

ANTIOXIDANT ACTIVITY, AMINO ACID PROFILE AND MICROBIAL QUALITY OF *BEBONTOT*, A BALINESE TRADITIONAL FERMENTED CHICKEN MEAT PRODUCTS

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ABSTRACT

Antioxidant activity, amino acids profile and microbial quality of raw *bebontot* of chicken meat of spent laying hen were investigated. The samples were prepared using meat dices (1.5 x 2.0 cm) mixed with fresh ground coriander, garlic, galangal, white pepper, salt, sugar and coconut oil then wrapped in *Areca catechu palm* dried sheaths and finally fermented spontaneously by drying under the sun for 5 days. The results showed there was a decreasing in pH value, moisture content and 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity of final products, while its total phenolic content was increasing. Glutamic acid was the most abundant amino acid in products after 5 days fermentation, followed by tyrosine, aspartic acid, lysine, leucine and histidine. The total plate count and lactic acid bacteria counts were decreasing to 9.39 log cfu/g and 8.98 log cfu/g; the *Micrococcaceae* was decreased to 5.31 log cfu/g; the yeast and moulds counts were increased to 8.58 log cfu/g and 6.51 log cfu/g at the final stage of fermentation. It can be concluded that *bebontot* chicken meat in this study is a good source of natural phenolic antioxidant, and the present microorganisms will provide the source for the selection of strains well adapted to the environment and able to compete with contaminant bacteria.

Keywords: antioxidant activity, amino acids, microbial profile, bebontot

AKTIVITAS ANTIOKSIDAN, PROFIL ASAM AMINO DAN KUALITAS MIKROBA *BEBONTOT* DAGING AYAM PRODUK FERMENTASI TRADISIONAL BALI

ABSTRAK

Aktivitas antioksidan, profil asam amino dan kualitas mikroba *bebontot* daging ayam petelur afkir diselidiki. Sampel disiapkan dengan menggunakan daging (ukuran 1,5×2,0 cm) dicampur dengan bumbu ketumbar, bawang putih, lengkuas, lada putih, garam, gula dan minyak kelapa, kemudian dibungkus dengan pelepah pinang (*areca catechu*) kering dan difermentasi secara spontan dengan pengeringan di bawah sinar matahari selama 5 hari. Hasil penelitian menunjukkan terdapat penurunan nilai pH, kadar air dan aktivitas antioksidan produk akhir, sementara kadar fenolik total meningkat. Asam glutamat adalah asam amino yang paling banyak dalam produk setelah 5 hari fermentasi, diikuti oleh tirosin, asam aspartat, lisin, leusin dan histidin. Jumlah total mikroba dan jumlah bakteri asam laktat menurun menjadi 9,39 log cfu/g dan 8,98 log cfu/g; micrococcaceae berkurang menjadi 5,31 log cfu/g; total ragi dan kapang meningkat menjadi 8,58 log cfu/g dan 6,51 log cfu/g pada tahap akhir fermentasi. Dapat disimpulkan bahwa *bebontot* daging ayam dalam penelitian ini merupakan sumber antioksidan fenolik alami yang baik, dan keberadaan mikroorganisme dalam produk dapat digunakan sebagai sumber untuk pemilihan strain yang baik dan disesuaikan dengan lingkungan yang mampu bersaing dengan bakteri kontaminan.

Kata kunci: aktivitas antioksidan, profil asam amino, mikroba, bebontot

INTRODUCTION

Poultry meat from spent laying hen chicken is very popular in Bali as an excellent and an increasing important source of high-value animal protein. Okarini *et al.* (2013) reported that spent laying hen and Bali indigenous chicken breast fillet contain higher protein and lower fat compared to broiler breast fillet. Furthermore the essential amino acid content between Bali indigenous chicken with spent laying hen breast fillet was not significantly difference, however the antioxidant activity of broiler fillet was significantly lower compared to either spent hen and Bali indigenous chicken.

Bioactive meat protein (the skeletal muscle tissue of red meat, fish and poultry) as well as bioactive peptides are part of a balanced diets, which could also be nutritionally beneficial as a source of essential amino acids (Udenigwe and Aluko, 2012). These bioactive peptides can be released by using specific microorganisms, especially protease from LAB in fermentation food processing, and enzyme-catalyzed proteolysis *in vitro* or in the digestive tract after human consumption (Arihara, 2006), because bacterial cultivation costs are relatively low (short time of maturation). Furthermore LAB and their products are considered safe (Agvei and Danquah, 2011). Rediscovery of traditional fermented meat products as functional food is an interesting direction, because the original characteristic of raw material (meat, fat, spices and starter culture) and technology (salting, fermentation, ripening drying, fermentation and drying procedures) are changed remarkably resulting in product with improved functionality (Arihara, 2006; Zhang *et al.*, 2010). It is possible due to many proteins are hydrolyzed during fermentation and ripening and to accumulate quantities of biologically active peptides with the enhancement of the physiological activity or many peptides are multifunctional and exhibit more than one activity of the products (Arihara, 2006; Iroyukifujita *et al.*, 2000; Jahan-Mihan *et al.*, 2011; Liu *et al.*, 2010).

