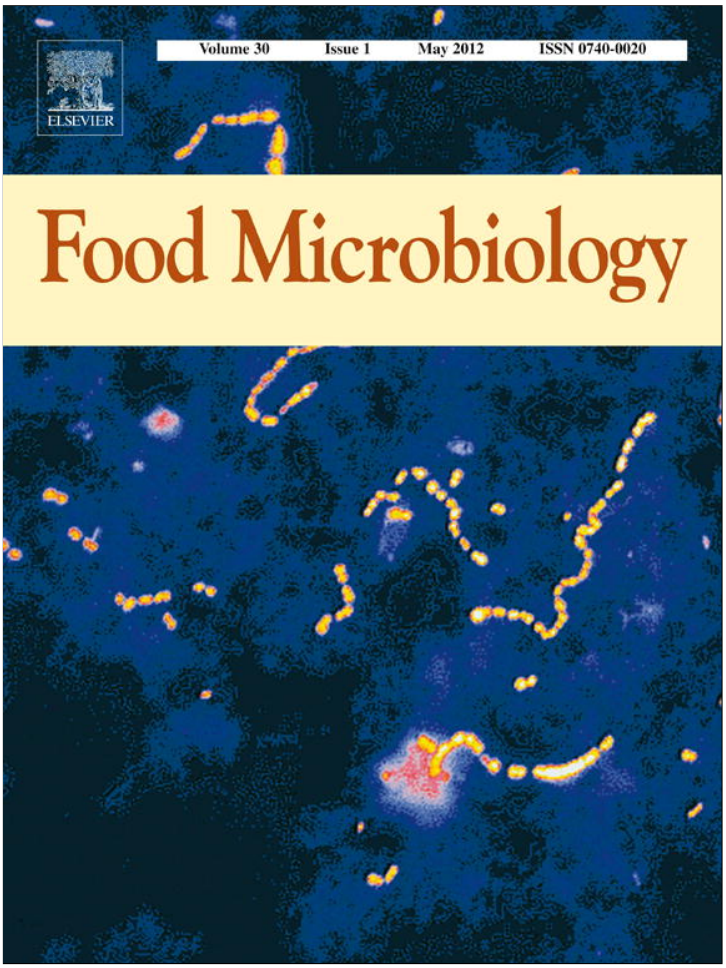

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Antimicrobial activity of cyclodextrin entrapped allyl isothiocyanate in a model system and packaged fresh-cut onions

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ABSTRACT

The aim of this work was to determine the antimicrobial effect of allyl isothiocyanate (AIT) entrapped in alpha and beta cyclodextrin inclusion complexes (ICs). In model experiments, AIT formulations were applied to filter paper discs fixed inside the lid of Petri dishes, where the agar surface was inoculated with the target organism (*Penicillium expansum*, *Escherichia coli* or *Listeria monocytogenes*). Solid phase microextraction coupled with gas chromatography was used to determine static headspace concentrations of AIT formulations. The antimicrobial effect of beta IC was determined during aerobic storage of packaged fresh-cut onions at 5 °C for 20 days. AIT entrapped in beta IC exhibited a significantly ($p < 0.05$) better antimicrobial effect compared to unentrapped AIT. AIT vapour concentrations in the static system were highest for unentrapped AIT followed by beta IC and alpha IC. Application of beta IC (200 μl/l) to packaged fresh-cut onions effectively decreased numbers of *L. monocytogenes*, which were also decreased at slower rates to undetectable levels on untreated cut onion. After 10 days, total aerobic counts were *ca.* 4 log CFU/g lower on onions treated with beta IC (100 and 200 μl/l) compared to untreated controls. This work demonstrates the utility of beta IC as an antimicrobial treatment with potential applications in packaged fresh-cut vegetable products.

1. Introduction

Allyl isothiocyanate (AIT) is a pungent, volatile, antimicrobial substance derived from mustard seed that is commonly used as a flavouring (Fenwick et al., 1983). It is largely insoluble in aqueous systems and reacts readily with peptides and proteins (Cejpek et al., 2000). AIT in the vapour phase affects microorganisms, with bactericidal concentrations of 2–8 μl/l air in glass jars (Delaquis and Sholberg, 1997), and inhibitory effects caused by 0.1 μl/l air in sealed glass Petri dishes (Isshiki et al., 1992). Lin et al. (2000) showed that AIT vapour was bactericidal to bacterial pathogens on tomatoes, however, very high AIT concentrations of 100 μl/l were needed.

