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The Effect of Sterility Values and Retort Temperatures on the Change of Physical and Sensory Properties of a Canned Mushroom Product

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Abstract

This research aimed to study the effect of sterility values on physical quality (drained weight, brown colour index, and texture) and sensory properties of canned *Agaricus bisporus* mushrooms processed at different retort temperatures (115, 121, and 130 ^{o}C) and processing times (2-97 minutes). Mushrooms in brine solution media packaged in 300x407 cans were heated in industrial-scale horizontal static retorts at different retort temperatures for specific processing times to reach different F_0-values. The canning process was carried out following commercial production procedures in one of the mushroom canning factories. Measurement of heat penetration into the product was carried out using a protocol established by the Institute of Thermal Process Specialists (IFTPS), and the sterility values (F_0) were calculated. Our results indicated that the physical and sensory properties of canned mushrooms were not only affected by sterility value but also by the combination of temperature and time used to process the product. At the same level of sterility, a higher retort temperature (130 ^{o}C) resulted in canned mushroom with a lower browning rate, an improved texture profile (decreased hardness, increased chewiness, and shear force), a sweeter taste, and increased intensity of umami taste. However, the canning process at a temperature of 130 ^{o}C resulted in a greater reduction of the drained weight as compared to that of canning at 115 ^{o}C and 121 ^{o}C.

Keywords: *Agaricus bisporus*; Canning; Browning colour index; Drained weight; Sensory; Texture

1 Introduction

Agaricus bisporus is one of the most popular edible mushrooms (Kumar et al., 2017) and accounts for 30% of total mushroom production in the world (Royse, 2014). This mushroom has a high economic value because of its nutritional content, functional properties from its bioactive compounds, and unique taste (Zhang et al., 2018). Mushrooms are a potential source of essential nutrients, such as carbohydrates, proteins, dietary fibre, phenolic compounds, polyunsaturated fatty acids, vitamins, and minerals (Ramos et al., 2019). However, fresh *A. bisporus* is highly perishable by nature and has a short

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shelf-life in the ambient environment, owing to its high moisture content, lack of physical protection to avoid water loss or microbial attack (Fernandes et al., 2012; Joshi et al., 2018) and extreme sensitivity to heating (Xue et al., 2017). Thermal processes, including canning, are widely applied by the mushroom processing industry to extend the shelf-life, which is cost-effective (Tola & Ramaswamy, 2018). The primary concern when designing a sterilization process is the inactivation of pathogenic microorganisms to protect public health. The desired lethal effect on pathogenic microorganisms can be achieved through a wide range of time-temperature combinations. However, the heat process targeted to inactivate pathogenic microorganisms also results in various physical and sensory quality degradations. The optimum sterilization process is required to maintain the quality of the product without compromising food safety aspects (Ling et al., 2015).

During the sterilization process, the effect of temperature and heating time on pathogenic microorganisms is commonly represented by the F_0-value. It can be defined as the equivalent heating time at a temperature of 121.1 ^{o}C. Previous researchers have reported the impact of commercial sterilization or canning on physical and sensory properties. Anantheswaran et al. (1986) reported that heating mushrooms at the temperature of 110-129.4 ^{o}C for 8-46 minutes decreased the whiteness, yield, and texture. Moreover, Jaworska et al. (2010) showed that sterilization of different mushrooms at atemperatures of 100-121 ^{o}C caused a significant decrease in hardness of up to 92%. Sun et al. (2014) also reported that pressure-cooking significantly affects the texture and chemical composition of processed products. On the other hand, the change of organoleptic attributes as the impact of high-temperature processes was also reported by Jaworska et al. (2011) and Liu et al. (2014). However, it is still arguable whether the change of physical quality and sensory attributes due to sterilization could also be solely described as a function of the F_0-value. Limited data is available to systematically explore the impact of time-temperature on mushrooms. Therefore, this research aimed to study the effects of sterility values (F_0-values) and retort temperatures of various time-temperature combinations on physical quality (drained weight, colour, texture) and sensory properties of canned *A. bisporus* mushrooms.

2 Materials and Methods

2.1 Materials

Fresh white button mushrooms (*A. bisporus*) cultivated in Probolinggo, East Java, Indonesia were kept fresh by storing at 4 ^{o}C for one day before being processed. A horizontal static retort with a diameter of 1.25 m and a length of 2.35 m (Chi Yinfa, Taiwan) was used as the equipment. Temperature measurement and recording were done using the OM-CP-Hitemp140 data logger (Omega Engineering, Norwalk, Connecticut, USA), which could measure temperatures up to 140 ^{o}C, with an accuracy of $\pm$0.1 ^{o}C.

2.2 Canning procedure

Preparation of *A. bisporus* samples before canning followed the procedure used in one of the mushroom canning factories (PT. Suryajaya Abadiperkasa, Probolinggo, East Java, Indonesia). Mushrooms were stored one day at a temperature of 3-5 ^{o}C before the canning process. In general, the canning steps were material preparation, blanching, filling into cans, filling medium (citric acid, ascorbic acid, and NaCl), exhausting, seaming, sterilizing, cooling, and storing (Figure 1). The retort consisted of three baskets loaded randomly with the cans. Each basket was filled with 700 cans (full capacity conditions). Control mushroom samples were taken shortly after the seaming process or just before the retorting process. In contrast, the treated product samples were taken after the sterilization process according to the design of the experiment at different retort temperatures (115, 121, and 130 ^{o}C). The canning process was carried out in duplicate.

2.3 F_0-value calculation

F_0-value was calculated based on heat penetration data. Heat penetration tests were carried

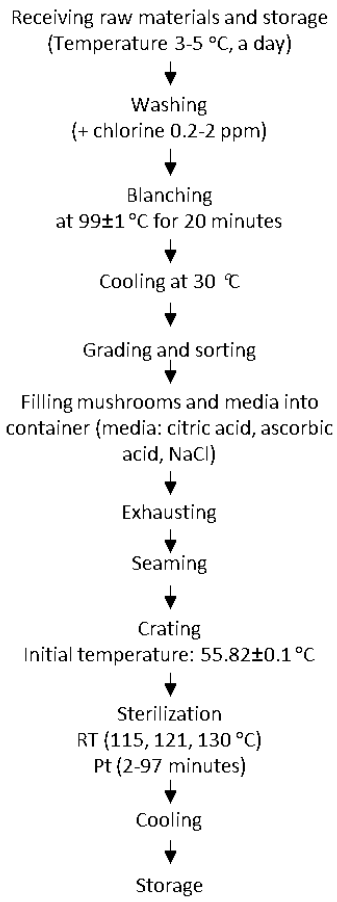

Figure 1: Flow chart of the canning process of the *A. bisporus* mushroom (Pursito et al., 2020)

out by placing eleven data loggers (OM-CP-Hitemp 140, Omega Engineering, Norwalk, Connecticut, USA) in the slowest heating area. Data loggers were located at the centre of the cans, and their sensors were inserted into the mushrooms. Sterility value was expressed as a F_0 calculated using the General Method (Holdsworth & Simpson, 2016), as shown in Equation 1.

$$F_0 = \int_0^t 10^{\frac{T-T_{ref}}{z}} dt \tag{1}$$

where F_0 was the equivalent heating time (in minutes) at a constant temperature of 121.1 ^{o}C to inactivate *C. botulinum* spores, T was the product's temperature at any given time; T_{ref} was a reference processing temperature (121.1 ^{o}C), and z was 10 ^{o}C. Datalogger placement schemes and samples on a static horizontal retort are shown in Figure 2.

2.4 Drained weight calculation

Drained weight was measured in triplicate (AOAC International, 1984). The samples were drained on an eight-mesh stainless steel filter (Fisher Scientific Company, USA) for two minutes with a 20^o slope angle at room temperature (25 ^{o}C). The calculation of drained weight was based on Equation 2.

$$Drained\ weight = \frac{yield}{net\ weight\ of\ can\ content} \times 100 \tag{2}$$

2.5 Texture analysis

The texture measurement was performed by using the TA-XT2i Texture Analyzer (Stable Micro Systems Ltd., Godalming, Surrey, UK) with a cylindrical probe (diameter of 35 mm), by performing texture profile analysis (TPA), as shown in Figure 3A. The mushroom samples were measured in triplicate. Samples were cut to have uniform dimensions of 20 mm x 20 mm x 18 mm. For the TPA test, the samples were pressed twice at a crosshead speed of 1 mm sec^{-1}, deformation level 60%, and a time interval of 20 seconds between compressions. Textural parameters of hardness and chewiness were recorded from the force–deformation curve. Shear force test was carried out by using a Warner-Bratzler Shear Cell (Figure 3B). Texture parameters from TPA and shear force tests were obtained using Texture Expert 1.22 software (Stable Micro System Ltd, Scarsdale, NY).

2.6 Colour Analysis

Analysis of visual colour was performed by using CR310 Chromameter (Konica, Minolta, Tokyo, Japan). Hunter's colour parameters (L, a, and b values) for the surface of treated mushroom samples (caps) were recorded (Mihalcea et al., 2016). The colour was measured in triplicate. The L-value indicated lightness, a, the red (+) or green

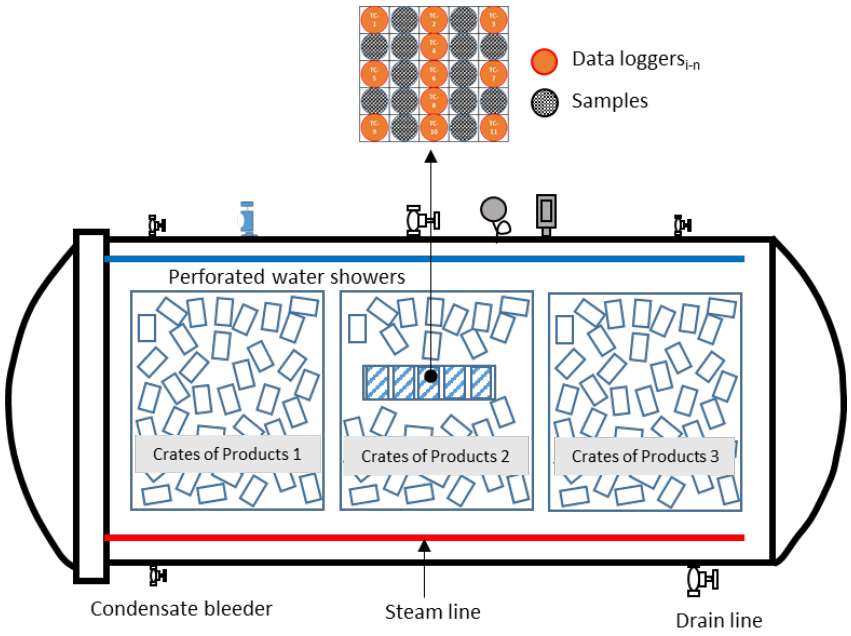

Figure 2: Datalogger placement schemes and samples on static horizontal retort (Pursito et al., 2020)

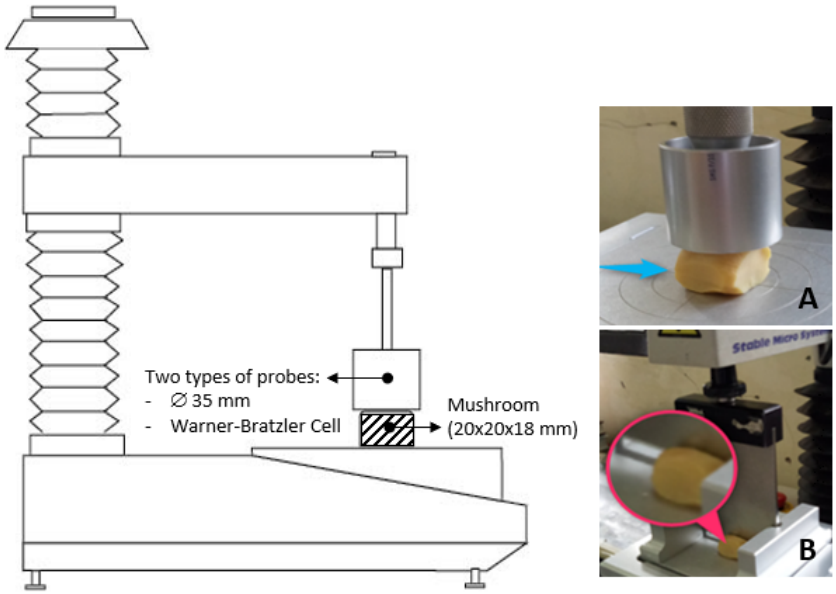

Figure 3: Measurement with a texture analyzer model XT2i type probe with diameter of 35 mm (A) and Warner-Bratzler (B).

(-) coordinate, and b, the yellow (+) or blue (-) coordinate. The Browning Colour Index (BCI), representing the purity of brown colour (Gao et al., 2014), was calculated from equation 3.

$$BCI = [100(x - 0.31)]/0.172 \quad (3)$$

Where x= (a+1.75L)/(5.645L+a-3.012b)
"L" is the brightness (lightness), "a" is the colour of reddish-green (greenness/redness), and "b" is the colour of bluish yellow (yellowness/blueness).

2.7 Sensory Evaluation

The control samples were thawed and conditioned at room temperature (25 ^{o}C). Samples were boiled for 10 minutes, cooled at 25 ^{o}C, and blended for 20 seconds. Samples (30 g each) were presented on white plates, each with a total of 4 test samples and one reference (control) sample together with water, white bread, and tissue paper (Figure 4). Each sample container was given a three-digit random number as the sample code. Each sample was tested triplicate and repeated on different days.
Sensory tests were carried out in a sensory laboratory at room temperature conditions, with sufficient light, and ventilation. Based on the screening tests, panellists were selected from professional assessors. Eleven trained panellists consisting of nine females and two males, 24-46 years old were involved in this sensory test. There was a discussion and re-training on scalar scoring methods in all group discussions on the basic quality parameters of taste (umami, salty, sour, sweet, and bitter). Determination of the standard value/reference (control) of taste was determined based on focus group discussion on the taste of salty, sour, sweet, bitter, and umami. In particular, umami taste intensity was trained to differ in the gradations of 6 concentrations of MSG standard solutions 0.03, 0.09, 0.15, 0.21, 0.27, or 0.30 g/100 mL (Phat et al., 2016). Panellists evaluated the intensity of flavours using a non-structured line scale of 1-15. The sensory assessment results were transformed on a scale of 0-100 where 0 = no taste and 100 = extreme intensity.

2.8 Statistical Analysis

The physical quality and sensory evaluations were analyzed by regression and descriptive analysis methods using MS Office Excel (2016) for each temperature (115, 121, and 130 ^{o}C) and sterility values. The rate of change of the values of the quality parameters (drained weight, colour, texture, and sensory attributes) were determined from the gradient of each regression line.

3 Results and Discussion

3.1 Drained Weight

The canning of mushrooms induces significant changes in their physical and chemical properties. One of the most critical changes is water loss from the mushroom, which causes a loss of weight and a reduction of the net weight in the can. These losses have a significant economic repercussion (Vivar-Quintana et al., 1999). Figure 5 shows the effects of retort temperatures (115, 121, and 130 ^{o}C) an F_0-values on the drained weight. Samples with a value of F_0=0 minutes are control samples, which were taken before the retorting process. The drained weight decreased exponentially with increasing F_0-values at all sterilization temperatures. The result also showed that retort temperature significantly influenced the change in the drained weight of the product. The rate of decline at retort temperature of 130 ^{o}C was slightly higher than that at the retort temperatures of 121 and 115 ^{o}C. At the same F_0-value, for example 10 minutes, the percentage of drained weight at 115, 121, and 130 ^{o}C were 48.84%, 48.45%, and 47.86%, respectively. Retorting the product at 115 ^{o}C and 121 ^{o}C is expected to give the drained weight reduction of 0.18 – 0.19% from that of the control sample. On the other hand, retorting the product at 130 ^{o}C would result in a drained weight reduction of 0.99%.
A decrease in drained weight occurred at higher temperatures probably because the higher retort temperature (130 ^{o}C) caused more cell wall damage as compared to the lower retort temperature. At higher temperatures, the cell and tissue

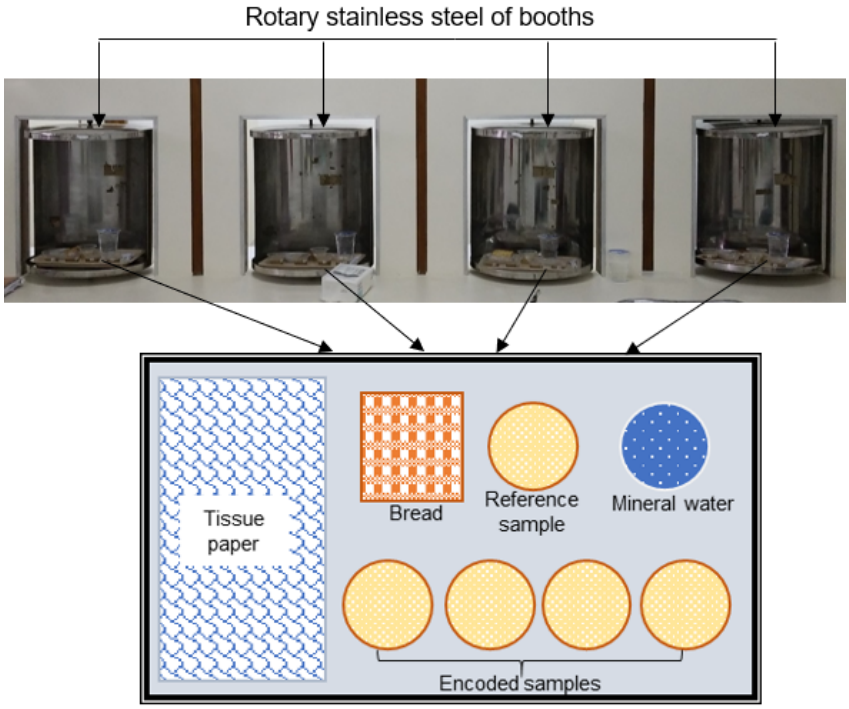

Figure 4: Sensory booths and details of sample presentation

structure shrinks and releases water contained in the tissue (Almonacid et al., 2012). Canning also causes protein denaturation and leads to decreased yield. A decrease of drained weight due to canning (high temperature) was also reported by Anantheswaran et al. (1986) and Paudel et al. (2016). The loss of water holding capacity after heat treatment was correlated mainly with the loss of cell membrane integrity.

3.2 Colour

The quality of the mushrooms is determined by the colour, which is one of the most crucial parameters that influence consumers' choices and preferences of foods. Browning on mushrooms reduces the selling value of the product. In general, the brighter colour of mushrooms is preferred by consumers so that it has higher economic value (Chen et al., 2018). The change in food colour during a thermal process is influenced by various mechanisms, such as degradation of pigments, oxidation of ascorbic acid, enzymatic browning, and non-enzymatic browning (Ling et al., 2015). Figure 6 shows the effect of F_0-values at different retort temperatures (115, 121, 130 ^{o}C) on the Browning Colour Index (BCI) of canned *A. bisporus*.