Fermentations as an ancient method of extending shelf-life of meat product, while fermented sausages play a major role in meat industry (Zhang *et al.*, 2010), because a significant number of biochemical, physical and microbiological reaction take place during the fermentation process (Flores *et al.*, 2004). Whilst meat processing operation could increase oxidative stress by introducing oxygen, removal of nature antioxidant, destruction of endogenous antioxidant, and increasing pro-oxidative factors (Wojciak and Dolatowski, 2012). The ability of proteins to inhibit lipid oxidation makes them an important component of the antioxidant defence of biological tissues from which foods are produced, because protein are somewhat unique in this

way compared to other food antioxidants, where they can potentially act as multifunctional antioxidants that can inhibit several different lipid oxidation pathways (Elias *et al.*, 2008).

Arihantana (1981) noted that a Balinese traditional fermented product known as *Bebontot* or *buntilan* are prepared using mixed lean and fat pork meat, chopped into 1.5-2 cm cube and mixed with fresh ground spices consist of tumeric, galangal, aromatic ginger, ginger, garlic, chillies, pepper and salt, and this mixture was then sundried. While Hartawan (2002) reported that this product was prepared traditionally in small-scale enterprises and in each region of Bali difference spices mixture used to produce *bebontot*, where it is also related to the drying environment condition. However, there are no reports on some quality traits of fermented spent laying hen meat *bebontot*, therefore the aims of this study were to measure the antioxidant activity (total phenolic content and DPPH radical scavenging activity), amino acids profile and microbial quality of *bebontot* using spent hen layer breast meat produced in Bali island.

MATERIALS AND METHODS

Bebontot or Buntilan Chicken Breast Meat Preparation

Spent laying chicken (ISA-brown) after a period of laying of 76 weeks obtained from commercial poultry farms in central region of Bali were used as raw material in *Bebontot* preparation. All chickens were slaughtered at the university farm at the same time after a fasting period for 12 h, and the skinless breast fillet (*Musculus Pectoralis Superficialis*), left and right breast muscles of each bird from those chicken were packed in polyethylene pouches and stored at 4°C for 24 h before used for *bebontot* processing. Meat chopped into 1.5-2 cm cube and mixed with fresh ground spices consist of coriander (12.5-15%), garlic (25%), galangal (35-37.5%), in the part 1:2:3, white pepper (2%), salt (8%), sugar (10%), and coconut oil (5%) on weight raw material. Then it was wrapped with *Areca catechu palm* dried sheaths and finally dried under of the sun for 5 days (the afternoon temperature was 35-39°C and 28-33°C at night).

Moisture content of *bebontot* samples was measured following method no. 950.46 AOAC, (2000). While pH of the samples were determined (method no. 981.12 AOAC., 2000; Jang *et al.*, 2008) by homogenizing 5 g of samples with 25ml of distilled water, then homogenates were filtered and furthermore the pH of samples was measured with a pH meter (Model 720 P Istek, Inc.) at room temperature.

Total Phenolic Content

Total phenolics content were determined calorimetrically using *Folin-Ciocalteu* reagent as described by Jang *et al.* (2008) with slight modifications. The *bebontot* chicken meat samples (5g) in distilled water (15 mL) was homogenized at 3000 rpm for 2 min. Chloroform (9 mL) was added to the homogenates and the mixture was shaken vigorously 2 to 3 times to separate the lipids. A 1-mL aliquot of diluted sample (1:4, vol/vol) was added to the *Folin-Ciocalteu* reagent (500 µL), followed by addition of 1 mL of sodium carbonate solution (5%). The reaction mixture was vortexed and the absorbance was measured with a spectrophotometer (SP-870 TURNER Barnstead USA) at 700 nm after incubation for 1 h at room temperature. Samples were measured in three replicates (0 day and 5 days), each replicate were of 3 different *bebontot* samples and absorbance measured in two readings. Quantification was done based on the standard curve generated with gallic acid, and the standard calibration curve of gallic acid solution (10, 20, 30 and 40 mg/L) was prepared using the similar procedure.

DPPH Radical Scavenging Activity

The Radical-Scavenging Assay: *1,1-Diphenyl-2-picrylhydrazyl* (DPPH) radical scavenging activity was estimated with the aqueous supernatant obtained from raw of *bebontot* chicken meat according to the method of Blois as described by Jang *et al.* (2008), with slight modifications. 0.1ml quantity of aqueous supernatant diluted to 5ml of water, then take into test tube 1.5 ml and was added 1.5 mL of methanolic DPPH solution (0.2 mM). The mixture was vortexed and left to stand at room temperature for 30 min. A tube containing 1.5 mL of methanol and 1.5 mL of methanolic DPPH solution (0.2 mM) served as the control. The absorbance of the solution was measured at 517 nm (SP-870 TURNER Barnstead USA). Each sample of 0 day and 5 days were prepared three replicates (each replicate was from three different *bebontot* samples) and absorbance measured in two replicate readings. The percentage of DPPH radical scavenging activity was obtained from the following equation:

Radical-scavenging activity (%) =

$$\left(1 - \frac{\text{absorbance value of testing solution}}{\text{absorbance value of control solution}}\right) \times 100$$

Microbiological Analysis

The *bebontot* or *buntilan* chicken meat samples were assayed for (1) aerobic plate counts, (2) lactic acid bacteria, (3) *Micrococcaceae*, (4) yeasts, (5) moulds and (6) coliforms. Ten grams of each sample were aseptically transferred to a sterile stomacher bag with 90 ml of saline water (Sodium chloride 0.85%). The preparation was mixed for 3 mins in a stomacher. Additional decimal dilutions were prepared, and the following analyses were

carried out: (1) aerobic plate counts on Plate Count agar incubated for 48 h at 37°C (2) LAB on de Man-Rogosa Sharpe (MRS) agar incubated for 48 h at 37°C under restricted oxygen conditions (2 Gas-Pack envelopes; BBL, Baltimore, Maryland, USA) in an anaerobic jar (3) *Micrococcaceae* on mannitol salt agar (MSA, Pronadisa, cat.1062) incubated for 48 h at 37°C (4) Yeasts and Mould on Malt Extract Agar (MEA, Pronadisa cat.1038) incubated for 48 h at 30°C for yeasts, (5) for moulds incubated to 72 h at 30°C, and (6) coliforms on Eosin Metilen Blue Agar (EMBA, Pronadisa, cat.1039) incubated for 24 h at 37 °C. After counted, means and standard deviations were calculated.

