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REGULAR ARTICLE

Properties of soy protein isolate antimicrobial films and its application in preservation of meat

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ABSTRACT

The soy protein isolate (SPI) antimicrobial films were made with Nisin (N), sodium lactate (NaL), EDTA (E) and their optical combination (C). The C film showed a significant ($p < 0.05$) antimicrobial effect on three food pathogens, *E. coli, Salmonella* and *Bacillus cereus*, which inhibitory zone is 32mm, 33.33mm and 32.33mm, respectively. The addition of N and NaL to SPI film reduced its mechanical properties, that is, the tensile strength (TS) was reduced from 10.8716MPa to 8.1405MPa (N1) and 3.2715MPa (NaL3) and the elongation at break (E) was increased from 3.03% to 4.73% (N3, N4) and 11.21 % (NaL4). While the addition of EDTA and combination increased TS from 10.8716MPa to 17.0600MPa (E4) and reduced E from 3.03% to 2.36% (E1). The water vapor permeability (WVP), oxygen permeability (OP) and total color difference (ΔE) of films had changed with the addition of antimicrobial agents. The FTIR analysis showed no specific interaction between active groups of Nisin with functional group of control film. However, the intensity of peaks in the spectrum of NaL and EDTA increased, indicating interactions between NaL, EDTA and SPI. The appliction experments showed that the SPI antimicrobial film (C) did have a bacteriostatic preservation effect on the meat antibacterial preservation and extend the shelf life to 3-6 days compared with SPI film and ordinary wraps. The SPI antimicrobial film broadens the application of SPI film.

Keywords: Antimicrobial SPI film; Nisin; Sodium lactate; EDTA; Food pathogenic bacteria

INTRODUCTION

Food safety is a global priority and also one of the main objective of the current food legislation(Quintavalla et al., 2002). Particularly, bacterial contamination of ready-to-eat products is of concern to human health (Pranoto et al., 2005). What can we do in the face of this tricky problem? There are three methods to reduce or prevent the growth of the bacteria in food product currently: (1) incorporation into the foodstuff; (2) dipping or pulverization; (3) incorporation into a film. There are already many studies about antimicrobial packaging films. The antimicrobial and physical properties of chitosan with Nisin were demonstrated by Wang et al. (2015), Li et al. (2006) and Pranoto et al. (2005). Wang et al. (2015) reported that the chitosan-based films incorporating sodium lactate could effectively inhibit the growth of E.coli. Dutta et al. (2009) demonstrated the potential of chitosan in the study of antimicrobial films. Theinsathid et al. (2011) reported that the biobased film incorporating sodium lactate were effective on the inhibitory of Listeria monocytogenes. Resa et al. (2016) and Pattanayaiying et al. (2015) all demonstrated that the starch antimicrobial edible films had potential in the application of food packaging for the preservation of food, such as ready-to-eat muscle foods and refrigerated argentinian port salut cheese. However, the majority of current researches on antimicrobial packaging films are about chitosan and starch based films, and the researches about SPI antimicrobial films are rarely seen.

SPI has been widely used in hydrogel, adhesives, plastics, films, coatings, and emulsifiers (Tian et al., 2010; Tian et al., 2008; Kumar et al., 2002; Gennadios et al., 1993) and has wide application in producing an edible antimicrobial film to employ the antimicrobial agents on the surface of the food products (Park et al., 2002; Eswaranandam et al., 2004; Gallagher et al., 2004; Silva et al., 2007). The antimicrobial and mechanical properties of the SPI films with various natural antimicrobials have been identified (Park et al., 2002; Ko et al., 2001). Zhao et al. (2013) reported that the SPI films can prevent the growth of bacteria and its useful life was prolonged with

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the help of antimicrobial agents-$AgNO_3$. Kim (2016) explored the inhibitory effect of SPI films incorporating cinnamaldehyde on the growth of microbals. Ko et al. (2001) and Eswaranandam et al. (2004) have demonstrated the antimicrobial and physical properties of SPI films with various natural antimicrobials.

Bacteriocins, particularly Nisin (N), contain the natural antimicrobial substances that can be incorporated into the edible films to control the food-borne pathogens (Were et al., 1999; Yildirim et al., 1997; Dawson et al., 2003; Gennadios et al., 1996). Nisin is a food-grade bacterium (Quintavalla et al., 2002), which can be incorporated into the edible films to control various Gram-positive microbial growth including food-borne pathogens (Were et al., 1999; Yildirim et al., 1997; Dawson et al., 2003; Gennadios et al., 1996; Jin and Zhang, 2008). Nisin binds to the precursor of peptidoglycan and lipid II to inhibit the cell wall biosynthesis. It then forms the pores within the cell membrane that leads to the release of essential ions and, ultimately, it causes the cell death (Hsu et al., 2004). Nisin has been appoved by the Food and Drug Administration (FDA) used as a food preservative. Sodium lactate is the sodium salt of the low molecular weight organic acids. It can control the growth of microbial (Sallam, 2007) andwas used to extend the shelf life of many products (Long and Phillips, 2003; Juneja, 2006), which is non-toxic and commonly available (Schelegueda et al., 2012). EDTA is believed to release a huge Gram-negative lipo-polysaccharides from the outer membrane and expose the hydrophobic phospholipids which are able to increase the susceptibility to hydrophobic and cell wall degrading agents (Helander et al., 1997; Walsh et al., 2003). It improves the activity of nisin against Gram-negative bacteria, including E. Coli O157: H7 and S. Typhimurium (Stevens et al., 1991).

The development of the packaging material-related antimicrobial agents is an active area of research as a complementary method to inhibit the growth of food pathogens. The objectives of this research are: (1) to produce antimicrobial protein films for food coating and packaging from soy protein isolate; and (2) to determine their antimicobial properties, mechanical properties, barrier properties and color difference and to evaluate the potential of SPI films incorporated antimicrobial agents (nisin, sodium lactate, EDTA) for use as a antimicrobial coating/film material. And the application experiments verified the feasibility of antimicrobial SPI-based films in food fresh packaging. In conclusion, the properties and application of incorporation of three antimicrobial in SPI films were discussed in the present study.

MATERIALS AND METHODS

Materials

Pathogenic bacteria *Escherichia coli* (CMCC 44103), *Salmonella* (CMCCB 50041) and *Bacillus cereus* (CICC 21261) were obtained from BNCC (Bena Culture Collection) Biological Technology Co., Ltd, China. Microbial media and nutrient agar (Hopebio Biological Technology Co., Ltd, Qingdao, China), soy protein isolate (SPI) (GS5100) (GUSHEN Biological Technology Co., Ltd, Shandong), glycerol, nisin, sodium lactate and EDTA (Sinopharm Chemical Reagent Co.,Ltd). All materials used are analytical reagent.

