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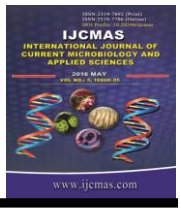

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Gouda Cheese Microbial Controlling During Ripening Using Composite Edible Film Containing Lysozyme

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A B S T R A C T

The purpose of this research was microbial controlling of gouda cheese during 8 weeks ripening using composite edible film containing modified lysozyme. Modified lysozyme content (control, 0,05 and 0,1%) in composite edible film and cheese ripening (1 day, 2 weeks, 4 week) using Nested Design with Group Randomized Design. The variables were: microorganism quality (Aerobic plate count, Lactic acid bacteria, *Enterococcus*, Coliform, *E. coli*, *Salmonella*, *S. aureus* and yeast/mold). The result showed that modified lysozyme addition in composite edible film did not gave significant effect (P>0.05) on aerobic plate count, lactic acid bacteria, enterococcus, coliform, E. coli, salmonellae and S. aureus, but cheese ripening nested in modified lysozyme addition gave highly significant effect (P<0.01) on aerobic plate count, lactic acid bacteria, enterococcus, coliform, and E. coli. Gouda cheese coated with composite edible film containing modified lysozyme decrease population of native microbial gradually but population of artificial pathogen contamination decrease faster at the beginning of cheese ripening. Antimicrobial composite edible film containing modified lysozyme effective to inhibit microorganism growth both at the surface and inside region of gouda cheese during ripening.

Introduction

Cheese is a food susceptible to microbiological deterioration throughout storage, distribution (Cha and Chinnan, 2004), processing and ripening due to its high water content and favorable pH for microbial growth (Conte *et al.,* 2013). The cheese composition and environmental conditions during ripening often promote extensive mould/yeast and bacteria development at cheese surface, which considerably reduces its quality (Cerquira *et al.,* 2009). Coatings can act as carriers of antimicrobials for protect cheese surfaces (Cerqueira *et al.*, 2009).

Addition of antimicrobial agents reduces growth of pathogenic and spoilage microorganisms (Franssen *et al.,* 2004). Cheeses offer a suitable environment for the survival and growth of microorganisms (Melo *et al.,* 2015). One of the frequently isolated spoilage yeast in this type of food is Saccharomyces cerevisiae. Its presence causes an undesirable flavour, affects visual appearance and reduces the shelf life of food (Corsetti *et al.,* 2001; Welthagen and Viljoen, 1998). Penicillium is the genera of moulds most frequently isolated from naturally contaminated cheese. All these microorganisms comprise strains with psychrotrophic characteristics that could increase in number during cold storage (Sorhaug and Stepaniak, 1997).

In recent years, active packaging systems for food have gained much attention mainly due to increased demands on product safety, shelf life extension, environmental issues, and consumer convenience (Ahvenainen, 2003). Recently, the research showed an increasing interest in active edible films supporting antimicrobials for the purpose of enhancing food safety and extending food shelf life due to their potential to decrease antimicrobial diffusion rate from the surface to the bulk of the product, thus assisting in the maintenance of high concentrations of the active ingredient where it is required. Also, edible films can be an alternative source for packaging materials development due to their biodegradability (Pires *et al.,* 2008; Fajardo *et al.,* 2010; Kristo *et al.,* 2008; Resa *et al.,* 2013; Ture *et al.,* 2011).

Different edible films can be used to incorporate antimicrobial agents (Appendini and Hotchkiss, 2002), including whey protein isolate (WPI). In general, the resistance to water vapour transmission of protein films is limited because they are highly polar polymers with a high level of hydrogen bonding and hydroxyl groups (Ko *et al.,* 2001). Furthermore, in high humidity environments the water vapour barrier properties are subsequently reduced because of protein films´ susceptibility to moisture absorption and swelling.

In recent years, consumer demand for natural food ingredients has increased and, the use of natural antimicrobials from natural sources has begun to be explored (Gould, 1997; Tiwari *et al.,* 2009). The enzyme lysozyme (EC 3.2.1.17) is a naturally occurring antimicrobial protein in hen egg white. Lysozyme is a peptidoglycan N-acetylmuramoylhydrolase that is primarily effective against gram-positive bacteria. Lysozyme hydrolyzes the peptidoglycan component of the bacterial cell wall and thus lyses the cell (Masschalck and Michiels, 2003). Hen egg white lysozyme is is added during the production of cheese to protect against the development of Bacillus cereus, Clostridium tyrobutyricum a known food pathogen (Lo´pez-Pedemonte *et al.,* 2003; Bottazzi *et al.,* 1996; Danyluk and Kijowski, 2001; Masschalck and Michiels, 2003).