BCI values increased with the increase of F_0-values at all sterilization temperatures (115, 121, 130 ^{o}C). At the same F_0-value, for example, at the F_0-value of 10 minutes, retort temperatures of 115, 121, and 130 ^{o}C, the BCI values were 10.54, 11.86, and 10.52, respectively. Therefore, sterilization at a retort temperature of 130 ^{o}C gave a more favourable colour compared to the other two retort temperatures. The difference of BCI can be measured by comparing the BCI of certain F_0-value (for example, at the F_0-value of 10 minutes) and initial BCI (F_0-value of 0 minutes) at 115, 121, and 130 ^{o}C. At an F_0-value of 10 minutes, it was found that the BCI values of 115, 121, and 130 ^{o}C were 10.54, 11.86, and 10.52, respectively (Figure 6). Therefore, ther-

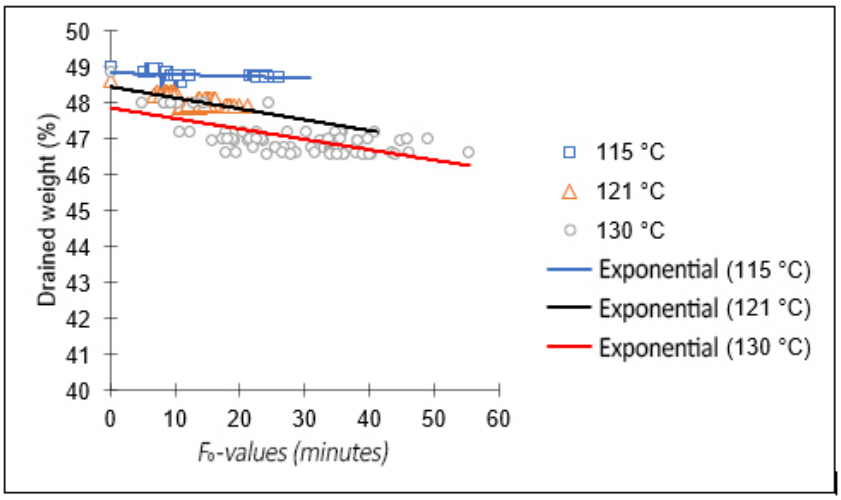

Figure 5: Effect of F_0-value at different retort temperatures (115, 121, and 130 ^{o}C) on drained-weight of canned *A. bisporus* mushroom packed in 300x407 can. The lines are lines of best fit.

mal treatment at 115 and 121 ^{o}Cwould give an increment of BCI of as much as 14.9 - 22.9%. However, retorting at 130 ^{o}Cwould result in a BCI increment of 4.8%. This result showed that sterilization at a retort temperature of 130 ^{o}C resulted in less colour change compared to that of the other retort temperatures.

The browning rate at retort temperature of 130 ^{o}Cwas lower than other retort temperatures because to achieve the same F_0-value, a higher temperature (130 ^{o}C) required less processing time compared with other retort temperatures (115 and 121 ^{o}C). Conversely, to achieve the same F_0-value, sterilization at retort temperatures of 115 and 121 ^{o}C required a longer time. A previous study by Pursito et al. (2020) showed that the times needed to achieve an F_0-value of 10 minutes at temperatures of 115, 121, and 130 ^{o}C were 39.32, 11.22, and 1.30 minutes, respectively.

An increase in BCI-values (Figure 6) may have been due to the impact of the retort temperature and processing times on non-enzymatic Maillard reaction. Ames (1990) and Jing and Kitts (2002) stated that the rate, extent, and course of Maillard reactions are influenced by several factors including, but not limited to, type of reactants, temperature/time combinations, pH, and water activity. An increase in temperature increases the rate of Maillard reaction. This reaction occurred in the canning process because mushrooms contain amino acids and reducing sugars, which are ingredients needed for the Maillard reaction. Maillard reactions led to changes in food colour, organoleptic properties, protein functionality, and protein digestibility (Lund & Ray, 2017). The Maillard reaction is of primary importance to the food manufacturer since it is frequently responsible for the aromas and colours that develop during the heating or storage of food products. Studies of model systems showed that an increase in temperature and/or time of heating resulted in increased colour development (Ames, 1990). Colourless solutions characterize the initial Maillard reaction, but after several reactions, a brown or black insoluble compound called melanoidin is formed (Awuah et al., 2007). Maillard browning can be inhibited by decreasing moisture to minimum levels or by increasing dilution, lowering pH, and temperature if the product is in the form of a liquid.

Due to the differences between *z-value* of *Clostridium botulinum* (z = 10 ^{o}C) with D_{121} = 0.25 minutes and the *z-value* of browning reaction z = 32 ^{o}C (Toledo et al., 2018), it is suggested that the activation energy of browning is lower than the deactivation of microbial activity

(*C. botulinum*). This value implies that the rate of destruction of microorganisms will be much higher than the rate of destruction of colour attributes at a higher temperature. Thus, the thermal processing of food products at higher temperatures can achieve commercial sterility with better retention of colour quality.

3.3 Texture

Hardness, chewiness, and shear force are essential attributes of mushroom texture among several other attributes. Figure 7 shows the effect of retort temperatures (115, 121, and 130 ^{o}C) and F_0-values on hardness. Compared with control samples, the hardness decreased quadratically with increasing F_0-values at all sterilization temperatures (115, 121, 130 ^{o}C). The reduction of hardness at 130 ^{o}C was lower than that at 121 and 115 ^{o}Cretort temperatures. The difference of hardness can be measured by comparing the hardness at a certain F_0-value (for example, at the F_0-value of 10 minutes) and initial hardness (F_0-value of 0 minutes) at 115, 121, and 130 ^{o}C. At an F_0-value of 10 minutes, it was calculated that the hardness values of 115, 121, and 130 ^{o}C were 5441.94 *gf*, 5480.59 *gf*, and 5738.59 *gf*, respectively (Figure 7). Therefore, treatment at 115 and 121 ^{o}C reduced the hardness by 11.6%, whereas the treatment of 130 ^{o}C reduced the mushroom hardness by 7.6%. It can be said that sterilization at a temperature of 130 ^{o}C caused less damage to hardness compared to other retort temperatures.

It was found (Figure 8) that the chewiness increased at higher F_0-values, which may have been due to the heat-induced cell decomposition and fragmentation during sterilization. At the same F_0-value (F_0-value of 10 minutes) at 115, 121, and 130 ^{o}C, the chewiness values were 2.084 *gf*, 2.077 *gf*, and 2.06 *gf*, respectively. Conversely, Jaworska et al. (2010) reported the reduction of chewiness after canning, but sterilization was carried out at temperatures up to 100-121 ^{o}C, 5-12 minutes of *Boletus edulis*. The canning process led to changes in the textural parameters depending on the species of mushroom.

The result of the texture testing using Warner-Bratzler shear cells is shown in Figure 9. The use of this blade is intended to resemble human incisors when used to cut mushrooms. Compared to the previous parameters of texture, the difference of shear force can be measured by comparing the shear force of certain F_0-value (for example at the F_0-value of 10 minutes) and initial shear force of control samples (F_0-value of 0 minutes) at 115, 121, and 130 ^{o}C. At an F_0-value of 10 minutes, the shear force of 115, 121, and 130 ^{o}C were 32,430, 31,852, and 24,088 *gf*, respectively (Figure 9). Therefore, thermal treatments at 115 and 121 ^{o}C would give a reduction of shear force of more than 140%, whereas the treatment of 130 ^{o}C would give a reduction of shear force less than 100% (78.6%). It can be seen that mushroom sterilization at a temperature of 130 ^{o}C would result in less shear force reduction compared to that of lower retort temperatures. Compared with control samples, this increasing trend of shear force was similar to the findings of a study reported by Anantheswaran et al. (1986). Generally, increased processing temperatures significantly affect mushroom texture (Zivanovic & Buescher, 2004). Figure 5 shows that a higher retort temperature (130 ^{o}C) resulted in a more significant effect on the reduction of drained weight due to cell integrity. Higher temperatures or longer canning processes cause more damage to the cell walls of mushroom tissue. It could impact changes in WHC during thermal processing, whereas, among the cell wall components in mushrooms, both chitin and protein contribute to water retention by providing mechanical strength to the cell wall (Paudel et al., 2016).

Furthermore, Jasinki et al. (1984) observed using transmission electron microscopy (TEM), that high temperatures during canning caused coagulation of cytoplasmic material and disruption of the intracellular membranes. Therefore, retorting might probably cause the loss of WHC of the tissue, which also affected the hardness, chewiness, and shear force (Figures 7, 8, and 9). For practical application, our findings agreed with those of Tang et al. (2014) who report that the higher sterilization temperature improves the textural as well as the colour and sensory acceptability of the retort processed product.

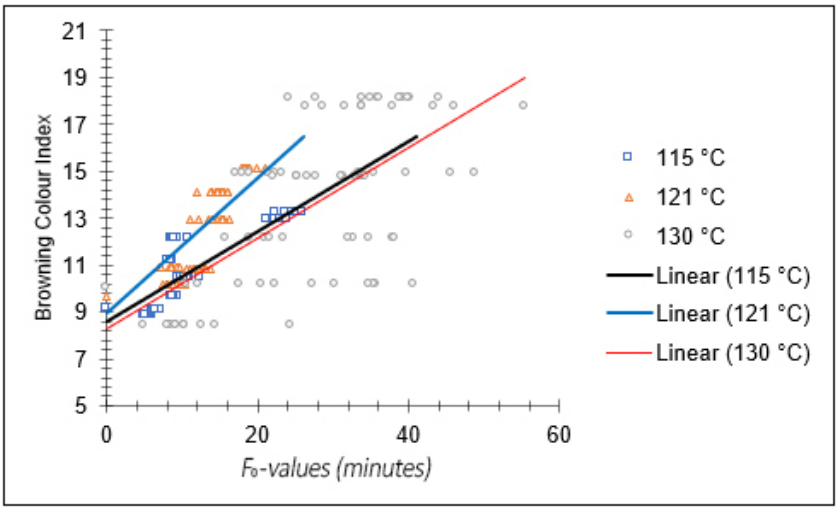

Figure 6: Effect of F_0-value at different retort temperatures (115, 121, and 130 ^{o}C) on the browning index of canned *A. bisporus* mushroom. The lines are lines of best fit.

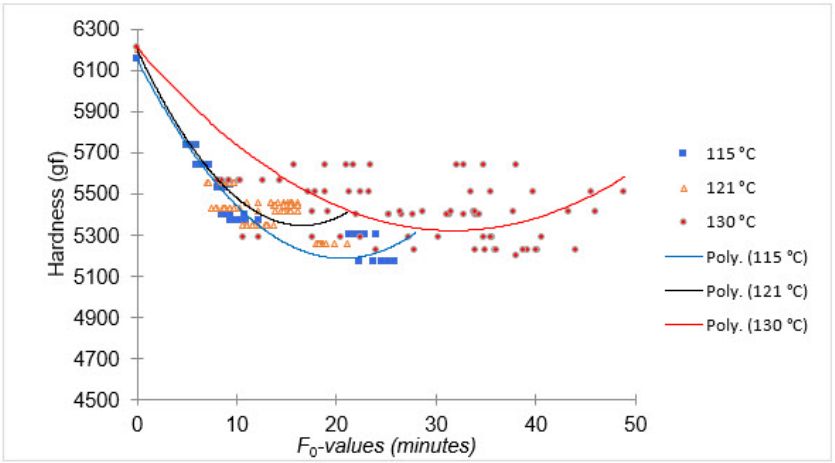

Figure 7: Effect of F_0-value at different retort temperatures (115, 121, and 130 ^{o}C) on the hardness (TPA) of canned *A. bisporus* mushroom. The lines are lines of best fit.

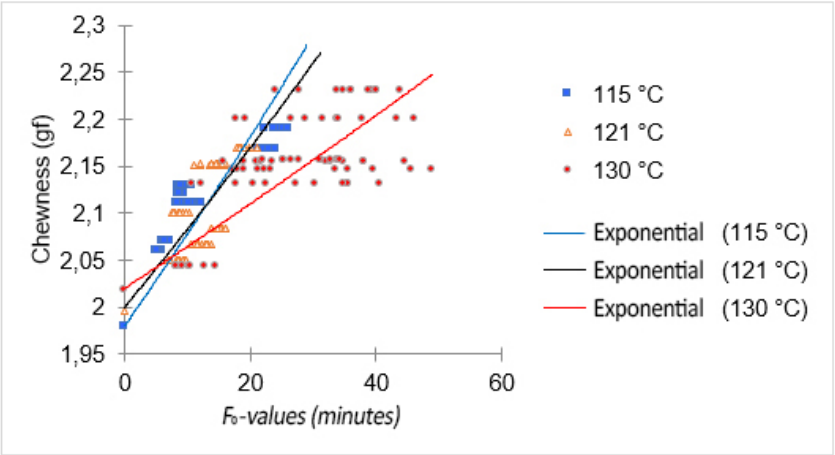

Figure 8: Effect of F_0-values at different retort temperatures (115, 121, and 130 ^{o}C) on the chewiness (TPA) of canned *A. bisporus* mushroom. Lines are lines of best fit.

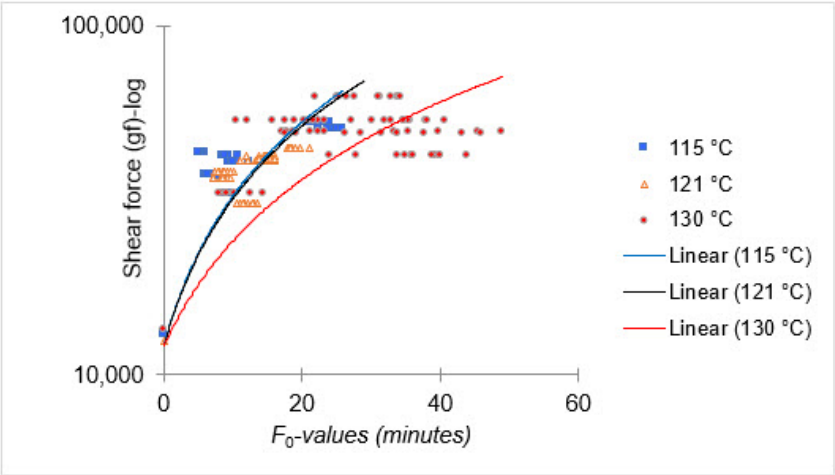

Figure 9: Effect of F_0-value at different retort temperatures (115, 121, and 130 ^{o}C) on the shear force of canned *A. bisporus* mushroom (Warner-Bratzler shear cell). The lines are lines of best fit.

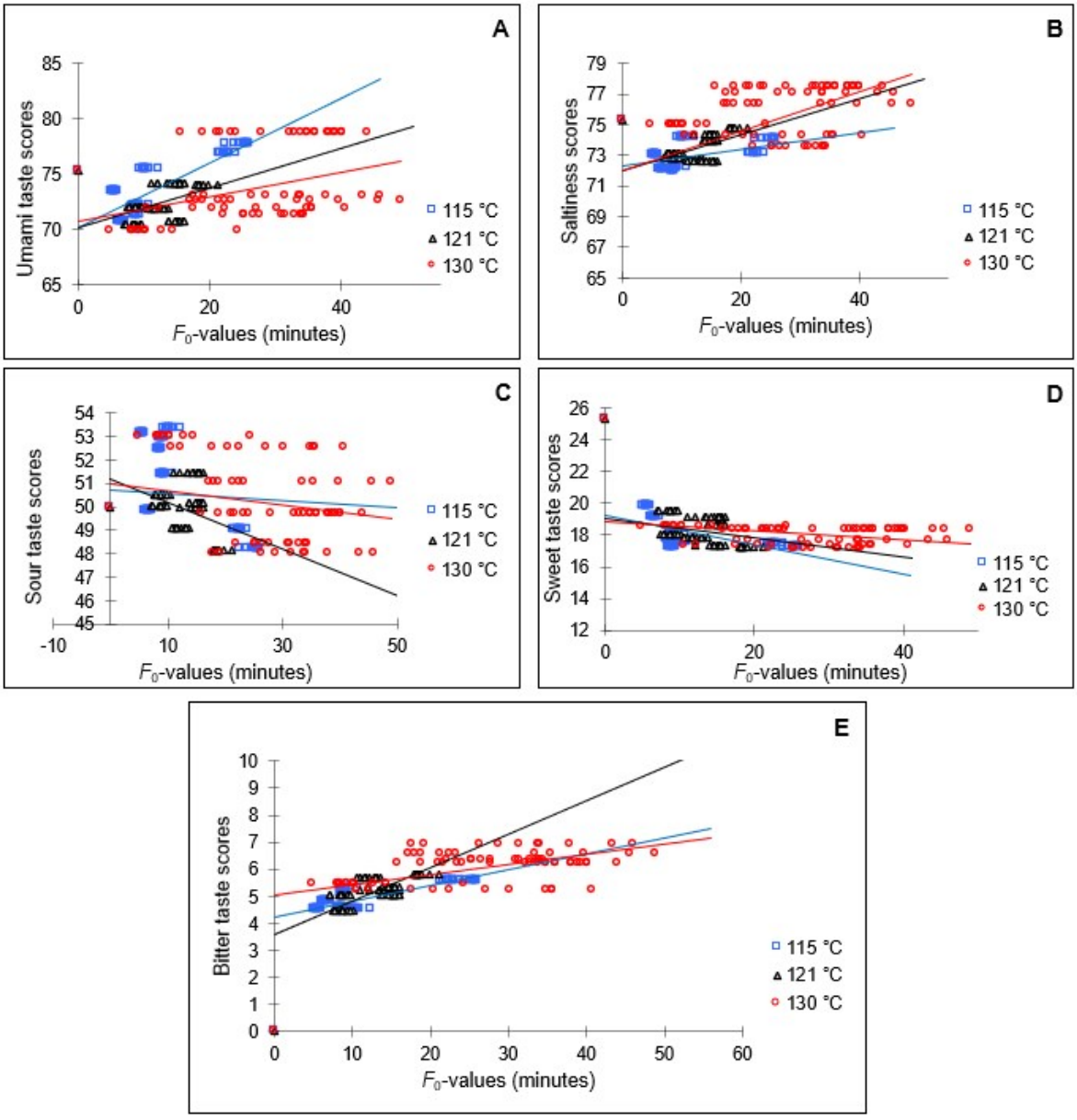

Figure 10: Effect of F_0-value at different retort temperatures (115, 121, and 130 °C) and on the taste intensity of canned *A. bisporus* mushroom (A=umami taste, B=saltiness, C=sour taste, D=sweet taste, E=bitter taste). The lines are lines of linear best fit

3.4 Sensory Attributes

Mushrooms have long been used as food or flavouring ingredients because of their unique and delicate taste (Chang & Wasser, 2012; Kurihara, 2009). Taste is an essential aspect for consumers in choosing mushroom products. Figures 10A-E show the effect of different F_0-values and retort temperatures (115, 121, and 130 ^{o}C) on the intensity of canned mushroom taste. At the same sterility value (F_0=10 minutes), regression analysis revealed that all the sensory attributes were significantly affected by the retort temperature.

Figure 10A shows that the umami score increases with increasing F_0-values for all retort temperatures. At the same F_0-value, the umami score of mushrooms processed at 130 ^{o}C was lower than the retort temperatures of 115 and 121 ^{o}C. The umami score of 130 ^{o}C was lower than other retort temperatures because to achieve the same F_0-value, a higher temperature (130 ^{o}C) needed less processing time compared with other retort temperatures (115 and 121 ^{o}C). The effect of sterility value and retort temperature on the umami taste intensity (Figure 10A) and the brown colour change (Figure 6) are interrelated. The increase of browning colour is probably due to the Maillard reaction during the sterilization process. More extended processing or retorting time has a more significant impact on the formation of umami taste as a result of the occurrence of non-enzymatic reactions during the sterilization process.