When evaluating AIT as a food preservative, it is necessary to consider its reactivity, odour and volatility, as well as taste. Also, in packaged food systems AIT vapour may diffuse with time out through the polymer materials (Lim and Tung, 1997). Entrapping AIT in cyclodextrins (CD) may be a way to reduce odour, decrease unwanted reactivity and control release similar to results obtained for some CD entrapped volatile flavour compounds (Madene et al., 2006). CDs are cyclic structures, comprised of 6 (alpha), 7 (beta) or 8 (gamma) glucose monomers linked by alpha 1-4 bonds. CDs have a relatively apolar central cavity that can be used to form a stable interaction with hydrophobic "guest" compounds such as antimicrobials, flavouring, or pharmaceutical substances (Del Valle, 2004; Szejtli, 2004). These guest–host complexes are called inclusion complexes (ICs) (Szejtli, 2004). Release and vapourization of AIT entrapped in alpha or beta CD occurs in the presence of water and is dependent on temperature and relative humidity (Ohta et al., 1999; Shiga et al., 2000). Other workers have proposed that controlled release of volatile antimicrobial compounds from ICs in packaged food products could be used in food preservation (Ayala-Zavala et al., 2008). Recently, that group demonstrated that thyme and garlic oil beta ICs inhibited the fungal growth *in vitro* and on tomatoes, and that the release of volatile antimicrobials from the beta ICs was related to the relative humidity (Ayala-Zavala and Gonzalez-Aguilar, 2010; Del Toro-Sanchez et al., 2010). Entrapment of AIT in CDs has previously been described (Li et al., 2007; Zhang et al., 2007), as has the inhibitory effect of AIT in an alpha IC preparation on *Penicillium* spp. (Plackett et al., 2006). However, the ability of AIT in beta IC formulations to elicit antimicrobial effects *in vitro* or in a food product has not been characterized.

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The objective of this work was to determine the antimicrobial effect of unentrapped AIT and its ICs (alpha and beta) on *Penicillium expansum*, *Escherichia coli* and *Listeria monocytogenes* in a model system; and of beta IC applied to an aerobically packaged fresh-cut onion product without or with prior inoculation with *L. monocytogenes*.

2. Material and methods

2.1. Preparation and characterisation of AIT inclusion complexes (ICs)

Modifying the method of Li et al. (2007), beta (Acros Organics, Morris Plains, NJ, USA) and alpha (Sigma Aldrich, Oakville, ON, Canada) cyclodextrins were dissolved in distilled water at 60 °C and cooled to 40 °C. A 1:1 (vol/vol) mixture of AIT (94% pure, Acros Organics) and ethanol was added in a 2:1 M ratio of AIT:CD and stirred for 3 h. These IC mixtures were cooled overnight, filtered (Whatman #4 filter paper, NJ, USA), rinsed with distilled water, and frozen at −40 °C until later use. ICs were stored frozen for less than two months and used before any detectable degradation of AIT. Three batches of each IC were analysed for AIT content by dissolving 0.1 g complex in a mixture of distilled water and hexane (1:1 ratio) followed by sonication for 20 min (Elmasonic EL 10, Elma, Germany). Hexane fractions were collected and injected into a gas chromatograph equipped with flame ionisation detection (GC, Perkin ElmerAutosystem II, MA, USA). A type VF I column (15 m × 0.25 mm) (Varian, CA, USA) was used for the analysis, with helium as the carrier gas. Inlet and detection temperature were set to 250 °C. The temperature program consisted of an initial temperature of 60 °C, increasing at 12.5 °C/min, and then held at 95 °C for 4 min. VarianStar software (Star Chromatography, CA, USA) was used to analyse the resulting peak areas. Peak areas and a standard curve were used to calculate AIT content of the ICs. ICs were oven dried (40 °C) to a constant weight to determine moisture content and CD mass. AIT concentration and CD mass per IC weight unit was then converted to moles in order to calculate the inclusion efficiency (%) = (mol AIT/mol CD) × 100.