Film formation

SPI films were prepared using a casting method modified slightly from Wan et al. (2005). SPI solution (50g/L) and 0.4 g/g glycerol (based on SPI content) was added into the solution (Wang et al., 2016). It was stirred with a magnetic stirrer (78HW-1) for 30 min. Antimicrobials were then allowed to added to the solution before heating it in a water bath for 30 min at 60°C. The pH was adjusted to 7 by pH meter (SIN-PH-100). Before being heated, control; Nisin: N_1 (5000 IU/g SPI), N_2 (10000 IU/g SPI), N_3 (15000 IU/g SPI), N_4 (20000 IU/g SPI); Sodium Lactate: NaL_1 (0.5 g/g SPI), NaL_2 (1.0 g/g SPI), NaL_3 (1.5 g/g SPI), NaL_4 (2.0 g/g SPI); EDTA: E_1 (0.04 g/g SPI), E_2 (0.08 g/g SPI), E_3 (0.12 g/g SPI), E_4 (0.16 g/g SPI) and E_5 (0.20 g/g SPI) and their combination [N (5000 IU/g SPI)+NaL (1.0 g/g SPI)+E (0.08 g/g SPI)] were added into the film-forming solutions. All concentrations used in this study were selected based on our preliminary tests for formation of SPI films (Sivarooban et al., 2008). Then the solution was cooled to room temperature and then poured into the Plexiglas plates (230 mm×230 mm×30 mm) before dried in a vacuum drying oven for 12 h at 50°C. The dried films were peeled off and conditioned again at 43%RH and 25°C for 24 h prior to testing.

Film characterization

Antimicrobial analysis

The antimicrobial activity of SPI-based films was determined by agar diffusion method. In detail, the edible films were cut into a disc form of 20mm diameter by a compasses knife. On the Mueller Hinton agar plates (Merck, Darmstadt, Germany) are the film cuts. They had been seeded in advance with 0.1ml of inoculum, with the indicator microorganisms included in 10^5-10^6 CFU/ml. Afterwards, they underwent incubatation at 37°C for 24 h. The diameter or inhibitory zone surrounding film discs as well as contact area of films with agar surface were then measured.

Physical features of edible SPI film

Thickness

The average thickness (mm) of the edible film was measured by a hand micrometer at a few points (Aice, China). Based on the standard of the ASTM (American Society for Testing and Materials).

Tensile Strength (TS) and Elongation at break (E)

The TS and E of the films was measured by TA Plus texture analyzer (SMS TA, UK). The prepared films were cut into 1.5 cm×10 cm strips. The films were held parallel with an initial grip separation of 5cm and then pulled apart at a head speed of 25 mm/min. TS was calculated by dividing the maximum force at break (read from machine or computer contacted with the machine) by cross-sectional area of film.

$$TS = \frac{F \times 10^{-6}}{S}$$

Where F = maximum force at break (N); S = cross-sectional area of film (m^2).

Percent E was calculated based on the length extended and original length of the films.

$$E = \frac{\Delta G}{G} \times 100\%$$

Where ΔG = the length extended of the films (cm); G = original length of the films (cm).

Water Vapor Permeability (WVP)

The water vapor permeability (WVP) was determined gravimetrically using a modified ASTM procedure as used by Gontard et al. (1994). WVP was as below:

$$WVP = \frac{w \times x}{A \times t \times \Delta P}$$

where w = weight gain of the beaker (g); = film thickness (m); A = area of the exposed film (m^2); t = time of weight gain (s); and ΔP= water vapor partial pressure difference (Pa) across the two sides of the film calculated on the basis of relative humidity.

Oxygen permeability (OP)

The oxygen permeability (OP) was determined according to Wang et al. (2015).

Oxygen transmission rate (OTR, according to ASTMD1434) of film was determined at 23°C and 0%RH on a Labthink gas permeameter (Jinan Labthink Electrical and Mechanical Technology Co., Ltd, Shandong, China). Oxygen permeability (OP) was calculated from OTR ($cm^3m^{-2}d^{-1}kPa^{-1}$) as follows:

$$OP = OTR \times x$$

where OTR was gained from the gas permeameter, and x is the film thinkness (m).

The thickness and open testing area of each sample in the three parallel measurements were approximately 100μm and 50 cm^2.

Total color difference (ΔE)

Color was determined for soy protein films with and without the addition of antimicrobial agents to provide background information for comparison with color of Nisin, sodium lactate, EDTA and their combination. Color was determined by NR10QC and recorded in L*a*b* color system. Measurements were taken as a average of at least three points of each sample. Total color difference (ΔE) was calculated as follows:

$$\Delta E = \sqrt{(L^* - L)^2 + (a^* - a)^2 + (b^* - b)^2}$$

where L*, a* and b* are the standard values of white plate, L, a, and b are values of samples measured.

Analysis on the FTIR

At the Engineering College of Northeast Forestry University, China, the FTIR spectrometry (Nicolet Antaris II) at room temperature was applied to record the spectra of the SPI films (controlling and the three antimicrobial substances are incorporated). The light source of transmittance was in the middle range infrared between 600 cm^{-1} and 4000 cm^{-1}. The detector used was triglycine sulfate (TGS) with the resolution 4cm^{-1}. The gained spectra was applied to recognize the potential interactions of the function groups among SPI and Nisin, sodium lactate and EDTA.

Application experiment

Sample processing

The method for sample processing was a modified procedure as used by Zhao (2013).

Take fresh pork tenderloin and divide it into about 40g small pieces in good health conditions. Then packaging them with prepared SPI antimicrobial film [N (5000 IU/g SPI)+NaL (1.0 g/g SPI)+EDTA (0.08 g/g SPI)] and pure SPI film. The control group was a meat sample packaged with only ordinary wraps. All samples were placed in the refrigerator at 4°C for 24 hours. After that, the indicators were measured every day until samples are corrupt.

Determination of total bacterial count

The total bacterial count was determined according to Devlieghere et al. (2005).

Determination of total volatile basic nitrogen (TVB-N) value

Using Kjeldahl method to measure the TVB-N value of sample (Cheng et al., 2014).

