






Research Article

Influence of Fermentation Time on the Nutritional and Antioxidant Properties of Black Garlic

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Abstract: Black garlic (BG) is a nutritive food produced by subjecting fresh garlic (FG) to controlled thermal processing and humidity conditions for at least 4 weeks. To date, the effect of the fermentation period on the nutritional values of black garlic remains vague in Brunei Darussalam. Therefore, this study aimed to evaluate the nutritional compositions of BG fermented for 4, 6 and 8 weeks at 65 °C and relative humidity of around 70%. The salt, sugar, alcohol, protein, lipid content and antioxidant activity of BG were examined and compared with FG. The study showed that different fermentation periods demonstrated a significant effect ($p < 0.05$) on the salt, sugar, protein and lipid content of the garlic samples. No alcohol content was detected in all garlic samples. The present study also revealed that BG exhibited higher antioxidant properties, about 5-7 times higher as compared to FG. Our study indicated that the best treatment is black garlic fermented for 4 weeks (BG4) owing to its high protein content and antioxidant properties. Overall, BG is a promising high-value product that can be exploited by the food or nutraceutical industries.

Keywords: Black garlic, antioxidant, fermentation, functional food

1. Introduction

Spices, derived from various plant sources, are the essential building units of flavour in cuisines worldwide. The term 'spice' originates from the Latin word 'species', meaning specific kind. Spices have been well-known for their aromatic, pungent, and diverse beneficial properties. Their widespread use is attributed to their ability to stimulate appetite, enhance flavours, add texture, and create visual appeal in dishes [1-2]. Spices can be sourced from a variety of plant parts including seed (coriander), berry (black pepper), bark (cinnamon), stalk (lemongrass), rhizome (ginger), flower (saffron), bulb (garlic), and other sources. These spices can be used fresh, dried, or frozen, offering versatility in culinary applications [3]. In addition to its culinary uses, most spices are also commonly utilised in traditional medicines. For instance, black peppers (*Piper nigrum* Linn.) spice is used to treat insect bites, snake bites, and sunburn while cinnamon (*Cinnamomum verum*) spice has been known to alleviate headaches and neuralgia. Garlic (*Allium sativum* L.) is one of the oldest dietary vegetables with well-documented health benefits including its fibrinolytic capabilities, antimicrobial properties, immuno-regulatory activity, and its potential to lower blood cholesterol levels

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due to the presence of organosulfur compounds and bioactive enzymes [4-6]. Moreover, garlic has also been utilised as traditional remedy in the Middle East for local pain, where crushed garlic bulbs are applied to the affected site and secured with a bandage. This practice is widely employed by naturopathic physicians worldwide and as part of conventional Arabic medicine [7].

Despite its numerous health benefits, fresh white garlic has a strong pungent odor and intense flavour, which can be unpleasant for consumers. Besides, garlic may contain fructan, a compound that can irritate the digestive tract and potentially harm the stomach lining, causing stomach discomfort [8]. To address this issue, black garlic (BG), a processed garlic product, has been developed. BG can be produced using various processing methods, for instance aging, heat treatment, and fermentation. Typically, fermentation is carried out for 4 weeks at temperatures of 60-70 °C and relative humidity levels of 70-90%. The process duration can vary depending on the desired flavour and texture. This process helps mitigate the undesired aroma while enhancing palatability and improving its beneficial functionalities [9]. BG is characterised by its sweet-sour taste, coupled with a jelly-like texture. It contains various bioactive compounds such as phenols, flavonoids, pyruvate, thiosulfate, S-allylcysteine, and S-allylmercaptocysteine [10-11]. Previous study clearly demonstrated that BG exhibited stronger antioxidant properties and better efficacy in preventing metabolic diseases and alcoholic hepatotoxicity [4-5, 12-13].

