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**FINAL REPORT**

**INTERNATIONAL RESEARCH COLLABORATION**

**AND SCIENTIFIC PUBLICATION**

# ENHANCED METHOD FOR AMPLIFYING ANTIMICROBIAL ACTIVITY OF LACTOPEROXIDASE SYSTEM IN MILK AND DERIVED PRODUCTS BY CARROT EXTRACT AND BETA CAROTENE

Year 1 of plans 3 years

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## ABSTRACT

OSCN– has been known and well documented as natural antimicrobial agent generated from the lactoperoxidase system (LPOS) but the antimicrobial activity exerted from this system is too low for certain food such as milk and derived products (up to 1.2 log CFU/ml) resulting in the restriction on industrial development of LPOS. Our previous study concluded that involvement of carrot extract and beta carotene in LPOS significantly boosted the antimicrobial activity (up to 6 log CFU/ml) against *S. enteritidis*. This finding should be continued to the application on food. Since we found that LPOS generates low antimicrobial activity on milk and derived product, our research will be conducted on the application of LPOS plus carrot extract and beta carotene on milk and derived product. Because the high antimicrobial activity is needed for industrial purposes, this research may open the way for industrial development of natural antimicrobial agent from LPOS.

This research will be conducted in three steps of experiment: (1) ensuring the incredible antimicrobial activity of LPOS plus carrot extract and beta carotene against three poisonous tropical bacteria: *C. jejuni*, *S. enteritidis*, and *E. coli,* (2) utilization of LPOS plus carrot extract and beta carotene in milk: full cream and skimmed milk, and (3) utilization of LPOS plus carrot extract and beta carotene in milk derived product: yogurt and tropical fruity milk. The purification of LPO from bovine milk, the complimentary data analysis for generation of LPOS antimicrobial activity by the addition of carrot extract and beta carotene will be conducted in Japan and for the application in tropical poisonous bacteria, milk, and milk derived product will be conducted in Indonesia.

The outcomes of this research from three step of research are three international publications at international-scopus-indexed-journals : International Journal of Dairy Science and one of patent: “enhanced method for improvement of LPOS antimicrobial activity by involvement of carrot extract and beta carotene in milk and derived product”.



Keywords: Lactoperoxidase, antimicrobial activity, carrot extract, beta carotene, milk, derived products

# CHAPTER 1. INTRODUCTION

## Background

Fruits, vegetable, fresh meat, fresh fish are commonly washed with water containing chemical sanitizing agents such as chlorine, chlorine dioxide, trisodium phosphate, and hydrogen peroxide to decontaminate the surface of these foods. Chlorine is the most familiar among the chemical agents because it is most effective in reducing microorganisms. Nevertheless, chlorine provides side effect such as severe irritation of nose, throat and upper respiratory tract.

Natural antimicrobial agents have received attentions for preserving food without undesirable side effects. Lactoperoxidase (LPO) is the most abundant enzyme in milk and it accounts for approximately 1% of the total protein in cow's milk. LPO in combination with $H_2O_2$ and $SCN^-$, generate a natural antimicrobial agent, $OSCN^-$. The antimicrobial system is called lactoperoxidase system (LPOS). LPOS has been studied for the preservation of various dairy products including raw milk, cheese, and yogurt, and non-dairy products such as mango and vegetable juice.

Although LPOS has been applied successfully in various kinds of food, a problem still remained over the strength of LPOS in some food such as including skim milk, fresh milk, fruity milk and meat broth. Our previous experiment resulted that LPOS did not remarkably inhibit the proliferation of *Salmonella enteritidis* in skim milk, fresh milk, tropical fruity milk, and other derived milk product. This may be explained by the less power of $OSCN^-$ as LPOS output product and the inhibitor compound of LPO activity such as sugar. We proved that sugar highly inhibited LPO activity.

It is understood that $OSCN^-$ is the agent to kill bacteria, therefore the existence of this compound is a key for preserving food against bacteria. Our previous research in last year (2012) found the remarkable result that the involvement of carrot extract and beta carotene in LPOS exerts huge antimicrobial activity against *S. enteritidis* (published in Journal of Food Chemistry 130 (2012) 541–546). About 10 ppm of involvement generates about 3 log CFU/ml of antimicrobial activity. Our experiment using higher concentration of carrot extract and beta carotene (20 ppm) resulted in the extremely power of antimicrobial activity (6 log CFU/ml). All of the employed bacteria have been killed by this involvement. We call this involvement as lactoperoxidase system plus carrot extract and beta carotene. Using spectral analysis,

we concluded that the oxidized beta carotene is a responsible compound to enhance antimicrobial activity.

