

## NATURAL FOOD PRESERVATION SYSTEM AS ALLYL ISOTHIOCYANATE AND EDIBLE BROWN SEAWEED *LAMINARIA JAPONICA* AGAINST SELECTED ANAEROBIC FOODBORNE BACTERIA: *CLOSTRIDIUM PERFRINGENS* AND *CAMPYLOBACTER JEJUNI*

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### ABSTRACT

Allyl isothiocyanate (AITC) is basic compound in mustard essential oil and found in some other species of *Brassicaceae*. It has antimicrobial properties against wide range of foodborne pathogens. Its food industry use as a natural preservative is limited by its quick evaporation, taste pungency, and weak water solubility. The minimum inhibitory concentration (MIC) for AITC against the two tested bacteria; *Clostridium perfringens* and *Campylobacter jejuni* was determined by Microdilution assay, which were 0.078 and 0.039 mg/ml, respectively. According to the results of the same assay, bactericidal effect was recorded against both tested bacteria. By using vapor and solution deposition ways into *Laminaria japonica* de-oiled powder, the antimicrobial effect of AITC vapor was demonstrated against the selected bacteria; up to 91% of colony deactivation occurred within 72 h for *C. perfringens*, while 97% for *C. jejuni*, the decay behavior of both solution and vapor -deposited were parallel. At 72 h, the de-oiled *L. japonica* powder was adsorbed AITC twice that of the raw powder approaching 99% theoretical maximum amount. The continued antimicrobial activity of AITC after contact proves that AITC + de oiled *L. japonica* powder would be used as natural system for preserve food.

**Keywords:** Allyl isothiocyanate; *Laminaria japonica*; antimicrobial effect; anaerobic foodborne bacteria; *Clostridium perfringens*; *Campylobacter jejuni*

### INTRODUCTION

Managing and keep the food in ways that control food borne diseases is described as food safety. This contains some of protocols that should be applied to stop food borne diseases outbreaks. Three types of microorganisms can contaminate food which in many cases lead to death and cause public health problems: bacteria, molds, and yeasts. Yeast and molds cause spoilage of food so, they make the big economic problems in the food industry (<http://www.foodsafetysite.com>).

Food preservation by traditional strategies including physical preservation and chemical antimicrobial addition may lead to organoleptic changes in foods and/or the loss of its nutrients. In addition, chemicals have side effects on human health. These factors reduce consumer acceptance, needing a novel methods for food preservation, including new and natural preservatives (Tiwari *et al.*, 2009).

Middle Eastern peoples rely on vegetables and fresh products as their main source of carbohydrates, vitamins, processed food, fermented sausages, dairy products, meat and poultry as protein. All of these can get contamination by many microorganisms, just like anaerobic Gram negative bacteria *Campylobacter jejuni* (<https://www.who.int/news-room/fact-sheets/detail/campylobacter>) and anaerobic Gram positive bacteria *Clostridium perfringens* (Bennett *et al.*, 2013), which considered more dangerous on human and foodborne diseases (FBD) than aerobic one. These anaerobic bacteria don't need oxygen for growth, so it can contaminate many types of food; processed canned, fresh, and raw food. *C. perfringens* is the second microorganism responsible for causing foodborne diseases in the United States, after *Salmonella typhimurium*. It is Gram-positive spore-forming anaerobic rod and found in many environments (especially in the intestines of many animals and humans) (Brynestad and Granum, 2002). The areas which contaminated with human or animal fecal pollution contains the spores of this bacteria in its soil. *C. perfringens* is the most dangerous bacterial species that they have fatal toxins so; it causes food contamination during manufacture (Le Loir *et al.*, 2003). *C. perfringens* causes perfringens disease. *Campylobacter jejuni* is Gram negative anaerobic bacteria, which can reach to your system via eating insufficiently cooked poultry or you eat food that has touched raw or insufficiently cooked poultry. Animals' digestive system just like chickens and cattle is the place where *C. jejuni* live. Unpasteurized milk also has *Campylobacter*

bacteria, which the diarrhea is the main symptoms of campylobacteriosis. children and babies can get infected easily by *Campylobacter* than adults (<https://www.webmd.com/food-recipes/food-poisoning/what-is-campylobacter-infection#1>). Both bacteria can grow in a different environmental condition such as freezing and moderate high temperatures (2–45 °C).