Due to the increasing demand for BG products, extensive research has been conducted to enhance the nutritional and sensory properties of BG through various processing methods. Pakakaew and colleagues found that pretreating fresh garlic with a 2% calcium chloride solution and freezing before fermentation reduced processing time while preserving high levels of S-allylcysteine and antioxidant properties in BG products [14]. In addition, another study demonstrated that the application of high-pressure treatment enhanced the destruction of cellular structures and promoted enzymatic reactions in BG, leading to shortened production duration while enhancing the nutritional and sensory characteristics of BG [15]. Besides, a recent study also revealed that BG fermented using lactic acid bacteria (*Lactiplantibacillus plantarum* X7021, X7022 and *Limosilactobacillus fermentum* S1L23) led to the higher S-allylcysteine content, ACE inhibitory activity, antioxidant activity, and antagonistic activity against *Helicobacter pylori* of the BG [16]. Nevertheless, one aspect that remains relatively unexplored is the effect of fermentation time on the nutritional values and antioxidant properties of BG. Therefore, this study aimed to convert raw fresh garlic (FG) into BG using a home-based fermentation method in a rice cooker with different fermentation intervals (4, 6, and 8 weeks) [17-18]. The chemical compositions namely protein, fat, sugar, salt, and alcohol content of the BG and FG samples were determined and compared. Furthermore, the antioxidant activity of BG was also assessed across various fermentation durations in the present study.

2. Materials and methods

2.1 Materials

Fresh white garlic used in this experiment was purchased from a vegetable market in Brunei Darussalam. Physical examination of the raw material was carried out to ensure they were dust-free, disease-free and damage-free. The samples were then stored in a cool, dry location and utilized within 24 h. The chemicals used in the present study were of analytical grade.

2.2 Preparation of black garlic (BG)

About 0.5 kg of clean, fresh, unpeeled, and dry white garlic was first wrapped with aluminium foil to prevent moisture loss during fermentation. The wrapped garlic was placed in a rice cooker (85 cm diameter × 21.6 cm height) with an aluminium tray to avoid direct contact between the hot pot and the wrapped garlic. The fermentation was carried out at different fermentation periods (4, 6, and 8 weeks) at 65 °C which were referred to as BG4, BG6 and BG8, respectively. The fermentation periods were selected based on the common practices in the industry. At the end of fermentation, the BG was let to cool and then they were sliced into the appropriate thickness and oven-dried overnight at 50 °C until a constant weight was achieved. Then, the dried slices were ground, and the BG powder was produced. The samples were stored at -4 °C prior to analysis.

2.3 Extraction of BG

To prepare the BG extracts, 20 g of BG was weighed and homogenized with two times of 70% methanol. The mixture was vortexed for 60 s to ensure thorough mixing. The mixture was then centrifuged for 10 min at 20,000 rpm. The supernatant was collected and concentrated in rotary vacuum evaporator. The remaining methanol was removed by heating the extract at 45 °C using a hot plate [17]. The extract is stored in the refrigerator at 4 °C until further analysis.

2.4 Determination of protein content

The protein content was conducted using the Biuret method as described by Subroto and others. Prior to the analysis, Biuret reagent and 0.85 w/v% brine solution were prepared. About 0.5 ml of extracted sample was pipetted into vials with 1 ml brine solution and 1.5 ml Biuret reagent. The mixture of 1.5 ml of Biuret reagent and 1 ml of brine solution was used as the blank solution. Then, the solutions were incubated for 30 min. The protein content in the sample was determined using the UV-Vis spectrophotometer at a wavelength of 540 nm [19]. The bovine serum albumin (BSA) of known protein concentrations (100-700 ug/ml) was used for the standard curve. The protein concentrations (%) of samples were obtained from the standard curve.

2.5 Determination of crude lipid content

The crude lipid content was conducted in accordance with Rafe and Nadjafi [20]. The amount of crude lipid was determined using the solvent extraction method. Around 250 ml of hexane was poured into a round-bottomed flask, followed by the addition of 15 g of BG into the Soxhlet extractor. The extraction was carried out at 65 °C for 5 h. After that, it is cooled to room temperature and approximately 5 g of sodium sulphate was added to the flask to dry up the hexane mixture prior to vacuum filtration. The filtrate was collected and heated using rotary evaporator to remove the hexane from the solution [20]. The crude lipid content (%) was calculated using Equation (1).

$$\text{lipid content (\%)} = \frac{m_2 - m_1}{m_s} \times 100\% \quad (1)$$

where

m_2 = Mass of flask and lipid (after extraction);

m_1 = Mass of flask (before extraction);

m_s = Mass of sample used.

2.6 Determination of salt content

The salt content was evaluated using the protocol according to ASTM [21]. About 2.5 g of BG was diluted in 200 ml of distilled water. The few drops of the mixture were spread across the entire surface of the prism before closing the light plate. The temperature was allowed to compensate by leaving it to cool for 30 s. The meter was then held in the direction of the light source and the reading was taken by looking into the eyepiece [21]. The result was expressed in Brix percentage (%).