As mentioned above, we successfully applied LPOS in various milk and milk products but it still remained the problem: weak antimicrobial activity of LPOS. The various modifications of LPOS have been made, for example by increasing LPO activity and removing sugar from milk but it was failed to enhance the antimicrobial activity. Based on our research, the maximum antimicrobial activity of LPOS was 1.2 log CFU/ml. Consequently, for industrial purposes the enhancement of antimicrobial activity of LPOS is urgently required and lactoperoxidase system (LPOS) plus carrot extract and beta carotene may provide the answer. Since there was no documentation or publication in the practical use of lactoperoxidase system plus carrot extract and beta carotene in milk and its derived product, this research will be conducted.

In this study, practical approaches to utilize lactoperoxidase system plus carrot extract and beta carotene for preserving milk and derived product will be conducted and this experiment will be divided into three steps: (1) ensuring the extreme power of antimicrobial activity of lactoperoxidase system plus carrot extract and beta carotene in common food poisoning bacteria in tropical countries, (2) utilization of lactoperoxidase system plus carrot extract and beta carotene in various milk: full cream and skimmed milk, (3) utilization of lactoperoxidase system plus carrot extract and beta carotene in various milk products: yoghurt and fruity milk (milkshake).

Since the low cost of LPOS utilization is needed for industrial purposes, it is believed that this experiment may facilitate the possibility of LPOS utilization for industrial purposes as an effective natural disinfectant.

**Research Purposes**

Our research will be done for enhancing antimicrobial activity of LPOS by the involvement of carrot extract and beta carotene against deadly tropical bacteria in milk and derived milk product. The research will be conducted in three steps of experiment:

1. Ensuring the high antimicrobial activity of LPOS plus carrot extract and beta carotene in deadly tropical poisonous bacteria in food: *C. Jejuni, S. enteritidis, E. coli*
2. Utilization of LPOS plus involvement of carrot extract and beta carotene in milk (full cream and skimmed milk)

3. Application of LPOS plus carrot extract and beta carotene in milk products: yogurt and fruity milk

The improvement of antimicrobial activity may provide high benefit to suppress the cost of LPOS utilization. As a consequent, the much cheaper of the cost may open the way for utilization of LPOS for industrial purposes

# CHAPTER 2. LITERATURE REVIEW

The lactoperoxidase (LPO), hydrogen peroxide ($H_2O_2$), thiocyanate ion ($SCN^-$), that is known as lactoperoxidase system (LPOS) produces an inhibitor of bacterial growth. A number of bacteria metabolic activities are inhibited, including inhibition in the respiration process and glycolysis. The growth of certain bacteria is completely inhibited (Corbo et al., 2009, Thomas and Aune, 1977). The lactoperoxidase system (LPOS) should be consisted of three components: LPO, $SCN^-$and $H_2O_2$ to generate the antimicrobial product: hypothiocyanite ion ($OSCN^-$). This reaction product has a broad spectrum of antimicrobial effect (Seifu et al., 2004, Min et al., 2005, Reiter, 1985, Saad, 2008, Seifu et al., 2005, Wolfson and Sumner, 1993). The product inhibits microorganisms by the oxidation of sulphydryl groups of microbial enzymes (Kussendrager and Hooijdonk, 2000, Gurtler and Beuchat, 2007, Wit and Hooydonk, 1996). International Dairy Federation recommends the use of LPOS to preserve raw milk during transport in developing countries (FAO, 2005, Gurtler and Beuchat, 2007, FSANZ, 2002). Furthermore, LPOS has been studied as a means to control pathogens and spoilage microorganisms in pasteurized milk (Marks et al., 2001, Seifu et al., 2004), caprine milk (Seifu *et al.*, 2004), infant formula (Gurtler and Beuchat, 2007, Banks and Board, 1985), fruit and vegetable juices (Nguyen et al., 2005, Raybaudi-Massilia et al., 2009), beef (Elliot *et al.*, 2004) and poultry (Borch et al., 1989, Touch et al., 2004).

It has been documented that obtaining of LPO is not complicated by ion exchange process, however, it is costly for practical use in food industry (Touch et al., 2004, Al-Baarri et al., 2010b). Our previous experiment immobilized bovine whey, which is rich of LPO content, into SP-Sepharose Fast Flow (SP-FF) for repeating $OSCN^-$ production (Al-Baarri et al., 2010a). Even though SP-FF is able to reuse for multiple times, food industry may dislike since the initiation cost for SP-FF is too high. In addition, the utilization of LPOS in food is less in action especially in food containing high carbohydrate and protein such as fresh milk and the products of milk. Our previous findings proved that LPOS was able to reduce ±1 log CFU/ml of total bacteria in fresh milk (Villa et al., 2013). It was also studied in our experiment that LPOS reduces 1.2 log CFU/ml in fruity milk or milkshake (Al-Baarri et al., 2013). The study in the Indonesian milk product was also done for the milk derived food namely “dangke”. This food is similar to cheese that is produced from milk with the

casein coagulation by papain enzyme. It has been studied that LPOS has less of action to reduce pathogenic bacteria in "dangke" (±1.2 log CFU/g reduction) (Rasbawati et al., 2013).