Statistical analysis

All experiments were performed at least three times. All data were presented as means ± standard error of the mean. As for multiple group comparison, the significance of the differences among the treatment groups and their respective control groups were analyzed using origin 8.5 and SPSS 17.0 software. Statistical significance was assessed by either student's t-test or one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison. Differences between means were considered statistically significant if $p < 0.05$.

RESULTS AND DISCUSSION

Nisin, NaL and EDTA were found effective to fight against food-borne pathogens. Nevertheless, when these compounds are applied singly, they would be not adequate as a protection against food-borne pathogens. Presently, it was further studied that their combination into SPI film-forming solutions as a multiple hurdle approach against major food-borne pathogens, including *E. coli* (G-), *S. typhimurium* (G-), and *B. cereus* (G+) and their effect on the mechanical properties of the SPI edible films.

Antimicrobial activity

Table 1 and (Fig. 1a-c) showed the antimicrobial activity of the SPI edible films, combined with N, NaL, EDTA and their combination against *E. coli* (G-), *Salmonella* (G-), and *B. cereus* (G+). These three pathogens are also known as the ordinary meat products contaminants. According to the clear zone (Fig. 1), the inhibitory activity can be measured based on the strips of the circular film.

The values of inhibition zones were always higher than 20 mm, which was the diameter of the film strips, because the value included the diameter. Without surrounding clear zone, there would be no inhibitory zone. And the value would be defined as zero. The contact area was used to evaluate the growth inhibition under film discs in direct contact with target microorganisms in agar.

The control SPI film did not show inhibitory effect against three tested microorganisms in terms of no surrounding clear zone for the no inhibition nature of SPI molecules. The SPI film incorporating N showed an antimicrobial effect. The inhibitory zone were markedly high against *B.cereus* (G+) at the lowest level of 5000 IU/g SPI and the value is 29.58 mm. But it did not show inhibitory zone on *E.coli* (G-) and *Salmonella* (G-). It is to be expected as the cell war structures of these categories of bacteria are different and Gram-positive bacteria are more sensitive to these agents. However, the N-incorporated SPI film showed an inhibitory effect on the growth underneath film discs of these organisms. Higher level of N adding to the SPI films than 5000 IU/g SPI did not revealed a significant increased antimicrobial effect, which may probably due

Table 1: Antimicrobial activity of SPI edible films containing Nisin, sodium lactate (NaL) and EDTA against food pathogenic bacteria of *E. coli, Salmonella* and *B.cereus*

Antimicrobial agents	*E. coli* (G-)		*Salmonella* (G-)		*B.cereus* (G+)	
	Inhibitory	Contact	Inhibitory	Contact	Inhibitory	Contact
Control	0^{f}	−	0^{f}	−	0^{i}	−
Nisin (10^3 IU/g SPI)						
5	0^{f}	+	0^{f}	±	29.58 ± 0.77^{b}	±
10	0^{f}	±	0^{f}	±	29.31 ± 1.55^{b}	+
15	0^{f}	+	0^{f}	±	26.83 ± 1.84^{c}	+
20	0^{f}	+	0^{f}	+	26.97 ± 0.58^{c}	+
NaL (g/g SPI)						
0.5	28.50 ± 1.63^{b}	+	27.00 ± 1.22^{bc}	+	20.83 ± 0.59^{h}	±
1.0	28.50 ± 1.47^{b}	±	26.67 ± 1.03^{c}	+	23.48 ± 3.00^{de}	±
1.5	26.36 ± 1.51^{c}	+	27.00 ± 1.22^{bd}	+	22.72 ± 1.26^{g}	±
2.0	26.00 ± 2.48^{c}	+	25.17 ± 1.42^{e}	+	23.00 ± 4.24^{fg}	±
EDTA (g/g SPI)						
0.04	23.00 ± 0.41^{e}	±	27.00±1.22b^{c}	+	23.72 ± 0.97^{d}	±
0.08	25.72 ± 1.59^{c}	±	27.44 ± 1.13^{b}	+	23.17 ± 0.24^{efg}	+
0.12	24.33 ± 1.25^{d}	±	26.67 ± 1.03^{c}	+	23.22 ± 0.87^{ef}	+
0.16	23.33 ± 0.98^{e}	+	27.19 ± 2.76^{b}	+	23.94 ± 1.4^{d}	±
0.20	23.33 ± 0.85^{e}	+	25.89 ± 0.96^{d}	+	23.89 ± 1.84^{d}	±
Nisin (5000 IU/g SPI)+NaL (1.0 g/g SPI)+EDTA (0.08 g/g SPI)	32.00 ± 2.04^{a}	+	33.33 ± 0.62^{a}	+	32.33 ± 0.62^{a}	+

$^{a-i}$Mean±standard deviation (n=3). Means in same column with different superscript letters are significantly different ($p<0.05$). Inhibitory is inhibitory zone surrounding film discs, measured diameter in mm; contact is contact area under film discs on agar surface. - indicates growth in the area, + indicates no growth; control is a plain film disc without antimicrobial agent incorporation

to the maximum capability of SPI polymer to carry active agents beside the occurrence of functional groups interaction(Pranoto et al., 2005). Bari et al. (2005) had demonstrated that N and some organic acids can be used as antimicrobials in foods at lower levels without diminishing their inhibitory effects. As shown in Table. 1 and Fig. 1-b, SPI films incorporated with NaL had a relatively stable antimicrobial properties against *E.coli* (G-), *Salmonella* (G-) and *B.cereus* (G+). And at the level of 1.0 g/g SPI the addition of NaL showed a relatively high inhibitory effect against three micorganisms. The inhibitory effect of NaL-incorporated SPI films on B.cereus (G+) were significantly ($p<0.05$) lower than the N-incorporated SPI films. So the NaL could make up the shortage of N-incorporated SPI films which could not inhibit the G- bacteria. The SPI film incorporated with EDTA showed a little antimicrobial activity against three pathogens. The inhibitory effect of EDTA on bacteria may probably be contributed to the chelation of divalent cations found in the cell war (Ukuku and Fett, 2004).