Amino acids profile

The amino acid profile of *bebontot* chicken meat samples was determined by the method of Antoine *et al.* (2001) with slightly modification. The sample obtained were 3 mg protein or 0.015 g chicken breast meat / dry weight and then 1 ml 6 N HCL was added. The mixture was purged with N₂ and then heated in the oven at 110°C for 24 hours. A sample was prepared after the 6 N HCL hydrolysis then dissolved to 5 ml 0.01 N HCL and filtered using Millipore 0.45 µm filter membrane (WHATMAN ®) diameter 25 mm, followed by adding potassium-borate buffer (pH 10.4). A 5 µL quantity of hydrolysis protein sample was added with 25 µL of OPA reagent and then injected after 1 minute of derivatization.

Mobile phase A was made up of 0.025M sodium acetate buffer (pH 6.5) 0.5 g Na EDTA, 90 ml methanol and 10 ml THF (80:10:9:1) prepared from analytical grade dissolving to 1 L water Hi Pure. The pH of the acetate buffer (A buffer) was adjusted to 6.5 using NaOH solution, and mobile phase B buffer contain 95% methanol on Hi Pure water. The mobile phases were ultrafiltered through Millipore filter having a pore diameter of 0.45µm (WHATMAN ® diameter 25 mm) and degassed by sparging for 5 minutes with pure nitrogen. Gradient elution was generated using solvent delivery module Varian Pro Star Model Number 240 (Chromatography Systems, Walnut Creek, CA 94598 USA made in USA), was used for controlling the gradient and flow rate (1.0 ml/min) of the mobile phases.

OPA-thiol reagent was made up at least 24 hours before used by dissolving 50 mg o-phthalaldehyde in 4 ml methanol, and 0.025 ml mercapthoethanol was added. The mixture was thoroughly mixed, then 0.050 ml Brij-30 and 1 ml borax buffer solution were added. The OPA-thiol reagent stored in the dark bottle or in a tightly closed container at 4°C.

The amino acid profile of *bebontot* chicken meat were analyzed by cation exchange ICI Instrument High Performance Liquid Chromatography (HPLC) with column Ultra Techsphere ODS 3µ particle size, 4,6 mm x

7.5 cm (PARKER 316 SGE PTY. LTD Victoria Australia) and O-phthaldialdehyde (OPA) precolumn derivatisation. The amino acid standard H (L-Alanine, L-Arginine, L-Aspartic Acid, L-Glutamic Acid, Gycine, L-Histidine, L-Isoleucine, L-Leucine, L-Lysine, L-Methionine, L-Phenylalanine, L-Serine, L-Threonine, L-Tyrosine and L-Valine (PIERCE, Rockford Illinois 61105, USA) were used.

Amino acids were analyzed in three replicates (0, 1, 3 day and 5 days), each replicate was obtained from 3 different *bebontot* samples. The amino acid composition was expressed as mg of amino acid per 100g (%) of raw *bebontot*.

Statistical analysis

The experiments were carried out using completely randomized design with three replications, and the data obtained were analysed by using two-way analysis of variance (ANOVA) and Pearson Correlation. The differences among means were determined by Duncan Multiple Range test using SPSS version 13.0 (Chicago, Illinois, USA) at a level of significance of $p < 0.05$ (Pramesti, 2005). Data were presented as means \pm standard deviation and each analysis of *bebontot* product were replicated for total phenol content and DPPH radical scavenging activity ($n=3$), amino acids content ($n=3$) and for microbiological count ($n=3$) to each drying or fermentation.

RESULTS AND DISCUSSIONS

Results of the physico-chemical components of the *bebontot* or *buntilan* are shown in Table 1. The pH of *bebontot* was decreased within traditional fermentation cycle (drying under sun) from initial value of pH around 6.2 to 4.6 in final fermentation (Table 1), and it was lower of Mhom, a Thai traditional meat sausages (Samappito *et al.*, 2011); of Turkish sucuk, a Turkey traditional fermented sausages (Siriken *et al.*, 2009); and of naturally fermented Italian sausages produced in the North East of Italy (Cocolin *et al.*, 2001; Comi *et al.*, 2005) and of Vallo di Diano, a Southern Italy of traditional fermented sausages (Casaburi *et al.*, 2007), and it was higher of Androla, a Spanish traditional dry-

cured sausages (Lorenzo *et al.*, 2000). However, similar pH have been described for Turkish *sucuks* (Erkmen and Bozkurt, 2004); Northern Europe type sausages (Zanardi *et al.*, 2004); Nham, a Thai fermented pork sausage (Visessanguan *et al.*, 2006) and also of fermented sausages (21 days) as reported by Aro Aro *et al.* (2010). The decrease of pH in fermented meat products due to the decrease in the water-holding capacity of the meat proteins when pH approaches the isoelectric value. This condition not only improves its preservation, but also the drying process can bringing to extend the storage life to the high fermentation temperature. Especially in the afternoon 35-39°C with relative humidity (RH) 65-70%; and 28-33°C with RH 55-60% in the night time. In the present study a significant positive correlation between pH value with LAB ($R^2=0.609$) and total aerobic count ($R^2=0.622$) ($p < 0.05$); and between pH value with *Micrococcaceae* count ($R^2=0.935$), moisture content ($R^2=0.849$), total phenolic content ($R^2=0.996$) and radical DPPH scavenging activity ($R^2=0.987$) ($p < 0.01$) were observed.