The increasing panellist scores also occured in salty taste (Figure 10B) and bitter taste (Figure 10E). The bitter and salty tastes increased as the F_0-value increased for all retort temperatures (Figure 10B and 10E). The rate of increase in saltiness score at 130 ^{o}C was higher than that at retort temperatures of 115 and 121 ^{o}C. As shown in Figure 10B, at the same F_0-value (10 minutes), the salty taste scores at retort temperatures of 115, 121, and 130 ^{o}C were 72.88, 73.17, and 73.28, respectively. Figure 10E demonstrated that the canning of mushroom at a retort temperature of 130 ^{o}C at a certain F_0-value, would result in the lowest bitter taste score compared to other retort temperatures (115 and 121 ^{o}C). The influence of non-enzymatic reactions may cause an increased bitter taste. The temperature of the retort significantly affected the intensity of the bitter taste. This phenomenon is in line with the change of BCI. As was described in Section 3.2., higher temperature shorter time resulted in less colour change. At the same F_0-value, the use of higher temperatures in the retorting process caused browning reactions to be much slower than the inactivation of *C. botulinum*. Slower browning reactions will produce fewer melanoidin or compounds which provide a lower bitter taste. Therefore, the temperature of 130 ^{o}C caused the taste of the mushrooms to be sweeter compared to the temperature of 121 and 115 ^{o}C. The z-value had a high value in the browning reaction, so the D-value did not decrease much when the process temperature was raised. The higher D-value indicated that the reaction rate was lower (D = 2,303/k). On the other hand, there was a slight decline in the sourness (Figure 10C) and sweetness (Figure 10D) with the increase in F_0-value for all retort temperatures. The decline of the sour taste at 115 ^{o}C was slower than that of other retort temperatures (121 and 130 ^{o}C). The sourness probably originated from the addition of ascorbic acid and citric acid to the mushroom media to improve or maintain the colour quality. Ascorbic acid is highly susceptible to losses during processing due to its solubility in water and because it is highly oxidizable (Chen & Ramaswamy, 2012). Figure 10D shows that sweetness scores tended to decrease with the increase in F_0-value for all retort temperatures. The rate of decline in the sweet taste at 115 ^{o}C was higher than that of the retort temperatures of 121 and 130 ^{o}C. It can be implied that, at the same sterility level, mushroom sterilization at higher retort temperature resulted in sweeter products. Generally, the sweet taste in mushroom products is favoured by consumers.

Overall, as the F_0-value rose, perceptions of umami, saltiness, and bitterness taste tended to intensify, and perceptions of sourness and sweetness tended to decrease at all retort temperatures. Commercial sterilization applications with a retort temperature of 130 ^{o}C indicate several sensory advantages, such as lower bitterness and higher sweetness compared to retort temperatures of 115 and 121 ^{o}C.

4 Conclusions

This study demonstrated that the canning process of *A. bisporus* mushroom at various F_0-values and different sterilization temperatures of 115, 121, and 130 ^{o}C resulted in changes in product quality and sensory attributes. Our findings showed that the canning process led to changes in the drained weight, colour, texture profile, and sensory attributes, which depended not only on F_0-value but also on retort temperature. At the same F_0-value (e.g., 10 minutes), we found that retorting the product at a higher temperature of 130 ^{o}C would provide benefits such as a lower browning rate, more sweetness, and also still provide an increase in the umami taste. Moreover, selecting the higher retort temperature (130 ^{o}C) also resulted in higher drained weight loss (0.99%), lower shear force, and lower BCI development. In addition, our findings showed the relationship between umami and browning colour trends, both of which are suspected to be linked to the Maillard reaction. The use of a higher temperature and shorter time is more favourable compared to that of lower temperature and longer time of retorting with respect to the physical and sensory properties.

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Impact of Different Drying Methods on Nutritional, Colour Change, Solubility and Microbial Count of Selected Herbal Plant Powders

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Abstract

The research aimed to study the effect of drying processes (spray and freeze drying), and feed concentrations (80%, 65% and 50% of plant in water, w/w) on physicochemical and microbiological characteristics of star gooseberry (*Sauropus androgynus*), ceylon spinach (*Basella alba*), and cowslip creeper (*Telosma minor*). After drying, the powder recovery of herbal plant powders was up to 77.47%. The aw and moisture content of spray-dried powder (SDP) were lower than that of freeze-dried powder (FDP). The drying method did not significantly affect nutritional values of both powders, whereas the feed concentrations markedly affected the nutritional values of the powders. The fibre and fat contents of powder prepared from 80% feed concentration had the highest values ($p<0.05$). The dried star gooseberry powder was rich in protein (13.01-16.81%) and fibre (5.03-5.52%). The colour of FDP represented a smaller change than that of SDP. The dried powders prepared by 80% showed the highest solubility, up to 85.44%. The microbial counts of SDP were lower than that of FDP. The colour might well have been preserved by freeze drying, whereas a low moisture and microbial count were likely due to the spray drying.

Keywords: Spray drying; Freeze drying; Feed concentration; Star gooseberry; Ceylon spinach; Cowslip creeper

1 Introduction

In Southeast Asia, there are many species of herbal plants that have been used for dietary and medicinal purposes since ancient times. Some of these plants, e.g. star gooseberry (*Sauropus androgynus*), ceylon spinach (*Basella alba*), and cowslip creeper (*Telosma minor*), are types of tropical herb which have also been used extensively as ingredients for cooking and alternative treatments of various diseases, such as genito-urinary diseases, cardiovascular diseases, and cancers (Adhikari et al., 2012; Khoo et al., 2015). These herbal plants are generally grown by small-scale production units and with home-grown vegetables around the region. *S. androgynus* is an alternate single leaf plant with a dark green leaf, having a high nutritional value, especially high in dietary protein, fibre, carbohydrates and vitamin C. It also has high antioxidant activity as it contains containing phenolic compounds that could be used for medicinal, and colouring agents in foods. The secondary metabolites include phenolic compounds such as phenolic acids, tannin,

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Nomenclature

SDP	Spray-dried powder	FDP	Freeze-dried powder

lignin, flavonoids, coumarins, and stilbenes, terpenes such as plant volatile, carotenoids, sterol, and nitrogen-containing compounds such as alkaloids and glucosinolates (Bunawan et al., 2015; Chaimat et al., 2007; Gireesh et al., 2013; Wang & Lee, 1997). The extract of *S. androgynus* consisted of various phytochemicals, and vitamin C was found as major component (Bose et al., 2018), exhibiting antimicrobial, antibiofilm, antipathogenic, and antifungal activities (Kusumanegara et al., 2017; Kuttinath et al., 2019). *B. alba* is a single-herbaceous creeper with thick, soft leaves and sticky mucus, and is used as an herbal medicine due to it being a good source of calcium, iron, vitamin A and C, as well as being rich in phenolics, peptides, and mucus polysaccharides, and is known as a vegetable that contains mucilage (Chatchawal & Nualkaew, 2009; Jaichuen & Samutsri, 2014). The leaves of *B. alba* are very low in fat, but high in phytonutrients, including enzymic and non-enzymic antioxidants, that show potential uses as antioxidants, antibacterials, anti-inflammatories, nephroprotective and giving wound healing properties (Singh et al., 2016).

The major phytonutrient presented in *B. alba* is flavonoid such as kaempherol (Adhikari et al., 2012; Yang et al., 2008), and its mucilage extract is composed of polysaccharide with D-galactose that can be used as a cosmetic and for treatment of skin diseases. Some amino acids such as arginine, leucine, isoleucine, lysine, threonine and tryptophan are also found in *B. alba* leaves (Adhikari et al., 2012; Murakami et al., 2001). *T. minor* has clustered flowers in bunches or axillary buds and yellow petals;, it is fragrant and sweet and its extract has the ability to be an excellent antioxidant (Kongchantree, 2011), as a radical scavenger and an inhibitor of lipid peroxidation (Teerarak et al., 2018) due to a high content of phenolic compounds and flavonoids. Its extract also showed anti-microbial properties against pathogenic bacteria (Krasaekoopt & Kongkarnchanatip, 2005).

Therefore, these herbal plants have a potential to be developed into health food products and become a valuable marketed product.

Drying is an important process to extend the shelf-life for preserving food. The drying process could convert the food solutions into dry solid form (Ratti, 2001). Spray drying is one of the most widely used for drying due to a short contact time for materials exposing to high temperature. It extensively used to preserve juices as high quality powder by spraying the feed into a hot drying chamber. In general, the spray-dried powder (SPD) retains high nutritional values, low water activity and reduced weight, resulting in easy storage and transportation, and its reconstituted form gives a fresh-like original juice (Shishir & Chen, 2017; Sonia et al., 2015; Tontul & Topuz, 2017). Freeze drying is non-thermal method that water in solid form is sublimated under vacuum at low temperature, resulting a porous structure in the dried product. This drying method is slower and with higher costs compared to spray drying (Guiné, 2018), whereas it was found to be the best method retaining superior functional properties (Lili et al., 2015) and high phytonutrient content (Agudelo et al., 2017; Ghirisan & Miclaus, 2017). After freeze drying, the original colour, taste and shape of the sample are maintained, but the texture becomes crisp, spongy and soft. The structural, physical, functional and nutraceutical of freeze-dried (FDP) powder are dependent on the feed and process condition (Valentina et al., 2016). Also, the microbiological quality depends on the initial quality of the fresh vegetable by growing, harvesting, transportation and market shelf, as well as the treatment methods before consumption. Therefore, the current study aimed to contribute to

better understanding of the effect of spray drying and freeze drying on the colour, solubility, nutritional and microbiological characteristics of selected herbal plants with the intent to use them as dietary supplements.

2 Materials and Methods

2.1 Materials and sample preparation

Star gooseberry (*S. androgynus*) leaves, ceylon spinach (*B. alba*) leaves, and cowslip creeper (*T. minor*) flowers were obtained from a local market in Thailand. They were immediately processed by water cleaning for 5 min to remove undesirable residues from their surfaces, draining for 5 min, and slicing into small pieces. After that, each of the plant species was mixed with water in proportions of 80%, 65% and 50% in a blender for 5 min, filtered through a stainless-steel sieve (mesh size 63 μm) to provide a feed solution at various feed concentrations (80%, 65% and 50%) for further drying.

2.2 Spray drying process

Prior to the spray drying operation, water was pumped into the spray dryer (Buchi, Mini Spray Dryer B-290) to adjust the inlet and outlet air temperatures for 30 min before the feed was introduced through feed pipe. The prepared feed solutions were spray-dried using a nozzle under an inlet temperature of 180 ^{o}C and a constant feed rate of 8 mL/min to obtain the SDP. The powder was sieved thought a 250 μm screen (Retsch, Germany), and immediately sealed in aluminium foil at -18 ^{o}C.

2.3 Freeze drying process

The samples were completely frozen at -18 ^{o}C and then freeze-dried in a laboratory-scale freeze dryer (Christ, Gamma 2-16LSC) equipped with a round bottom freeze drying flask, under vacuum condition at a pressure of 0.01 mbar and condenser temperature of -50 ^{o}C for 48 h at ambient temperature.

After that, the FDP was sieved thought a 250 μm screen (Retsch, Germany), sealed in aluminium foil to prevent water uptake, and stored at -18 ^{o}C.

2.4 Determinations of nutritional values

Proximate analysis

Proximate analysis of the samples, including moisture (AOAC Official Method 934.06), protein (AOAC Official Method 991.20), fat (AOAC Official Method 991.36), fibre (AOAC Official Method 962.09), and ash (AOAC Official Method 900.02) was carried out according to standard methods (AOAC, 2000).
The carbohydrate content was determined as the difference between 100 and the sum of the percentages of moisture, protein, fat, fibre and ash.

Vitamin C

Vitamin C (ascorbic acid) of the dried powder was estimated by titration of sample with 2,6-dichlorophenolindophenol (adapted method from (Benazzouz et al., 2020). Each determination was done in triplicate. Standard ascorbic acid solution was prepared and then vitamin C content was calculated equivalent to the amount of ascorbic acid (mg/100 g).

2.5 Determinations of physical properties

Powder recovery

The percentage of powder recovery of the dried sample was calculated by the weight of obtained dried powder divided by the total soluble solids and multiplied by 100. Each determination was done in triplicate.

Water activity

Water activity (a_w) of the samples was determined at 25 ^{o}C using a water activity meter (Aqualab 4TEV). Each determination was done in triplicate.

Total colour change

Colour parameters (Hunter L*, a* and b* values) of the samples were measured and repeated 3 times using a spectrophotometer-colourimeter (HunterLab, UltraScan VISs/n: USVIS 1406). Total colour change was then computed using Equation (1) (Ferrão et al., 2019; Maskan, 2006).

$$TCD = \sqrt{(L_0 - L)^2 + (a_0 - a)^2 + (b_0 - b)^2} \quad (1)$$

Equation (1) means L_0^*, a_0^*, and b_0^* denoted as the colour parameters of the original plants, and L*, a* and b* mean the colour parameters of the dried sample.

Water solubility

For water solubility determination, 1 g of the dried sample was weighed into a centrifuge tube, 10 mL of distilled water was added, and homogeneously mixed. Afterwards, the mixture was then incubated in a water bath (NH 03801, USA) at 37 ^{o}C for 30 min, and centrifuged at 3,000 rpm for 10 min using a centrifuge (2-16PK, Gerhardt, Germany). The residue was dried at 105 ^{o}C for 3 h. The water solubility was computed from Equation (2) (Que et al., 2008).

$$Solubility(\%) = \frac{Residue\ weight\ (g)}{Sample\ weight\ (g)} \times 100 \quad (2)$$

2.6 Determination of microbiological properties

The total plate count (AOAC Official Method 990.12), and yeast and mold (AOAC Official Method 2014.05) of the samples were determined using the pour plate technique as standard methods (AOAC, 1990). The number of microorganisms was expressed as colony-forming units per 1 g of sample (CFU/g). Duplicates were done for each dilution.

2.7 Statistical analysis

Significant differences among means of all treatments were subjected to analysis of variance (ANOVA) by new Duncan's multiple range test; the confidence limits used in this study were based on 95% ($p \leq 0.05$).

3 Results and Discussion

3.1 Effect of feed concentration on powder recovery of spray-dried and freeze-dried powders

The powder recovery or process yield, which is an important factor relating to production cost and efficiency of drying process, of the dried star gooseberry (*S. androgynus*), ceylon spinach (*B. alba*), and cowslip creeper (*T. minor*) is shown in Figure 1. It was found that the feed concentrations significantly affected the powder recovery. At the high feed concentration (80%), the powder recovery of all samples was higher than that of the powders obtained from the feed concentration at 50% and 65%, respectively. It was due to the higher feed concentration giving a higher solids content in feed, and therefore the solids in feed solution that became the powders after drying. The powder recovery of the FDP was significantly higher than those of SDP. Some dried powders stuck in the spray drying chamber, which caused a loss of powder. The results were in agreement with the results of Santo et al. (2013).

In addition, a higher feed concentration of plant resulted in increased powder recovery due to the entire soluble solid of fresh plants. The powder recovery of the dried ceylon spinach (*B. alba*), and star gooseberry (*S. androgynus*) was 69.63-77.47%, while the powder recovery of the dried cowslip creeper (*T. minor*) was 50.79-58.41%. However, the powder recovery of all dried samples was more than 50%, showing the successful drying in the laboratory-type drier (Bhandari et al., 1997).

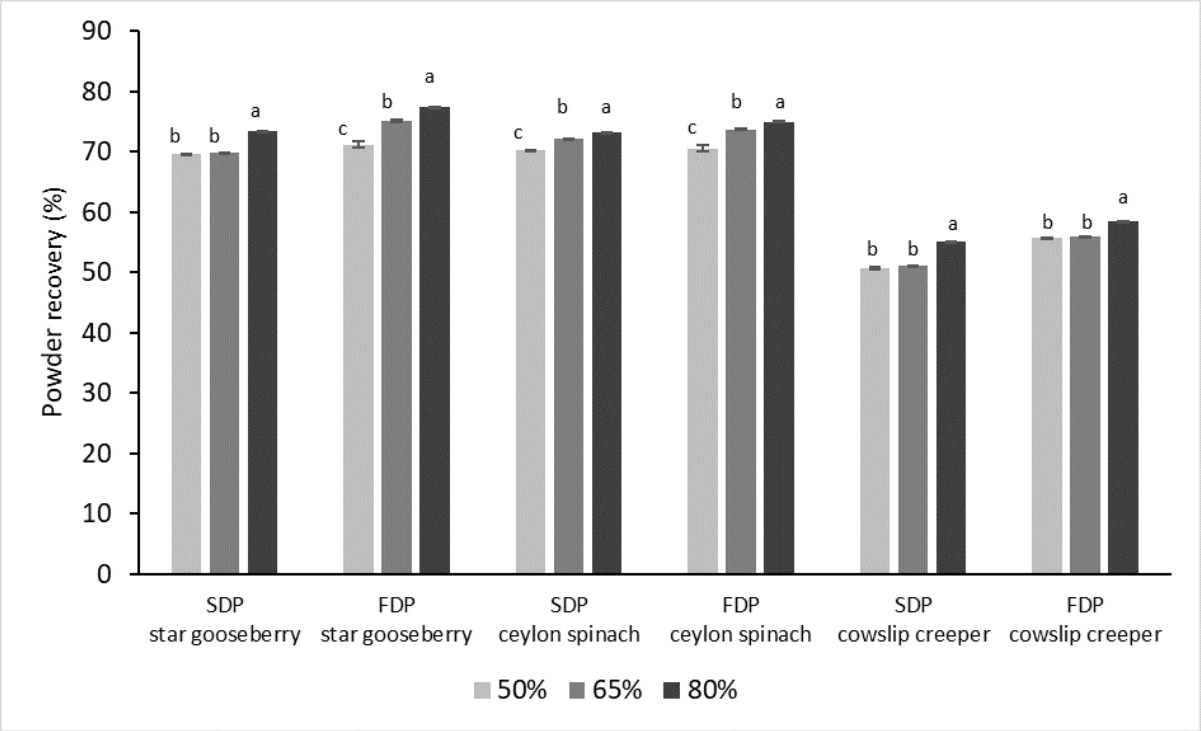

Figure 1: Percentage of powder recovery of selected herbal plants after spray and freeze drying at various plant feed concentrations of 50%, 65% and 80%. The bars with the same letter for each group were not statistically different ($p>0.05$)

3.2 Effect of feed concentration on nutritional values of spray-dried and freeze-dried powders

After drying, the moisture of some selected plant powders was much less compared to the fresh plants from 77.56-92.34% down to 3.17-5.85% (Table 1). The moisture content of FDP was higher than that of SDP ($p\leq0.05$) because of a high temperature during spray drying. Santo et al. (2013) also concluded that higher moisture contents were achieved with freeze drying comparative to spray drying. However, the moisture of the powder might have increased during storage due to hygroscopicity, which is the ability of materials to absorb moisture when exposed to the environment. The lower hygroscopicity, with the addition of maltodextrin into the feed, caused the stickiness and coagulation of the powders (Chen et al., 2014).