However, the SPI films incorporated with the combination of N, NaL and EDTA had a significant ($p<0.05$) antimicrobial effect against *E.coli* (G-), *Salmonella* (G-) and *B.cereus* (G+), inhibitory zone from 28.50mm (0.5, 1.0 g NaL/g SPI), 27.44 mm (0.08 g EDTA/g SPI) and 29.58 mm (5000 IU N/g SPI) to 32.00 mm, 33.33 mm and 32.33 mm. Branen and Davidson(2004), Kun

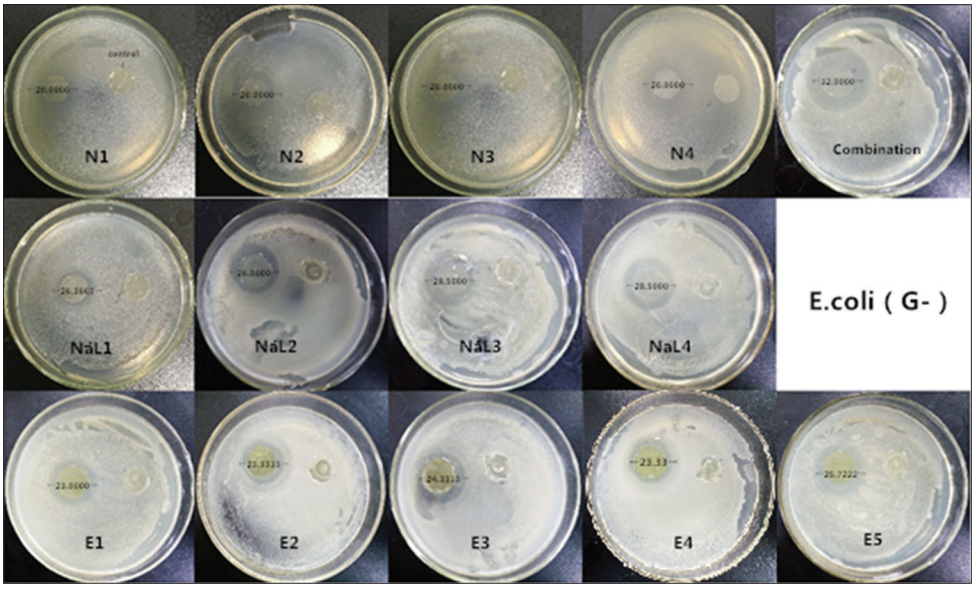

Fig 1-a. Inhibitory zone of SPI edible films containing Nisin, sodium lactate and EDTA against food pathogenic bacteria of *E.coli* (G-).

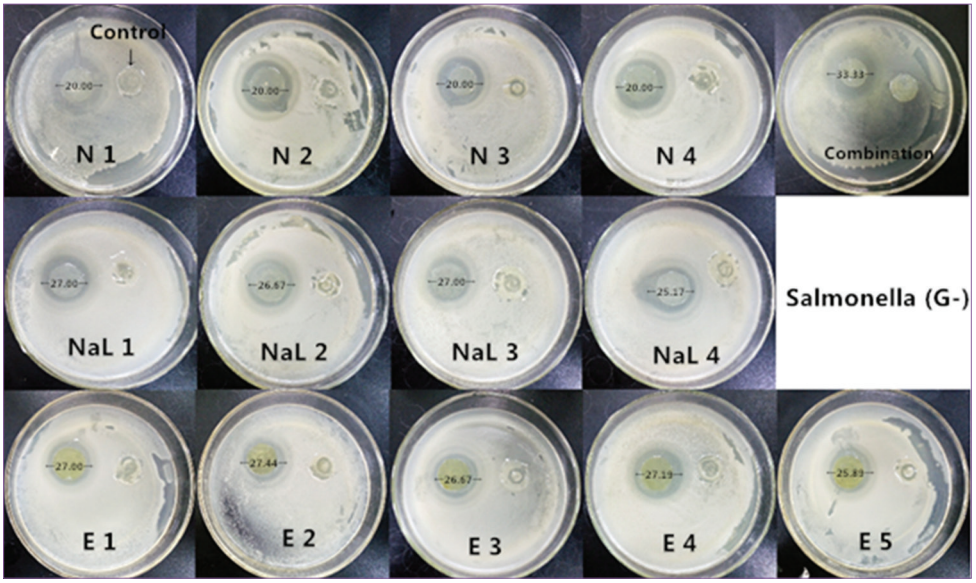

Fig 1-b. Inhibitory zone of SPI edible films containing Nisin, sodium lactate and EDTA against food pathogenic bacteria of *Salmonella* (G-).

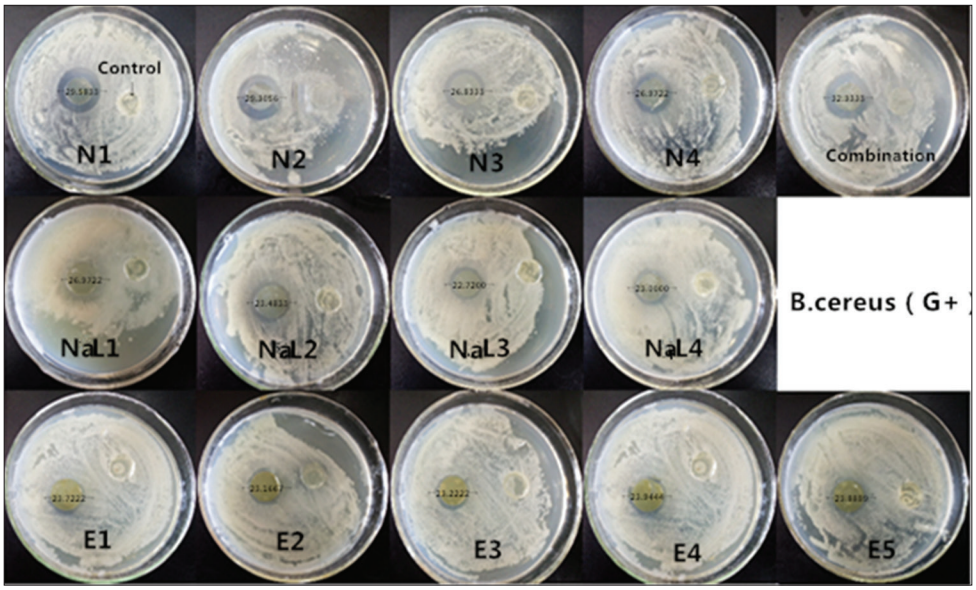

Fig 1-c. Inhibitory zone of SPI edible films containing Nisin, sodium lactate and EDTA against food pathogenic bacteria of *B.cereus* (G+).