Moisture content of *bebontot* chicken meat decreased continuously during fermentation from 61.5% to 42.4% (Table 1). The results indicated that higher fermentation temperature caused faster dehydration and it is probably due to muscle proteins denaturation during fermentation. Siriken *et al.* (2009) reported that moisture content of Turkish sucuk sausage were between 30-47% with average 40.9%; and for Spanish sausage were in the range of 18.2 – 62.2% (Lorenzo *et al.*, 2000) and 40-46% for Italian sausage (Comi *et al.*, 2005). In the present study a significant positive correlation between moisture content with LAB ($R^2=0.771$), total plate count (TPC) ($R^2=0.754$), *Micrococcaceae* ($R^2=0.965$), coliform ($R^2=0.723$) and pH value ($R^2=0.849$) ($p < 0.01$) were also observed.

The rapid decrease in pH and moisture content were also directly related proporsionally to the increase of fermentation temperature and the higher growth rate of LAB. On other the hand Leroy *et al.* (2006) reported when glucose concentration is above a certain level, increased amounts of fermentable sugar which derived from the dissimilation of endogenous glucose in the batter primarily influence acidification rate, the final

Table 1. The pH, moisture content, total phenolic content and radical scavenging activity of *bebontot* chicken meat

Time of fermentation (hours)	pH*	Moisture (%)*	Total phenolic* (GAE=mg/kg)	DPPH RSA (%)*
0	6.200 \pm 0.006 ^a	61.4971 \pm 0.014 ^a	25.269 \pm 0.533 ^c	52.111 \pm 0.180 ^a
24	4.930 \pm 0.008 ^b	56.3944 \pm 0.064 ^b	22.283 \pm 0.533 ^d	31.626 \pm 0.090 ^d
72	4.670 \pm 0.004 ^c	45.8820 \pm 0.064 ^c	32.363 \pm 1.067 ^b	35.580 \pm 0.090 ^c
120	4.640 \pm 0.006 ^d	42.3965 \pm 0.041 ^d	45.429 \pm 0.533 ^a	38.140 \pm 0.045 ^b

* a-d, Mean \pm Standard deviation ($n=3$) values with different superscripts within same column differ significantly ($P < 0.05$), the results are expressed on fresh matter to each time of fermentation or drying.

pH but neither the lag phase nor the rate of lactic acid formation. However the extent of pH drop also depends on the specific LAB, the manufacture technology such as temperature, raw materials, ripening time and the water activity of the drying sausage (Lorenzo *et al.*, 2000; Cocolin *et al.*, 2001; Comi *et al.*, 2005; Casaburi *et al.*, 2007; Lebert *et al.*, 2007). In addition not only sugar added on formulation cause pH fall, but some herbs and spices (antioxidant and antimicrobial effect) could be used by indigenous LAB to help to rise an acidifying of meat products during fermentation (Hagen *et al.*, 2000; Garcia, *et al.* 2001; Barriere *et al.*, 2001). Hagen *et al.* (2000) reported that some herbs and spices have been found to stimulate the growth of LAB and thereby the rate of lactic acid formation to varying degrees dependent on the concentrations especially the content of manganese ions in the herbs or spices, and the bacterial strain.

Antioxidant potential of bebontot spent laying chicken breast meat.

The total phenolic content of *bebontot* products (Table 1) was lower than origin spent laying hen breast fillet as reported by Okarini *et al.* (2013), and increase continuously after 24 h until the end (5 days) of fermentation process. In the present study positive correlation between total phenolic content and total yeast ($R^2=0.760$), mould count ($R^2=0.917$) ($p<0.01$) toward amount (12) of amino acids (one letter) as DESHGTYVILK ($p<0.01$) and F ($p<0.05$) include essential amino acid (TVFILK), except RAM amino acids were observed. The high total phenolic content of *bebontot* products compared to fresh spent breast meat fillet, was possibly due to a number of spices and herbs were added to the formulation (coriander: garlic: galangal = 1: 2: 3). According to Mahae and Chaiseri (2009) and Neveu *et al.* (2010) the highest total polyphenols content were in dried coriander (2260.0 mg/100g fresh weight) and white pepper (780.0 mg/100g fresh weight); while total phenolics content of galangal in water extract was 8.25 mg GAE/g extract and its essential oil were 5.01 mg GAE/g extract, it also contained catechin 0.73 mg/g extract and myricetin 14.60 mg/g extract. These compounds could interact with essential oil (volatile phenolic compounds) of herbs and spices together with protein of meat and some compounds of dried sheaths as wrapping material. A dried *Arecha cathecu palm* sheaths and their nuts contained arecoline, arecaidine, arecolidine, guvacine, choline, about 15% red tannin, about 14% fat (Anon, 1960), therefore this palm sheaths could be used as bioactive packaging.