It has been understood that LPO was inhibited by certain compound in milk such as casein (Singh et al., 2009) resulting in the less action of LPOS. The inhibition of LPO activity by sugar was also studied (Al-Baarri et al., 2011a). LPOS was found to be especially effective against *S. enteritidis* in tomato and carrot juices (Touch et al., 2004). The potent LPOS antimicrobial activity attracted us an interest in the bio-chemical components in these vegetables, for some vegetable components may enhance the antimicrobial activity of LPOS. Tomato and carrot contain high concentrations of carotenoid, especially lycopene for tomato and b-carotene. Thus, we conducted the experiment by the involvement of carotenoids in the enhanced antimicrobial activity of LPOS. We investigated the effects of carrot extract and beta carotene, one of major pigments in carrot, on LPOS antimicrobial activity against *S. enteritidis*. Furthermore, we also investigated the molecular roles of beta carotene, as an enhancer of LPOS antimicrobial activity by spectroscopic approaches. As a conclusion, there was a remarkably effect of this involvement on LPOS antimicrobial activity (Hayashi et al., 2012).

Addition of 20-fold diluted carrot extract improved LPOS antimicrobial activity from 1.4 to 3.8 log units. The carrot extract with the dilution ratio lower than 10-fold dilution enhanced the antimicrobial activity to 6.0 log units, indicating that all the inoculated bacteria were killed. We concluded that these improvement was due to the oxidation of beta carotene by OSCN, which was generated by LPOS.

Since our previous research on the utilization of LPOS in milk and and its product resulted in the low antimicrobial activity (1.2 log unit), the improvement of antimicrobial activity of LPOS is required. Since we found the method of improvement of LPOS antimicrobial activity, this research will be focused on the application of LPOS plus carrot extract and beta carotene in milk and derived product.

The improvement of antimicrobial activity was very excellent. It was more than 4 times higher than no addition of carrot extract and beta carotene against *S. enteritidies*. Unfortunately, we have no information and there was no publication yet on the improvement of LPOS antimicrobial activity by carrot and beta carotene against other pathogenic bacteria especially tropical deadly bacteria such as *Campylobacter jejuni*, *Staphilococcus aureus*, *L. monocytogenes*, and *Escherichia*

*coli*, therefore our experiment will be conducted using such mentioned bacteria. This research will be devided in three step for three year of experiment: (1) The enhancement of antimicrobial activity of LPOS plus carrot extract and beta carotene against *Campylobacter jejuni*, *Staphilococcus aureus*, *L. monocytogenes*, and *Escherichia coli*, (2) utilization of LPOS plus carrot and beta carotene on indegenous varieties of fresh milk (cow milk and goat milk) against *Campylobacter jejuni*, *Staphilococcus aureus*, *L. monocytogenes*, *S. enteritidis* and *Escherichia coli,* and (3) utilization of LPOS plus carrot extract and beta carotene on milk derived products tropical fruity milk (or tropical milkshake).

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## CHAPTER 3. MATERIAL AND METHOD

### Purification of LPO

Two liters of fresh cow's milk will be defatted by centrifugation at 10,300 × g at 10˚C for 30 min. The skim milk will be clotted with 0.02% (w/v) rennet and 2 ml lactic acid per liter milk at 30˚C for 30 min. Whey will be obtained by removing curd and filtration through filter paper under vacuum conditions. The whey will be dialyzed against 10 liters of 10 mM sodium phosphate buffer (PB), pH 6.8, overnight. The resulting solution will be loaded to SP Sepharose Fast Flow. The resin will be washed with 500 ml of 10 mM PB (pH 6.8) containing 0.1 M NaCl. LPO will be eluted with 500 ml of 10 mM PB (pH 6.8) containing 0.2 M NaCl. The eluate will be collected (5 ml/tube) and the protein concentration of each fraction will be determined by measuring absorbance at 280 nm. An extinction coefficient of 1.5 cm2 mg–1 will be used to estimate the protein concentration of LPO. The purity of LPO will be checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The protein solution was concentrated by ultrafiltration using a 10,000 MW cutoff membrane, and the retentate was filtered through a 0.22 µm filter unit. The purified LPO will be stored at -80 ˚C (Al-Baarri et al., 2011a).