(2012) and Yun et al. (2006) have demonstrated that the antimicrobial activity of N can be enhanced in the combination with EDTA. Ukuku and Fett (2004) reported that treatment of whole and fresh-cut cantaloupe and honeydew melon with N-EDTA significantly reduced the natural microflora and extended the shelf-life and that N in combination with NaL, EDTA caused a significant reduction of *Salmonella app*. population the pathogen transferred to fresh-cut pieces during cutting. EDTA might enhance the activity of N and NaL against these pathogens by chelating with the macro-molecules such as salt bridge divalent cations between lipo-polysaccharides on the cell membrane and destroying the stability of the bacterial cell (Vaara et al., 1999). So as to increase hydrophobic interactions between N or NaL and cell membrane (Yang et al., 2002).

Physical properties of SPI-based films

During handling and storage, it is expected that the edible SPI films have adequate strength and maintain constant integrity on the food products (Sivarooban et al., 2008). And the interaction between protein and other additives, including water, plasticizers and antimicrobial agents determined the mechanical and physical properties of SPI-based films (Park et al., 2002).The interaction of SPI and plasticizer which is glycerol here had an important effect on the mechanical properties of SPI-based film.

Mechanical properties

The formation of the protein film results from polymerizing the heat-denatured proteins with disulfide and hydrophobic bonds which are the main forces that can maintain the film network (Fukushima and Buren, 1970). Also, the electrostatic interaction between protein molecules and antimicrobial agents which can form the film network is possible to have resulted into the mechanical change of films (Ko et al., 2001).

The Mechanical properties of SPI-based films are shown in Table 2.

In the different concentration of antimicrobial agents studied, a significant ($p<0.05$) reduction of TS was shown by incorporating N and NaL compared with EDTA. Incorporating NaL at the level of 1.5 g/g SPI significantly ($p<0.05$) reduced TS from 10.8716 to 3.2715 MPa, while with N at 5000 IU/g SPI reduced it from 10.8716 to 8.1405 MPa. The results are consistent with the outcome of the report by Cagri et al. (2001), who had concluded earlier that incorporation of additives other than cross-linking agents generally lowers TS value.

However, a significant ($p<0.05$) improvement of TS was revealed by addition of EDTA at the level of 0.16 g/g SPI that raised TS value from 10.8716 to 17.0600 MPa. And the addition of the combination of three antimicrobial agents improved the TS value of SPI-based films from 10.8716 to 15.0834 MPa. The improvement of the TS value may be attributed to the addition of EDTA. It probably be due to the cross linking reactions between EDTA and the macromolecules in the SPI film (Singh et al., 2012).

The addition of N and EDTA in the concentrations studied and the combination of three antimicrobial agents into the SPI-based films didn't have a significant ($p<0.05$) effect on the E value of the films (Table 2). However, incorporating NaL in the range of concentrations studied significantly ($p<0.05$) increased E value from 3.03% to 11.21%, about

Table 2: Tensile strength (TS) , elongation at break (E), water vapor permeability (WVP) and total color difference (ΔE) of SPI films incorporated with Nisin, sodium lactate (NaL) and EDTA

Antimicrobial agents	TS (MPa)	E (%)	WVP (gm/m^2 daykPa)	OP (cm^{3}m^{-2}d^{-1}kPa^{-1})	ΔE
Control	10.8716±1.3481^{e}	3.03±1.50^{k}	0.0116±1.06×10^{-3j}	0.0052±0.99×10^{-3e}	7.01±0.01^{l}
Nisin (10^3 IU/g SPI)					
5	8.1405±2.5356^{j}	4.24±2.31^{h}	0.03701±1.17×10^{-3h}	0.0055±0.21×10^{-3e}	7.28±0.02^{k}
10	9.4105±3.1479^{h}	4.52±1.59^{g}	0.04012±1.06×10^{-3g}	0.0070±0.31×10^{-3d}	9.90±0.12^{g}
15	9.9602±4.5670^{g}	4.73±1.80^{f}	0.04713±0.16×10^{-3d}	0.0082±0.21×10^{-3cd}	7.28±0.10^{k}
20	8.2794±4.6685^{i}	4.73±2.38^{f}	0.0487±0.09×10^{-3c}	0.0085±0.67×10^{-3c}	6.01±0.07^{m}
NaL (g/g SPI)					
0.5	6.3902±2.2749^{k}	7.97±0.10^{d}	0.0431±0.16×10^{-3f}	0.0071±0.21×10^{-3d}	8.14±1.21^{h}
1.0	4.3799±1.3443^{l}	8.73±0.59^{c}	0.0437±0.32×10^{-3e}	0.0073±0.25×10^{-3d}	6.95±1.47^{l}
1.5	3.2715±2.0850^{n}	10.27±1.74^{b}	0.0626±0.06×10^{-3b}	0.0098±1.27×10^{-3b}	8.01±0.65^{i}
2.0	3.8586±4.2358^{m}	11.21±0.81^{a}	0.0728±0.38×10^{-3a}	0.0125±1.50×10^{-3a}	7.80±0.93^{j}
EDTA (g/g SPI)					
0.04	11.7894±4.5592^{d}	2.36±1.09^{l}	0.0095±0.80×10^{-3k}	0.0047±0.35×10^{-3ef}	24.21±3.35^{e}
0.08	15.4586±9.0313^{b}	3.21±1.15^{j}	0.0074±0.04×10^{-3l}	0.0037±0.30×10^{-3fg}	29.64±3.48^{b}
0.12	10.6509±1.2867^{f}	3.03±0.53^{k}	0.0062±0.07×10^{-3m}	0.0033±3.2×10^{-3g}	30.34±4.44^{a}
0.16	17.0600±4.5251^{a}	4.24±1.02^{h}	0.0059±0.59×10^{-3n}	0.0029±2.5×10^{-3g}	25.86±3.10^{d}
0.20	15.1317±2.7445^{c}	3.85±0.23^{i}	0.0052±0.15×10^{-3o}	0.0026±0.26×10^{-3g}	28.10±6.72^{c}
Nisin (5000 IU/g SPI)+NaL (1.0 g/g SPI)+EDTA (0.08 g/g SPI)	15.0834±1.4672^{c}	5.42±0.46^{e}	0.0205±0.85×10^{-3i}	0.0050±0.26×10^{-3e}	12.07±2.10^{f}

$^{a-o}$Mean±standard deviation (n=3). Means in same column with different superscript letters are significantly different (p<0.05)

four times. The result was probably due to the special properties of NaL, similar to the glycerol, as a slightly viscous liquid (Cui, 2009). The incorporating of NaL into the SPI-based film may cause the rearrangement of the disulfide and hydrophobic bonds or more protein-protein interactions, which both result the E value of SPI films increased significantly (p<0.05) (Chao et al., 2013). Choi et al. (2001) reported that increasing the concentration of glycerol in the film decreased tensile strength and elastic modulus, and increased elongation and water vapor permeability (WVP).