The high drying or fermentation temperature, released the water content and decrease the pH value, and this condition could promote interaction or is delivery to

enzymatic activity between essential oil (volatile phenolics compound) of spices mix and meat proteins which could blocked of the hydrophilic amino groups and stimulate yeasts and moulds to grow. Kroll and Rawel (2001) noted that the reaction of myoglobin with the derivative substances is accompanied by a corresponding blocking of the hydrophilic amino groups. It indicated that enzymatic reaction activity of raw material, endogenous and microorganisms enzymes at high temperature, because the optimal temperature for enzymatic hydrolysis process at 35-40°C. On the other hand the used of garlic, which according to Neveu *et al.* (2010) contained manganese (Mn) about 1.67 mg/100g; vitamin C about 8.21 mg/100g fresh weight, added during preparation of *bebontot* could stimulate acid production. Hagen *et al.* (2000) reported that herbs and spices contain especially manganese ions, and is the primary factor to stimulate acid production to varying degrees depending on the concentration and present natural microorganisms strains. In the presence of micronutrients, especially manganese of herbs and spices may have a positive influence on the metabolism of natural microorganisms during fermentation, especially the growth of LAB and *Micrococcaceae* and thereby the rate of lactic acid fermentation to supply nutrients for stimulate the growth of yeasts and moulds. Bertonecelj *et al.* (2007); Decker (2009) and Dimitrios (2006) reported that polyphenolic compounds play a wide range of biological effects including antibacterial, anticarcinogenic, inhibition of atherosclerosis, antiviral, antithrombotic and these functions have been attributed to their antioxidant activity.

The radical scavenging activity of DPPH of *bebontot* is presented in Table 1. The initial value was 52.11% of the batter (before wrapping) and decrease first day fermentation to 31.63% then increasing until final stage of fermentation (5 days) were 38.14%, and it is higher than origin spent chicken breast meat fillet (20.77%). In the present study a significant positive correlation between radical scavenging activity of DPPH and pH value ($R^2=0.987$) ($p<0.01$), and also of the *Micrococcaceae* count ($R^2=0.681$) ($p<0.05$). It was probably due to the higher of essential oil as volatile phenolic compounds of herbs and spices which added during preparation of *bebontot* as described above causes protein oxidation due to high fermentation temperature at the beginning of the fermentation cycle, and increased proteolysis activity, and it is rapidly decreased pH value and water content. Santé-Lhoutellier *et al.* (2007) reported that the formation of aggregates changing both chemical and physical recognition sites showed that proteolysis susceptibility of protein increases at low oxidant level, in turn at a high oxidant level, and a decrease of proteolysis rate. The use of spices is very frequent in the case of

sausages, which addition of spices not only contribute to color and flavor development, but also become a source of external contamination and antioxidant effect, and in some cases they have an inhibitory activity on microbial growth (Garcia, *et al.* 2001), in the present study coliform count was decreased. The reduction of pH value could increased radical scavenging activity of DPPH and decreased *Micrococcaceae* count of *bebontot* at the final fermentation stage (5 days).

The *bebontot* products in this study showed a higher radical scavenging activity of DPPH of isolate LAB which were dominated by *Lactobacillus plantarum* and *Pediococcus pentosaceus* than of isolate LAB from the batter which were dominated by *Pediococcus acidilactisi*. The possible reason was the present of micronutrients, especially manganese of herbs and spices may have a positive influence on the metabolism of natural microorganisms during fermentation, especially the growth of LAB and *Micrococcaceae* (Igarashi *et al.*, 1996; Hagen *et al.*, 2000; Barriere *et al.*, 2001; Garcia *et al.*, 2001; Dimitrios, 2006; Martinez *et al.*, 2006). Although *Micrococcaceae* are frequently mentioned as component of meat starter cultures as an important catalytic function that contributes to color stability and somehow prevention of lipid oxidation, this term generally refers to members of *Staphylococcus* genus, where the characterization of catalase and superoxide dismutase (SOD) in *Staphylococcus carnosus* and *Staphylococcus xylosus* have been reported (Barriere *et al.*, 2001). Two groups of LAB which were a heme-dependent catalase and nonheme Mn-containing catalase (Mn-dependent SOD) as reported by Igarashi *et al.* (1996). These probably due to that LAB isolates of *bebontot* contained the both of a heme-dependent catalase and nonheme Mn-containing catalase (Mn-dependent SOD) from spices was added. Furthermore, Neveu *et al.* (2010) noted that addition of garlic which contain manganese (Mn) about 1.67mg/100g fresh weight; iron (Fe) about 1.70 mg/100 fresh weight and coriander which also contain manganese (Mn) about 16.3 mg/100g fresh weight could effected the antioxidant activity of food. Frankel and Meyer (2000) also reported that some authors reported that antioxidant action becomes more complicated in food systems and the various mechanisms including free radical chain breaking, oxygen scavenging, metal chelating, and inhibition of oxidative enzymes, are effective. However, hydrophilic antioxidant are more active in bulk oil systems (to the case of *bebontot* product) but lipophilic antioxidant are more active in lipid suspended in aqueous systems like an oil-in-water emulsion (Frankel and Meyer, 2000), whilst Kim *et al.* (2007) reported several amino acids could have a function as antioxidant precursor.