The nutritional values of dried star gooseberry (*S. androgynus*), ceylon spinach (*B. alba*), and cowslip creeper (*T. minor*) powders are shown in Table 1. The feed concentrations affected the contents of ash and carbohydrate of *B. alba* and *T. minor* dried samples. An increased of feed concentration led to an increase of ash content and a decreased of carbohydrate of the samples. Moreover, the feed concentration also had an effect on protein and fibre contents of *S. androgynus* dried sample. The dried star gooseberry (*S. androgynus*) contained high protein (13.01-18.61%) and fibre (5.03-5.52%) due to its original source, suggesting a potential protein and fibre source of plant-based powder. The vitamin C contents of SDP were higher than that of FDP because of a short processing time of spray drying compared to freeze drying (including freezing

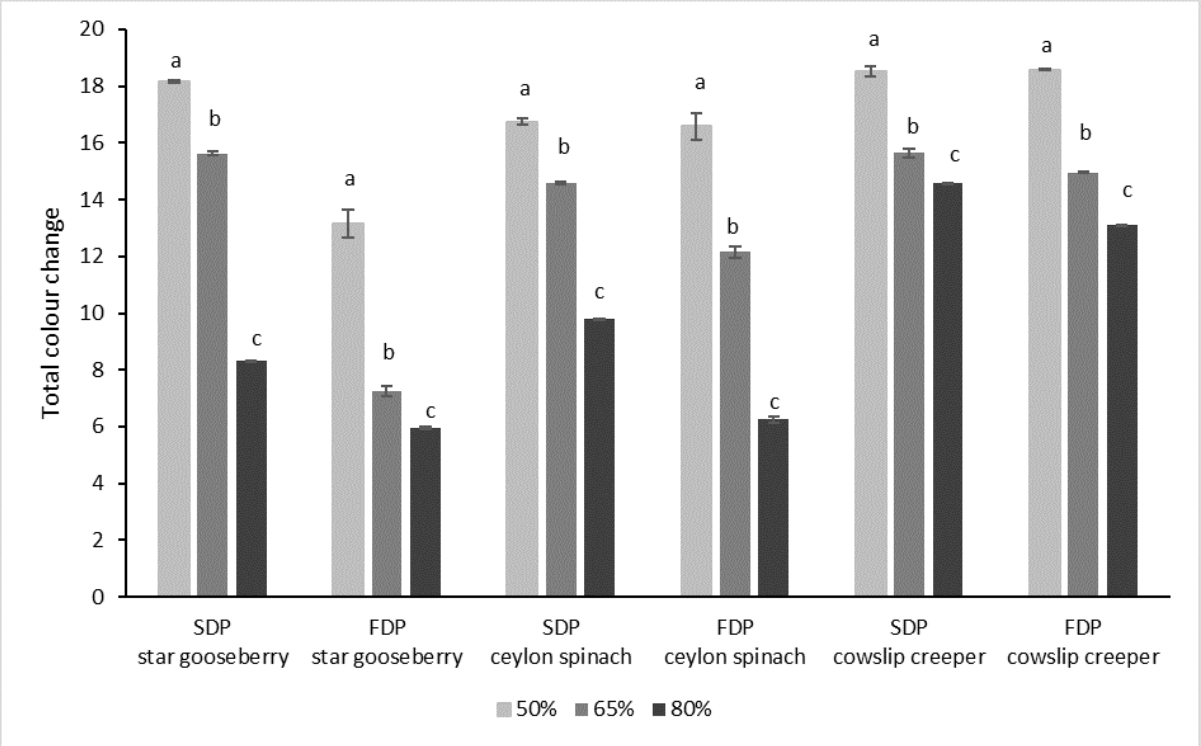

Figure 2: Total colour change of spray-dried and freeze-dried powders at various plant feed concentrations of 50%, 65% and 80%. The bars with the same letter for each group were not statistically different ($p>0.05$)

and drying process times). It has been reported that spray drying techniques could be used to preserve natural juice in a powder form as a potential source of nutritional values (Badmus et al., 2016), and freeze drying should also preserve the nutrients because of a short exposure of nutrients to oxygen, which resulted in fewer oxidation/degradation reactions (Silva-Espinoza et al., 2019).

3.3 Effect of feed concentration on physical properties of spray-dried and freeze-dried powders

Figure 2 illustrates the total colour change of SDP and FDP, showing the value in the range between 5.96 and 18.58 compared to the original samples, and showing a clear difference colour of the dried powder in comparison to the fresh plant. It has been reported that a larger value of total colour change denotes greater colour change from the reference material (fresh sample). A typical scale for evaluation of the colour difference is as follows: total colour change value in the range between 0.0 and 2.0 corresponds to unrecognizable differences, in the range between 2.0 and 3.5 corresponds to differences possible to recognize by an experienced observer, and over 3.5 corresponds to clear differences of colour (Ferrão et al., 2019). In the study, a high concentration of plant (80%) led to a small total colour change value, showing a fresh-colour similar to the original feed, followed by the 65% and 50% of plant, respectively. Moreover, the colour of FDP was greener than the SDP powder, showing lower total colour change value compared to SDP. Freeze

Table 1: Nutritional values (wet basis) of spray-dried and freeze-dried powders at various plant feed concentrations (50%, 65% and 80%) compared to the fresh plant

Plant sample	Nutritional values (%)						Vitamin C
	Moisture	Protein	Fibre	Ash	Fat	Carbohydrate	(mg/100 g)
Star gooseberry (*Sauropus androgynus*)							
Fresh	77.56+1.25^{a}	8.25±0.03^{e}	3.46±0.02^{f}	1.87±0.02^{d}	0.19±0.01^{d}	8.67±0.15^{f}	9.04±0.02^{c}
SDP (50%)	3.75±0.02^{d}	13.25±0.01^{c}	5.03±0.01^{e}	9.83±0.01^{c}	0.58±0.02^{c}	67.56±0.01^{a}	8.95±0.02^{d}
SDP (65%)	3.74±0.05^{d}	14.32±0.02^{b}	5.38±0.04^{c}	10.36±0.03^{b}	0.56±0.02^{c}	65.64±0.04^{c}	11.84±0.03^{b}
SDP (80%)	3.47±0.07^{e}	16.81±0.01^{a}	5.52±0.02^{a}	12.07±0.03^{a}	0.64±0.02^{a}	61.49±0.06^{e}	15.72±0.01^{a}
FDP (50%)	5.85±0.03^{b}	13.01±0.06^{c}	5.08±0.01^{d}	9.30±0.02^{c}	0.59±0.01^{c}	66.17±0.02^{b}	7.67±0.02^{f}
FDP (65%)	5.75±0.03^{b}	14.52±0.02^{b}	5.31±0.03^{c}	10.36±0.01^{b}	0.61±0.01^{b}	63.45±0.02^{d}	7.67±0.01^{f}
FDP (80%)	5.54±0.01^{c}	16.42±0.01^{a}	5.48±0.01^{b}	11.12±0.02^{a}	0.63±0.02^{a}	60.81±0.04^{e}	7.83±0.06^{e}
Ceylon spinach (*Basella alba*)							
Fresh	92.34±1.13^{a}	2.12±0.02^{e}	0.84±0.01^{g}	1.56±0.01^{g}	0.23±0.01^{f}	2.91±0.06^{g}	5.16±0.02^{c}
SDP (50%)	5.58±0.01^{b}	4.37±0.01^{c}	3.17±0.03^{f}	9.56±0.02^{e}	1.12±0.01^{d}	76.20±0.02^{b}	3.41±0.01^{e}
SDP (65%)	5.25±0.02^{d}	4.49±0.02^{b}	3.41±0.02^{d}	12.68±0.02^{c}	1.14±0.01^{c}	73.03±0.01^{d}	5.87±0.00^{b}
SDP (80%)	5.15±0.01^{e}	4.68±0.02^{a}	3.84±0.02^{b}	15.72±0.01^{b}	1.25±0.02^{b}	69.36±0.02^{e}	7.73±0.03^{a}
FDP (50%)	5.46±0.02^{c}	4.31±0.01^{d}	3.27±0.02^{e}	9.06±0.01^{f}	1.06±0.01^{e}	76.84±0.01^{a}	3.13±0.06^{f}
FDP (65%)	5.42±0.02^{c}	4.32±0.01^{d}	3.52±0.01^{c}	12.05±0.03^{d}	1.15±0.02^{c}	73.54±0.02^{c}	3.97±0.06^{d}
FDP (80%)	5.10±0.02^{e}	4.31±0.01^{d}	3.97±0.01^{a}	16.34±0.02^{a}	1.31±0.01^{a}	68.97±0.01^{f}	4.00±0.10^{d}
Cowslip creeper (*Telosma minor*)							
Fresh	81.54±1.08^{a}	5.08±0.03^{e}	0.76±0.02^{f}	2.12±0.02^{e}	1.17±0.01^{f}	9.33±0.31^{f}	7.58±0.03^{b}
SDP (50%)	3.21±0.00^{a}	8.35±0.03^{c}	4.06±0.01^{d}	9.22±0.00^{d}	3.24±0.01^{e}	71.92±0.02^{a}	4.38±0.02^{d}
SDP (65%)	3.20±0.02^{a}	8.42±0.01^{b}	4.28±0.02^{b}	10.47±0.02^{c}	3.64±0.02^{b}	69.99±0.01^{b}	7.15±0.04^{c}
SDP (80%)	3.17±0.02^{a}	8.58±0.02^{a}	4.33±0.02^{a}	13.26±0.01^{a}	3.91±0.02^{a}	66.75±0.02^{d}	9.26±0.02^{a}
FDP (50%)	5.82±0.02^{b}	8.31±0.01^{d}	4.01±0.01^{e}	10.43±0.02^{c}	3.32±0.01^{d}	68.11±0.01^{c}	3.07±0.06^{g}
FDP (65%)	5.69±0.01^{e}	8.31±0.01^{d}	4.21±0.01^{c}	11.50±0.01^{b}	3.45±0.02^{c}	66.84±0.01^{d}	3.87±0.06^{f}
FDP (80%)	5.39±0.02^{g}	8.33±0.01^{cd}	4.31±0.02^{a}	13.25±0.03^{a}	3.87±0.02^{a}	64.85±0.02^{e}	4.03±0.06^{e}

Data within column for each plant species followed by the same letter were not statistically different (p>0.05)

drying then seemed to be a suitable drying technique for preserving the colour of dried product (Liaotrakoon et al., 2012; Santo et al., 2013). It is because of non-thermal processing, therefore the losses of colour, flavour and volatile components were minimal, resulting in a stable in colour and flavour of the dried product (dos Santos et al., 2018).

The a_w of SDP and FDP herbal plants is shown in Table 2. An increase plant concentration led to a decrease of a_w of dried powders, and also found that the SDP and FDP of all plant species at the high plant concentration (80%) provided the lowest a_w value ($p \leq 0.05$), followed by the 65% and 50% of plant, respectively. A low a_w (0.19-0.37) of the studied samples could inhibit the growth of microbes of the dried product. However, the study also found that the a_w value of SDP was significantly lower than that of FDP ($p \leq 0.05$).

It was found that when the feed concentration of the samples was increased, the solubility values of FDP and SDP of the dried plants also increased (Table 2). Solubility of all plant species at the high plant concentration (80%) was higher than that of the plant concentration of 65% and 50%, respectively ($p \leq 0.05$). The solubility values of both FDP and SDP *B. alba* and *T. minor* were comparable in value, while the solubility values of FDP *S. androgynus* was higher than that of SDP *S. androgynus* ($p \leq 0.05$). The dried star gooseberry (*S. androgynus*) powder had a higher solubility (70.73-85.44%) than the others (47.96-65.07%). On the other hand, the solubility of SDP lime powder was 59.54-76.84% (Chuacharoen, 2017).

Table 2: Physical properties of spray-dried and freeze-dried powders at various plant feed concentrations of 50%, 65% and 80%.

Plant powders	$\mathbf{a}_w$	**Water solubility (%)**
Star gooseberry (*Sauropus androgynus*)		
SDP (50%)	0.32 ± 0.00^b	70.73 ± 0.21^e
SDP (65%)	0.24 ± 0.02^c	77.28 ± 0.11^d
SDP (80%)	0.21 ± 0.02^d	82.65 ± 0.13^b
FDP (50%)	0.37 ± 0.00^a	71.20 ± 0.50^e
FDP (65%)	0.36 ± 0.01^a	79.76 ± 0.26^c
FDP (80%)	0.31 ± 0.00^b	85.44 ± 0.20^a
Ceylon spinach (*Basella alba*)		
SDP (50%)	0.23 ± 0.01^c	51.85 ± 0.15^c
SDP (65%)	0.24 ± 0.00^c	58.24 ± 0.10^b
SDP (80%)	0.21 ± 0.01^d	64.51 ± 0.22^a
FDP (50%)	0.31 ± 0.01^a	52.61 ± 0.41^c
FDP (65%)	0.26 ± 0.02^b	59.08 ± 0.35^b
FDP (80%)	0.21 ± 0.01^d	65.07 ± 0.30^a
Cowslip creeper (*Telosma minor*)		
SDP (50%)	0.29 ± 0.00^b	48.36 ± 0.12^b
SDP (65%)	0.21 ± 0.00^d	48.95 ± 0.15^b
SDP (80%)	0.19 ± 0.00^e	51.20 ± 0.24^a
FDP (50%)	0.31 ± 0.01^a	47.96 ± 0.10^b
FDP (65%)	0.25 ± 0.00^c	48.47 ± 0.15^b
FDP (80%)	0.20 ± 0.01^d	50.00 ± 0.22^a

Data within column for each plant species followed by the same letter were not statistically different ($p>0.05$)

3.4 Effect of feed concentration on microbial counts of spray-dried and freeze-dried powders

A decrease of microbial counts clearly showed in dried powders compared to the fresh plants, especially for total plate count (reduced from 10^5-10^6 (fresh) to 10^2 (powder) CFU/g) due to a drying processing (Table 3). The total plate count of both SDP and FDP was found to be less than $3x10^2$ CFU/g with a range between $1.3x10^2$ and $2.7x10^2$ CFU/g, and yeast and mold of the samples was less than 10 CFU/g.

The feed concentration tended to have no effect on microbiology properties of the dried samples, while the SDP seemed to have lower microbial counts compared to the FDP. The results were found to relate to the lower aw values (Table 2) and moisture contents (Table 1) of the SDP samples compared to the FDP. This was because the SDP was dried under a high temperature (180 ^oC) with a short contact time, which caused a reduction in the number of microbes. The freeze drying also produces a very low moisture product that can prevent the growth of microorganisms, including bacteria, yeasts and molds. However, dos Santos et al. (2018) reported that freeze drying preserves the microbiological and sensory characteristics of yoghurt when compared to spray drying.

Table 3: Microbiology properties of spray-dried and freeze-dried powders at various plant feed concentrations of 50%, 65% and 80% compared to the fresh plant

Plant powders	**Total plate count (CFU/g)**		**Yeast and mold (CFU/g)**	
	SDP	**FDP**	**SDP**	**FDP**
Star gooseberry (*Sauropus androgynus*)				
Fresh	$2.7x10^5$		≤ 10	
50%	$1.3x10^2$	$1.6x10^2$	≤ 10	≤ 10
65%	$1.6x10^2$	$1.7x10^2$	≤ 10	≤ 10
80%	$1.6x10^2$	$1.9x10^2$	≤ 10	≤ 10
Ceylon spinach (*Basella alba*)				
Fresh	$3.2x10^5$		≤ 10	
50%	$1.5x10^2$	$2.7x10^2$	≤ 10	≤ 10
65%	$1.6x10^2$	$2.5x10^2$	≤ 10	≤ 10
80%	$1.7x10^2$	$2.5x10^2$	≤ 10	≤ 10
Cowslip creeper (*Telosma minor*)				
Fresh	$1.5x10^6$		≤ 10	
50%	$1.3x10^2$	$1.5x10^2$	≤ 10	≤ 10
65%	$1.3x10^2$	$2.0x10^2$	≤ 10	≤ 10
80%	$2.4x10^2$	$2.4x10^2$	≤ 10	≤ 10

4 Conclusions

The drying processes (spray drying and freeze drying) and feed concentrations of selected plant (80%, 65% and 50%, w/w) significantly affected the colour change, solubility, nutritional and microbiological characteristics of dried star gooseberry (*S. androgynus*), ceylon spinach (*B. alba*), and cowslip creeper (*T. minor*). The powder recovery was 50.79-77.47%, and the total colour change value of plant powders at a high feed concentration (80%) was the lowest value, followed by the plant concentration of 65% and 50%, respectively, and it was found that the colour change of FDP was lower than that of the SDP ($p<0.05$). At high feed concentration, the solubility of plant powders was significantly increased. The SDP provided lower a_w and moisture content than that of FDP ($p<0.05$).

A potential source of protein and fibre were found in the dried star gooseberry (*S. androgynus*) powder. The microbial counts of both SDP and FDP were <300 CFU/g of total plate count, and <10 CFU/g of yeasts and molds.

According to the main results, it could be concluded that the high ratio of feed plant (80%) is suggested to prepare samples prior to drying, and that stickiness may occur during the spray drying process. Freeze drying tended to preserve the colour of the powder, whereas spray drying seemed to reduce the moisture, aw and microbial count compared to the other one. Therefore, the dried selected herbal plants could be considered as an excellent source of nutrition, and also the FDP of star gooseberry (*S. androgynus*) and ceylon spinach (*B. alba*) could be suggested to be a (green) natural colouring.

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Optimization of Bromelain Isolation from Honi Pineapple Crown

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Abstract

Bromelain is a proteolytic enzyme that can be found in all parts of pineapple plant varieties. Pineapple crown (PC) has higher bromelain activity than other pineapple wastes such as peels and leaves. This study isolated bromelain from one variety of PC, namely Honi, and determined the optimum drying temperature as well as concentration of ammonium sulphate to obtain the best bromelain characteristics such as protein content, unit activity and specific activity. Honi PC was dried at various drying temperatures (35, 40, 45, 50, and 55 ^{o}C), and then extracted and purified using ammonium sulphate in various concentrations (20, 40, 60, and 80%), in order, to get bromelain. Furthermore, the yield of isolated bromelain was calculated and the protein content, unit activity and specific activity of bromelain characterised. The highest yield of crude bromelain (CB) was achieved at 35 ^{o}C. However, the highest protein level, unit activity and specific activity of CB were achieved at 55 ^{o}C ($p < 0.05$). Purification of CB using concentrations of ammonium sulphate in the range 40 to 80% resulted in a higher protein level ($p < 0.05$). The highest unit activity and specific activity of bromelain were achieved at a 60% concentration of ammonium sulphate ($p < 0.05$). 55 ^{o}C and 60% were the optimum drying temperature and concentration of ammonium sulphate respectively to achieve the best characteristics (2.16% protein level, 1.61 U/mL unit activity and 0.75 U/mg specific activity) for bromelain isolated from Honi PC. Honi PC isolated bromelain was shown to inhibit the browning reaction on apple fruits. The agroindustry waste product, Honi PC, has potential as a future alternative bromelain source.

Keywords: Optimum; Drying temperature; Characteristics; Bromelain; Pineapple crown

1 Introduction

There are several varieties of popular pineapple in the Indonesian fruit market such as Smooth Cayenne, Queen, and Honi (Yadi et al., 2020). Honi, which is a new variant of pineapple developed and introduced to the public in 2012 by Sunpride Research and Development located at East Lampung ("Honi, The Featured Pineapple," 2021), is the most popular variety. Lampung is a province in Sumatra, an island of Western Indonesia. People prefer Honi because of the sweeter taste and the fruit is fleshier. Genetically, Honi is a specific strain arising from the crossbreeding of two common strains, namely Smooth Cayenne and Queen ("9 Reasons You

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Should Try Honi Pineapple," 2020).
Nowadays, the total production of Honi pineapple in Indonesia is 1.39 million tons per year ("Top Pineapple Producing Countries," 2018). The high public consumption of pineapple results in abundant waste which is mostly pineapple crown (PC). The crown occupies up to 35% of the total pineapple weight (Campos et al., 2020). So far, the dominant use of PC as a material is for making natural fertilizers and animal feed (Prado & Spinacé, 2019). PC contains cellulose and bioactive components such as antioxidants and proteolytic enzymes, including bromelain (). PC contains lower levels of the bromelain enzyme than pineapple core and flesh. However, compared to other pineapple wastes such as peels and leaves, PC has higher bromelain activity and protein content (Manzoor et al., 2016). In the food Industry, bromelain is used for meat tenderisation, beer clarification, baking cookies, protein hydrolysate production and grain protein solubilisation, and as an anti-browning agent (Mohan et al., 2016). Stability of bromelain is influenced by temperature (40 to 65 ^{o}C) and pH (3.0 to 3.6). The optimum temperature for effectiveness of bromelain is between 50 and 60 ^{o}C (Sarkar et al., 2017).
In general, isolation of a proteolytic enzyme from several parts of pineapple involves a thermal drying process which can affect the yield and characteristics (Kurnia et al., 2018). Isolation and characterisation of bromelain from two PC strains (Morris and N36) has been carried out successfully without a drying stage (). The extraction of proteolytic enzymes from plant material, involving a thermal drying process, resulted in varying values of proteolytic activity and protein content (). In a previous study, 55 ^{o}C was the optimum drying temperature of Cayenne PC for CB production as a meat tenderising agent (Rizqiati et al., 2021).
In this study Honi PC was dried at various temperatures, CB was extracted and the bromelain resulting from a purification stage was characterised for protein content, unit activity and specific activity, and tested as an anti-browning agent on apple fruits. Utilisation of PC as a bromelain source can be expected to increase the added value of the pineapple agroindustry.