Although the growth of contaminating pathogenic bacteria is a concern in terms of consumer health and product quality, the ripening and development of the sensorial characteristics of cheese are associated with changes in the lactic acid bacteria (LAB) profile. The amplification of lysozyme expression in milk intended for cheese production therefore poses a potential threat to the ability of the LAB population to proliferate. However, observations of LAB challenged by lysozyme during Grana Parmesan cheese making indicate that the functionality of the LAB population is not impeded (Ottogalli *et al.,* 1983; Neviani *et al.,* 1996). Other work has also indicated that the presence of lysozyme does not have a negative impact on cheese making, because Grana cheeses manufactured with

the addition of lysozyme produced whey with lower titratable acidity than control cheeses but could proliferate in the presence of lysozyme (Grazia *et al.*, 1984).

The aim of this work was to investigate the effects of composite edible film containing lysozyme as gouda cheese edible coating with respect to microbial populations of cheese ripening.

Materials and Methods

Materials

The materials were gouda cheese (KPRI "Sejahtera Jaya" Malang District), hen egg white (local market) lysozyme extracts, SiO2 (PT. Panadia Corporation Indonesia), EDTA (Merck), glacial acetic acid (PT. Panadia Corporation Indonesia), whey protein concentrate (musclefeastindo), porang flour (PT. Perhutani), beeswax (local market), culture of *Salmonella typhi*, *Staphylococcus aureus*, and *E. coli* (culture collection of Microbiology Laboratory of Medical Faculty, Brawijaya University), sodium phosphate buffer (Merck) and trichloroacetic acid (TCA) (Brataco), culture Micrococcus lysodeikticus (Sigma), buffer sodium phosphate, sodium cyanoborohidrate, gouda cheese, PCA (oxoid), EMBA media (oxoid), MRS Agar (oxoid), VRBA (oxoid), SS (oxoid), Kanamycin aesculin azide agar (oxoid), BPA (oxoid). Some of the tools used in this research include glassware, analytical ballance (Ohaus BC series and Mettler Instrumente type AJ150L Switzerland), hot plate stirrer (Labinco), vortex (Vibrofix VF, Electronic), pH meter (Schoot Gerate), sentrifuge (Jovan, Japan), waterbath (Memmert Germany), incubator (Memmert Germany), oven (Memmert Germany), autoclave (Hirayama), sentrifuge (Bench top hettich centrifuge model microliter refrigerated micro centrifuge 22R), Spectrophotometer UV-2100 cole palmer.

Methods

The treatment were modified lysozyme content (control, 0,05 and 0,1%) in composite edible film and cheese ripening (1 day, 2 weeks, 4 weeks and 8 weeks) using Nested Design with Group Randomized Design. The variables were native microbial populatin in gouda cheese (Aerobic plate count, Lactid acid bacteria, Enterococcus, Coliform, yeast/mold) was analyzed according to Turkoglu et al. (2003) and artificially contaminated pathogen (*E. coli*, Salmonella, S. aureus) was analyzed according to Bellio et al. (2016).

Lysozyme Extraction

SiO2 (0.851 g) dissolved in 1 M sodium phosphate buffer, 20 ml of hen egg white was adjusted using 1 N acetic acid at pH 3, stirred for 5 min, dissolved in 60 ml of 0.5 M NH4Cl. The solution dissolved in SiO2 solution, stirred for 5 min, left over night at 4°C then stirred for 5 min, centrifuge at 6000 rpm, 4°C for 20 s (Sharegi *et al.*, 2012).

Lysozyme Modification

Lysozyme and EDTA at ratio 11.14:11.14 mg/mL prepared and homogenized for 5 min, heated at 50°C for 20 min (Apriliyani *et al.*, 2014; Susanto *et al.*, 2013).

Prepration of Composite Edible Film Whey Protein Porang Flour Containing Lysozyme

Edible film solution containing 3 g/ml of whey protein isolate and 3 g/ml of porang flour was heated at 90oC and stirred at 250 rpm for 30 min, cooled down until 30oC. The solution of modified lysozyme added to composite edible film solution (Manab, 2008).