2.2. In vitro vapour assays to determine the antimicrobial effect of AIT and its ICs

P. expansum (Isolate #1525, postharvest pathology culture collection, Agriculture and Agri-Food Canada, Summerland, BC, Canada) was grown on Potato Dextrose agar (Potato Dextrose Broth, PDB, HiMedia, Mumbai, India, supplemented with 15 g/l Bacto agar [Difco, Oakville, ON, Canada]) at 25 °C for 5 days. Conidia were harvested by swirling 5 ml of sterile distilled water over mature growth plates. Conidia were then inoculated on fresh PDA plates at a concentration of *ca.* 4 log CFU/plate. Aqueous suspensions of IC complexes and AIT were vigorously mixed and immediately pipetted onto sterile filter paper discs (2 cm in diameter, Whatman #4) placed inside of the Petri dish lids to yield initial concentrations ranging between 0.1 and 5 μl AIT/l air as calculated by diving the AIT concentrations with the Petri dish headspace volume, i.e., dish volume minus 20 ml to account for the volume of the agar. The Petri dishes were then assembled with the dish lid at the bottom and the inoculated agar surface above facing the lid's AIT infused paper disc, and closed with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA). Plates were incubated at 5 and 10 °C for 12 days, or 25 °C for 5 days followed by re-incubation (after removal of the AIT or IC infused filter paper discs) for the same time and temperature combinations to observe fungistatic and fungicidal effect, respectively, of the treatments. Duplicate samples were prepared for each treatment and the experiment was repeated three times. Controls consisting of cyclodextrins (CDs), water and ICs prepared without AIT (non-AIT ICs) were also assayed.

The Gram positive *L. monocytogenes* (ATCC 19115, serotype 4 b, human isolate) and the Gram negative *E. coli* (ATCC 25922) were grown overnight in tryptic soy broth (Difco), diluted in peptone saline (PS, 0.1% peptone, 0.85% NaCl) and spread on tryptic soy agar plates (TSA, Difco) at a concentration of 4 log CFU/plate. Plates were assembled as described above with initial AIT loads ranging from 5 to 50 μl/l and incubated for 3 days at 25 °C followed by the assessment of growth to determine the bacteriostatic concentration. After removing the filter paper discs, plates were re-incubated (3 days at 25 °C) to determine the bactericidal concentration. AnaeroIndicator tablets (Mitsubishi Gas Chemical Co., Japan) were used in separate controls to confirm that the environment in the Petri dishes remained aerobic during the incubation. Controls (CDs, water and non-AIT ICs) were assayed in duplicate.

Fungistatic or bacteriostatic effects were determined as a ≥100-fold reduction in CFU/plate compared to the control after incubation. Bactericidal or fungicidal effects were noted as lack of regrowth or maintenance of a ≥100-fold reduction in CFU/plate compared to the control after AIT was removed from the system and plates were re-incubated. Damaged cells may have been viable but were not culturable.

2.3. Release of AIT vapour from aqueous solutions of AIT and the ICs

To determine the headspace concentration of AIT released from alpha IC, beta IC and AIT in aqueous suspension, three separate batches of ICs were examined (experiment performed once in triplicate). Aqueous suspensions of the test substances with AIT concentrations, yielding a final initial theoretical load of 5 μl AIT/l air if release of 100%, were pipetted onto filter paper discs placed in 55 ml glass vials sealed with Teflon® closures. Vials were incubated at 25 °C for 5 days. On sampling days, the solid phase microextraction (SPME) fibres (100 μm polydimethylsiloxane fibre, Supelco, Sigma Aldrich, Oakville, ON, Canada) were exposed to vial headspace for 30 min at 22 °C, followed by desorption of AIT in the GC injector held at 235 °C. A SAC-5 gas chromatograph column (30 m × 0.25 mm, Supelco) with helium as the carrier gas was used for the analysis. The GC oven temperature was heated from 60 °C to 230 °C at 4 °C/min, with the flame ionisation detector held at 235 °C. The peak areas represent AIT headspace concentrations.