The increasing of side effects of the synthetic food preservatives has needed to use natural preservatives instead (Hyldgaard *et al.*, 2012). In past, some spices and plants were used to keep the food preserved for long time, but at the same time, they have an organoleptic effects which they could not overcome (Han, 2013). Allyl isothiocyanate (AITC) is the basic effective compound in mustard essential oil which has bactericidal and bacteriostatic effects against many species of bacteria and also fungicidal against many types of fungi. Although in Japan now, AITC is added to food for increase its shelf life, but AITC is permitted as a food preservative due to it contains some residual chloride compounds and its strong organoleptic effect in food (Isshiki *et al.*, 1992). The US FDA approved that AITC is a safe food additive, when set at a maximum of 25 mg/kg/day for human's body (Isshiki *et al.*, 1992), however, it has not been approved as a food preservative yet.

There are challenges of using AITC as antimicrobial preservative because of its physical properties, which it is highly evaporated in liquid phase. Adsorption and desorption of AITC from porous delivery solids such as powder of mustard seeds (Dai and Lim, 2014), beads of calcium alginate (Kim *et al.*, 2008), powder of maize (Paes *et al.*, 2011), and silica mesoporous carrier (Park and Pendleton, 2012) used to avoiding this problem. Each of these systems acted as system for increasing shelf life of food and keep it away from any contamination or spoilage. Sihaan *et al.* (2014) used *Saccharina japonica* as porous carrier system for AITC, they demonstrated that AITC has antimicrobial activity towards *Salmonella Typhimurium*, *Escherichia coli* and *Bacillus cereus* after desorption from the brown seaweed. Also, we used in our previous study other natural food preservative system (AITC as natural compound carried on edible seaweed *L. japonica*) against some Gram-positive foodborne bacteria (*Bacillus subtilis*, *Listeria monocytogenes*, and *Micrococcus luteus*), and we proved it was an effective system to suppressed and controlled these food borne bacteria (El Fayoumy *et al.*, 2017).

This research demonstrated the antimicrobial effect of allyl isothiocyanate (AITC) on two dangerous anaerobic foodborne bacteria which make contamination easily to raw and processed food (*Clostridium perfringens*, and *Campylobacter jejuni*). So, we used this natural food preservative system consist of allyl isothiocyanate (AITC) compound which is main constituent of mustard essential oil and another carrier, edible brown seaweed, *Laminaria japonica* which found in the sea waters of the Middle East, Japan and other countries around the world. This natural food preservative system proved before in our other research, it can overcome the annoying physical properties of AITC which limit its using as food preservative (El Fayoumy *et al.*, 2017). The de-oiled powder of *L. japonica* was examined as a suitable carrier for AITC, with subsequent antimicrobial tests of vapor released activity after solution and vapor deposition methods against the two tested anaerobic foodborne bacteria. Siahaan *et al.* (2014) used two different diameters of their particle carrier in their work (900 and 500  $\mu\text{m}$ ), and they found that; The larger particle diameter of the delivery carrier for AITC led to greater amount of AITC adsorbed. So according to this, the current work aimed to finding a more economic process and test its antimicrobial activity in a small amount, so we used the smaller particle size (500  $\mu\text{m}$ ) as a carrier made by vapor and solution phase AITC deposition.

## MATERIALS AND METHODS

### Materials

Brown seaweed *L. japonica* was getting from Guemil-eup, Wando-gun, South Korea. Both dimethyl sulfoxide (DMSO) and AITC were 99.9 % grade. The media of the bacterial growth were Tryptose sulfite cycloserine (TSC) agar, Tryptose sulfite cycloserine (TSC) broth, Mueller Hinton broth + CSS (*Campylobacter* selective supplement) and Mueller Hinton agar (MHA) +CSS, all were bought from Sigma Aldrich, Australia.

### Preparation of selective media

Tryptose sulfite cycloserine (TSC) agar is selective media for isolation and enumeration the spores of *Clostridium perfringens* in food (Harmon *et al.*, 1971), S.F.P. Agar base media (Shahidi – Ferguson Perferingens) which consist of tryptose, soya peptone, yeast extract, sodium bisulphite, ferric ammonium citrate and agar was bought from Sigma Aldrich, Australia, then suspend 42 gm in 1 liter distilled water. Boil to dissolve the media. Sterilize by autoclaving at 15 lbs. Pressure (121°C) for 15 min. Cool to 50 °C. Add 400 mg of rehydrated D-cycloserine (Sigma Aldrich, Australia) to 1 liter of SEP Agar base media as selective agent for *C. perfringens* growth, which appeared as black colonies. TSC broth media is the same composition but without agar. For growth *Campylobacter jejuni*, one vial of CSS which consist of sodium pyruvate, vancomycin, cefoperazon and cycloheximide was bought from Sigma Aldrich, Australia, then added to sterilized 500 ml of Mueller Hinton agar (MHA) (Sigma Aldrich, Australia), and the same for preparation broth Mueller Hinton media (Bolton *et al.*, 1983).