Bacterial Species

Salmonella typhi, *Staphylococcus aureus* and *E. coli* species were used separately. Species are available as liquid culture from culture collection of microbiology laboratory, Medical Faculty, Brawijaya University.

Preparation of Inocula

Salmonella typhi, *Staphylococcus aureus* and *E. coli* was grown on nutrient broth, incubated at 37°C for 24 h. Each bacterial suspension spried on the surface of young gouda cheese after young gouda cheese coated using solution of composite edible film containing modified lysozyme.

Cheese Coating

Gouda cheese coating with composite edible film based whey protein, porang flour and beeswax containing modified cheese by immersion (during 60 s), the residual coating was allowed to drip off and cheeses were left for 2 h at 4°C allowing the coating to dry (Yildirim *et al.*, 2006).

Population of Native Microbial

Young gouda cheese immersed in the solution of composite edible film containing modified lysozyme. Gouda cheese ripened for 8 weeks at 10°C and and 85% RH. Microbial enumeration analyse at 1 day, 2 weeks, 4 weeks and 8 weeks and 8 weeks.

Population of Artificial Pathogen Contamination

Young gouda cheese immersed in the solution of composite edible film containing modified lysozyme. The batches were artificially contaminated with an inoculum of *Salmonella typhi*, *Staphylococcus aureus*, *E. coli* separately depending on the treatment by sprying microorganism suspension (106/ml) on the surface of coated young gouda cheese. For each species of the three pathogens, three different batches were prepared. Artificially contaminated gouda cheese ripened for 8 weeks at 10°C and 85% RH. Microbial enumeration analyse at 1 day, 2 weeks, 4 weeks and 8 weeks.

Data Analysis

Data obtained on the research analyzed with variety analysis (ANOVA), if there are significant effect followed by Distance Multiple Duncan Test (DMDT).

Results and Discussion

Native Microbial Population of Coated Gouda Cheese

The result showed that modified lysozyme addition in composite edible film did not gave significant effect ($P>0.05$) on aerobic plate count, lactic acid bacteria, enterococcus, coliform, but cheese ripening nested in modified lysozyme addition gave highly significant effect ($P<0.01$) on aerobic plate count, lactic acid bacteria, enterococcus and coliform. According to Table 1 all native microbial population of coated gouda cheese using composite edible film based whey protein containing modified lysozyme showed a declined trend during 8 weeks cheese ripening.

Aerobic plate count showed a decreased at 4 weeks ripening and fairly stable at 4 log cfu/gram from 4 weeks until 8 weeks. Lactic acid bacteria showed a gradually decreased from 7 log cfu/gram at the beginning of ripening until 4 log cfu/gram at 8 weeks.

Table.1 Native Microbial Population of Coated Gouda Cheese During Cheese Ripening (Log Cfu/Gram)

Lysozyme (%)	**0**				**0.5**				**1.0**			
Ripening time	**1day**	**2weeks**	**4weeks**	**8weeks**	**1day**	**2weeks**	**4weeks**	**8weeks**	**1 day**	**2weeks**	**4weeks**	**8weeks**
Aerobic Plate Count	8.9773	5.8521	4.9250	4.0161	8.7600	5.9376	6.0023	3.9872	8.7358	5.6106	4.9395	4.1848
Lactic acid bacteria	7.8411	7.3610	6.1159	4.5530	8.0217	7.2990	6.1648	5.2549	7.7699	7.4163	6.1610	4.9886
Enterococcus	7.3100	4.0005	3.4306	2.8719	7.0684	3.7493	3.5653	2.9411	6.8642	3.7469	3.4198	2.6225
Coliform	4.4529	2.7530	2.5475	2.3389	4.5571	2.9784	2.6680	2.5764	4.4923	3.0487	2.4029	2.6275
Mold/yeast	3.7957	4.3812	3.4659	2.1427	3.5500	4.2720	3.7074	3.1179	3.8376	4.4598	3.3518	3.4894

Table.2 Artificial Pathogen Contaminated Population of Coated Gouda Cheese during Cheese Ripening (Log Cfu/Gram)