2.4. Antimicrobial effect of beta IC in packaged fresh-cut onions

Freshly chopped yellow onions (*Allium cepa*) were obtained from Nova Agri Inc. (Centreville, NS, Canada) during the months of May–August, 2010. The effect of AIT entrapped in beta CD on the endogenous onion microflora and on the fate of inoculated *L. monocytogenes* 568 (serotype 1/2a, food isolate, Hefford et al., 2005) was followed during storage trials for 20 days at 5 °C. Briefly, overnight cultures (37 °C, 24 h) of *L. monocytogenes* 568 were harvested, washed in PS and resuspended to 6 log CFU/ml. Onion (100 g, ~0.5 × 1 cm pieces) was inoculated with 1 ml PS or listerial suspension to yield an initial concentration of 4 log CFU/g and thoroughly mixed. Twenty five gram samples of onions were placed into 6 × 8 cm polyethylene bags (oxygen transmission 2946 cm^2/m^2 day, material identical with packaging material used by the company) equipped with sterile filter paper discs containing water or beta IC to yield 0, 100 and 200 μl AIT/l bag headspace. Bags were then heat sealed and incubated at 5 °C. On sampling days, onions (25 g) were aseptically removed from the bags and diluted with 225 ml PS and homogenized for 1 min (Stomacher 400 Lab Blender, Seward Laboratory, London, UK). Enumeration was done by spot plating (5 × 20 μl spots to yield 100 μl per dilution, Miles

et al., 1938) appropriate serial dilutions on Oxford Agar (Oxoid, Nepean, ON, Canada) and Plate Count Agar (Difco) for *Listeria* counts and total aerobic counts, respectively, following incubation for 48 h at 35 °C. In samples, where *Listeria* numbers approached the spot plate method's detection limit (100 CFU/g), a 3 tube MPN method, where aliquots of 0.1, 1.0 and 10 ml from the 10^{-1} dilution were enriched in Fraser broth (Oxoid) followed by detection on Palcam agar (Oxoid), was used to determine the fate of the inoculated bacteria. Each treatment was done in duplicate and the entire experiment repeated twice. Statistical comparisons of the treatment effect were made with the Holm-Sidak multiple comparison test using a 5% significance level (Sigmaplot 11, Systat Software Inc., Germany).

3. Results

3.1. Characteristics of AIT inclusion complexes (ICs)

Both the AIT and moisture contents were significantly ($p < 0.05$) higher in beta IC (110 μl AIT/g dry matter and 33%) than in alpha IC (86 μl AIT/g dry matter and 3%, Table 1). Inclusion efficiencies for the wet IC preparations were found to be 86% and 128% for alpha and beta ICs, respectively, indicating that for beta IC some AIT was associated with the surface. Both inclusion complexes had a faint AIT odour after filtration and washing; however, this odour was reduced compared to the unentrapped AIT. The IC preparations were left undried in a hydrated state suitable for direct application onto filter paper discs for delivery into the vapour phase of the petri dish or packages of fresh-cut onion.

3.2. In vitro antimicrobial vapour assays

AIT entrapped in beta CD exhibited the most antimicrobial effect, with minimum initial concentrations with fungistatic effect of 0.5–1 μl AIT/l air and bacteriostatic concentrations of 25 and 50 μl AIT/l for *E. coli* and *L. monocytogenes*, respectively (Tables 2 and 3). Alpha IC also effectively inhibited *P. expansum* with a minimum initial concentration with fungistatic effect of 1 μl/l, while AIT alone inhibited growth at 5 μl/l air (Table 2). Neither alpha IC nor unentrapped AIT inhibited *E. coli* or *L. monocytogenes* when applied at 50 μl/l (Table 3). Beta IC and alpha IC were fungicidal at initial concentrations of 5 μl/l air, whereas AIT was not (Fig. 1). The target bacteria were less susceptible and only beta IC (50 μl AIT/l) was bactericidal, while the same concentration of AIT and alpha IC had no effect (Table 3). AIT tended to be more inhibitory at 5 °C than at 10 and 25 °C for the fungus (Table 2). No growth reduction was observed in the control treatments (CD and ICs without AIT).