### Bacterial cultures preparation

Cultures of two tested bacterial strains *Clostridium perfringens*, and *Campylobacter jejuni* were made by streaking stocks of culture on to (TSC) agar and (MHA) +CSS media respectively inside anaerobic chamber which has oxygen removing system (Model BJPX-G-1), which A hydrogen (H<sub>2</sub>) gas mixture is circulated through a heated palladium catalyst to get rid of oxygen (O<sub>2</sub>) by forming water (H<sub>2</sub>O), and provided 0.25 % CO<sub>2</sub> for *C. perfringens*, which small amount of CO<sub>2</sub> is enough for its growth (Reilly, 1980), but provided for *C. jejuni* 10% CO<sub>2</sub> for its growth (Park, 2002). then left to grow in anaerobic incubator inside the chamber until appearance of visible colonies (48 h, 37 °C) for both tested bacteria. One colony of each bacteria was then picked up to start seed cultures in their selective broth media (5 mL, 16 h and 37 °C) anaerobically in an incubator shaker at 180 rpm to perform next tests.

### Determination of MIC for AITC against the selective bacteria

This assay aims to know the minimum inhibitory concentration (MIC) of the AITC against the tested microorganisms, representing the lowest concentration that completely inhibits the growth of microorganisms. Stock solutions of 20 mg/ml of AITC were prepared with DMSO (20%) as a solvent. 96 wells sterilized plate was used for the assay. To each well on the plate, 100  $\mu\text{l}$  of selective media broth for each tested bacterium was added in the first row, 100  $\mu\text{l}$  of the stock solution was added. Subsequent rows were filled by transferring 100  $\mu\text{l}$  by serial dilution resulting in AITC concentration from 10 to 0.0097 mg/ml across the 96-well microtiter plates. DMSO (20%)-media broth with no tested compound acted as a control in the wells of the last row of the plate. Then inoculated each well with 100  $\mu\text{l}$  inoculum of each tested bacterium, using a 16-h culture adjusted by the McFarland standard. Additional dilution was made with the selective broth media for each bacterium to make approximately 10<sup>6</sup> CFU.ml<sup>-1</sup>. The total volume of each well in the plate, 200  $\mu\text{l}$ , was then

incubated anaerobically at 37 °C, 16 h at 150 rpm agitation for both bacteria. Experiments were carried out triplicate. By HT Multi-detection Microplate Reader (Bio-Tek, UK) at 540 nm, we compared the absorbance values before and after incubation, so the inhibition of bacterial growth was recorded. The MIC is defined as the lowest concentration of the antimicrobial compound make inhibition for any visible growth (Andrews, 2001).

### Type of Antimicrobial effect of AITC against the tested bacteria

This test was performed to determine the type of effect AITC had against the tested bacteria, i.e., if it was bacteriostatic or bactericidal.

This assay depended on the results of the previous micro-dilution assays. The definitions used in this work were those established by others (Smith-Palmer and Fyfe, 1998). Aliquots of 15  $\mu\text{l}$  from the wells that the bacteria inhibited and/or failed to grow were plated onto the selective media agar plates for each tested bacterium, then the plates were incubated for 24 h at 37 °C anaerobically. Three times of each micro assay were performed.

### Food preservative system consists of AITC and *L. japonica* as edible carrier

#### Powder of *L. japonica* preparation

Fresh de oiled and raw *L. japonica* samples were used as medium carriers for AITC and were prepared according to El Fayoumy *et al.* (2017). Supercritical carbon dioxide (SC-CO<sub>2</sub>) used for extraction the lipid content of *L. japonica* (El Fayoumy *et al.*, 2017).

#### AITC adsorption by *L. japonica* powder

The adsorption of AITC by *L. japonica* was performed according to El Fayoumy *et al.* (2017), which used vapor and solution adsorption. For both ways, the amount of AITC loaded was calculated by recording weight increasing of the samples with time (4, 8, 12, 24, 48, and 72 h).