Barrier properties

Water vapor permeability (WVP) and oxygen permeability (OP) is the barrier properties of SPI-based films.

WVP is used to measure the ease of moisture to penetrate and pass through a material (Pranoto et al., 2005). Permeability, depending on the composition and the molecular structure of the filmogenic matric, may be a complex phenomenon (Li et al., 2015).

The addition of N and NaL significantly (p<0.05) increased the WVP value from 0.0116 gm/m^{2}day kPa to around 0.0487 gm/m^{2}daykPa (20000 IU N/g SPI) and 0.0728 gm/m^{2}daykPa (2.0 g NaL/g SPI), respectively (Table 2). It can be seen that higher agents of N and NaL could lead to increasing the WVP value. The result is consistent with the conclusion of Roy et al. (2000). and Diop (2011), who reported that the natural materials are hydrophilic materials with polar groups in their molecular structures and the interactions of the polar groups with permeating water molecules causes the WVP to depart from the ideal behavior. However, the WVP value of the SPI film decreased as the concentration of the EDTA was higher from 0.0116 gm/m^{2}daykPa to 0.0052 gm/m^{2}daykPa (0.20 g EDTA/g SPI). The EDTA, as a hydrophobic chelating agent, increased hydrophobic composition in the SPI-based films (Diop, 2011) and cause the film structure to be more compact, thus making the WVP value decreased. The combination of bacteriostatic agent increased slightly the WVP value from 0.0116 gm/m^{2}daykPa to 0.0205 gm/m^{2}daykPa. The result is coherent with Yong et al. (2007), who concluded that increasing the flexibility of the material, plasticizers generally contribute to the increase of gas and WVP of films. Relatively low WVP value of SPI-based films contributes to the antibacterial properties of the packaging film (Chen, 2010).

Oxygen permeability (OP) of food packaging is generally considered since it related to the development of off-favors, off-odors and nutritional loss associated with oxidation in food stuffs (Ozdemir and Floros, 2005). It means that lower oxygen permeability (OP) of films is better (Ou and Kwok, 2004). As could be seen from Table 2, the OP and WVP value of films were positively correlated. Similar to WVP, the OP values of SPI-based films incorporating N and NaL had significantly (p<0.05) increased, from 0.0052 to 0.0085 (20000 IU N/g SPI) and 0.0125 cm^{3}m^{-2}d^{-1}kPa^{-1} (2.0 g NaL/g SPI), respectively. However, the OP value of the SPI-based films was decreased as the addition of EDTA, from 0.0052cm^3

$m^{-2}d^{-1}kPa^{-1}$ to 0.0026 $cm^3m^{-2}d^{-1}kPa^{-1}$ (0.20 g EDTA/g SPI), and the OP value was decreased as the concentration of EDTA was higher. The OP value of SPI-based film incorporating with the combination of three antimicrobial agents is similar to the value in the pure SPI film, which is 0.0052 $cm^3m^{-2}d^{-1}kPa^{-1}$ (Table 2).

Color difference (ΔE)

Color of the films is essential for a product, which may affect the consumer acceptability. From the ΔE value, it can be seen that the color is totally different which was formulated by a, L and b values, representing green to red, black to white and blue to yellow. The color reference should be the white plate. Rhim et al. (2000) said that various of the additional compounds structurally bind with the film-forming solution which could make the native color of the soy protein film change. The color of the SPI films incorporating N, NaL, EDTA, and their combination were evaluated. As seen in Table 2, the N and EDTA within the concentration studied influenced significantly ($p<0.05$) the color of SPI films compared with the control (Table 2). As the antimicrobial agents were incorporated into the films, its transparency was reduced. The value of pure SPI film was 7.01. And the SPI-based films incorporated with NaL did not significantly ($p<0.05$) change the ΔE value. The N and EDTA affected ΔE of the SPI film produced. The EDTA incorporating into SPI film at 0.04g/200ml had a significant influence on ΔE value already, which increased the ΔE from 7.01 to 30.34 (0.12 g/g SPI) (Table 2). The incorporation of their combination into the SPI film reduced the effect of EDTA on color of SPI-based films, reducing from 30.34 to 12.07.

FTIR Analysis

The interaction between the antimicrobial agents and SPI have been studied using FTIR. Figs.2 a-c showed the SPI films spectrum incorporating with different antimicrobial agents at varying levels in the study. All of the spectrum demonstrate the patterns sharing similarity when it peaks at 3269.75 cm^{-1} and 1041.73 cm^{-1}. It is indicated according to the absorption in this area that O-H and N-H bonds are stretched at 3269 cm^{-1}, and C-O bonds at 1041cm^{-1}. Also, the absorption gains the highest at 2928 cm^{-1}, 1625 cm^{-1}, and 1550 cm^{-1} which are correspondent to the C-H stretching, carboxyl groups (-COO-), as well as an amine group ($-NH_2$). The above mentioned indicates that major structural changes do not take place in the SPI polymer.

Figs. 2-a depicts the spectrum of the control SPI film and the films incorporated N at varying levels used in the study. The spectrum of control film and antimicrobial films incorporated with N showed the same pattern on their informative peaks as the control film (Theinsathid et al., 2011). As expected, this behaviour could be considered

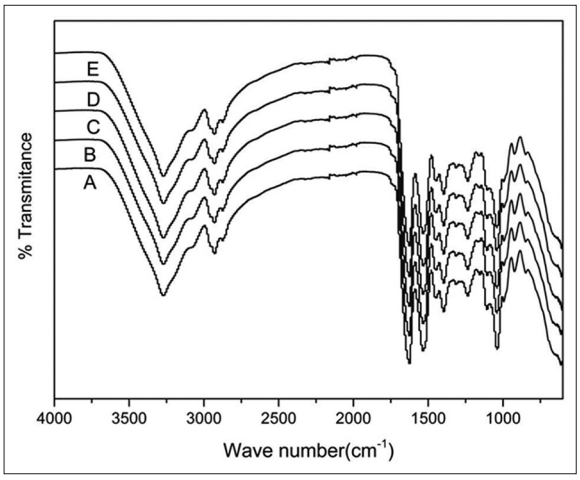

Fig 2-a. Spectra of Fourier Transform Infrared (FTIR) of SPI edible films. (A) SPI film,(B) SPI film incorporated with nisin 5000 IU/g SPI, (C) SPI film incorporated with nisin 10000 IU/g SPI, (D) SPI film incorporated with nisin 15000 IU/g SPI, (E) SPI film incorporated with nisin 20000 IU/g SPI.