3.3. Release of AIT from aqueous suspensions of unentrapped and entrapped AIT

The release of AIT vapour differed significantly ($p < 0.05$) between AIT formulations. AIT headspace levels reached an equilibrium after 1 day and remained stable over 5 days, with the concentration in the static headspace over suspensions of unentrapped AIT being 10 folder greater than that of beta IC which in turn was tenfold greater than that over alpha IC (Fig. 2).

Table 1
Properties of inclusion complexes (ICs) consisting of allyl isothiocyanate (AIT) entrapped in alpha or beta cyclodextrin.

Inclusion complex (IC)	Total AIT (μl/g dry matter)	Moisture (% wet basis)	Inclusion efficiency (%)
Alpha IC	86.2[a] ± 6.6	3 ± 1	86 ± 7.8
Beta IC	110.3 ± 8.7	33 ± 4	128 ± 8.0

[a] Means ($n = 3$) ± standard deviation.

Table 2
Inhibition of *Penicillium expansum* by allyl isothiocyanate (AIT) vapour released from filter paper discs loaded with different initial concentrations of alpha IC, beta IC and unentrapped AIT during incubation at 5, 10 or 25 °C ($n = 6$).

Sample	Temperature (°C)	Minimum initial AIT concentration with fungistatic effect (μl/l air)	Minimum initial AIT concentration with fungicidal effect (μl/l air)
Alpha IC[a]	5	1	5
	10	1	>5
	25	1	5
Beta IC[a]	5	0.5	5
	10	1	5
	25	1	5
AIT	5	5	>5
	10	>5	n/a[b]
	25	>5	n/a

[a] Inclusion complex (IC) consisted of AIT entrapped in alpha or beta cyclodextrin.
[b] Not available, as the highest initial concentration of AIT (5 μl/l air) had no fungistatic effect.

3.4. Antimicrobial effect of beta IC on packaged fresh-cut onions

Addition of beta ICs yielding initial concentrations of 100 μl AIT/l air significantly ($p < 0.05$) reduced aerobic plate counts compared to the control from days 6 through to 12, and 200 μl AIT/l significantly ($p < 0.05$) reduced microflora levels from day 3 through 14, with levels never reaching more than 5 log CFU/g for either treatment (Fig. 3). In the absence of AIT, aerobic plate counts increased from initial levels of 3.8 log CFU/g to levels of 8 log CFU/g after 8 days after which levels gradually decreased back to 4 log CFU/g at the end of the aerobic storage period.

Inoculated *L. monocytogenes* showed a slow decline from initial levels of 3.5 to 3 log CFU/g after 12 days in onion samples treated with no AIT or 100 μl AIT/l, indicating that *L. monocytogenes* is incapable of growth on raw cut onions (Fig. 4). On onion treated with 200 μl AIT/l *L. monocytogenes* numbers dropped to 1.4 log CFU/g after 8 days, with significant reductions ($p < 0.05$) compared to the control on days 8, 10, 12 and 14. On day 20, all samples approached the MPN detection limit (Fig. 4). *Listeria* spp. were not found in uninoculated control samples.

Table 3
Bacteriostatic and bactericidal effect of allyl isothiocyanate (AIT) vapour released from filter paper discs loaded with different initial concentrations of alpha IC, beta IC, and unentrapped AIT on *E. coli* and *L. monocytogenes* during incubation at 25 °C ($n = 6$).

Sample	Minimum initial concentration with bacteriostatic effect (μl/l air)	Minimum initial concentration with bactericidal effect (μl/l air)
E. coli		
Alpha IC[a]	>50	n/a[b]
Beta IC[a]	25	50
AIT	>50	n/a
L. monocytogenes		
Alpha IC	>50	n/a
Beta IC	50	50
AIT	>50	n/a

[a] Inclusion complex (IC) consisted of AIT entrapped in alpha or beta cyclodextrin.
[b] Not available, as the highest initial concentration of AIT (50 μl/l air) had no bacteriostatic effect.