#### AITC antimicrobial assay

#### Initial growth determination for bacterial suspensions

The growth of *Clostridium perfringens*, and *Campylobacter jejuni* were occurred anaerobically on selective liquid media (15 mL) (TSC) agar and (MHA) +CSS media respectively with shaking (200 rpm) for 18 h at 37 °C. These two cultures acted as inoculum. the optical density of the overnight liquid samples was measured at 540 nm by using spectrophotometer (Fisher Scientific). Commonly the bacterial turbidity measured in the range of (420 nm – 660 nm), because the bacterial cells in this range of UV will not get affected or dead, which the UV causes mutations in the bacterial cell and destroying its genes. At wavelength 420 nm, spectrophotometer gave blue ray and used if the media was colorless, spectrophotometer gave green ray at 540 nm and used if the media was yellow like media used in this research, but at 600 nm gave orange ray and used if the media was brown (Gunasekaran, 2007). The absorbance at OD<sub>540</sub> was increased when the culture concentration increased. The microorganism's growth reached to the required concentration by these absorbances. a 0.5 McFarland standard used to make same concentration of the bacterial growth. The same initial bacterial concentration was made by dilution the growth culture of each tested bacteria, which make it to 10<sup>6</sup> CFU/mL. 10<sup>-1</sup> to 10<sup>-8</sup> serial dilution of each tested bacterial culture (1 mL) were made by suspending them in a sterile saline solution to make 10% v/v for each dilution factor. Samples (1 mL) of each tested bacterium were then spread on their selective media agar plates for each one three times for calculating the means according to their dilution factors. Then we incubated them for 72 h at 37 °C anaerobically. For each colony, initial cell counts were made.

#### Antimicrobial assay of released AITC vapor

Three vacuum desiccators (10 L at 25 °C) were prepared as containers for each tested bacterium. The first vacuum desiccator contained AITC loaded on *L. japonica* via vapor adsorption, the second one, AITC loaded on *L. japonica* via solution adsorption, and the last, served as a control, which there is no AITC or *L. japonica*. Glass tube containing (2 mL) overnight-grown selected bacterial cultures was put in each desiccator. Samples tubes were taken periodically after 4, 8, 12, 24, 48, and 72 h. On removal, the samples were diluted and (1 mL) was inoculated on the selective media agar plates for each bacterium to determine the effect of exposure to AITC vapor (released from *L. japonica* carrier) on further microorganism growth. Then, we incubated these plates at 37 °C anaerobically for 72 h. Any further growth, determined as a colony count on the spread plate, was adjusted for its corresponding dilution factor. Three replicates of each assay were made.

**Statistical analysis**

All required measurements were carried out in triplicate and the results were calculated as mean ± standard deviation, in addition to statistical analysis using the one-way (ANOVA), the mean difference is significant at P < 0.05 level by using SPSS version 22.

**RESULTS AND DISCUSSION**

AITC antimicrobial activity against two selected anaerobic foodborne bacteria, *Clostridium perfringens*, and *Campylobacter jejuni* was proved in the discussion below. These bacteria have been found as pollutants in some processed and raw food consumed all over the world. The antimicrobial compound (AITC) inhibit, and destroys many bacteria and fungi (Park and Pendleton, 2012). Since, an edible natural carrier was being used in our previous paper against some aerobic gram positive foodborne bacteria, and it didn't have any reducing effect on AITC antimicrobial activity (El Fayoumy et al., 2017), but it was necessary to establish if this carrier has any reducing effects on AITC antimicrobial activity towards the selected anaerobic foodborne microbes in the present study.

**Determination of MIC for AITC against the selective bacteria**

This assay for calculating MIC (minimum inhibition concentration) of AITC for each bacterial strain, and it is known as the minimum concentration that completely prevent the bacterial growth (Andrews, 2001). MIC for AITC against *C. jejuni* (0.039 mg/ml) was lower than MIC of AITC against *C. perfringens* (0.078 mg/ml), that means, *C. jejuni* is more sensitive towards AITC than *C. perfringens*, and this because of the ability of Gram +ve bacteria *C. perfringens* to form endospore in the hard stress conditions, which gave it more resistant towards AITC and other antimicrobial compounds than Gram -ve bacteria. In addition, Gram +ve bacterial cell membrane can be as much as 20-fold thicker than the cell membrane of Gram -ve bacteria, also Gram +ve bacteria have a greater volume of peptidoglycan, which makes the thick outer covering and give more protection than Gram -ve one (<http://info.achs.edu/blog/gram-positive-gram-negative-bacteria>) Table 1.

Lu et al. (2016) demonstrated that the minimum inhibition concentration (MIC) of AITC against foodborne microbes was at range 10 to 100 µg/ml. Also, Shofran et al. (1998) found in their research that MICs of AITC were (0.05 to 1 mg/ml) in broth culture for bacterial species and that is very close to our results.