2.7 Determination of sugar content

The sugar content was determined using a refractometer in Brix (%). A 2.5 g BG was diluted in 200 ml distilled water. A few drops of the mixture were placed in the sample spot of the meter. The instrument allows the automatic reading by giving a numerical value for sucrose content [22].

2.8 Determination of alcohol content

The alcohol content was determined using a hand-held digital density meter. About 1 g sample was shaken

vigorously for 1 min with 10 ml distilled water. The filter paper was used to separate the residual and filtrate components. After that, the filtrate was used to determine the alcohol content in the samples [22]. The result was expressed in percentage (%).

2.9 Antioxidant activity

The antioxidant activity of BG was analysed using the DPPH assay [23]. Firstly, 1 ml of BG extract was dissolved in a 10 ml volumetric flask. Then, 2.85 ml of methanolic DPPH reagent (0.002 g/50 ml) and 0.15 ml of the sample solution were mixed. Negative control was prepared by mixing 0.15 ml water with 2.85 ml methanolic DPPH reagent. The mixtures were incubated for 30 min in the dark at room temperature. The absorbance was analysed immediately against a blank of absolute methanol at the wavelength of 517 nm. Ascorbic acid solution (100-700 µg/ml) was used for the calibration curve. Free radical scavenging activity (RSA) was expressed as inhibition percentage and was calculated using Equation (2).

$$\text{RSA (\%)} = \frac{\text{Ac} - \text{As}}{\text{As}} \times 100\% \quad (2)$$

where

Ac = Absorbance of negative control;

As = Absorbance of sample.

2.10 Statistical analysis

Data analysis will be carried out using SPSS software (version 19) (IBM SPSS Statistics, Chicago, IL, USA). All analyses will be conducted in triplicate. One-way analysis of variance (ANOVA) will be used to assess the significant difference between the means with a significant level of 0.05. Mean comparison will be performed using Tukey's test.

3. Results and discussions

3.1 Visual observation of BG



Figure 1. (a) Black garlic bulbs and (b) Black garlic cloves and (c) Black garlic powder

The conversion of white FG to black colouration is attributed to non-enzymatic browning and Maillard reaction (Figure 1) [17-18]. This phenomenon can be explained by the chemical reaction or condensation reaction between amino groups of proteins, peptides, and amino acids with carbonyl groups present in reducing sugars, which lead to the formation of Schiff bases, as well as Amadori and Heyns rearrangement products. Maillard browning significantly affects the various aspects of food, including its physicochemical properties, organoleptic qualities, colour, and protein

functionality. Temperature-time characteristics utilised during food preparation are used to create unique aroma profiles [24]. Browning reactions can contribute to desired changes such as the development of delicate flavours, but they can also result in undesirable quality changes, especially if the browning reactions are very prominent, creating bitter and burnt flavours [24]. Consequently, the fermentation duration holds paramount importance in this study, as this parameter significantly influences the quality of the BG samples.

3.2 Protein content

The chemical composition and antioxidant properties of FG and BG samples are presented in Table 1. FG showed the lowest protein content ($1.6 \pm 0\%$). The highest protein content was recorded in all BG samples with $4.3 \pm 0\%$. There was a significant difference ($p < 0.05$) between BG samples and FG, meanwhile no significant difference ($p > 0.05$) for all BG samples regardless of the fermentation periods.

Table 1. Chemical composition and antioxidant properties of BG and FG

Samples	Protein content (%)	Crude lipid content (%)	Salt content (%Brix)	Sugar content (%Brix)	Alcohol content (%)	DPPH scavenging activity (%)
BG4	4.3 ± 0.0^a	0.58 ± 0.06^d	1.0 ± 0.0^a	0.9 ± 0.0^a	n.d.	63.6 ± 1.5^a
BG6	4.3 ± 0.0^a	1.70 ± 0.43^c	0.6 ± 0.0^b	0.7 ± 0.1^b	n.d.	61.4 ± 1.7^a
BG8	4.3 ± 0.0^a	2.70 ± 0.13^b	0.4 ± 0.0^c	0.4 ± 0.1^c	n.d.	57.1 ± 2.3^a
FG	1.6 ± 0.0^b	3.66 ± 0.10^a	0.7 ± 0.1^b	0.7 ± 0.0^b	n.d.	11.9 ± 5.0^b

* Data are expressed in average and standard replication ($n = 3$).

** n.d. means not detected.

^{a-d} Different letters within the same column indicate significant ($p < 0.05$) differences.