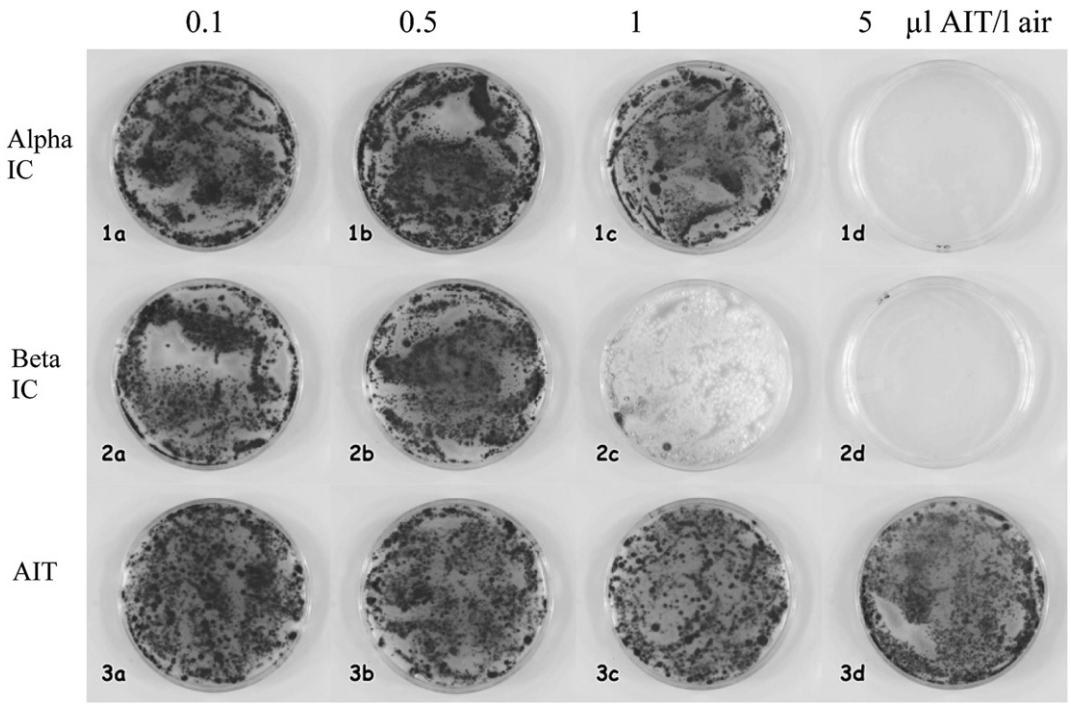

Fig. 1. Fungicidal effect of allyl isothiocyanate (AIT) vapour on *Penicillium expansum* at 5 °C. AIT was applied as aqueous suspensions of alpha IC (1 a–d), beta IC (2 a–d) and pure unentrapped AIT (3 a–d) to filter paper discs which were placed in the lids of Petri dishes incubated upside down. Initial concentrations were $a = 0.1$, $b = 0.5$, $c = 1$, and $d = 5$ µl AIT/l air. The inclusion complex (IC) consisted of AIT entrapped in alpha or beta cyclodextrin. Plates were incubated with AIT for 12 days, followed by removal of AIT from the system, and re-incubation without AIT for 12 days to allow damaged/injured cells to grow.

4. Discussion

Alpha IC contained the expected amount of AIT (86% inclusion efficiency), which concurs with the results of Li et al. (2007). Beta IC had higher than expected amounts of AIT (>100% inclusion efficiency, with ~90% expected according to results of Li et al., 2007). This could be explained by the hydrated preparation of beta IC containing some unentrapped, surface associated AIT. In the current study, we elected to use washed hydrated ICs to allow for easy mixing with the appropriate amount of water to obtain a predetermined AIT concentration, and this diluted IC suspension was in turn applied to the filter paper disc based delivery system to allow for AIT release into the vapour phase of the food package or Petri dish model system.