2 Materials and Methods

2.1 Preparation and Purification of Honi PC Powder

This research was conducted from July to December 2020 at the Food Chemistry and Nutrition Laboratory and the Food and Agricultural Products Engineering Laboratory, Diponegoro University, Semarang, Indonesia. Honi variety pineapple crown (HVPC) powder was produced by first cutting HVPC into small pieces, placing the crowns on a tray in a cabinet dryer (Maksindo), drying until the water content reached 8 to 10%, at 55 ^{o}C, 50 ^{o}C, 45 ^{o}C, 40 ^{o}C and 35 ^{o}C, and then crushing the dried crowns with a grinder (HR-40B, China) into a fine powder. The supernatant of the bromelain enzyme extract from HVPC was produced by dissolving 20 g of the finely ground crown powder sample in 180 ml of a sodium citrate cold buffer and stirring it until homogeneous. The mixture was filtered and the filtrate was centrifuged (DLab-DM0412 Clinical Centrifuge) at 4500 rpm for 25 minutes to separate the supernatant from the precipitate. The supernatant obtained was frozen at - 20 ^{o}C. Purification of the pineapple crown extract supernatant used a method reported by Hartesi et al. (2020), with modifications. Ammonium sulphate at concentrations of 20%, 40%, 60% and 80% was added to the enzyme solution and stirred until homogeneous. The resulting solution was stored at 4 ^{o}C, overnight or for 24 hours. The solution was centrifuged at a speed of 3500 rpm for 25 minutes. This solution formed a precipitate of the enzyme bromelain. The precipitate was dissolved using sodium citrate buffer, with a pH of 6.5, and homogenized for further dialysis, overnight or for 16 hours.

2.2 Experimental Analysis

Protein content of the crude extract of bromelain enzyme from pineapple crown was determined using the Lowry method (Febriani et al., 2017) with modifications. Bovine Serum Albumin (BSA) standards were prepared at several concentration points by mixing 0.5 ml of BSA with 5 ml of Lowry reagent, vortexing (vortex

mixer, TRSWIX VT02) and incubating for 10 minutes to let the protein binding reactions occur. 0.5 ml of Folin-Ciocalteu reagent was added to the solution and left to stand for 30 minutes. The absorbance of the solution was analysed using a UV-Vis spectrophotometer (Shimadzu UV-1601, Germany), with a wavelength of 650 nm, and a standard value curve of BSA was formed. The supernatant sample of bromelain enzyme extract from HVPC was first diluted 15 times. Then, 0.5 ml of the diluted supernatant was added to 5 ml of Lowry reagent, vortexed and incubated for 10 minutes. The sample solution was added to 0.5 ml of Folin-Ciocalteu reagent and allowed to stand for 30 minutes. The absorbance of the solution was read using a UV-Vis spectrophotometer with a wavelength of 650 nm. The protein content of the sample was determined by linear regression on the obtained BSA standard curve.

Enzyme unit activity testing was carried out using a method reported by Simamora and Sukmawati (2020), with modifications. The sample solution containing 0.5 ml of crude bromelain enzyme extract was diluted 15 times with 0.5 ml casein and 0.5 ml phosphate buffer (pH 6.6). The solution was then incubated using a water bath (Memmert WNB14, Germany) at 40 ^{o}C for 20 minutes. 1 ml of TCA (trichloroacetic acid) 10% was then added to the sample solution and incubated for 10 minutes at room temperature to stop the reaction. Samples were centrifuged at 5000 rpm for 10 minutes to separate the sediment. After obtaining a clear supernatant, the absorbance of the sample was read using a UV-Vis spectrophotometer with a wavelength of 275 nm. The standard solution was prepared in the same way except that the sample was replaced with a bromelain solution from bromelain tablets. Then the absorbance was read using a UV-Vis spectrophotometer with the same wavelength. The enzyme unit activity was calculated according to Sumardi et al. (2019).

The specific activity of the enzyme was calculated by dividing the unit activity (U ml^{-1}) with the protein content (mg ml^{-1}) (Kahiro et al., 2017).

Evaluation of the bromelain activity as an anti-browning agent was performed on apple fruits. Apple slices were soaked (1%, w/v) in Honi PC bromelain and the other natural anti-browning agents, namely onion juice, chili pepper juice, honey, and lemon. As a control, one slice of apple was immersed in distilled water. All treatments were incubated for 12 h at 25 ^{o}C, and the colour change on the surface of each apple slice was observed and recorded in a photograph.

2.3 Statistical Analysis

All data generated were analysed statistically using Analysis of Variance to determine the effect of the given treatment. Further analysis was performed using a Duncan Multiple Range Test to explain the significant difference between each treatment group.

3 Results and Discussion

Drying temperature (35 ^{o}C to 55 ^{o}C) for the Honi PC had a significant effect on the yield (Fig. 1). The highest yield was achieved at a drying temperature of 35 ^{o}C. Above 35 ^{o}C significantly less CB was produced ($p < 0.05$). A decrease in yield with increasing drying temperature was caused by a moisture factor. The higher temperature, the easier it is for water to be released from the materials' surface (Ramos et al., 2016). For dry powdered food products, the maximum water content is 10% so that microbes and fungi can't grow (Zambrano et al., 2019).

3.1 Characteristics of Crude Bromelain (CB)

Drying temperature for the Honi PC significantly influenced the CB characteristics of protein level, unit activity and specific activity (Fig. 2). The highest protein level, unit activity and specific activity of CB were achieved at 55 ^{o}C ($p < 0.05$). The protein content increased in line with increasing drying temperature of PC (Shu et al., 2016). The higher drying temperature caused a lower PC moisture content which resulted in a higher protein content. Extraction of bromelain from PC with high protein content resulted in CB with high protein content as well. Enzyme activity will continue to increase as drying temperature increases until the optimum drying

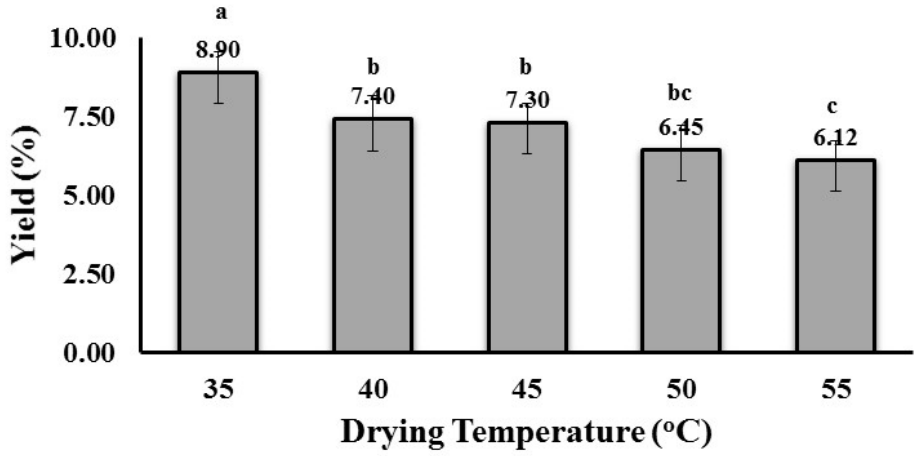

Figure 1: Yield of crude bromelain extract from Honi pineapple crown at various drying temperatures

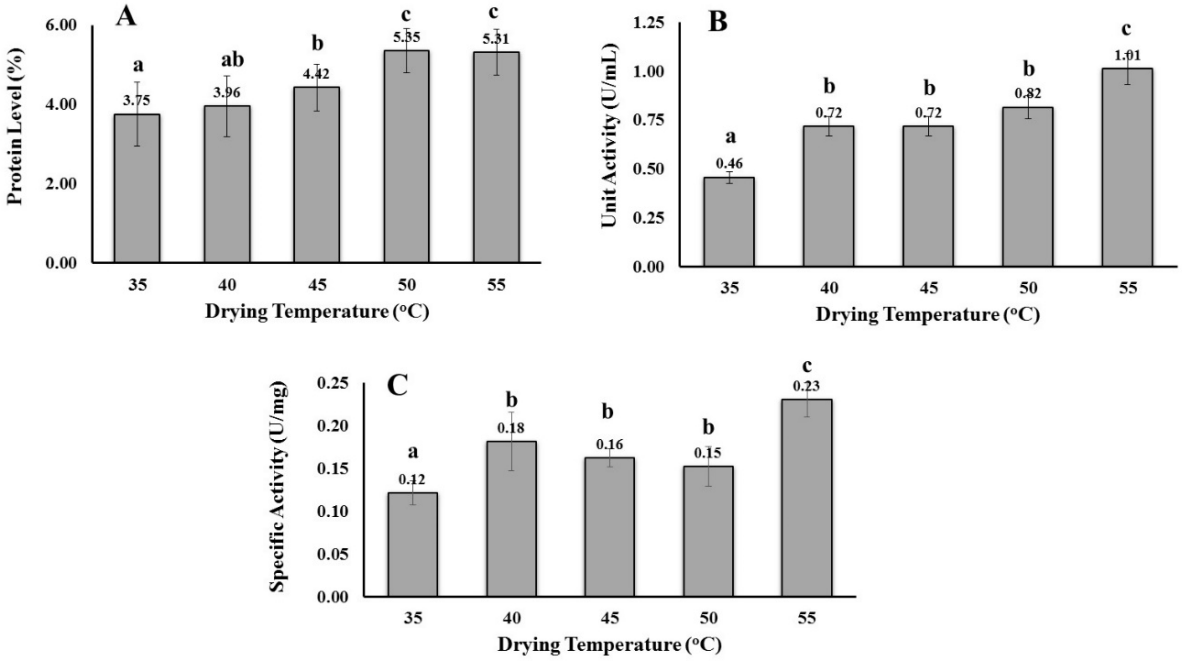

Figure 2: Protein content (A), unit activity (B) and specific activity (C) of crude bromelain extract from Honi pineapple crown at various drying temperatures

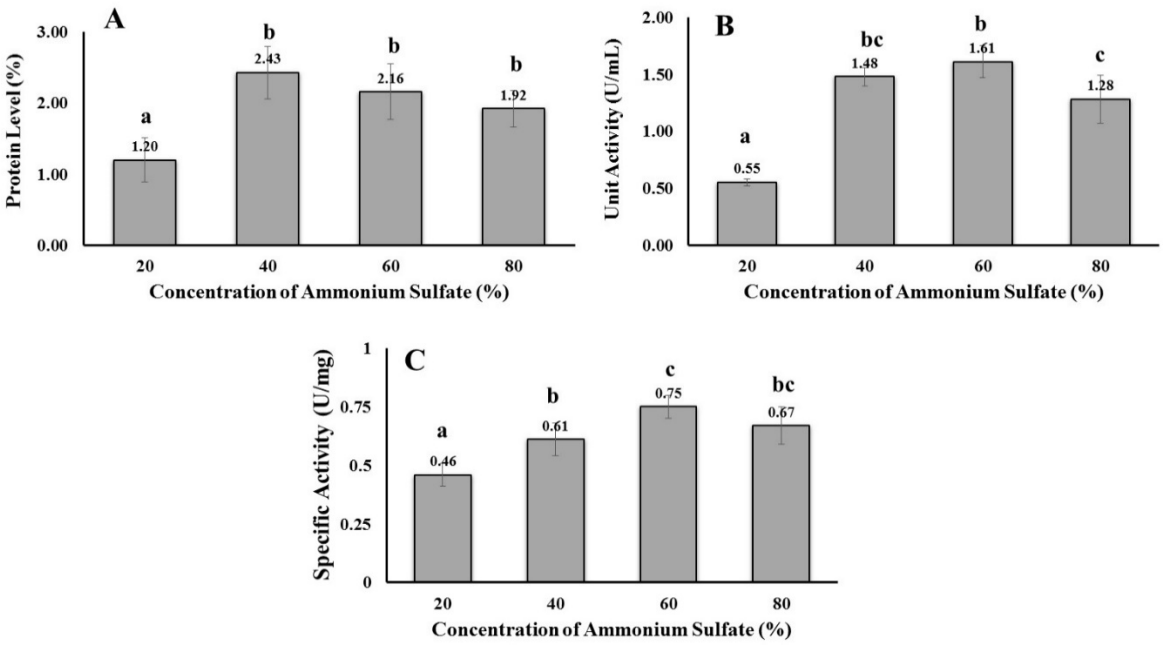

Figure 3: Effect of concentration of ammonium sulphate on the protein content (A), unit activity (B) and specific activity (C) of bromelain from Honi pineapple crown

temperature for isolation of the bromelain enzyme is reached between 50 ^{o}C and 60 ^{o}C. Above that drying temperature range, the activity of the bromelain enzyme will decrease (Manzoor et al., 2016). High temperatures can accelerate the reaction and activity of the enzyme but if it exceeds the optimum temperature, it will reduce the activity of the enzyme (Poba et al., 2019). The results of the higher unit activity value of the CB show the higher the number of enzymes that can actively catalyse protein breakdown reactions.

This specific activity value aims to determine the amount of bromelain enzyme present and is obtained from a comparison of protein levels with enzyme activity. Ramli et al. (2017) stated that the specific activity of the bromelain enzyme can show the amount of the existing bromelain enzyme expressed in U mg^{-1} which is the unit of enzyme activity per milligram of the total enzyme protein. The samples with a drying temperature of 55 ^{o}C showed the highest activity because temperature affects the work of the enzyme. Enzymes at low temperatures have reactions that tend to be slow and if the temperature is increased, the reaction will be faster and will be maximised if it reaches the optimum temperature. As the temperature increases to the optimum, there will be an increase in kinetic energy which will accelerate the motion of the enzymes and the substrate so that there is an increase in the intensity of the collision between the substrate and the enzyme which will facilitate the formation of the enzyme-substrate complex and increase product formation. If the temperature is too high the enzyme will denature, leading to a conformational change in the substrate, and the enzyme activity decreases (Shu et al., 2016). The protein content value will not always be directly proportional to the specific activity because the protein contained in the crude extract of the bromelain enzyme is not specific and there are still other proteins.

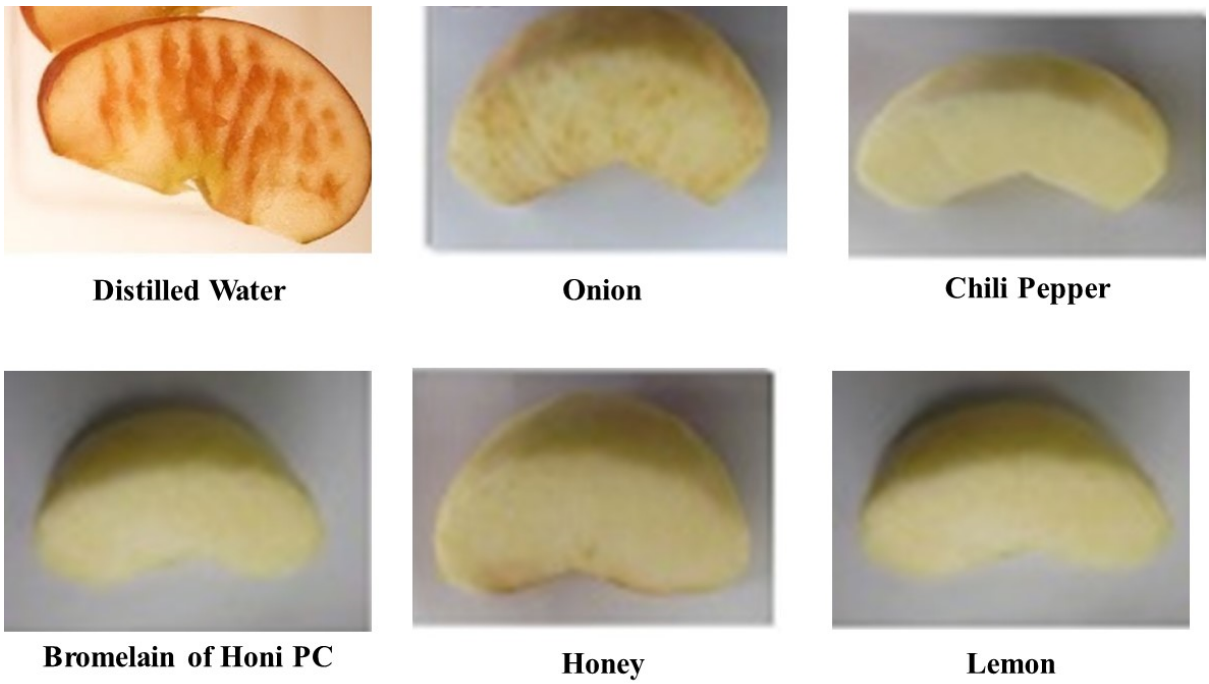

Figure 4: The anti-browning activity of bromelain from Honi PC compared to other natural anti-browning agents

3.2 Characteristics of Bromelain

Bromelain in this study was obtained using ammonium sulphate as a separating agent in the purification of crude bromelain extract. The best CB resulted from PC dried at 55 ^{o}C which was then purified using various concentrations of ammonium sulphate (20, 40, 60 and 80 %). The concentration of ammonium sulphate significantly influenced the bromelain characteristic of protein content, unit activity and specific activity (Fig. 3). 60% ammonium sulphate was determined as the optimum concentration to obtain bromelain with higher protein content, unit activity and specific activity ($p < 0.05$). The addition of the ammonium salt will cause the salt ions to compete with the protein to bind water molecules. As the solubility of salt ions is greater than protein, the protein in the enzyme will form clods and settle (Liliany et al., 2018). The 60% concentration of ammonium sulphate produced the highest unit and specific activity ($p < 0.05$). High activity of bromelain was produced by purification with 50 to 80% ammonium sulphate (Setiasih et al., 2018). The water of CB was bound to the ammonium sulphate salt without disturbing the existing protein nor disrupting the enzyme activity. All proteolytic enzyme proteins are optimally soluble and precipitate at a saturation concentration of ammonium sulphate of 60%. At a concentration of 80%, the enzyme proteolytic cannot dissolve and precipitate again because the solution has reached its saturation point (Abd-ElKhalek et al., 2020). The higher the specific activity of the bromelain enzyme, the purer the sample is because at a certain level of salt saturation, the amount of protein in the enzyme bromelain is more than other proteins. The crude extract of the bromelain enzyme has the highest specific activity in fractionation using ammonium sulphate at a concentration of 50 to 80% (Febriani et al., 2017). Bromelain isolated from the purification process using 60% ammonium sulphate showed a reduction in in-

tensity of brown colour formation on the surface of apple fruits compared to the other natural anti-browning agents of honey, chili pepper, lemon, and onion, and to the control of distillated water (Fig. 4). Bromelain, as one of the protease enzymes, can inhibit enzymatic browning by inactivating polyphenol oxidase (PPO) of fruits (Atrooz, 2008).

4 Conclusions

55 ^{o}C and 60% were the optimum drying temperature and concentration of ammonium sulphate respectively to achieve the best characteristics (2.16% protein level, 1.61 U/mL unit activity, and 0.75 U/mg specific activity) for bromelain isolated from Honi PC. Honi PC isolated bromelain was shown to inhibit the browning reaction on apple fruits. The agroindustry waste product, Honi PC, has potential as a future alternative bromelain source.