Amino acid content of bebontot spent laying chicken breast meat

Amino acids content in present study significantly increased during fermentation of *bebontot*, except arginine and alanine (Table 2). It is interesting to note that on final fermentation of *bebontot* were significant highest amount of glutamic acid and tyrosine while aspartic acid and lysine were observed higher at 0 day (in the batter). It was similar to the results reported by Aro Aro *et al.* (2010) on 3 days fermentation of sausages without starter culture. The other authors were also suggested the similar increased amount of amino acids (glutamic acid, lysine, histidine, leucine, threonine, isoleucine, phenylalanine) of dry fermented sausages with used of commercial proteases (pronase E from *Streptomyces griseus*, aspartyl proteinase from *Aspergillus oryzae*) at 5 days ripening (Diaz *et al.*, 1997; Bruna *et al.*, 2000). While glutamic acid, tyrosine, aspartic acid, lysine, leucine, histidine, threonine, isoleucine, phenylalanine, valine, serine, and glycine were also observed higher concentration in the final (5 days) fermentation compared to the batter (before wrapping, 0 day). In the present study predominance of glutamic acid, and a similar pattern was also reported by other authors in different fermented sausages (Diaz *et al.*, 1997; Bruna *et al.*, 2000). This condition had been attributed to high fermentation temperature applied during drying process indicating that the highest enzymatic activity of the raw materials (spent chicken meat, concentration of herbs and spices and the wrapping materials of the batter), rather than from microorganisms as described by Lücke, (2000). Several authors have reported a major protein breakdown during fermentation of sausages resulting or release of free amino acids by muscle endopeptidases (cathepsins B, D) and microbial (*Micrococcaceae*, yeast and mould) proteinases, bound either to the cell wall or to the cell membrane (Lücke, 2000; Hughes *et al.* 2002; Candogan *et al.*, 2009)). It is known that such activity in muscle show considerable variation, related to anatomical location, gender, genetic, animal age, and postmortem rate of pH drop (Toldrá and Flores, 2000; Claeys *et al.*, 2000).

In the case of arginine, it is high concentration (3.16%) of spent laying hen breast fillet and significant lower than of Bali indigenous chicken (Okarini *et al.*, 2013), but after fermented product, *bebontot* used meat of spent hen, arginine content is low (Table 2), and it is probably due to arginine conversion to ornithine via arginine deiminase (ADI) pathway and supports the growth of lactobacilli in the latter stage of meat fermentation or were transformed to other compounds by action of the mould and yeast dependent of strains. A similar research results were reported by Sanz and

Table 2. Amino acids profile of *bebontot* chicken meat

Amino Acids*	Time fermentation of bebontot			
	0 (hrs)	24 (hrs)	72 (hrs)	120 (hrs)
Aspartic Acid	2.26 ± 0.20 ^b	2.02 ± 0.51 ^{bc}	3.00 ± 0.11 ^{ac}	3.34 ± 0.04 ^a
Glutamic Acid	5.19 ± 0.32 ^b	3.87 ± 0.04 ^b	6.33 ± 0.08 ^{ab}	7.21 ± 0.17 ^a
Serine	0.92 ± 0.03 ^b	1.06 ± 0.15 ^a	1.12 ± 0.02 ^a	1.25 ± 0.04 ^a
Histidine	1.29 ± 0.07 ^b	1.54 ± 0.05 ^{ab}	1.85 ± 0.07 ^a	2.02 ± 0.10 ^a
Glycine	0.81 ± 0.07 ^c	0.88 ± 0.00 ^{bc}	1.07 ± 0.02 ^{ab}	1.20 ± 0.08 ^a
Threonine	1.24 ± 0.14 ^c	1.28 ± 0.04 ^{bc}	1.66 ± 0.04 ^{ab}	1.83 ± 0.06 ^a
Arginine	0.93 ± 0.03 ^b	1.80 ± 0.69 ^{ac}	1.13 ± 0.00 ^{bc}	1.19 ± 0.02 ^{bc}
Alanine	1.23 ± 0.07 ^a	0.72 ± 0.40 ^a	0.97 ± 0.24 ^a	0.90 ± 0.05 ^a
Tyrosine	1.97 ± 0.23 ^d	2.75 ± 0.12 ^c	3.91 ± 0.73 ^{bc}	5.02 ± 0.35 ^a
Methionine	0.65 ± 0.01 ^b	1.06 ± 0.44 ^a	0.76 ± 0.05 ^{ab}	0.93 ± 0.03 ^a
Valine	1.07 ± 0.08 ^b	1.20 ± 0.04 ^a	1.27 ± 0.00 ^a	1.48 ± 0.05 ^a
Phenylalanine	1.11 ± 0.07 ^b	1.49 ± 0.35 ^{ab}	1.39 ± 0.04 ^{ab}	1.61 ± 0.08 ^a
Isoleucine	1.21 ± 0.11 ^b	1.37 ± 0.03 ^a	1.53 ± 0.01 ^a	1.77 ± 0.04 ^a
Leucine	1.73 ± 0.16 ^c	1.70 ± 0.27 ^{cb}	2.12 ± 0.03 ^{ab}	2.48 ± 0.09 ^a
Lysine	1.52 ± 0.06 ^c	1.74 ± 0.18 ^{cb}	1.98 ± 0.12 ^{ab}	2.77 ± 0.23 ^a
Total	23.14 ± 1.65	24.48 ± 3.31	30.10 ± 1.56	35.00 ± 1.43

* a-c, Mean ± Standard deviation (n = 3) values with different superscripts with in same row differ significantly (P<0.05). The results are expressed as mg/100g of fresh matter to each time of fermentation or drying.

Toldrá (2002); Chaillou *et al.* (2005); Hüfner and Hertel (2008) whom had characterised arginine-specific aminopeptidase activity in *Lactobacillus sakei* during sausage fermentation, which is important to release of amino acids for used of energy production, when the environmental and ecological to stress condition where sugar concentration declines. A similar results was also reported by Diaz *et al.* (1997) and Bruna *et al.* (2000).