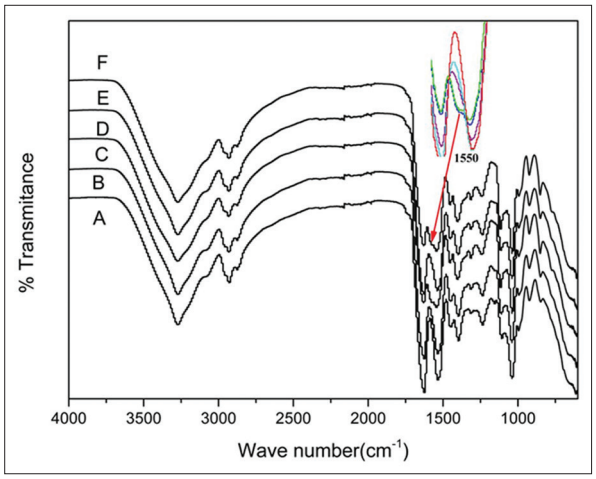

Fig 2-b. Spectra of Fourier Transform Infrared (FTIR) of SPI edible films. (A) SPI film,(B) SPI film incorporated with sodium lactate 0.5g/g SPI, (C) SPI film incorporated with sodium lactate 1g/g SPI, (D) SPI film incorporated with sodium lactate 1.5g/g SPI, (E) SPI film incorporated with sodium lactate2 g/g SPI.

to be no specific interaction between active groups of N with functional group of SPI. Similar scheme was presented by Liu et al. (2010). However, the result is different from Shiroodi et al. (2016). They reported that the peak indensity of whey protein isolate (WPI)-based and pea protein isolate (PPI)-based films increased as the concentration of N higher. The difference between the results may be due to the physical and chemical difference in the matrix, which is WPI, PPI, and SPI.

Fig. 2-b showed the spectrum of the control SPI films and the films incorporated NaL at different levels. The indensity of peaks in SPI-NaL films was slightly increased. And as we can seen from Fig 2-b, there is a change in the amide I

band at 1550 cm^{-1} appears obviously. Absorption peak at 1550 cm^{-1} was assigned to the stretching vibration of C=C.

Figs. 2-c depicts the spectrum of the control SPI film and the films incorporated EDTA (E) at varying levels used in the study. Similar to the spectrum of SPI-NaL films, the intensity of peaks was increased. In the SPI-based films the intensity of peaks increase in amide I,II, III regions, indicating immobilization of NaL and EDTA onto film surface (Shiroodi et al., 2016). The increase was more significant as the concentration of EDTA higher. At the level of 0.2 g EDTA/g SPI, there were obvious peaks at 1358 cm^{-1} and 1183 cm^{-1}, indicating the interaction between EDTA and SPI.

The SPI films are characterized by its mechanical and antimicrobial properties when they are incorporated with three antimicrobial agents, which were supported by the FITR infrared spectral data. When SPI films were incorporated with N, and the SPI functional groups would have no modification. Thus, the mechanical properties have no significant changes. When the hydroxyl groups and conjugated double bonds are available in the reactive groups, the efficacy of the natural extracts can be determined on the pathogen inhibition. The active compound of N freely inhibits the microorganisms in the antimicrobial test (Pranoto et al., 2005; Wang et al. 2015). Relatively, the incorporation of NaL and EDTA into the SPI film had a significant effect on the functional group's change of the SPI film. Therefore, they changed much on the mechanical properties of SPI films produced but they did not show significant inhibitory effect caused by the unavailability of the free antimicrobial group.

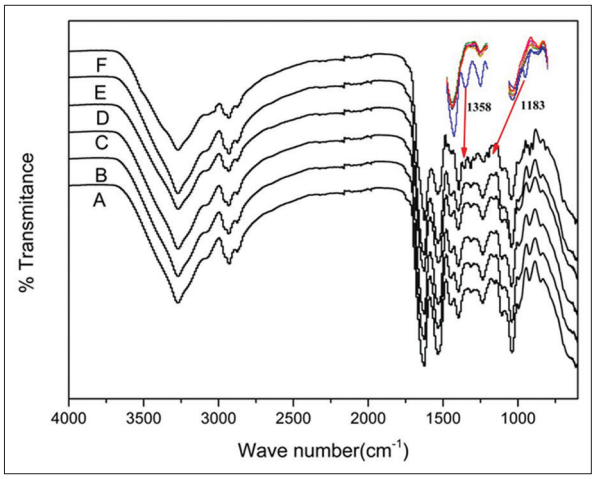

Fig 2-c. Spectra of Fourier Transform Infrared (FTIR) of SPI edible films.(A) SPI film, (B) SPI film incorporated with EDTA 0.04g/g SPI, (C) SPI film incorporated with EDTA 0.08g/g SPI, (D) SPI film incorporated with EDTA 0.12 g/g SPI, (E) SPI film incorporated with EDTA 0.16g/g SPI, (F) SPI film incorporated with EDTA 0.2g/g SPI.

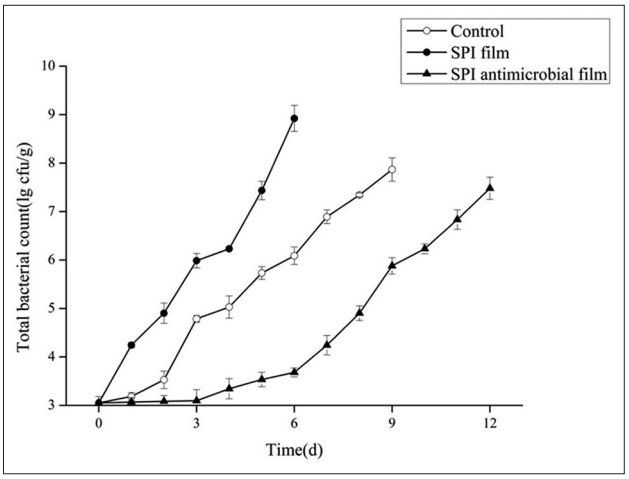

Fig 3-a. Evolution of total bacterial count during storage at 4°C.