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Handling Practices and Quality Attributes Along the Supply Chain of *gboma (Solanum macrocarpon)*: A Leafy Vegetable in Southern Benin

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Abstract

Gboma is a leafy vegetable commonly consumed in Benin, which plays an important role in food security. This study assessed the handling and preservation practices as well as quality attributes of *gboma* along the supply chain in Southern Benin. A survey among 785 people (285 producers, 180 wholesalers/retailers, and 326 consumers) living in four Departments of Benin was conducted to evaluate the consumption frequency, the transportation mode, the preservation methods and quality attributes of *gboma* during purchasing. The survey revealed that, in general, *gboma* sauce is consumed twice to three times a month in all the departments covered. Different practices, including humidification, were used to preserve the leaves during selling. Traditional baskets or old clothes were used for the transportation and storage of *gboma*. Important quality attributes mostly used by actors during purchasing were freshness and color followed by physical damage and, to a lesser extent, weight and leaf surface at different degrees. *gboma* is currently sold in informal markets; however, improving handling and selling practices could preserve the freshness of this leafy vegetable that could then be placed in conventional supermarkets. Thereby, the selling and consequently the production level will increase, with positive impact on the income of producers.

Keywords: *Solanum macrocarpon*; leafy vegetables; quality criteria; transportation materials; frequency of consumption

1 Introduction

Vegetables are annual or perennial horticultural crops with certain parts such as roots, stalks, flowers, fruits, leaves, are consumed by humans. They are important for human nutrition and health because of their content in bioactive nutrients such as vitamins, minerals, phytochemical compounds and dietary fiber (Ilodibia et al., 2016). Adequate vegetable consumption can be protective against some chronic diseases (diabetes, obesity, metabolic syndrome, ...), and reduce risk factors related to these diseases (Grubben et al., 2014; Oyenuga & Fetuga,

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1975). Leafy vegetables, especially dark leafy green vegetables, are important sources of minerals (iron and calcium), vitamins (A, C, and riboflavin) and fiber sources (Ülger et al., 2018). Young fresh leaves contain more vitamin C than mature plants, while thinner and greener leaves are most nutritious and usually have lower calories (Ülger et al., 2018).

In Africa where the daily diet is dominated by starchy staples, indigenous leafy vegetables are generally used to balance the nutrition value of such diet. They are reported to play an important role in income generation and subsistence (Schippers, 2002). Leafy vegetables are crucial commodities for poor households because they are readily available everywhere and their prices are relatively affordable when compared to other food items. Besides, they provide essential sources of employment for those people outside the formal sector in peri-urban areas since this activity is generally short labour-intensive, with low levels of investment and a high yield (Schippers, 2002).

Among leafy vegetables produced in Benin, *Solanum macrocarpon* L. (Solanales: Solanaceae), commonly called *gboma* (Figure 1), is widely consumed in the form of *gboma* sauce that is well appreciated by the people (Baco, 2019; Dougnon et al., 2012). It is mainly produced in urban and peri-urban zones by both men and women. After production, *gboma* is purchased in the production sites by wholesalers, and sold in different local markets (Achigan-Dako et al., 2010; MAEP, 2016). Most studies have focused on the production system, the nutritional value as well as the consumption level of this leafy vegetable in West Africa (Baco, 2019; Chinedu et al., 2011; Dougnon et al., 2012; Houngla et al., 2020; Kwenin et al., 2011). For instance, apart from its richness in macronutrients, *gboma* also contains calcium (391 mg/100 g) and phosphorus (49 mg/100 g); and its composition is comparable to that of other dark green leafy vegetables (Chinedu et al., 2011).

However, there is a lack of information on some aspects related to the handling practices of *gboma*, from the production sites to consumers' houses; this could help develop relevant practices to extend its shelf life as well as its quality attributes. Additionally, such information is important to improve the preservation of the integrity of *gboma* during transportation and selling. The present study fills this gap by assessing the handling practices and preservation conditions of *gboma* along the supply chain in southern Benin. It also evaluated the quality criteria that guide buyers and consumers during *gboma* purchasing.

2 Materials and Methods

2.1 Study area and sampling of respondents

A survey was conducted from July to September 2018 in some production locations of *gboma* identified in four Departments of Southern-Benin, namely Atlantique (Abomey-Calavi, Allada and Ouidah), Littoral (Cotonou), Mono (Bopa, Grand-Popo, and Lokossa), and Ouémé (Avrankou, Porto-Novo, and Sèmè-Podji) (Figure 2). Three groups of actors involved in the *gboma* value chain were targeted in this study: producers, wholesalers/retailers, and consumers. Adequate sampling size for respondents was determined by using the equation N. 1

$$Ni = \frac{4pi(1-pi)}{d^2} \quad (1)$$

where Ni is the total number of actors surveyed in department I; pi is the proportion of actors implied in the value chain of *gboma* (producers, wholesalers and consumers), to the total number of populations i; and d is the expected error margin in the conclusion, which was fixed at 0.05 (Dagnelie, 1998). The number of persons interviewed in each group of actors in each Department was proportional to its number in the total population (case of consumers) or in the total agricultural population (for other groups of stakeholders).

Based on this formula, 178 people were selected in Atlantique Department , 200 people in the Littoral, 140 in the Mono, and 267 in the Oueme, giving a total of 785 respondents among which 279 producers, 180 wholesalers/retailers and 326 consumers.

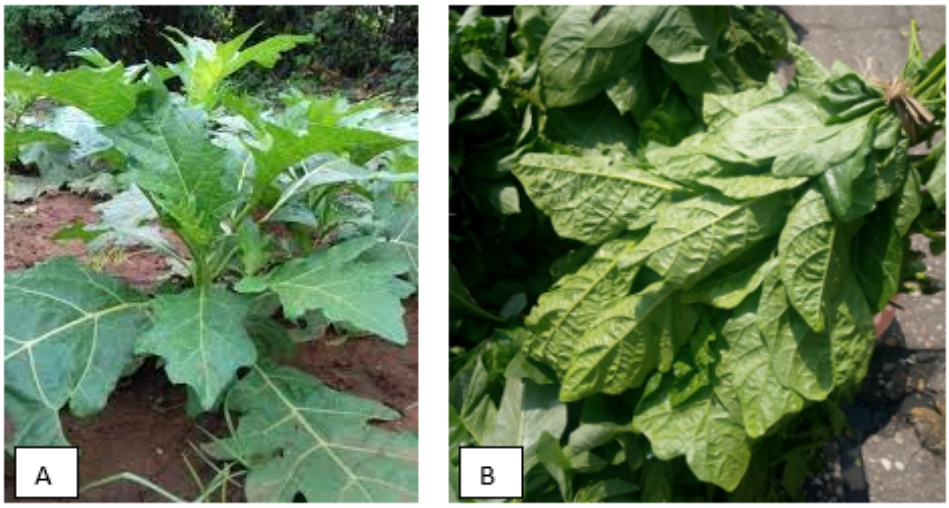

Figure 1: A: *Gboma* (*Solanum macrocarpon*) plant; B: *Gboma* tuft sold in market

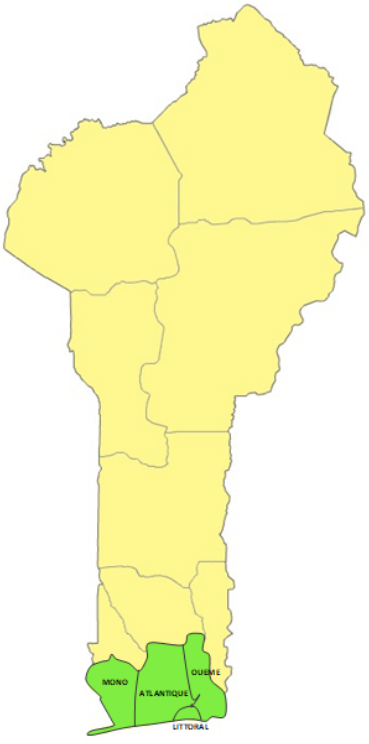

Figure 2: Map of Benin showing in color the study departments

2.2 Questionnaire content and data collection

Specific questionnaires were developed for each type of actor. They were tested and adjusted if needed before the survey. In each location, respondents were randomly selected and face-to-face interviews were conducted in the language or dialect that was best understood by the respondents, with translation when necessary.
The questionnaires developed for the different stakeholders covered the following aspects:

For the producers' level questions were related to their socio-demographic characteristics, production and handling practices (sites and period of production in a year, selling sites, packaging materials used by buyers for handling), criteria to determine *gboma* price, and characteristics of good quality of *gboma*.

For the wholesalers/retailers' level questions were linked to their socio-demographic characteristics, purchasing practices including the quality attributes considered during purchasing (places of purchase and sale, periods of high and low

availability, quality criteria retained during purchasing, criteria to determine the price, quality perception of the purchased *gboma*, packaging materials used for handling and selling, transportation conditions).

For the consumers' level questions addressed the following aspects: socio-demographic characteristics, purchasing practices including the quality attributes considered during purchasing (places of purchase, quality criteria during purchasing, quality perception of the purchased *gboma*) and consumption frequency.

2.3 Data Analysis

The raw data was saved in the Sphinx software (5.1.0.4). Descriptive statistics were processed using SPSS IBM 2020. Quality criteria identified were ranked and ordered according to actors.

3 Results and Discussion

Socio-demographic characteristics of actors indicated that all wholesalers/retailers interviewed and more than 97 % of consumers were females (Table 1). Women are less involved in *gboma* production than men: 68 % of producers were male. Respondents had various ages and diverse educational backgrounds. Most of them were between 40 and 60 years old. The majority of wholesalers (67.6 %) had no formal education and 28 % of producers had finished primary school. In addition, more than 40 % of producers and wholesalers had less than 10 years of experience in *gboma* production and selling activities.

3.1 Production and frequency of *gboma* consumption

gboma is produced all the year in the four Departments surveyed with noticeable differences in the production levels. In most locations of the Departments of Atlantique, Littoral and Mono, *gboma* production is a regular activity undertaken throughout the year. However, in some locations in the Oueme Department, this activity is seasonal and it is done during the rainy season. In addition, the production period also depends on the customers' request. This figure was also observed by Achigan-Dako et al. (2010) who reported that *gboma* production is generally a full-time activity for most producers in Benin. The frequency of *gboma* consumption differed significantly among stakeholders. Globally, more than half of the interviewed persons consumed *gboma* at least 2 to 3 times a month, while around 4 % of consumers declared to rarely consume this product (less than once a month) (Table 2). High consumption level was observed in the Littoral Department in which 44 % of respondents consumed *gboma* sauce at least once a week, followed by Mono and Atlantique Departments. These observations could be explained by the fact that the Department of Littoral is represented by the city of Cotonou which is the largest urban city of Benin. Moreover, most of the inhabitants of Cotonou, not only know the importance of fruit and vegetable consumption on the health, but are also willing to pay for these products regardless of the price and the season. In fact, vegetables and specifically leafy vegetables are rich in micronutrients and health-promoting phytochemicals, and the increase in their consumption is critical to alleviate worldwide incidence of nutritional deficiencies (Yang et al., 2006). In contrast, low level of consumption frequency was noticed in Oueme Department where this situation could be attributed to the seasonal production of *gboma* in certain locations of the Department. The consequence is that the product is sometimes unavailable and also the resulting high cost during these periods of the year reduce its consumption. Similar patterns of *gboma* consumption frequencies were also found in other *gboma* producing countries in the West-African sub-region such as Ghana, Nigeria, and Togo (Bonsu et al., 2002; Schippers, 2002). Comparison among locations indicated that high frequencies consumptions (at least once a week) were observed in Allada (45 %) in Atlantique Department, Cotonou (44 %) in Littoral Department, Grand-Popo (41 %) and Lokossa (41 %) in Mono Department (Table 2). These observations could be explained by several factors such as: food habits, production levels of vegetables in general, and the purchasing power.

Table 1: Socio-demographic characteristics of respondents by actors

	Producers (N=279)	Wholesalers/retailers (N=180)	Consumers (N=326)
		Gender	
Male	68.0	0.0	2.8
Female	32.0	100.0	97.2
		Age (year)	
<25	2.9	2.2	11.0
25–29	11.8	5.6	9.5
30–39	26.8	14.5	25.8
40–60	49.3	62.6	46.0
> 60	9.2	15.1	7.7
		Education level	
No formal education	39.9	67.6	42.5
Primary school	28.0	20.7	23.7
Secondary school (1st cycle)	18.8	8.4	15.4
Secondary school (2nd cycle)	8.9	2.8	8.0
University	4.4	0.6	10.5
		Experience in the activity (year)	
0–10	42.3	41.1	
11–20	34.9	31.7	
21–30	13.6	18.3	
31–40	6.6	6.7	
More than 40	2.6	2.2	

"Values presented correspond to % of respondents"

In fact, in most of these municipalities, leafy vegetables are included in their food habit for a long time, and this practice is perpetuated from generation to generation regardless of their evolution of the living cost. Next to that, the production of leafy vegetables in these locations is done throughout the year, irrespective of the season; thereby encouraging people to consume *gboma* regularly. However, due to the health benefit from the consumption of fruits and vegetables, and to promote the consumption of vegetables, the Word Health Organization recommends the consumption of at least three different vegetables species per day (WHO, 2003).

3.2 Quality attributes of *gboma* by actors of the supply chain

Quality criteria identified by different stakeholders in the *gboma* value chain are presented in table 3. Among the six criteria identified, freshness and color were the major selection criteria used by wholesalers and consumers during purchasing. These two criteria were also the main factors mentioned by producers for *gboma* quality characterization. Freshness represents an important criterion for leafy vegetables that is measured by the shininess or brightness, and the vigor of the leaves (Bonsu et al., 2002; Kwenin et al., 2011; Schippers, 2002). Consumers usually purchase fresh products driven by their visual appearance, while additional other components of quality such as texture and aroma make them to claim for the same product whenever they are in the need (Kader, 2001).

Color is generally the most important attribute used by consumers to evaluate the quality of the vegetable, and plays, therefore, a decisive role in the acceptability of such products. Color change is the first visible symptom of senescence in many

Table 2: Consumption frequency of *gboma* among respondents by locations and departments

Municipalities	2 - 3 times/week	Once / week	2 - 3 times / month	Once month	Less than once/month
			Atlantique department		
Abomey-Calavi (N=66)	13.6	25.8	24.2	31.8	4.6
Allada (N=11)	18.2	27.3	18.2	36.4	0.0
Ouidah (N=10)	0.0	20.0	20.0	60.0	0.0
Total in Atlantique (N=87)	12.6	25.3	23.0	35.6	3.4
			Littoral department		
Cotonou (N=106)	16.0	28.3	24.5	26.4	4.7
			Mono department		
Bopa (N=08)	12.5	25.0	12.5	50.0	12.5
Grand-Popo (N=22)	9.1	31.8	13.6	27.3	18.2
Lokossa (N=17)	17.6	23.5	29.4	23.5	5.9
Total in Mono (N=47)	12.8	27.7	19.1	29.8	10.6
			Oueme department		
Avrankou (N=15)	0.0	13.3	33.3	53.4	0.0
Porto-Novo (N=50)	10.0	10.0	36.0	40.0	04.0
Seme-Kpodji (N=21)	0.0	23.8	28.6	47.6	0.0
Total in Oueme (N=86)	5.8	14.0	33.7	44.2	2.3
Total (N=326)	12.0	23.6	25.8	34.0	04.6

"Values presented correspond to % of respondents"

horticultural crops and may compromise their economic value. Additionally, the dark green color of the leaves is the color criterion preferred by all actors (Figure 2). Color may be considered as an index for estimating the antioxidant properties of the leafy vegetables (Ali et al., 2009; Tijskens et al., 2001). Indeed, the green coloration of leafy vegetables is generally associated with the presence of chlorophyll which indicates the presence of antioxidant activities (Ali et al., 2009; Limantara et al., 2015).

Apart from these two criteria, leaf width or area and appearance were other important parameters that all actors used to appreciate the quality of *gboma*, but at different degrees. Appearance is refered to the integrity of the leaves. Wholesalers and consumers preferred *gboma* with a wide surface without any physical damage (Figure 3). Physical damage of the leaves is generally associated with insect and pests' infestation or inappropriate use of synthetic pesticides during the cultivation (James et al., 2007). In contrast, odor and texture of the leaves of *gboma* are not the main concerns for all actors.

In summary, all the actors investigated believed that *gboma* must be fresh (shiny and firm) with a large area without any physical damage.

3.3 Handling practices and preservation

Generally, producers sell their products to wholesalers or retailers; but sometimes, they are in direct contact with consumers who come to their garden for purchasing. Wholesalers directly bought plots of *gboma* which they harvested by themselves. After harvesting, different materials were used to handle the leaves from the production sites to markets or selling places (Table 4). Apart from baskets and bowls, respondents also used other materials for transportation because of their ease of transport on motorcycles. So, many old clothes or recycled rice bags are sewed together to get a large surface capable of carrying a large amount of *gboma* leaves. These materials (clothes and recycled rice bags) are widely used

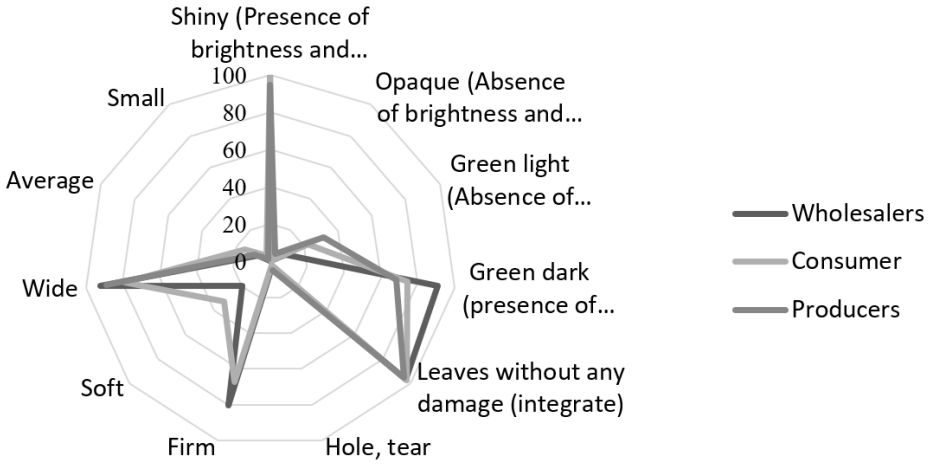

Figure 3: Rank of quality attributes among actors.

Table 3: Quality attributes of *gboma* among actors

Actors	Freshness	Color	Appearance	Texture	Odor	Leaves width
Producers (N=279)	23.6	23.7	19.8	13.1	0.4	19.3
Wholesaler (N=180)	26.6	22.9	19.3	9.9	0.1	21.2
Consumers (N=326)	25.5	22.7	22.3	10.5	0.5	18.5

"Values presented correspond to % of respondents"

Table 4: Materials used by wholesalers and retailers for *gboma* transportation

	Old clothes	Recycled rice bags	Baskets	Plastic bags	Leaves[1]	Large bowls
Wholesalers/retailers (N=180)	92.0[2]	88.4	36.0	18.2	12.6	09.8

"Values presented correspond to % of respondents"; [1] *Tectona grandis* leaves wrapped with palms of *Elaeis guineensis* leaves; [2]Sum> 100 because several answers were possible

Table 5: Preservation methods of *gboma* among actors

Actors	Method 1*	Method 2	Method 3	Method 4	Method 5
Wholesalers/retailers (N=180)	79.5	12.2	8.2	0.0	0.0
Consumers (N=326)	15.7	51.4	12.9	18.6	1.4

"Values presented correspond to % of respondents"; *: Method 1: water preservation (soaking in water, water spray); Method 2: dew preservation (dew exposition); Method 3: aeration preservation (shade and air exposition); Method 4: cold preservation (refrigeration and deep freezing). Method 5: parboiling preservation

Table 6: Producers and wholesalers/retailers reactions when *gboma* do not satisfy consumers' requirements

Actors	Purchase price reduction	Rejection of the whole lot	Sorting and rejection of spoiled leaves
Producers (N=279)	51.7	5.9	42.4
Wholesalers/retailers (N=180)	72.6	6.7	20.7

"Values presented correspond to % of respondents"

by wholesalers due to their affordability and also because they are easy to carry on while travelling. However, some of these packaging materials (baskets and clothes) often lead to physical deterioration of the *gboma* leaves which, according to Salunkhe and Desai (1988), thereby causing severe loss in the nutritional quality of fresh vegetables during subsequent handling operations such as distribution, storage, and marketing. Thus, proper packaging system and appropriate handling materials should be designed for an easy handling of the post-harvest *gboma* to prevent physical damage prior to selling.