### Enzymatic activity assay

LPO enzymatic activity will be determined using ABTS as a substrate. The reaction solution will be prepared by mixing 50 µl of LPO (0.2 U/ml), 50 µL of sugars (up to 4.0%) in 10 mM PB (pH 7.0), and 450 µl of 1 mM ABTS in 0.1 M acetate buffer (pH 4.4). The enzymatic reaction will be initiated by adding 450 µl of

0.55 mM H2O2 to a reaction solution. Immediately, the absorbance of the solution will be monitored at 412 nm for 20 s at 25˚C. One unit of LPO enzymatic activity will be expressed as the amount of enzyme needed to oxidize 1 µmol ABTS/min. The ABTS molar extinction coefficient at 412 nm was 32,400 M–1 cm–1 (Al-Baarri et al., 2011b).

**Determination of residual [SCN−]**

**Determination of residual [SCN−]** in LPOS. The LPOS solution that will be used in the experiments includes 0.3 mM KSCN, 0.3 mM H2O2, and 0.01 U/ml of LPO in 10 mM PB (pH 7.0) containing 0.1–0.3% sugar. Determination of the residual [SCN−] in LPOS will be carried out according to the procedure previously described by Al-Baarri et al. (2010b). Ten grams of ferric nitrate (Fe(NO3)3·9H2O) will be dissolved in 20 ml of concentrated nitric acid and diluted with pure water to a final volume of 200 ml. This solution will be used as the stock solution of ferric nitrate. One ml of LPOS solution containing sugar was added to 2.0 ml of the ferric nitrate stock solution. The absorbance at 460 nm will be monitored to determine the residual [SCN−] in LPOS solution. The [SCN−] will be calculated using a standard curve, which will be generated with ferric nitrate solution in the range 0.05–3.2 mM.

**Determination of residual [H2O2]**

**Determination of residual [H2O2] in LPOS** will be conducted according to the method described by Al-Baarri (2012). Two ml of mixture containing 1.23 mM ABTS and horseradish peroxidase (6.0 µg/ml) in 0.1 M PB (pH 6.8) will be prepared. The enzymatic reaction will be initiated by the addition of 1.0 ml LPOS solution. The absorbance at 412 nm at 25˚C will be monitored for 30 s. The [H2O2] will be estimated from an established standard curve of ABTS, which will be generated in the range 0.05–3.2 mM.

**Determination of [$OSCN^-$]**

$OSCN^-$ concentration will be determined according to the method of Al-Baarri et al. (2011b) with minor modifications. The principle of the method is based on the oxidation of Nbs to Nbs2. Nbs stock solution will be prepared by adding 2.0 µl of mercaptoethanol to 10.0 ml of Nbs solution diluted to 0.5 mM with 0.1 M PB (pH 7.2) containing 5.0 mM EDTA (PBE). The Nbs stock solution will be prepared fresh daily and kept on ice. Before OSCN− determination, H2O2 present in a sample will be removed by adding 20 µl of 1.0 mg/ml catalase solution to 1.0 ml sample. Four

milliliter of PBE will be added to 0.1 ml of the H2O2-free sample solution, followed by the addition of 0.5 ml of Nbs stock solution. Immediately, the absorbance of the mixture will be measured at 412 nm. The concentration of remaining Nbs will be calculated from the absorbance reading, with assumption of a molar absorption coefficient of 13,600 M-1 cm-1 for Nbs.

**Preparation of carrot extract**

Fresh carrots will be peeled and homogenized with 5-fold weight of sterile 0.1 M PB (pH 7.0), containing 0.15 M NaCl. The suspension will be centrifuged at 8000g, at 4 °C for 15 min. The resultant supernatant (carrot extract), will 2–20 times be diluted with the same buffer. The carrot extract stock solution, and its diluted solutions, will be used for further experiments. All the processes of preparation is done under aseptic conditions (Hayashi et al., 2012).

**Beta carotene concentration assay**

**Beta carotene concentration** will be determined based on our previous research (the method of Hayashi et al. (2010)). First, beta carotene aqueous solution will be prepared as follows: 25 mg of beta carotene and 0.9 ml of Tween 20 will be dissolved in 25 ml of chloroform. One milliliter of beta carotene solution will be evaporated to dryness *in vacuo*. The resultant residue will be dissolved immediately in 10 ml of 0.25% EDTA and filtered through an Advantec grade No. 2 qualitative filter paper. Thereafter, 40 ml of 10 mM sodium acetate buffer (pH 4.6) will be added to the filtrate. The solubilised beta carotene aqueous solution (2.7 ml) will be mixed with 100 µl of 8.25 mM H2O2, 100µl of 11.3U/ml of LPO, and 100µl of 10 mM KSCN in a 10x10 mm crystal cuvette, and an absorption spectrum of the mixed solution was immediately monitored from 360 to 550 nm.