**Table 1** MIC for AITC against each tested bacterium in the microdilution assay

Bacteria	MIC <sup>a</sup> ± SE
<i>Clostridium perfringens</i>	0.078 mg/ml ± 1
<i>Campylobacter jejuni</i>	0.039 mg/ml ± 1.2

<sup>a</sup> Values are the mean and standard error (SE) of three replicate trials.

**Type of the Antimicrobial effect of AITC against the tested bacteria**

AITC activity showed a bactericidal effect against both tested bacteria *C. perfringens* and *C. jejuni*. When Lu et al. (2016) used Wasabi essential oil in their research (which AITC is the main effective compound), displayed higher bactericidal activity against many tested foodborne bacteria. AITC showed a great bactericidal effect toward foodborne microbes on food products (Lin et al., 2000b). Lin et al. (2000a) demonstrated that the bactericidal effect of AITC because of its damage to the cell membrane of the bacteria which caused leakage of the components outside the cells.

**Food preservative system consists of AITC and *L. japonica* as edible carrier**

Park and Pendleton (2012) showed that, the desorption of antimicrobial compound from a porous materials and controlling release of this antimicrobial agent for the inhibition activity occurred via two consecutive processes; first: beginning burst of the antimicrobial compounds to make a high concentration in small time, and second: a continuous, but slower rate of desorption to keep a good concentration for the antimicrobial activity of released materials. In our previous research, we used *L. japonica* as an edible carrier, so there are not any bad effects if touched or contacted with the food. It is widely existing in large amount on the sides of marine waters of the Middle East countries, so it can be harvested easily, cheaply and legally (El Fayoumy et al., 2017). After we proved the success of this natural preservative system against some Gram-positive foodborne bacteria (El Fayoumy et al., 2017), in this work, we used the same natural system but against other species of anaerobic foodborne bacteria. Siahaan et al. (2014) which used other type of seaweeds as carrier for AITC, proved via Fourier transform infrared red spectroscopy (FTIR) measurements that, the antimicrobial activity of AITC was not reduced or get affected because of its contact with the seaweed surface, and that is due to the interactions between AITC and the seaweed were physically only with no chemical

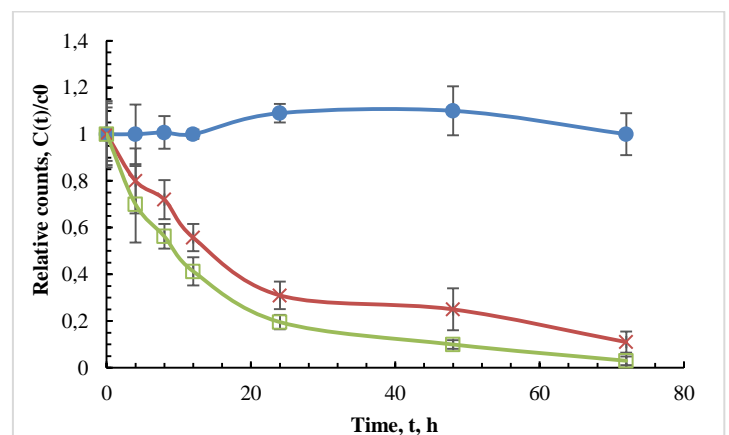
interactions occurred, which can cause any changes in the AITC chemical structure, so no effect occurred on AITC's antimicrobial effect.

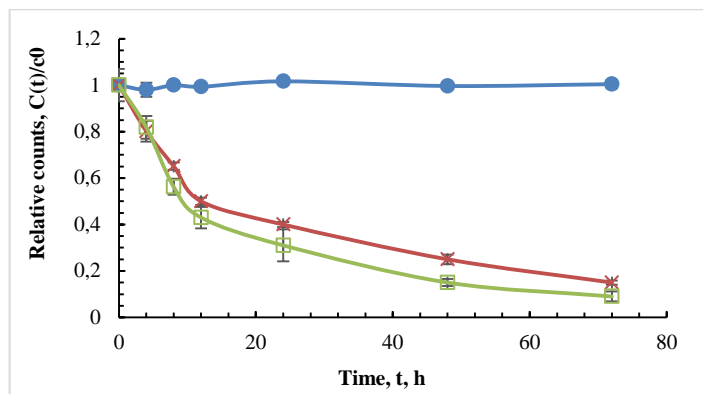
**AITC adsorption by *L. japonica* powder**