The *in vitro* antimicrobial vapour assay, where the antimicrobial is applied to a disc of filter paper placed inside a package (here a polystyrene Petri dish closed with parafilm) together with agar containing water, protein and carbohydrates, can be thought to conceptually represent an aerobic model food package. Higher levels of unentrapped AIT were needed to inhibit growth in this

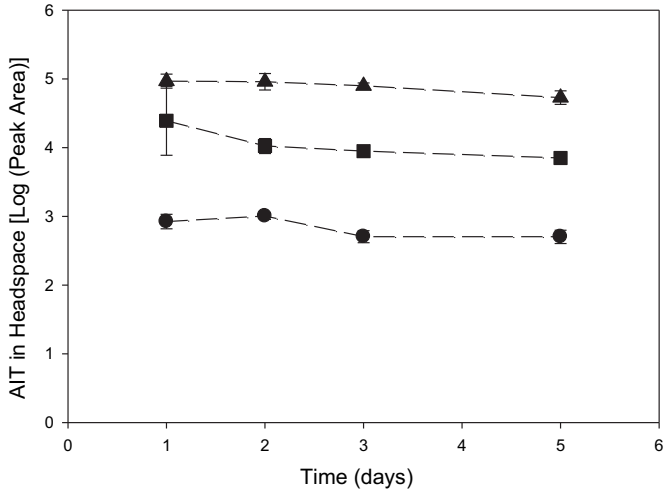

Fig. 2. Allyl isothiocyanate (AIT) vapour concentrations (Log_{10} Peak Area) released from aqueous suspensions of alpha IC (●), beta IC (■), or AIT (▲) at 25 °C in a static hermetically sealed system initially loaded with 5 µl AIT/l air. Vapour AIT concentrations were determined by solid phase microextraction followed by gas chromatography ($n = 3$, means ± standard deviation). The inclusion complex (IC) consisted of AIT entrapped in alpha or beta cyclodextrin.

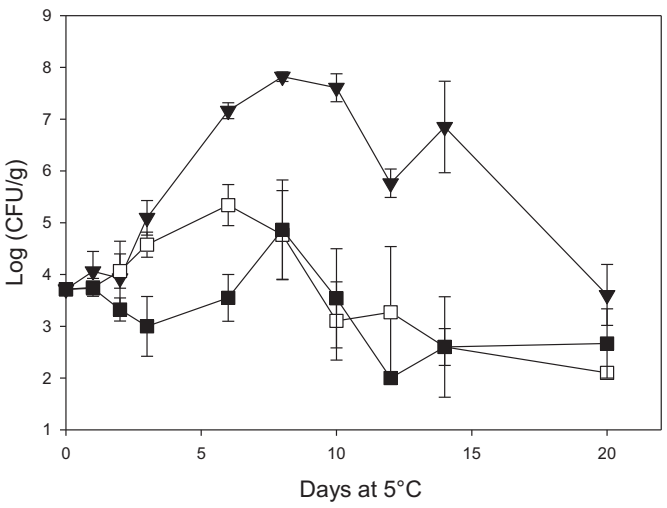

Fig. 3. Development of total aerobic counts in fresh-cut onions packaged with filter paper discs containing beta inclusion complexes (beta ICs) during refrigerated storage. Beta ICs consisted of AIT entrapped in beta cyclodextrin. Individual packages contained initial AIT levels of 0 (▼), 100 (□) or 200 (■) µl AIT l^{-1} air. Means (log [CFU/g]) represent $n = 4$ ± standard error.

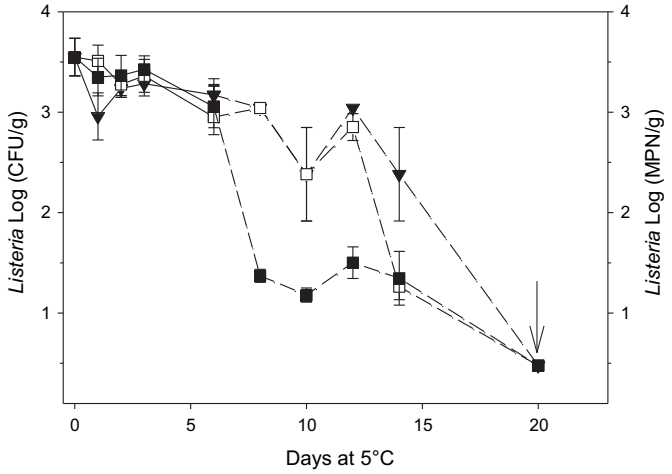

Fig. 4. Survival of *L. monocytogenes* inoculated on fresh-cut onions packaged with filter paper discs containing beta inclusion complexes (beta ICs) yielding initial allyl isothiocyanate (AIT) levels of 0 (▼), 100 (□) or 200 (■) µl AIT/l air during refrigerated storage. Beta ICs consisted of AIT entrapped in beta cyclodextrin. Data connected with solid lines were obtained by plating on *Listeria* Selective Agar (average ± standard error, $n = 4$), while data connected by dotted lines were obtained using the Most Probable Number (MPN) assay (average ± standard error, $n = 2$) in order to lower the detection limit to 3 MPN/g. On day 20, *Listeria* MPN/g was as indicated by the arrow below the detection limit for all treatments.