Table 1 reveals a significant increase in garlic protein content following fermentation ($p < 0.05$). The observation can be attributed to the reduction in water content, which is utilised in chemical reactions to form various compounds and undergoes evaporation throughout the fermentation process [25]. Previous study indicated that the increase in amino acid levels, such as aspartic acid, threonine, arginine, and leucine, is probably a result of protein or peptide hydrolysis that happens through enzymatic or non-enzymatic reaction [26]. The protein content for BG4, BG6, and BG8 was maintained. This might be due to the use of constant operating conditions throughout the fermentation process. A research study carried out by Herlina et al. [25] demonstrated that BG exhibited its highest protein content at a processing temperature of $80\text{ }^\circ\text{C}$, whereas the lowest protein content was observed at $60\text{ }^\circ\text{C}$. Nevertheless, further investigations are required to explore the relationship between BG protein content and various fermentation durations.

3.3 Lipid content

Our results highlighted that there was a significant difference in lipid content between the samples ($p < 0.05$). FG had the highest crude lipid content with $3.66 \pm 0.10\%$ followed by BG8 ($2.70 \pm 0.13\%$). BG6 showed higher crude lipid content with 1.70% as compared with BG4 with only 0.58% . Based on Table 1, there was an increasing trend of crude lipid content between BG4 to BG8.

Resende and co-workers reported that when the fresh white garlies were subjected to the fermentation process for 22 days, their crude lipid content increased nearly fourfold from 0.11% to 0.43% , respectively [27]. The observation was consistent with the research carried out by Choi et al. where the crude lipid content of BG increased significantly from 0.18% to 0.58% in garlic products. This indicates that the conversion of FG to BG led to changes in lipid profiles due to various chemical interactions [9]. In contrast, a study conducted by Lu reported a decrease in crude

lipid concentration from 0.33% (FG) to 0.16% (BG) [28]. The inconsistencies could be attributed to the variations in garlic species and fermentation conditions, particularly in terms of relative humidity and heat temperature during the fermentation process. Hence, the effects of different fermentation durations of BG should be investigated further in the future as its documentation is rarely reported.

3.4 Salt content

The BG8 was found to have the lowest salt content (0.4%) as compared to BG4 and BG6 with 1% and 0.6%, respectively. As for FG, it was recorded to have 0.73% of salt and it has no significant difference ($p > 0.05$) with BG6. However, there were significant differences ($p < 0.05$) among BG4, BG8 and FG. We hypothesised that the decrease in salt concentration could be attributed to the presence of moisture during fermentation, causing gradual dissolution of salt components over time. After 4-week fermentation period, a slight increase of 0.3% Brix in salt concentration was observed. This phenomenon may be attributed to the gradual release of salt from the BG4 sample, followed by progressive dissolution facilitated by moisture throughout the fermentation process, ultimately resulting in a reduction in salt content by the eighth week. However, there is a lack of existing literature discussing the salt content of BG, highlighting the need for future research to elucidate the synthesis or changes in salt content in BG throughout different fermentation periods.

3.5 Sugar content

The present study showed that all samples were found to possess sugar but less than 1%Brix. The highest sugar content was observed in BG4 ($0.9 \pm 0.0\%$), followed by BG6 ($0.7 \pm 0.1\%$), FG ($0.7 \pm 0.0\%$) and lastly BG8 ($0.4 \pm 0.1\%$). There was a significant difference ($p < 0.05$) between BG4 and the others whereas there was no significant difference ($p > 0.05$) between both BG6 and FG.

The sugar content in the garlic product increased by 0.2%Brix following a 4-week fermentation. A study conducted by Liang et al. found that the content of fructose sugar increased in BG after 5 and 25 days (4 weeks). This was due to the degradation of fructan polysaccharides in garlic which caused the fructose level in BG to rise as the fructan polysaccharides were destroyed, allowing the fructose level to rise [26, 29]. In addition, Liang and others also demonstrated that the sugar component of fructose decreased when the fermentation period was above 25 days (4 weeks). Another similar study demonstrated that the decrease was due to the presence of reducing sugar such as glucose and fructose [25]. When BG reached a level of reducing sugars consumed, surpassing the accumulation rate, a decline in reducing sugars was observed subsequently [25]. This is supported by Ding and others who mentioned that temperature and storage time could reduce the composition of garlic sugars due to the activity of a fructan extrahydrolase that hydrolyses fructosan. The authors also mentioned that the reduction in sugar content could be related to the Maillard reaction that utilises reducing sugar during the fermentation process [30].