In the present study significant positive correlation between 12 amino acids and total phenolic content was observed, and this is possibly due to the presence of herbs (galangal, garlic) and spices (coriander seed, white pepper) with their essential oils (volatile phenolic compounds) on the recipe formulation and material wrapping (*Areca cathecu* sheets) of *bebontot*. Therefore it could be considered as sources of natural phenolic antioxidants. It probably as described by Elias *et al.* (2008) that many amino acids with antioxidant potential can be buried within the protein core where they are inaccessible to prooxidants. Whilst Kroll and Rawel (2001) reported that reactivity of the phenolic compounds is influenced by the number and the position of the hydroxyl group, where free amino group of proteins were blocked, and the reaction is very pH-dependent conditions. The colour stability of *bebontot* (at final pH 4.64), was probably as a result of interaction of phenolic promotion with blocking of the hydrophilic amino groups. Kroll and Rawel (2001) noted that the reaction of myoglobin with the derivative substances is accompanied by a corresponding blocking of the hydrophilic amino groups. Anyway, both amino acid fermentation and antioxidant characteristic are probably more important as selective criteria for flavor-

enhancing starter bacteria than their lipolytic and proteolyses properties (Lücke, 2000).

Beneficial of amino acids of *bebontot* as antioxidant in the present study was showed to the increase amount of glutamic acid until 60% and tyrosine 6.5 folds higher, where both have the function as neurotransmitter and free radical scavenging. However the others amino acids amount also increased until final fermentation e.g. aspartic acid (67%), serine (36%), glycine (58%), methionine (82%), isoleucine (54%), histidine (60%) compared to the original spent laying breast meat fillet. In the present study 30% of total hydrophilic amino acids were increased at 5 days of the fermentation. Especially the highest tyrosine (*alpha-amino-p-hydroxyhydrocinnamic acid*) amount in *bebontot* chicken meat in this study (Table 2) was probably due to high galangal concentration on spices and herb mixture. Mahae and Chaiseri (2009) was also noted that there was high amount of similar compound (*p-hydroxy cinnamaldehyde*) found in the chloroform extract of galangal rhizome.

Microbiological quality of bebontot spent laying chicken breast meat

The microorganisms count of *bebontot* is presented in Table 3, and the initial total plate count or total aerobic mesophilic flora showed were 9.55 log cfu/g (before wrapping) and a slight decrease to 9.39 log cfu/g at the end of fermentation, it is higher of the results as reported by Cocolin *et al.* (2001); Comi *et al.* (2005); Aro Aro *et al.* (2010) and Samappito *et al.* (2011). These differences were probably due to the variations in type and amount of raw material, fermentation and drying condition lead to an extended diversity concerning

the dominating microorganisms, given rise to a wide range of products with unique sensorial traits. Lebert *et al.* (2007) reported that type of microorganism development in traditional meat products is related to the diversity in formulation, and to the fermentation and ripening practices, it could be very different in terms of raw material, temperature, duration and relative humidity. In the present study positive correlation between total plate count and decrease of moisture content ($R^2=0.754$) ($p<0.01$), pH ($R^2=0.622$), coliform ($R^2=0.617$) and *Micrococcaceae* count ($R^2=0.629$) ($p<0.05$) during fermentation was also observed.

The average LAB counts were 9.56 log cfu/g (before wrapping), and then a slight decrease to 8.98 log cfu/g, respectively on 5 days fermentation (Table 3). This result was quite similar to those reported by Aro Aro *et al.* (2010) and Cocolin *et al.* (2001); and higher than the one reported by Comi *et al.* (2005) and Samappito *et al.* (2011) at the final fermentation stage of their meat product samples. The LAB constitute the major or dominant microorganisms at the end of the fermentation or ripening stage, and it is well known that high acidification rates are usually accompanied by fast LAB growth rates in the fermented sausages (Lebert *et al.*, 2007). However, increased lactic acid production by LAB in the presence of glucose has been noticed in spite of an unchanged specific growth rate, whilst the additional energy obtained from direct fermentation of glucose is used for functions other than growth (Leroy *et al.*, 2006). A significant positive correlation between pH value with LAB ($R^2=0.609$) ($p<0.05$) was observed in present study and it was in agreement with the one described by Cocolin *et al.* (2001).

The initial amount of *Micrococcaceae* on *bebontot* chicken meat were 6.43 log cfu/g and decreased after 5 days to 5.31 log cfu/g (Table 3). This result was similar to the one at the end of seasoning of natural fermented Italian sausages reported by Comi *et al.* (2005) and Cocolin *et al.* (2001) and slightly higher compared to the one as reported by Samappito *et al.* (2011). These differences probably due to the growth of *Micrococcaceae* appeared to be significantly affected by acidification of *bebontot* or by the inability to compete with the natural flora. The *Micrococcaceae* strains produce proteases which is able to induce meat protein degradation resulting in the release of peptides and amino acids, and neutralize the organic acids produced by LAB in first stages of the fermentation (Cocolin *et al.*, 2001). A positive correlation between *Micrococcaceae* count and pH ($R^2=0.935$) and also between *Micrococcaceae* count and moisture content ($R^2=0.965$) ($p<0.01$) were also observed in this study. This was in agreement with Casaburi *et al.* (2007) results who considered acidification, higher

temperature and anaerobic condition to be the main cause of *Micrococcaceae* inhibition in dry fermented sausages.