The below is the components that will be brought to Indonesia:

1. LPO powder
2. Carrot extract powder
3. Beta carotene
4. *C. jejuni* and *S. enteritidis*

**Sample preparation of milk**

Fresh milk will be obtained from a faculty's farm. This fresh milk then converted to full cream and skimmed milk using laboratory equipment and sterilized using autoclave

**Application of LPOS on Milk and Milk Product**

This method is adopted from experiment of Azizah et al. (2013) with minor modification. Nine milliliters of milk or its derived product will be added into 0.95 ml of LPOS (5.6 U/ml LPO, 1.25 mM KSCN, 0.25% Glc, and 0.87 U/ml GO) were mixed with 0.05 ml of diluted carrot extract solutions or beta carotene solution. The mixture was incubated at 30 °C for 2 h. Thereafter, the mixture will be diluted with 0.15 M NaCl and streaked onto a DHL agar plate. The plate will be incubated at 37 °C for 24 h. The number of bacterial colonies formed on the plate was counted. All the processes of preparation will be done under aseptic conditions.

**Antimicrobial activity assay**

Four hundred microliters of LPOS (5.6 U/ml LPO, 1.25 mM KSCN, 0.25% Glc, and 0.87 U/ml GO) will be mixed with 50 µl of diluted carrot extract solutions or beta carotene solution. After adding 50 µl of $10^7$ cfu/ml bacteria solution, the mixture will be incubated at 30°C for 2 h. Thereafter, 100 µl of the LPOS-treated bacterial suspension, dilute to countable cell number with 0.15 M NaCl, will be streaked onto a Desoxycholate Hydrogen Sulfide Lactose Agar plate and incubated at 37°C for 24 h. The number of bacterial colonies formed on the plate will be counted. Control is the treatment using Milli-Q water instead of the diluted carrot extract or beta carotene solution and LPOS. Antimicrobial activity of LPOS is expressed as log N0/N1, where N0 = cfu/ml of control and N1 = cfu/ml of sample (Hayashi et al., 2012)

**The preparation of bacteria**

*C. jejuni and S. enteritidis* (from the Institute for Fermentation, Osaka, Japan) and *E. coli* (from Institute Food and Nutrition, Gadjah Mada University, Yogyakarta, Indonesia) will be cultured overnight in a medium containing 1% polypeptone, 0.5% yeast ex- tract, 0.3% glucose, 1% NaCl, 0.1% MgSO4 7H2O, and 1.5% agar at pH 7.0 and 37˚C. The bacteria will be allowed to grow overnight at 37˚C in flasks containing pre-sterilized tryptic soy broth with 1.25% glucose. The bacteria will be

harvested by centrifugation with pellets being washed with 0.1% sterile peptone water (PW). The cells will be resuspended in the same solution to measure the optical density of the growth. The bacterial density, expressed as cfu/ml, will be estimated from the absorbance at 600 nm using a standard curve that relates each bacteria cfu/ml to absorbance (Touch et al., 2004).

# CHAPTER 4. RESULT AND FURTHER RESEARCH

## Result

**DATA LPO PURIFICATION**

| | |
|---|---|
| LPO (ml) | 90 |
| Source (milk in ml) | 3000 |
| Activity before ultrafiltration (U/ml) | 11 |
| Activity after ultrafiltration using 10KDa MWCO (U/ml) | 105 |
| Band before ultrafiltration using SDS PAGE | 5 |
| Band after ultrafiltration using SDS PAGE | 1 |
| Purity index (%) | 90 |
| LPO storage | 0.1 mM Phosphate Buffer pH 7.0 |
| Ambient termperature storage | -20˚C |

## Formula of Optimized LPO System

| LPO System formula | Non Beta Carotene | Plus Beta Carotene |
|---|---|---|
| LPO activity (U/ml) | 4.5 | 4.5 |
| KSCN (mM) | 0.3 | 0.3 |
| H2O2 (mM) | 0.3 | 0.3 |
| Hypothiocyanate (mM) | 0.27 | 0.28 |

**Remaining Hyphothiocyanite (mM)**

| Time (minute) | Non Beta Carotene | Plus Beta Carotene |
|---|---|---|
| 10 | 0.26 | 0.27 |
| 20 | 0.26 | 0.28 |
| 30 | 0.26 | 0.27 |
| 40 | 0.25 | 0.27 |
| 50 | 0.24 | 0.25 |
| 60 | 0.25 | 0.26 |
| 70 | 0.22 | 0.25 |
| 80 | 0.21 | 0.26 |
| 90 | 0.21 | 0.27 |
| 100 | 0.2 | 0.26 |
| 110 | 0.17 | 0.27 |
| 120 | 0.16 | 0.25 |