system than was reported for an assay in sealed glass vessels (Isshiki et al., 1992; Delaquis and Sholberg, 1997). This may be due to AIT vapour rapidly being released from unentrapped AIT and escaping the Petri dish before an antimicrobial effect can be observed. Application of beta IC resulted in significantly ($p < 0.05$) greater inhibitory or killing effects compared to alpha IC. This difference in the antimicrobial effect between beta and alpha ICs in the vapour system is thought to be due to their release patterns. The static headspace experiment showed that ten-times less AIT was released from alpha IC than from beta IC in a sealed glass vial (Fig. 2). However, the presence of unentrapped AIT in the beta IC preparation may also have increased the AIT release. Previous research showed that beta IC released AIT more quickly than alpha IC due to beta CD's larger diameter (Ohta et al., 1999; Li et al., 2007). This phenomenon would cause alpha ICs to yield lower headspace concentrations in the Petri dish system and less antimicrobial effect. In contrast, beta IC would release AIT at higher levels to result in a larger antimicrobial effect, as was observed in the antimicrobial vapour model system. These observations suggest that an optimal balance is required where release of AIT entrapped in an IC can either be too slow, or in the case of unentrapped AIT too fast (Zhang et al., 2007). In our study, beta IC in our open aerobic model system achieved the best balance.

The finding that bacteria are more resistant to AIT than the fungus agrees with previous observations (Isshiki et al., 1992; Delaquis and Sholberg, 1997), however, the reason for this is unknown. It has been hypothesized that strict aerobes are most sensitive to AIT, possibly due to the antimicrobial acting as an uncoupler of oxidative phosphorylation (Delaquis and Sholberg, 1997). Lower temperatures appear to increase AIT activity when applied to the fungus, but this could be due to slower fungal growth.

Beta IC released sufficient AIT to cause a large, sustained antimicrobial effect on onions packaged in a plastic material with high oxygen permeability and stored at 5 °C. Beta IC formulations with garlic oil have recently been reported to inhibit the microflora on tomatoes (Ayala-Zavala and Gonzalez-Aguilar, 2010), indicating the possible scope for use of beta ICs with different antimicrobials. The slower release of beta IC causes headspace levels to be lower than for unentrapped AIT. This should correspond to less AIT odour when opening packages with beta IC, making it less objectionable to consumers. The necessity to use more antimicrobial agent when moving from *in vitro* testing to a food product is common, as antimicrobials may dissipate from packaging, undergo chemical reactions with food components, and target cells being more resistant due to stress response. Application of beta IC (200 µl/l) effectively inhibited or killed inoculated *L. monocytogenes*; however, results also revealed that endogenous onion antimicrobials inhibited growth which is in agreement with a recent report by Santas et al. (2010).

In our work, we delivered vapourized AIT to a chopped vegetable product. Another way to apply the AIT ICs may be to mix them directly into the food matrix. A similar method was used to deliver AIT entrapped in acacia gum to finely chopped beef and sausages, and was shown to effectively inhibit *E. coli* O157:H7 at concentrations not detrimental to the sensorial quality of the products (Chacon et al., 2006a and 2006b).

In conclusion, the beta IC formulation, where AIT is entrapped in beta CD, inhibited *P. expansum*, *E. coli* and *L. monocytogenes* more effectively than unentrapped AIT and alpha IC. The fungus was more susceptible to beta IC than the two bacteria tested. Beta IC also inhibited the growth of the aerobic microflora and reduced survival of *L. monocytogenes* in packaged fresh-cut onions. Cost, efficacy against pathogens, increased shelf life, and consumer acceptance all play a role in the development of antimicrobial technologies in food products. Further work is necessary to explore the potential use of AIT as beta IC as a food preservative.

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