Lysozyme	**0**				**0.5**				**1.0**			
Ripening	**1 day**	**2 weeks**	**4 weeks**	**8 weeks**	**1day**	**2weeks**	**4weeks**	**8weeks**	**1 day**	**2weeks**	**4weeks**	**8weeks**
E. coli	4.2648	2.8977	2.3389	2.3453	4.2891	2.6478	2.5968	2.3545	4.2913	3.1576	2.7095	2.2322
Salmonella sp	3.7214	2.5502	2.2440	2.3030	3.6192	2.4671	2.3404	2.2336	3.2581	1.9484	2.8316	3.0247
S. aureus	4.0458	4.0965	4.2455	2.1471	4.1159	4.2891	4.2432	3.5916	3.5170	4.4603	4.3745	2.6330

Enterococcus showed a declined at 2 weeks and a gradually decreased from 2 weeks until 8 weeks. Coliform showed a decreased at 2 weeks and fairly stable at 2 log cfu/gram from 2 weeks until 8 weeks. Mold/yeast count showed increased 1 log cfu/gram at 2 weeks, but decreased gradually from 4 weeks until 8 weeks cheese ripening.

Modified lysozyme in the composite edible film decreased microbial growth of native microbial population of coated gouda cheese including lactic acid bacteria, enterococcus, coliform and mold/yeast gradually. It stated these microorganism caused lipolytic and proteolytic activities (Var *et al.,* 2006) and could be used as an indicator of contamination during production and ripening of cheese that caused decrease cheese quality (Fajardo *et al.,* 2010). Decreasing growth this microorganism could be used as an indication that modified lysozyme released from coating and diffused to inner of cheese and act as inhibitory agent of these microorganism.

Incorporation of modified lysozyme on composite films could act as an additional post-processing safety measure, once the inhibitory effect on microbial growth is expected to provide protection against a broad spectrum of microorganisms (Fajardo *et al.,* 2010; Manab *et al.,* 2016). Lysozyme in active packaging mainly inhibition of the Gram-positive pathogenic bacteria (Duan *et al.,* 2007; Min *et al.,* 2005), however, when lysozyme is combined with ethylene-diaminetetraacetic acid (EDTA), the outer membranes of Gram-negative bacteria are destabilized by EDTA and show antibacterial activity against on pathogenic bacteria including *E. coli* O157:H7 and *Salmonella typhi*murium (Gucbilmez *et al.,* 2007; Mecitog˘lu *et al.,* 2006; Padgett *et al.,* 1998; Unalan et al. 2011).

Artificial Pathogen Contaminated Population of Coated Gouda Cheese

The result showed that modified lysozyme addition in composite edible film did not gave significant effect ($P>0.05$) on *E. coli*, salmonellae and S. aureus, but cheese ripening nested in modified lysozyme addition gave highly significant effect ($P<0.01$) on *E. coli* (Table 2). *E. coli* showed a decreased at 2 weeks ripening and fairly stable at 2 log cfu/gram from 2 weeks until 8 weeks. Salmonellae count showed a decreased at 2 weeks ripening and fairly stable at 2 log cfu/gram from 2 weeks until 8 weeks. *S. aureus* count showed a fairly stable at 4 log cfu/gram from the beginning until 4 weeks but decreased at 8 weeks ripening.

These results showed that edible coating containing modified lysozyme was successful in preventing the growth of artificial pathogen contaminated population of coated gouda cheese during cheese ripening especially at the beginning of cheese ripening but from 2 weeks until 8 weeks fairly stable at 2 log cfu/gram. These results indicated that at the beginning of cheese ripening, amount of lysozyme in the surface of cheese is enough to inhibit the growth of artificial pathogen contaminated, but may be after 2 weeks some lysozyme released to inner region of cheese caused inhibitory activities at cheese surface decreased. Antimicrobial packaging incorporating antimicrobials agent can retard the release of antimicrobials into the food, target mainly the food surface on which microbiological growth occurs (Appendinni *et al.,* 2002; Devlieghere *et al.,* 2004; Lopez, 2007).

It concluded that gouda cheese coated with composite edible film containing modified lysozyme decreased population of native

microbial gradually but population of artificial pathogen contamination decrease faster at the beginning cheese ripening. Antimicrobial composite edible film containing modified lysozyme effective to inhibit microorganism growth both at the surface and inside region of gouda cheese during ripening.

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