**Antibacterial Activity**

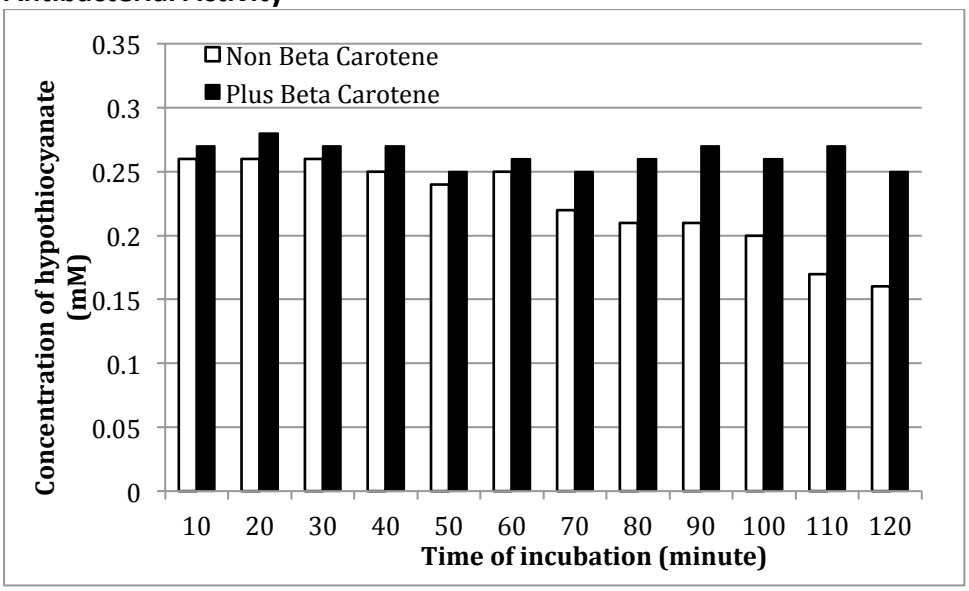


Figure 1. Production of hypothiocyanite from the LPOS solution which was consisted of LPO (10 U), KSCN (0.3 mM) and $H_2O_2$ (0.3 mM) with and without extract carrot addition against time of incubation at 37˚C. Values are means of three sets of experiments.

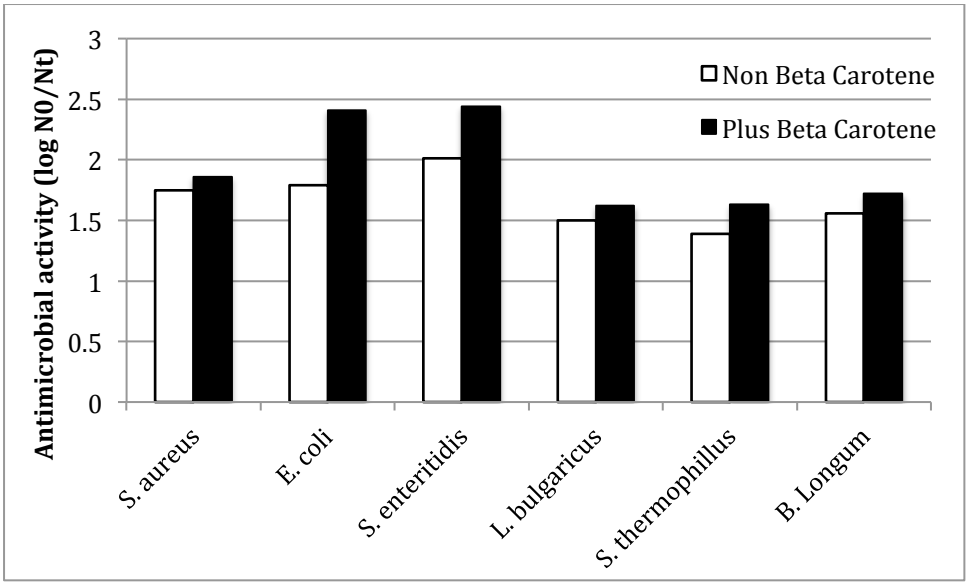


Figure 2. Antimicrobial activity of the LPOS against pathogenic bacteria: *S. aureus, E. coli, S. enteritidis,* and non-pathogenic bacteria: *L. bulgaricus, S. thermophillus, B. longum*. All bacteria (±5 log CFU/ml) were innoculated to LPO solution and incubated for 4 h at 37˚C. Values are means of three sets of experiments.