The profiles of time-dependent adsorption for AITC onto de-oiled and raw *L. japonica* powder by vapor and solution depositions were obtained in our previous study against some Gram positive foodborne bacteria (El Fayoumy et al., 2017), which showed that de-oiled *L. japonica* samples adsorbed AITC more than the raw samples. El Fayoumy et al. (2017) demonstrated that, the maximum amount adsorbed at 72 h by the de-oiled powder was approximately twice that of the raw powder, approaching 99% theoretical maximum. This difference was back to that AITC is controlled and restricted to diffuse into the cells containing lipids, because of lipids' hydrophobic nature, which providing weak water-lipid interfacial transfer. (El Fayoumy et al., 2017; Siahaan et al., 2014).

**Antimicrobial assay of released AITC vapor**

SPSS analysis reveals that high significant effect (P < 0.05) of the exposure time to the AITC vapor released from *L. japonica* powder on the count reduction of the selective bacterial cell. When AITC adsorbed via silica (Park and Pendleton, 2012), *S. japonica* (Siahaan et al., 2014) surfaces, and de-oiled *L. japonica* powder (El Fayoumy et al., 2017), it was only by dispersion forces, which no evidence for any chemical reactions between AITC and *L. japonica*. So there is no any changes in AITC's composition and according to this, the release of AITC vapor provided an antimicrobial atmosphere. El Fayoumy et al. (2017) studied before the antimicrobial effect of AITC desorption from *L. japonica* powder against some Gram-positive pathogenic bacteria, in this study, we will test against two dangerous anaerobic foodborne bacteria (*Clostridium perfringens* and *Campylobacter jejuni*). Data of these experiments are spread in Fig. 1. The antimicrobial effect was demonstrated by two different ways; vapor and solution loaded *L. japonica* powders and a control system without AITC or *L. japonica* powder. El Fayoumy et al. (2017) compared the deactivation curves by making the count reduction of bacteria with time, *c(t)*, and the initial count, *c<sub>0</sub>*, with time, *t*. Fig. 1 show that the mean values and their standard deviation (SD). The shape of each curve in Fig. 1 proves that the decay behavior of vapor loaded systems is paralleling with the solution loaded systems. The control systems without AITC or *L. japonica* stayed a constant and same relative bacterial count. The beginning burst in AITC released at the first 12 hours led to 50-59% reduction in both tested bacteria. After 24 h exposure, 81% of anaerobic gram -ve bacteria (*C. jejuni*) was inhibited, but just 69% only of anaerobic gram +ve bacteria (*C. perfringens*) was inhibited. Exposure up to 72 h led to 97% total reduction of *C. jejuni* and 91% of *C. perfringens*. The data of this system was agreed with AITC vapor deposited from silica (Park and Pendleton, 2012). *C. jejuni* was more sensitive than *C. perfringens*. These findings were compatible with our MICs data, which proved that Gram +ve bacteria are more survival to AITC than Gram -ve bacteria. Finally, we concluded that, there were parallelism between the decay behavior of AITC vapor toward the microbial growth for both vapor and solution loaded, and the different in the inhibition of the both tested bacteria couldn't be noticeable and very small (Fig.1), these results are agreed with our previous results (El Fayoumy et al., 2017), so the using of vapor as loading way would be more cheaper and safety, in addition to more economic than solution deposition. (Fig. 1).





**Figure 1** Antimicrobial activity of AITC vapor on the two tested bacteria released from de-oiled powder *L. japonica*, by vapor (×), solution (□) adsorption and control (●), (A) For *Campylobacter jejuni* and (B) for *Clostridium perfringens*

## CONCLUSION

In the present work, we demonstrated that AITC has a bactericidal effect against anaerobic foodborne bacteria *Clostridium perfringens* and *Campylobacter jejuni*. The de-oiled *L. japonica* powder adsorbs AITC more than the raw powder, which produces a suitable antimicrobial atmosphere inhibiting the growth of these two pathogenic bacteria. Since the decay behavior of AITC vapor which loading via vapor- and solution-deposition were parallel, and the different in the inhibition of all tested bacteria couldn't be noticeable and very small between the both loading ways, so the vapor loading way would be better to use in this system than solution one according to the healthy and economic viewpoints. De-oiled *L. japonica* powder would be safe, natural edible carrier for the antimicrobial agents to prevent the food contamination or food spoilage by different range of foodborne aerobic and anaerobic bacteria, so it can be used as natural food preservation system enhancing safety and security of food during its transportation and storage processes.

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