Moreover, to optimize the quality and to prolong the shelf life of *gboma* after harvesting, it is important to maintain optimal temperature and relative humidity by avoiding solar exposure during the transport and selling period. Proceeding as such could help reduce the degenerative processes that occur during the post-harvest stages, thereby increasing the shelf life of the products (Yang & Tsou, 2006).

After packaging, the leaves of *gboma* are transported either on motorcycles (92 % of wholesalers interviewed) or by car (4 % of wholesalers) to the markets or selling sites. Few wholesalers (5 %) used pirogues for *gboma* transportation, a practice that is common in swampy locations in the Oueme department during the rainy season. Elsewhere, *gboma* is generally sold unpacked in markets and consumers who kept their stuff in plastic bags on their way home.

Different methods were used by actors in the markets or selling places to preserve the freshness and color of the *gboma* (Table 5). It appeared that more than 79 % of wholesalers/retailers soaked the leaves in water or regularly sprayed them with water; some of them (12.2 %) left the leaves to the dew for their humidification during the night. When *gboma* is planned to be processed the day after purchasing, consumers left them to the dew (51.4 %) or in the fridge (18.6 %) to preserve their freshness. All these preservation's methods mentioned above have also been reported by Achigan-Dako et al. (2010) and Baco (2019) in Benin. These techniques are generally used to preserve the freshness of leafy vegetables (Vorster et al., 2003). However, some consumers also preserve the quality of the *gboma* using cooling systems (i.e. refrigeration or freezing), but this technique is more common in urban areas where electric power is available, and it is often consumers wealthy who choose this option.

In spite of all measures taken, the loss of quality of freshly harvested *gboma* leaves occurs in all groups of stakeholders. Wholesalers/retailers, sometimes, are confronted to quality deterioration (freshness, color, physical damage) that constraints them to sell their commodity at lower prices or to poor sales following rejection by the purchasers. Sometimes, *gboma* leaves are discarded when the supply exceeds the demand; this happens generally during rainy season. Several authors have investigated the causes of post-harvest losses of leafy vegetables and reported many causes including the use of local or auto-propagated seeds, excessive use of fertilizers, inadequate irrigation system, poor harvesting practices, inadequate handling conditions (poor transport and storage), attacks of insects and fungi (Able et al., 2003; Ahvenainen, 1996; Jacxsens et al., 2002). In addition, Salunkhe and Desai (1988) stated that significant changes in color, flavor, texture and nutritional quality of vegetables also occur during storage, as influenced by storage temperature and environment and could affect the quality of the vegetables, hence, their marketable value. With this as-

sumption, wholesalers and retailers need to preserve *gboma* during sales and storage to avoid losing the overall quality of their product.
When, unfortunately, some parts of the *gboma* production or stocks were rejected by the final consumers for one reason or another, producers and wholesalers are forced to adopt one of the strategies presented in Table 6. In that respect, most of the respondents (51.7 % of producers and 72.6 % of wholesalers) either reduce the quantity for sale or lowered the purchasing price. Some of them (42.4 % of producers and 20.7 % of wholesalers) sort and drop the spoiled leaves. In certain cases, producers sell to street food vendors or keep the spoiled products in the field, for them to rot into organic fertilizers.

4 Conclusions

This study showed that *gboma* is consumed at least two to three times a month by the majority (>60% of respondents) of the population in the Southern part of Benin. Freshness (brightness and vigor) and color (dark green) followed by appearance (leaves without any damage) and, to a lesser extent, leaf surfaces were important quality criteria mentioned by respondents. Practices used by stakeholders to preserve the freshness of *gboma* leaves during selling, include humidification and nightly exposure to dew. Post-harvest handling practices and preservation conditions during selling may significantly impair the quality of the vegetable product. Research is advocated to improve or develop appropriate materials for packaging and transportation to improve the shelf life of *gboma* thereby increasing the income of the different actors.

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Lactobacillus spp. Inhibits the Growth of HCT-116 and Reduces IL-8 Secretion by *Salmonella* typhimurium-Infected HCT-116 Colorectal Carcinoma Cells

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Abstract

Salmonella typhimurium causes symptoms resembling typhoid fever and gastroenteritis in humans. Its toxicity is due to an outer membrane consisting largely of lipopolysaccharides (LPS) which is responsible for the host immune response. The aim of this study is to evaluate the antimicrobial, anti-apoptotic ability of *Lactobacillus plantarum* and reduce *Salmonella*-induced pro-inflammatory cytokine IL-8 secretion. Adhesive tests were performed using lactobacilli co-cultured with the colon cancer cell line HCT-116 for 2 hours. The strains displaying the highest adhesion were selected for downstream 3- (4, 5- Dimethylthiazol -2-yl) -2, 5- diphenyltetrazolium bromide (MTT) tests to assess cytotoxicity. The supernatants of *Lactobacillus* cultured with HCT-116 cells for 24 and 48 h to evaluate the inhibitory effect. To determine Interleukin 8 (IL-8) secretion in colon cancer induced by *S.* typhimurium, we stimulated HCT-116 cells with *S.* typhimurium and co-cultured with lactobacilli for 24 h. Lactobacilli had the most significant inhibitory effects on cell growth, and their inhibitory effects were time-dependent. Strain No. 03-03-026 caused cancer cell deoxyribonucleic acid (DNA) fragmentation, and the anti-apoptosis protein (B-cell lymphoma 2) was reduced in the HCT-116 cells as determined. IL-8 production in colon cancer cells was significantly reduced by these lactobacilli. Our results suggested that lactobacilli maybe effectively reduce the numbers of *S.* typhimurium, IL-8 levels and the anti-apoptotic phosphorylated-p38 mitogen-activated protein kinase and B-cell lymphoma 2 proteins. *Lactobacillus* can be added to the diet as a food additive to prevent colorectal cancer and used to be the prophylactic agent against *S.* typhimurium.

Keywords: Colorectal cancer; *Lactobacillus*; Interleukin-8; Anti-apoptotic phosphorylated-p38; *Salmonella* typhimurium

1 Introduction

Salmonella, a Gram-negative bacterium, is widely present in the intestines of animals and humans and is excreted in the faeces, and it may cause enteritis. Transmission is via the faecal-oral route, spreading in water, food, and among organisms. *Salmonella* plays an important role in food hygiene and safety. Among potentially contaminated food sources, the key sources of human infection are animal feed and foods such as eggs and meat. Improper food handling may result in large-scale contamination and food poisoning cases (Schlosser et al., 2000). Some researches indicated *S.* typhimurium as the primary causative agent of food poisoning world-

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wide for the past two decades (Khakhria et al., 1997; Trémolières, 1996). Lactic acid bacteria (LAB) are specifically adsorbed by epithelial cells to establish and to outcompete other bacterial species in the gastrointestinal tract (Jiang et al., 2019). Lee and Salminen highlighted bacterial survival in the gastrointestinal tract and transient adsorption of intestinal epithelial cells (Lee & Salminen, 1995). In addition, Lee et al. proposed that probiotics with good adsorption capacity can prevent the adsorption of pathogenic bacteria such as *Salmonella* (Lee et al., 2000). Lee et al. also demonstrated that probiotics can invade intestinal cells and reduce gastrointestinal tract stimulation caused by pathogenic bacteria and infection (Lee et al., 2000). Intestinal mucus is an important site for the adsorption and colonization of probiotic strains, but it is difficult to perform probiotic biosorption tests in vivo (Mikelsaar et al., 1998). Therefore, the most commonly used method is an in vitro cell culture test. The cell lines commonly used include the human rectal epithelial cell Caco-2 (Hirano et al., 2003). The Caco-2 cell line is advantageous due to its ability to exhibit the type and functional differentiation in vitro, and this cell line possesses mature intestinal cells and functional microvilli (Hauri et al., 1985; Hidalgo et al., 1989; Jamyuang et al., 2019).

The pre-treatment of Caco-2 cells with the probiotic bacteria *L. plantarum* can reduce *Salmonella*-induced pro-inflammatory cytokine IL-8 secretion by nuclear factor κB (NF-κB) activation, which typically results in intestinal inflammation diseases (Lépine et al., 2018; Ren et al., 2013). A recent study corroborated these results in illustrating that pre-treatment with 1 $\times$ 10^6 CFU/mL *L. rhamnosus* GG for 12 h effectively suppressed IL-8 mRNA abundance, induced by TNF-α, in Caco-2 cells (Zhang et al., 2005). Roselli et al. utilized *Bifidobacterium animalis* MB5 and LGG to inhibit Caco-2-induced IL-8 expression in enterotoxigenic *E. coli* K88 (Roselli et al., 2006). IL-8 mRNA expression was significantly lower in Caco-2 cells co-cultured with *B. animalis* MB5 or LGG and *E. coli* than Caco-2 cells cultured with *E. coli* alone. A significant down-regulation of the anti-inflammatory cytokine TGF-β1 was also observed in the *E. coli*-only group.

At present, probiotics and their fermented products are beneficial to human health such as promoting anti-tumour growth, antimicrobial, antioxidant and intestinal health (Jang et al., 2019; Lee et al., 2000; Lee & Salminen, 1995). Probiotics have become popular in recent years and recommended as preventive strikes against rectal cancer (Zhang et al., 2005). However, different probiotic strains may yield differential anti-cancer or anti-tumour activity, and their mechanisms are unclear and require further study. Herein, we investigated plant-derived *Lactobacillus* using the colon cancer line HCT-116 to explore whether they can effectively reduce the anti-apoptotic phosphorylated-p38 mitogen-activated protein kinase (p-p38 MAPK) and B-cell lymphoma 2 (Bcl-2) proteins. Additionally, we investigated the impact of probiotics on IL-8 *S.* typhimurium dynamics in colon cancer lines HCT-116.

2 Methodology

2.1 Bacterial strains, cell lines, and culture conditions

Lactic acid bacteria (LAB) strains were isolated from fruit and fermented plant products, and the stock culture collection was maintained at -80 ^{o}C in 20 % glycerol. One hundred microliters of LAB cells were propagated twice in 5 mL Lactobacilli de Man, Rogosa and Sharpe (MRS) broth (Difco, Spark, MD, USA) containing 0.05 % L-cysteine for 18-20 h at 37 ^{o}C before experimental use. S. typhimurium (strain I50) was isolated from a patient with foodborne-induced diarrhoea obtained from the National Center for Disease Control, Taipei, Taiwan. For cultivation, one loopful of *Salmonella* cells was inoculated into 5 mL of tryptic soy broth and incubated at 37 ^{o}C for 12 h.

HCT-116 cell lines were obtained from the Bioresource Collection and Research Center (BCRC), Hsin-Chu, Taiwan. HCT-116 (BCRC 60349, human colorectal carcinoma) cells were grown in McCoy's 5A Medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 1% Nonessential amino acids (NEAAs), and 1.1 g sodium bicarbon-

ate. Cryostats containing the respective cell lines were transported from liquid nitrogen to a 37 ^{o}C water bath for rapid thawing. Cell suspensions were thawed in a 75 cm^2 cell culture flask or cell culture dish while a sterile pipette was used to add the appropriate amount of cell culture medium. Cells were incubated at 37 ^{o}C at 5 % CO_2.

2.2 Adhesion of *Lactobacillus* to HCT-116 cell lines

The method described by Tsai et al. was used (Tsai et al., 2019). HCT-116 cells were cultured in 75 cm^2 plastic tissue culture flasks (GIBCO). Cells were washed twice with PBS, then transferred (4×10^5 cells/ mL) with 0.05 % trypsin to 24-well plates containing fresh tissue culture medium without penicillin-streptomycin. Cells were kept at 37 ^{o}C in 5 % CO_2 until monolayers were achieved in each well. Prior to the adhesion test, all bacterial strains were washed twice with PBS and centrifuged for 10 min at 8000 × g (Hettich EBA 12R, Tuttlingen, Germany). Bacterial cells were resuspended in 1 mL McCoy's 5A Medium; suspensions (100 μL) were transferred to the 24-well plates (1×10^8 CFU/mL) containing the HCT-116 cells and incubated for 2 h at 37 ^{o}C in 5 % CO_2. After incubation, cells were washed twice with PBS, fixed with 5 % formalin for 30 min, washed four times with PBS, and then stained with crystal violet for 5 min.

2.3 Cell viability analysis

To understand the effects of the strain 03-03-026 and strain 03-03-027 on cell growth, the two strains were co-cultured with HCT-116 colorectal cancer cells, and cell viability was observed. HCT-116 cells were cultured for 12 h with strain 03-03-026 and strain 03-03-027 under the following conditions: (1) LAB cells grown to 10^7 CFU/mL or more, (2) *Lactobacillus* supernatants (SCS), (3) LAB supernatants at pH = 7.2, (4) Heat-treated (100 ^{o}C, 30 min) LAB supernatants, (4) Heat-treated LAB, (5) MRS medium containing LAB, and (6) Heat-treated MRS medium containing LAB.
Method described by Watson et al. was used (Watson et al., 2000). Briefly, 3- (4, 5-dimethylthiazol- 2-yl) - 2, 5- diphenyl tetrazolium bromide (MTT), a colorless, transparent tetrazolium salt, is reduced to yield a purple formazan crystal by mitochondrial dehydrogenase in living cells. In total, 100 μL (5×10^4 cells/mL) of cells were seeded onto a 96-well plate, and the cells were cultured overnight at 37 ^{o}C in a 5 % CO_2 incubator. Cells were gently washed twice with 1× PBS, and after discarding PBS, 100 μL of *Lactobacillus* suspensions were added to the wells. The results were subsequently analysed after 12, 24, 36, and 48 h. For the analyses, media solutions were aspirated from the 96-well plate, and the cells were gently washed twice with 1× PBS, followed by removal of PBS via aspiration. Second, 100 μL of MTT solution were added to the cells. After 2-h incubation at 37 ^{o}C in a 5 % CO_2 incubator, supernatants were removed and 200 μL of dimethyl sulfoxide (DMSO) were added to the wells, which was followed by continuous shaking for 10 min to solubilize the purple formazan crystals. An enzyme-linked immunosorbent assay (ELISA) reader (Model 680, BIO-RAD, Hercules, CA, USA) was used to read the absorbance at 570 nm.

2.4 Detection the level of IL-8 by Enzyme-linked immunosorbent assay (ELISA)

S. typhimurium (10^8 CFU/mL) was centrifuged (8,230 × g, 10 minutes), washed twice with 1 × PBS, and resuspended in cell culture media. An ultrasonic grinder (HOYU Ultrasonic 250, Taiwan) was then used (output power amplitude: 40 %, time: 6-8 hours), and cells were then filtered with a 0.22 μM filter and stored at -20 ^{o}C for later use.
HCT-116 cells were cultured in a 24-well tissue plate and incubated in medium with or without *S.* typhimurium and with either LAB strain No. 03-303-026 or No. 03-03-027 for 37 ^{o}C, 24 h. Afterwards, supernatants were collected and ELISA performed to assay IL-8 secretion using the manufacturer's instructions (BD Biosciences, CA, USA). 96-well Immuno-Maxisorp plates (Nunc, Roskilde, Denmark) were coated

with monoclonal antibodies for IL-8, and placed in an incubating buffer overnight at 4 ^{o}C. The plates were blocked and washed 3 times. Samples were added to the plates and incubated for 2 hours at room temperature. The plates were washed 3 times again, and biotinylated anti-human IL-8, along with horseradish peroxidase (HRP)-conjugated streptavidin, were added for the detection of IL-8, and incubated 1 hour at room temperature. The reactions were developed using 3, 3´, 5, 5´-tetramethylbenzidine (TMB) substrate for 30 min at room temperature. The colour reactions were stopped using 2N H_2SO_4 and absorbance was measured at 450 nm.

2.5 Apoptotic proteins p-p38 and Bcl-2 in HCT-116 cells

Apoptosis-associated protein expression in HCT-116 cells was determined via Western blot analysis. HCT-116 cells (1.5 × 10^6 cells/mL) were added to a 10 cm plate and cultured overnight at 37 ^{o}C and 5 % CO_2 to encourage cell attachment, division, and proliferation. Various concentrations of LAB strains No. 03-03-026 and No. 03-03-027 (10^5, 10^6, or 10^7 CFU/mL) mixed with cell culture media were added to the plate, and the cells were cultured for 24 h at 37 ^{o}C and 5 % CO_2. Cells were collected and then lysed in radio immunoprecipitation assay buffer for 30 min on ice. The cells were then centrifuged at 24,000 × g (Hettich EBA 12R, Tuttlingen, Germany) for 5 min at 4 ^{o}C. Resulting supernatants (extracts of cellular proteins) were subjected to quantitative protein analysis using the Invitrogen Qubit® fluorimeter (Life Technologies, Waltham, MA, USA). Cellular protein extracts were mixed with 5X protein loading dye, heated at 95 ^{o}C for 5 min, and loaded in SDS-PAGE wells. Following electrophoresis, proteins on the gel were transferred to a nitrocellulose membrane for 1 h at 37 ^{o}C, with the addition of 5 % skim milk as the blocking buffer. Subsequently, the membrane was washed in TBST [20 mM Tris-HCl, 137 mM NaCl, containing 0.1% (v/v) Tween-20, pH 8] three times and incubated with the primary antibody (either p-p38 or Bcl-2 monoclonal antibody) overnight at 4 ^{o}C. After three washes with TBST, the secondary antibody was added and incubated for 1 h, which was followed by TBST washes. Gel images were captured using a luminometer, and bands were observed.

2.6 Genomic DNA preparation and PCR amplification

Total chromosomal DNA of LAB cells cultured overnight in MRS broth was extracted with the Blood & Tissue Genomic DNA Extraction Miniprep System (Viogene Laboratories, Taipei, Taiwan) according to the manufacturer's instructions. Eluted DNA was stored at -20 ^{o}C and 1 μL was used for downstream PCR amplification. PCR primers are shown in Table 1. One microliter of DNA was mixed with 24 μL PCR buffer containing 1× PCR buffer (10 mM Tris-HCl, pH 8.8; 1.5 mM MgCl2; 50 mM KCl; 0.1 % Triton X-100); 200 μM each of dATP, dGTP, dCTP, and dTTP (Boeheringer Mannheim, Mannheim, Germany); 0.2 μM each of the PCR primers; and 0.4 units of Prozyme (PROtech Technology, Taipei). The 25 μL PCR mixture was heated at 94 ^{o}C for 1 min using a thermal cycler (GeneAmp PCR System 9600; Perkin-Elmer), followed by 35 PCR cycles. For each PCR cycle, denaturation, annealing, and extension were carried out at 94 ^{o}C for 35 s, 58 ^{o}C (for *L. pentosus*) or 65 ^{o}C (for *L. plantarum*) for 35 s, and 72 ^{o}C for 40 s, respectively. Final extension was carried out at 72 ˚C for 4 min. To detect the amplified product, 3.5 μL of the PCR product was electrophoresed on a 2 % agarose gel in 0.5× TAE buffer (10× TAE: 40 mM Tris acetate, pH 7.6; 10 mM Na_2EDTA). The gel was visualized, and PCR products were identified by comparison with the molecular weight markers of a 100 bp DNA ladder (Promega Co., Madison, WI, USA), and target DNA PCR length of *L. pentosus* is 247 bp, as well as *L. plantarum* is 270 bp.