3.6 Alcohol content

No alcohol content was detected in all BG samples. No enhancers, additives, or preservatives were employed in this study to speed up the fermentation process. This is also referred to as non-alcoholic fermentation [31]. As a result, no alcoholic components were detected in all garlic samples. This contrasts when the fermentation is performed by utilising the yeast (*Saccharomyces cerevisiae*). Yeast is essential in producing all alcoholic beverages and choosing appropriate yeast strains is critical not only to maximising alcohol yield but also to maintaining beverage sensory quality [31]. As a result, alcohol components are produced in the fermented food or beverages at the end of the fermentation. Another study showed that yeast metabolites could modify dietary components, potentially enhancing bioactivity by converting sugar to alcohol and lactic acid [32]. The authors reported that garlic could supply nutrients to the yeasts, triggering alcoholic fermentation.

3.7 Antioxidant activity

The highest DPPH radical scavenging activity (RSA) (%) was found to be BG4 ($63.6 \pm 1.5\%$), followed by BG6

with $61.4 \pm 1.7\%$. FG had the lowest RSA ($11.9 \pm 5.0\%$) as compared to BG8 ($57.1 \pm 2.3\%$). There was a noticeable trend that the antioxidant activity of BG samples decreased gradually with increasing fermentation durations. There was a significant difference ($p < 0.05$) between the BG and FG samples meanwhile all BG samples showed no significant difference ($p > 0.05$) from each other regardless of the fermentation periods. Similar observation was also reported by Choi et al. in which the DPPH free radical scavenging of BG ranged from 37.32%-74.48% and was significantly higher than FG (4.65%). The authors also reported that the DPPH scavenging activity of BG increased approximately 2-fold, from 37.32% on the 7th day to 74.48% on the 21st day, and then slightly decreased to 63.09% till the 35th day of aging. The values were significantly higher than that of raw garlic (4.65%) ($p < 0.05$).

A colour shift from purple to pale yellow serves as an early indication of the presence of antioxidant components. When a free radical scavenger is present, the DPPH radical absorbs an electron or hydrogen atom to produce a stable compound that alters the colour of the solution from deep purple to pale yellow, with the degree of discoloration being proportional to the amount of electrons present [33]. Previous literature shows that the antioxidative compounds in BG increased over the fermentation process, particularly polyphenol, flavonoids, and some intermediate Maillard reaction components, which have been known to exhibit antioxidant properties [34-35]. Choi et al. also indicated that the total polyphenols increased during heat treatment or fermentation, reaching a significant peak after 21 days. This explains that BG4 possessed the highest RSA which is 5 times higher than FG.

However, processing FG at high temperatures for an extended period might result in a decrease in phenolic content [36]. A similar finding was documented by Choi and colleagues, indicating that prolonged heating times led to a higher thermal load, causing a substantial reduction in phenolic acid content both initially and after 21 days of heating. Our study also demonstrated a reduction in antioxidant activity from 63.6% to 57.1% with prolonged fermentation durations. This could be attributed to the combination of high temperature and prolonged fermentation duration, which enhanced the permeability of the cell wall and increased solubility and diffusion of phenolic compounds from garlic cells, leading to the degradation of these antioxidative compounds [34]. Our results showed that BG offers significant antioxidant potential when compared to fresh white garlic.

4. Conclusion

In this study, the effect of fermentation intervals on the physiochemical properties of BG was investigated. Apparently, different fermentation periods on garlic altered the content of salt, sugar, and crude lipid in the samples. However, our study showed that different fermentation periods did not significantly change the content of alcohol, protein, and antioxidant activity ($p > 0.05$). However, our research clearly demonstrated that extended heating and fermentation durations can diminish the antioxidant properties of BG products. Hence, fermenting for a period of 4 weeks would be the optimal condition for producing high-quality BG. Additionally, compared to FG, BG offers several advantages, including high DPPH scavenging activity, low salt content and high protein content. Since garlic has long been consumed in human society and is recognized as one of the safe food substances, there will be no restrictions on developing black garlic products for functional food, food supplements, and medical purposes.

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Authors' contributions

Muhammad 'Akif Bin Shahrum and Eng-Tong Phuah: Conceptualization. Muhammad 'Akif Bin Shahrum: Investigation and Methodology. Muhammad 'Akif Bin Shahrum and Masmunira Rambli: Writing-Original draft. Eng-Tong Phuah: Supervision. Shyang-Pei Hong: Writing-Review and editing.

Conflict of interest

The authors declare no competing financial interest.

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