/apple. However, when Cosmic Crisp apples underwent the gaseous ozone treatment, *E. faecium* NRRL B-2354 exhibited higher resistance to ozone treatment compared to *L. innocua* throughout 36-week storage (Figures 3B & 8). At the conclusion of the 24-week storage, upon discontinuation of the ozone gas treatment, the implementation of ozone gas led to an additional reduction of 1.1-1.2 $\log_{10}$

CFU/apple for *E. faecium* NRRL B-2354, whereas *L. innocua* exhibited a more substantial reduction of 2.4-2.7 log10 CFU/apple (Figures 3B & 8). Data indicate *E. faecium* NRRL B-2354 serves as a suitable non-*Listeria* surrogate for assessing *Listeria* behaviors during commercial cold storage.

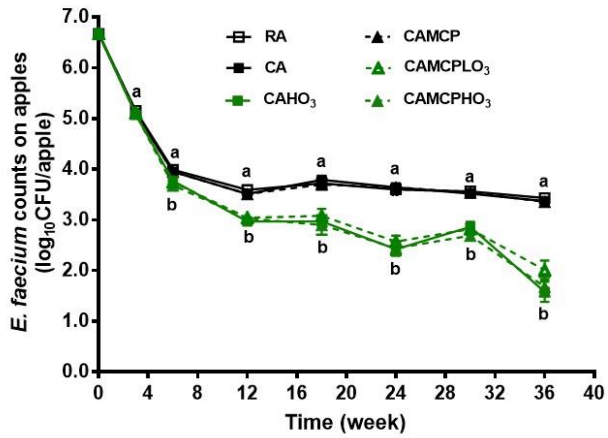


Figure 8. Fates of *E. faecium* NRRL B-2354 on Cosmic Crisp apples during 36 weeks of cold storage under different storage regimes. RA: refrigerated atmosphere; CA: controlled atmosphere; MCP: apples were treated with 1-methycyclopropene prior to cold storage; CAHighO_3: CA storage with continuous gaseous O_3 application at 78.2 ± 12.2 ppb; CAMCPHighO_3: CA storage with continuous gaseous O_3 application at 78.2 ± 12.2 ppb, where apples were treated with 1-methycyclopropene prior to cold storage; CAMCPLowO_3: CA storage with continuous gaseous O_3 application at 55.5 ± 8.8 ppb, where apples were treated with 1-methycyclopropene prior to cold storage. Different letters (a-b) at each sampling point indicated significant differences ($P < 0.05$).

EXECUTIVE SUMMARY

This comprehensive study explores the potential of gaseous ozone in mitigating *L. innocua* contamination on Granny Smith, Red Delicious, and Cosmic Crisp apples during extended commercial cold storage. The study revealed a higher die-off rate within the initial 6 weeks, showcasing reductions of 1.5-2.0 log for Granny Smith and Red Delicious, and a slightly elevated 2.8-2.9 log for Cosmic Crisp apples. Following the initial 6 weeks, the die-off rate diminished, resulting in a 2.2-3.0 log reduction for Granny Smith and Red Delicious, and a substantial 3.4 log reduction for Cosmic Crisp apples after 36 weeks of RA and CA storage. Additionally, the study demonstrated that gaseous ozone application at 50-87 ppb is a viable technology for controlling *Listeria*, leading to an additional 2.5~3.0 log CFU/apple reduction on apples compared to CA storage alone. Given the comparable antimicrobial efficacy of ozone gas at 50-87 ppb across all apple varieties, it suggests that a lower concentration within this range could be considered for practical applications in the apple industry. Data obtained from Cosmic Crisp apple storage revealed that gaseous ozone applied for the initial 24 weeks achieved a comparable anti-*Listeria* efficacy to Granny Smith and Red Delicious apples treated with 36 weeks of ozone gas, indicating potential for shorter duration in future industry applications. Furthermore, continuous low-dose ozone gas application at 50-87 ppb decreased yeast/mold counts on apples and did not adversely affect fruit quality or lead to internal and external disorders across all tested apple varieties. Our data further indicate that. *E. faecium* NRRL B-2354 can serve as a suitable non-Listeria surrogate for *L. monocytogenes* in apple cold storage studies.

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