The highest increase of yeast count of *bebontot* was observed at initial stage i.e. 6.80 log cfu/g and increased to 8.58 log cfu/g at the final stage of fermentation (Table 3). These results were slightly higher than those reported on Spanish fermented sausages (Encinas *et al.*, 2000); naturally fermented sausages produced in the North East of Italy (Comi *et al.*, 2005); and of Mhom, a Thai traditional meat sausages (Samappito *et al.*, 2011). The increase of yeasts was probably due to raw material on formulation of *bebontot* and also to the decreased of moisture content by the effect of high fermentation temperature and diameter of products. Encinas *et al.* (2000) also noted that high temperature during fermentation and size of samples could affected its moisture content. According to Bruna *et al.* (2000) and Bruna *et al.* (2003) the most influential variables were proteinase, aminopeptidase and a carboxipeptidase. In this study a significant positive correlation between yeast count and total phenolic ($R^2=0.760$, $p<0.01$) and also lysine content ($R^2=0.688$, $p<0.05$) were also observed. Kim *et al.* (2007) reported that function of lysine was important to transport of long-chain fatty acids into mitochondria for oxidation and storage of energy as acetylcarnitine.

The initial amount of moulds in *bebontot* was 2.48 log cfu/g and at final stage of fermentation was 6.51 log cfu/g (Table 3), and this result was similar to the one reported by Comi *et al.* (2005) at 0 day, but of variation as described by Samappito *et al.* (2011) at maximum levels of about 6.86 log cfu/g. In this study a significant positive correlation between moulds count and total phenolic content ($R^2=0.917$) ($p<0.01$) toward amount (12) of amino acids (one letter) as DESHGTYVILK ($p<0.01$) and F ($p<0.05$) include essential amino acid (TVFILK), except RAM amino acids were observed. The results indicated that increase of moulds count also followed by an increase of amino acids and total phenolic content at the final fermentation stage of *bebontot* products. Bruna *et al.* (2003) also observed a similar pattern on dry fermented sausages. The growth of yeasts and moulds in *bebontot* during spontaneous fermentation was probably due to an increased of metabolic activity of natural microorganisms present in raw material at low pH, and the high temperature of drying (on local climate conditions) as a consequence of higher availability of amino acids released by the action of enzymatic of wild strains of yeast and mould.

In the present study a significant positive correlation between decrease on coliform count (Table 3) at final fermentation (5.2 to 4.6 log cfu/g) and moisture content ($R^2=0.723$, $p<0.01$) was observed. Whilst lower of

Table 3. Microbial profile of *bebontot* chicken meat

Microbial profile*	Time of fermentation (hours)			
	0	24	72	120
LAB (log cfu/g)	9.56 ± 0.19 ^a	9.43 ± 0.02 ^a	9.41 ± 0.02 ^a	8.98 ± 0.01 ^b
TPC (log cfu/g)	9.55 ± 0.02 ^a	9.54 ± 0.02 ^a	9.18 ± 0.02 ^c	9.39 ± 0.02 ^b
Coliform (log cfu/g)	5.20 ± 0.02 ^b	5.85 ± 0.02 ^a	4.88 ± 0.02 ^c	4.62 ± 0.02 ^d
Micrococcaceae (log cfu/g)	6.43 ± 0.02 ^a	5.89 ± 0.02 ^b	5.59 ± 0.02 ^c	5.31 ± 0.02 ^d
Yeast (log cfu/g)	6.80 ± 0.02 ^b	6.64 ± 0.02 ^c	6.03 ± 0.01 ^d	8.58 ± 0.02 ^a
Mould (log cfu/g)	2.48 ± 0.02 ^c	2.30 ± 0.02 ^d	5.73 ± 0.02 ^b	6.51 ± 0.01 ^a

* a-d, Mean ± Standard deviation (n = 3) values with different superscripts with in same row differ significantly (P<0.05), the results are expressed on fresh matter to each time of fermentation or drying.

moisture content were also affected by lower of pH and higher of total phenolic content during drying or fermentation, and it is probably due to the high temperature of drying and addition of some herbs and spices mix on the formulation. Garcia *et al.* (2001) and Martinez *et al.* (2006) noted that herbs and spices could be a source of external contamination and antioxidant effect, and in some cases they have an inhibitory activity on microorganisms growth. The inhibition activity against microorganisms of herbs and spices during *bebontot* preparation could be the cause of reduction of coliform count. The coliform count in this study was slightly lower than the one reported by Comi *et al.* (2005) on the present of fecal enterococci in natural fermented sausages.

CONCLUSIONS

The natural fermentation of *bebontot* or *buntilan* of chicken breast meat from spent laying hen under the sun during for 5 days, suggested that bioactive components were assessed by increase of the total phenolic content, DPPH radical scavenging activity, total hydrophilic amino acids, and essential amino acids was related with the increase of LAB, TPC, yeasts, moulds and decrease of *Micrococcaceae*, coliform count, pH and moisture content. The highest content of glutamic acid, tyrosine, aspartic acid and lysine during fermentation of *bebontot* chicken meat of spent laying hen may account for its higher effectiveness as an antioxidant compare to the batter (before wrapping). This product is a good as source of natural phenolic antioxidant, they have nutritional importance and/or the potential for application in the promotion of health and prevention against damages caused by radicals. The present microorganisms of *bebontot* chicken meat in this study will provide the source for the selection of strains well adapted to the environment and able to compete with contaminant bacteria.

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