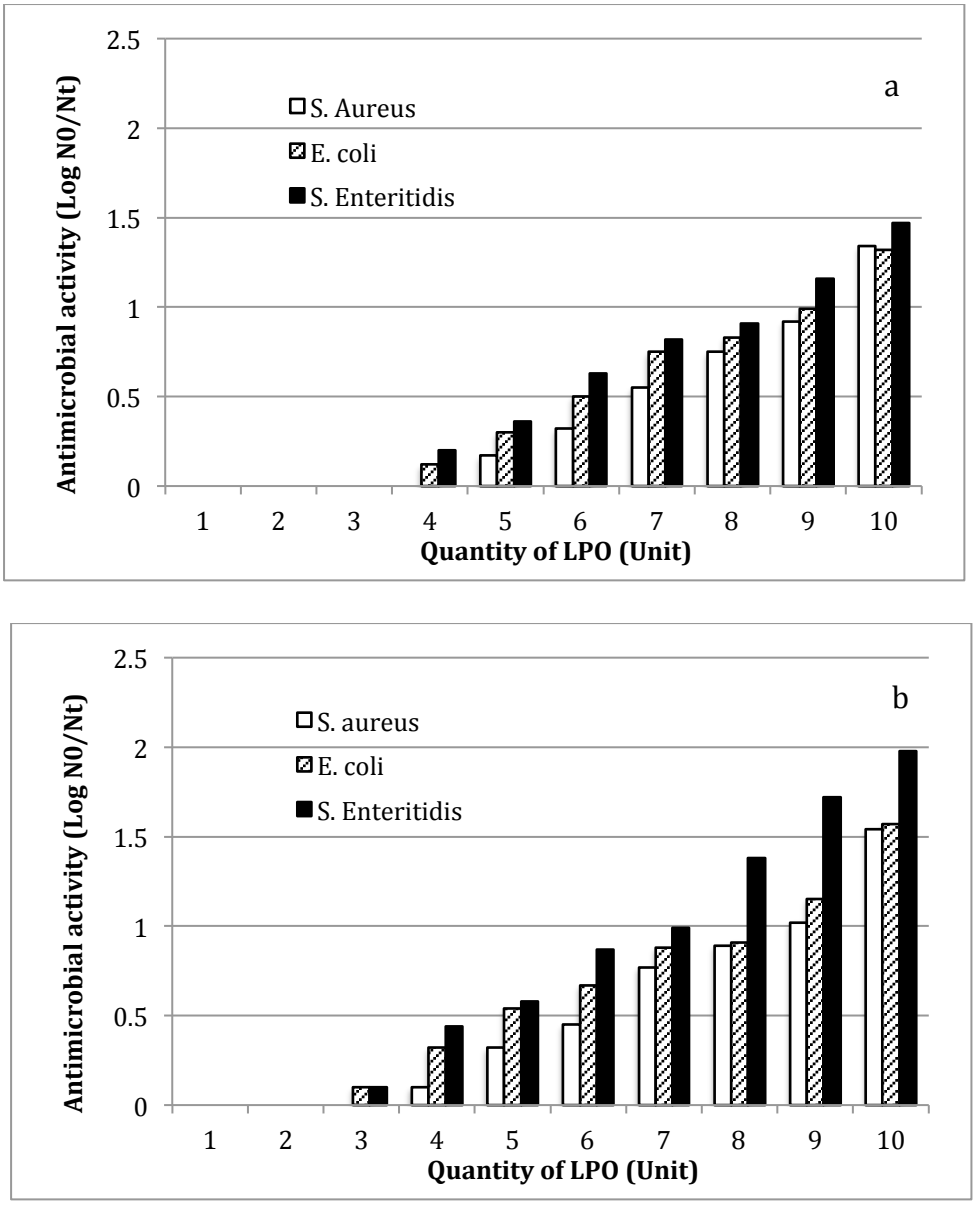


Figure 3. Antimicrobial activity of the LPOS against pathogenic bacteria: *S. aureus, E. coli, S. enteritidis*. All bacteria (±5 log CFU/ml) were inoculated to LPOS solution with various enzyme activities from 1–10 U and incubated for 4 h at 37˚C without (a) and with (b) the presence of carrot extract in the LPOS solution. Values are means of three sets of experiments.