Application of experimental results analysis

Total bacterial count of sample

The total bacterial count of meat during storage is shown in Fig. 3-a. With the increase in storage time, the total bacterial count in each meat had been increasing (Fig. 3-a). Bacteria of the pure SPI film group were breeding very quickly that the samples wrapped by the pure SPI film group were corrupt earlier than the samples wrapped by the other two films. In the first three days the value of the total bacterial count of sample rose to 5.98 lg cfu/g, which means that the meat sample had become minor fresh meat, and to the 6th day the total bacterial count value was 8.92 lg cfu/g, indicating the deterioration of the sample. While in the ordinary wraps group, the sample had been corrupt more than 3 days later. The total bacterial count of the sample wrapped by ordinary wraps was 4.78 lg cfu/g on 3rd day (Minor fresh), 5.98 lg cfu/g on 6th day (Minor fresh), and 7.86 lg cfu/g on 9th day (Deterioration). There was a significant difference ($P<0.05$) between the SPI group and control group in the statistical analysis of total bacterial count of sample from the 3rd day.

The total bacterial count of the sample wrapped by SPI antimicrobial film was 3.08 lg cfu/g on 3rd day (Fresh), 3.53 lg cfu/g on 6th day (Fresh), 5.87 lg cfu/g on 9th day (Minor fresh) and 7.47 lg cfu/g on 12th day (Deterioration). The significant difference in the value of total bacterial count value between the sample of three groups indicating that the bacterial breeding speed of sample packaged with SPI antimicrobial films relatively slow, which was still in a freshness in the 9th day. In short, it can be seen that the SPI antimicrobial film has a significant ($p<0.05$) inhibitory effect on the beeding of bacteria during meat preservation.

TVB-N value of sample

TVB-N value is the ammonia and amines and other nitrogen substances in the process of corruption and deterioration of protein due to animal foods' enzymes reacts with food surface micro-organisms, which is an important indicator of meat freshness (Duan, 2001).

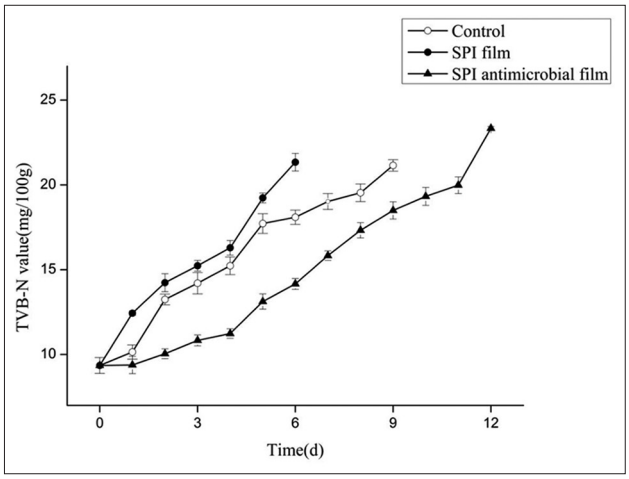

Fig 3-b. Evolution of TVB-N value during storage at 4°C

As shown in Fig. 3-b, with the increase of the storage time, the TVB-N values of the meat samples showed an increasing trend. On the 3rd day, the TVB-N values of the sample packaged with the pure SPI film, ordinary wraps and SPI antimicrobial film were 14.23, 14.20 and 10.83 mg/100g on the 3rd day, which means that the samples were in the first grade freshness and the sample of control group was the freshest. However, on the 6th day the value of samples wrapped by the pure SPI film and ordinary wraps were 21.34 and 18.09 mg/100g, indicating that the sample in two groups were rotten and in second grade freshness respectively. On the 9th day, the sample wrapped by ordinary wraps was rotten which TVB-N value was 21.15 mg/100g, whereas the sample of control group was still in second grade freshness (TVB-N value was 18.49 mg/100g). And the control group has become rotten on the 12th day and its TVB-N value was 23.33 mg/100g. Thus compared with pure SPI film and ordinary wraps, SPI antibacterial film in the secondary freshness extended for 6 and 3 days.

From the above analysis we can see that the effect of SPI film on the freshness of pork was inferior to ordinary wraps. It may be due to the protein matrix of the SPI that provides the nutrients needed for growth. While the SPI antimicrobial film was better than the two of the antibacterial freshness for the antimicrobial agents played a key role in the inhibiting the growth of microorganisms.

CONCLUSION

SPI film has great potential to improve its antimicrobial property by incorporating antimicrobial agents. N, NaL and EDTA at variable levels incorporating SPI film were effective in inhibiting the growth of E. Coli, *Salmonella* and B.cereus. N incorporating into SPI film increased the inhibitory zone on B.cereus to 29.58 mm. SPI films incorporated with NaL had a significant antimicrobial efficacy to *E.Coli* and *Salmonella*, the inhibitory zone from 0 mm to 28.5 and 27.0 mm. SPI film incorporated with EDTA showed a little antimicrobial activity against three pathogens. But it enhanced the physical properties of SPI film. The combination of N, NaL and EDTA increased significantly the inhibitory zone against three bacteria from 0 mm to 32.00, 33.33, and 32.33 mm. N, NaL and EDTA were found effective to fight against food-borne pathogens. And FTIR studies data supported the results obtained from other tests. In the application experiments, the SPI antimicrobial film (C) did have a bacteriostatic preservation effect on the meat antibacterial fresh and extend the shelf life to 3-6 days compared with pure SPI film and ordinary wraps. Overall, the incorporation of combination into SPI film had the desirable characteristic of acting as a physical and antimicrobial barrier to food contamination.

This finding has potential applications in various food products including raw and ready-to-eat product.

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AUTHORS' CONTRIBUTIONS

Guarantor of integrity of entire study: Yuanyuan Liu, Huajiang Zhang; Study concepts: Yuanyuan Liu; Study design: Yuanyuan Liu; Literature research: Yuanyuan Liu, Lina Xu; Experimental studies: Yuanyuan Liu, Lina Xu, Yongqing Wu, Wenhui Cao, Tong Li; Data acquisition: Yuanyuan Liu, Lina Xu; Statistical analysis: Yuanyuan Liu; Manuscript preparation: Yuanyuan Liu; Manuscript definition of intellectual content: Yuanyuan Liu; Manuscript editing: Yuanyuan Liu, Lina Xu; Manuscript revision/ review: Yuanyuan Liu; Manuscript final version approval: Huajiang Zhang,Yujie Chi.

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