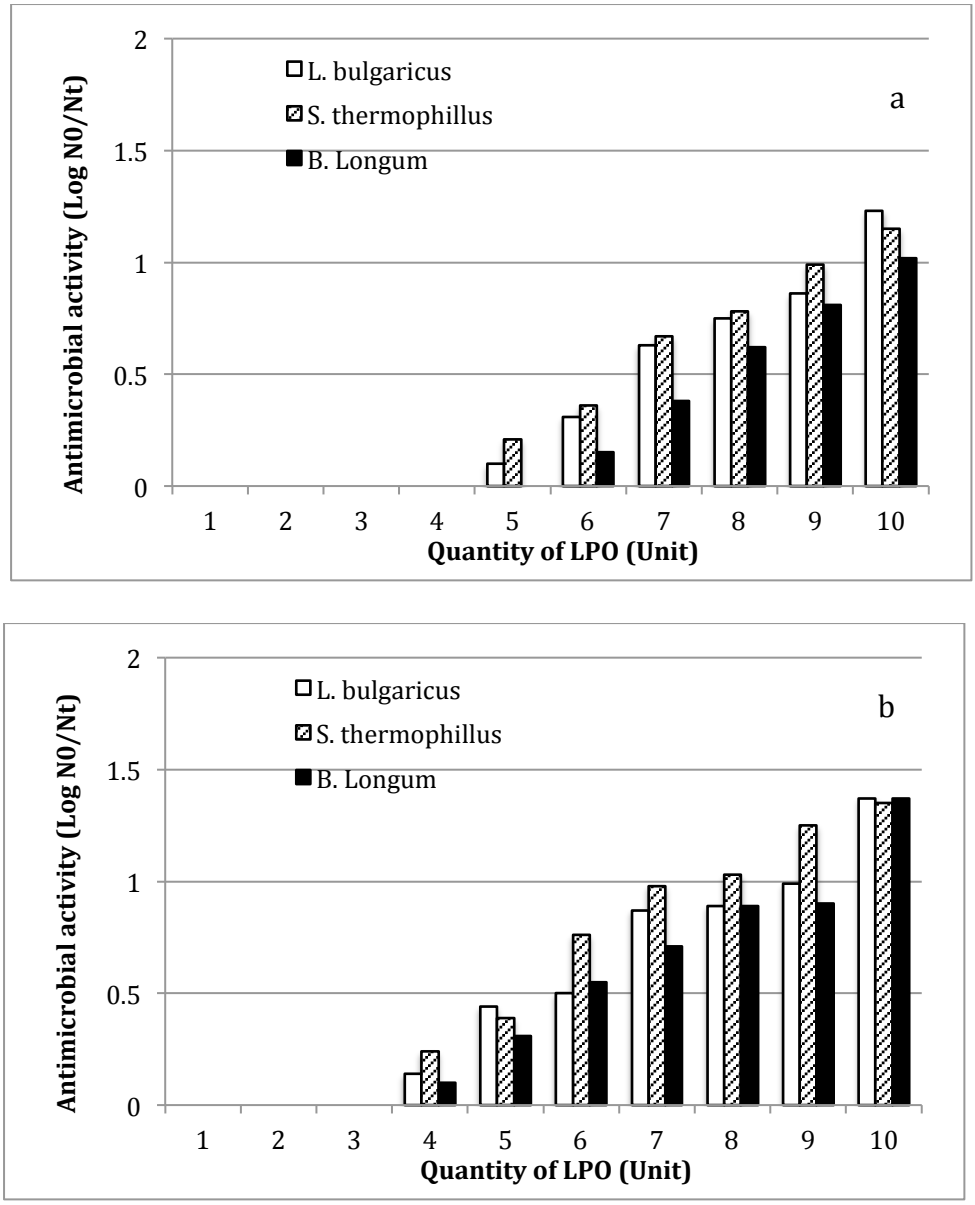


Figure 4. Antimicrobial activity of the LPOS against non-pathogenic bacteria: *L. bulgaricus, S. thermophillus, B. longum*. All bacteria (±5 log CFU/ml) were innoculated to LPOS solution with various enzyme activities from 1–10 U and incubated for 4 h at 37˚C without (a) and with (b) the presence of carrot extract in the LPOS solution. Values are means of three sets of experiments.

**Past and Next Work**

| Item | Month | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Preparation for the research: Internal meeting and consultation, order and booking for material and equipment, stationary purchase, latest literature searching, administrational work (sending letter, institutional coordination, task distribution) | done | | | | | | | | |
| Main material availability: Stock of tropical poisonous bacteria, medium, material for LPO purification | | done | | | | | | | |
| Experiment part A: Purification of LPO from bovine whey: high purity and high activity | | | done | done | | | | | |
| Experiment B: Carrot extraction and beta carotene preparation. This will continue to the involvement of these compounds to LPOS to produce oxidized beta carotene. High antimicrobial activity is the target. | | | done | done | | | | | |
| Experiment C: application of LPOS plus beta carotene to analyze antimicrobial activity against tropical poisonous bacteria | | | | | done | | | | |
| Internal discussion with Japan's research team and data analysis, drafting patent, drafting manuscript | | | | | | | | 80% | |
| Continuation of previous work (drafting drafting manuscript) | | | | | | | | | 90% |
| Manuscript submission | | | | | | | | | 90% |
| Administrational work for ending of the research (writing report and collecting the administrational prove) | | | | | | | | | 80% |

The target of this research has 89% done.

## Next Agenda

1. Internal discussion with Japan's research team and complimentary data analysis, drafting manuscript
2. Manuscript submission
3. Administrational work for ending of the research (writing report and collecting the administrational prove)

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