2.7 API 50 CHL system

Fermentation of carbohydrates was determined using API 50 CHL, a standardized system consisting of 50 biochemical tests for the study of carbohydrate metabolism by microorganisms. API 50 CHL was used for the identification

Table 1: PCR primers for the detection of *tuf* or *recA* genes in *Lactobacillus* spp

Target strain	Primer	Sequences	Accession No.a	Location	Length (bp)
L. pentosus	Lpen_rF	5'-AACAATTTCCAGCGGGTCAC-3'	AJ640079	*recA gene*	247
	Lpen_rR	5'-ATCTGGTTGTGAAAGTAACAAA-3'			
L. plantarum	Lpla_rF	5'-GTATATCGATGCCGAAAATGCAC-3'	AJ621666	*recA gene*	270
	Lpla_rR	5'-GTCCCTGATAACTTCCGGAGC-3'			

[a]The accession numbers of *tuf* gene were obtained from GenBank database

of *Lactobacillus* and related genera according to the manufacturer's instructions (BioMérieux, Marcy-l'Etoile, France).

2.8 Statistical analysis

Data were presented as means ± standard error (SEM) of the three replicates. The Statistical Package for the Social Sciences, version 12.0, and software package was used. One-way analysis of variance (one-way ANOVA) was used for each experimental group. The Duncan's new multiple range tests analysed the differences in the mean values among the experimental groups. $P < 0.05$ was considered statistically significant.

3 Results and Discussion

3.1 Absorptive colorectal cancer cells

In this study, strains were screened for their ability to colonize HCT-116 colorectal cancer cells. The average number of bacteria adsorbed on HCT-116 cells was 29.4 ± 7.1 for strain 03-03-026, whereas the average numbers of adsorbed bacteria were 43.1 ± 14.7 for strain 03-03-027 (Table 2).

Lactobacilli are natural colonizers of the human gastrointestinal tract and subdominant colonizers in the colon. *Lactobacillus* has certain characteristics to exert probiotic effect, such as bacterial adhesion and excluding enteric pathogens and their immunomodulatory effects (Kotzamanidis et al., 2010). Rinkinen et al. found that host-derived probiotics may also be effectively adsorbed on the intestinal mucosa of other hosts, and this adsorption was mainly based on the strain characteristics of the probiotics itself, not its host source (Rinkinen et al., 2003).

3.2 Cell viability analysis

After 12 h of HCT-116 cells treated with 03-03-026, significant inhibited the growth of HCT-116 cells compared to the control group (HCT-116 cells alone) (Fig. 1a). Strain 03-03-027 was co-cultured with HCT-116 cells for 12 and 24 h under the aforementioned conditions. Except when cell lines were co-cultured with 105 and 106 CFU/mL LAB, all other conditions significantly inhibited growth (Fig. 1b). In addition, when 03-03-026 and 03-03-027 were unadjusted, the pH values of the supernatants were 4.32 and 4.78 respectively, which may have reduced the cell survival rate. Therefore, the supernatant was adjusted to a pH value of 7.2, which significantly increased the cell survival rate.

O'Hara et al. used the MTT and Terminal deoxynucleotidyl transferase dUTP nick end abelling (TUNEL) assays to determine inhibition of cytokine-induced apoptosis in epithelial cells and confirmed that *L. plantarum* promotes the balance of intestinal epithelial cells and increases their survival (O'Hara et al., 2006). The adsorption capacity of LAB can hinder the absorption of many pathogenic and nonpathogenic bacteria and colonize the host's intestinal cells.

3.3 ELISAs to determine IL-8 secretion

We co-cultured *S.* typhimurium flagella-purified material with HCT-116 cells and found that the secretion of IL-8 was 1,876 ± 262 pg/mL (Fig. 2). After this experiment, we ultra sonicated

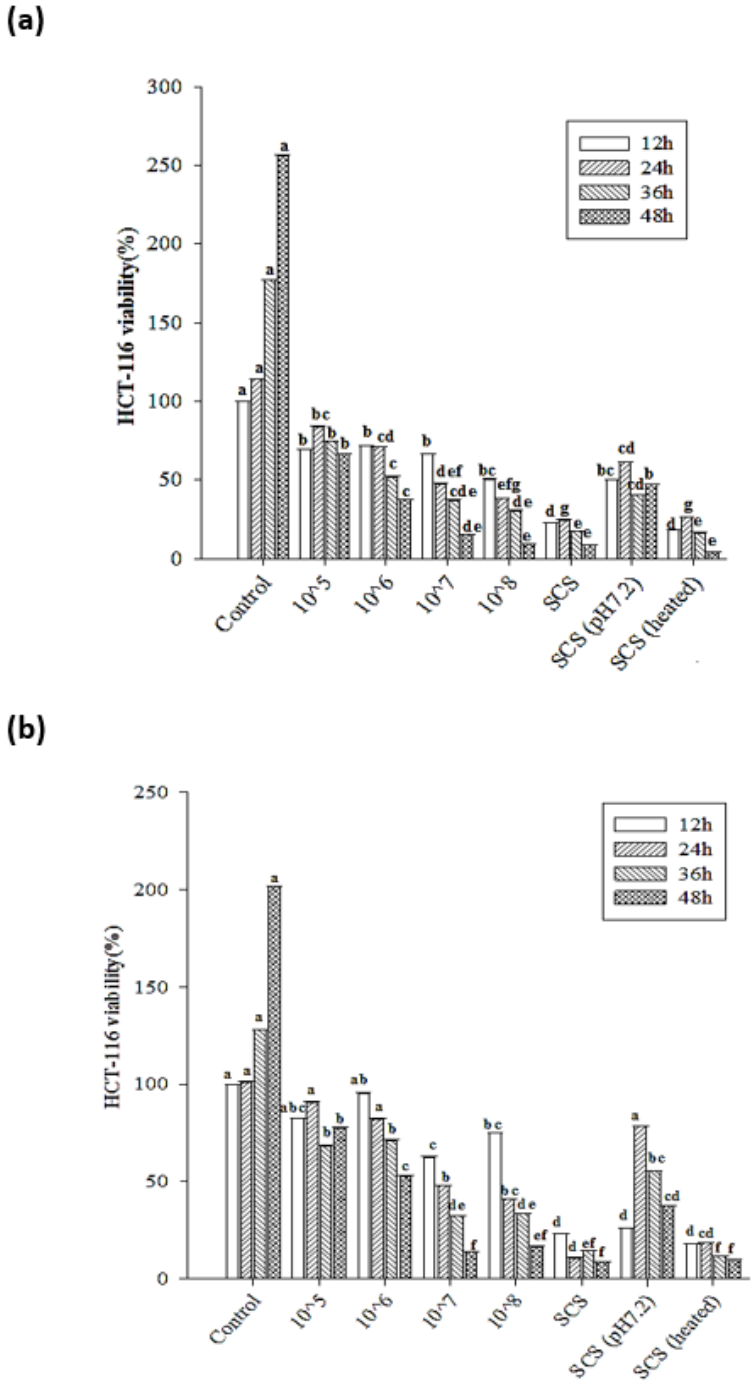


Figure 1: The effect of strain 03-03-026 on the viability of (a) HCT-116, and strain 03-03-027 on the viability of (b) HCT-116 for 12 h, 24 h, 36 h and 48 h. The viability of cell was determined by MTT assay. Each value is mean ± standard derivation of replicate analyses. Means with different superscript letters in the same bar are significant differences ($p<0.05$) using the Duncan's multiple rang test.

Table 2: Adhesion of various lactic acid bacteria strains onto the HCT-116 cell line.

Strain NO.	Adherence to the epithelial cells[a]
	HCT-116
03-03-026	29.4 ± 7.1
03-03-027	43.1 ± 14.7

Each adhesion assay was conducted in triplicate with cells from three successive passages. Adhesion assays were monitored after 2 h of incubation. The table presents mean numbers ± standard deviation of bacteria adhering per epithelial cells.

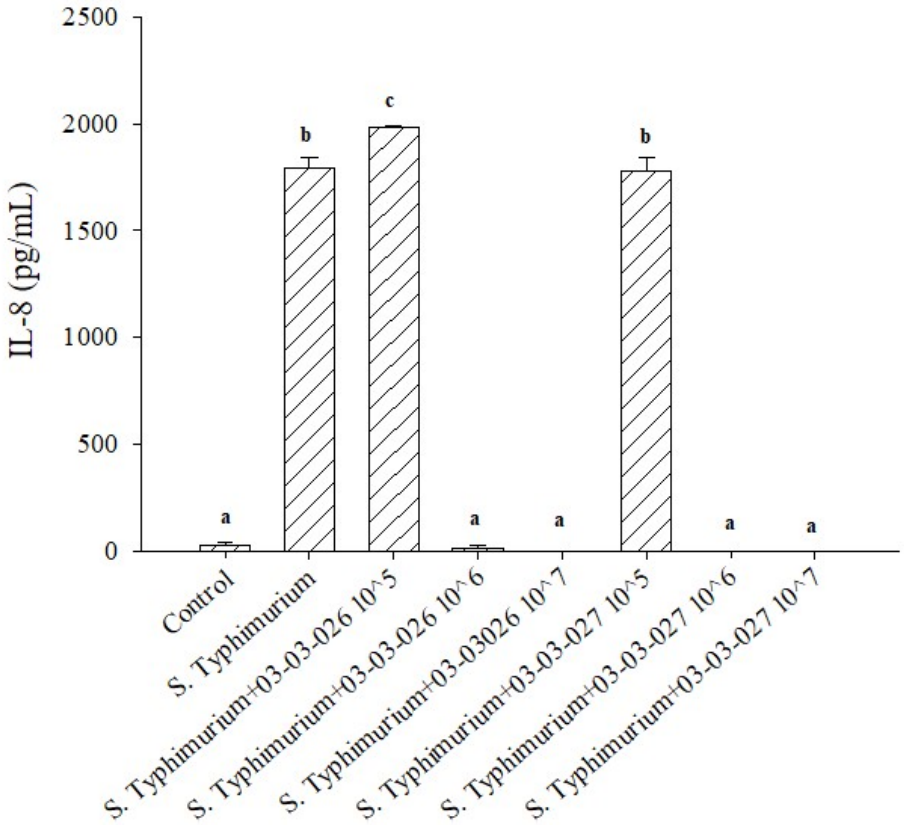


Figure 2: The effect of lactic acid bacteria on *S.* typhimurium I50 -induced IL-8 secretion by HCT-116 cells. Each value is mean ± standard derivation of replicate analyses. Means with different superscript letters in the same bar are significant differences ($p<0.05$) using the Duncan's multiple rang test.

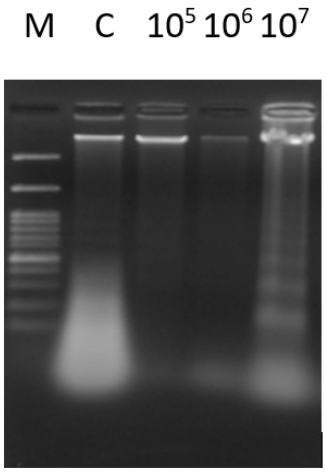


Figure 3: Effect of strain 03-03-026 on DNA fragmentation in HCT-116 cells for 48h.

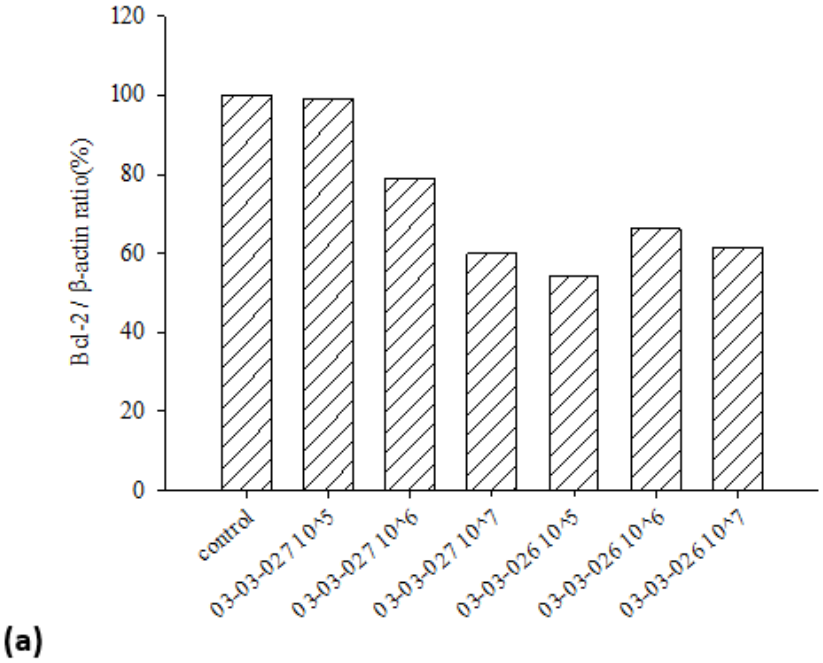
S. typhimurium and co-cultured with HCT-116 cells under the following conditions for 24 h to assess IL-8 secretion: (1) control group (only HCT-116 cells); (2) cells and ultra sonicated *S.* typhimurium only; (3) cells, ultra sonicated *S.* typhimurium, and 03-03-026 (10^5, 10^6, and 10^7 CFU/mL); (4) and cells, ultra sonicated *S.* typhimurium of different concentrations, and 03-03-027 (10^5, 10^6 and 10^7 CFU/mL). For co-cultures of HCT-116 cells and *S.* typhimurium, IL-8 secretion was 1,736.8 ± 15.7 pg/mL (Fig. 2). HCT-116 cell lines were significantly stimulated by *S.* typhimurium to secrete IL-8. When 03-03-026 and 03-03-027 reached concentrations of 10^6 CFU/mL and 10^7 CFU/mL, IL-8 secretion in HCT-116 cell lines were significantly inhibited (Fig. 2).

Xu et al. investigated the effects of LPS (1 μg/mL) on rat intestinal microvascular endothelial cells and found that adding lactate (7.5 μL/mL) to the medium decreased LPS-induced TNF-α and IL-6 mRNA expression (Xu et al., 2013). O'hara et al. showed that *S.* typhimurium UK1 rapidly induced IL-8 mRNA expression in HT-29 cells in 2 h, and IL-8 mRNA expression was regulated by NF-κB, which the authors determined was not activated due to *B. infantis* 35624 and *L. salivarius* UCC118 nor did these strains impact IL-8 production in HT-29 cells (O'Hara et al., 2006). In addition, the authors assessed *S.* typhimurium infection with *B. infantis* and *L. salivarius* in HT-29 cells for 2 h and observed that NF-κB significantly reduced binding activity. Since this result was only observed in confluent HT-29 cells, it suggests that the intact barrier function is important to normal epithelial immune activity.

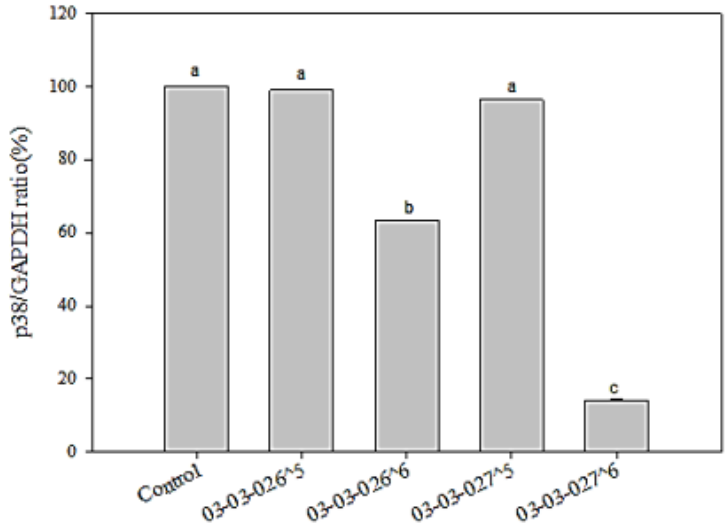
3.4 DNA fragmentation assay, protein expression of Bcl-2 and Phosphorylated-p38 (p-p38) dynamics

HCT-116 cells were co-cultured with 03-03-026 for 48 h to perform a DNA fragmentation assay. Fig. 3 shows that the strain 03-03-026 (10^7 CFU/mL) has DNA fragmentation, but not at 10^5 and 10^6 CFU/mL. Bcl-2 (B-cell lymphoma 2), encoded in humans by the BCL2 gene, is the founding member of the Bcl-2 family of regulator proteins that regulate cell death (apoptosis), by either inducing (pro-apoptotic) or inhibiting (anti-apoptotic) apoptosis. In addition, Bcl-2 expression was observed. Fig. 4(a) shows that Bcl-2 expression in HCT-116 cells decreased with co-cultivation of the two strains. The inhibitory effect was also apparent, indicating that the two lactic acid bacteria may induce apoptosis. Fig. 4(b) shows that when co-cultured with either of two strains, the expression of p38 kinase in HCT-116 cells significantly reduced; with the effect of 03-03-27 strain was significantly greater than that of 03-03-026. The increase in LAB concentration yielded an enhanced inhibitory effect. Additionally, we further found that phosphorylation of p38 in HCT-116 cells significantly increased. Our results indicated that the two LAB strains inhibited IL-8 secretion and may reduce intestinal cell inflammation via affecting p38 kinase.

Aggarwal et al. illustrated that DNA fragmentation is an indicator of apoptosis (Aggarwal et al., 2004). The Bcl-2 family proteins play different roles in regulating programed cell apoptosis in mitochondria by promoting or inhibiting the release of apoptotic molecules from the mitochondria into the cytoplasm (Marzo et al., 1998). This protein family regulates mitochondrial per-



(a)



(b)

Figure 4: The effects of lactic acid bacteria 03-03-026 and 03-03-027 on the expression of (a) Bcl-2 and (b) hosphorylated p38/ p38 kinase in HCT-116 cells after co-culture for 24 h

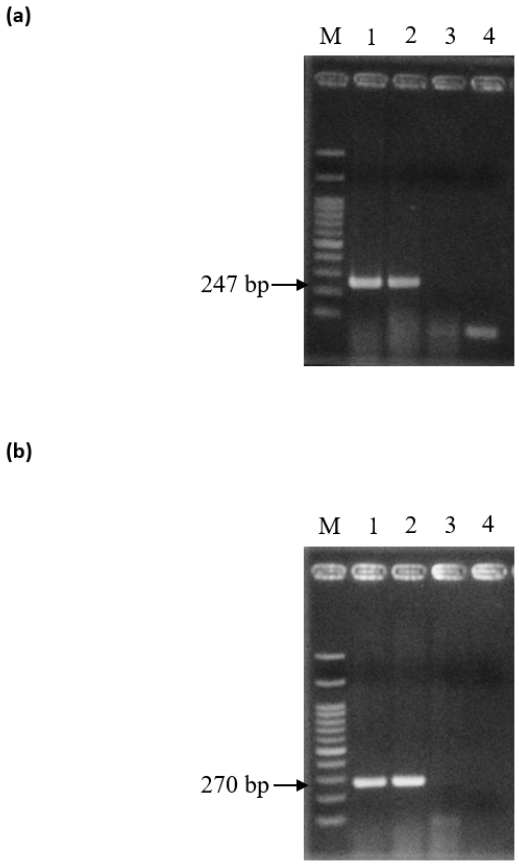


Figure 5: The PCR result of strain 03-030-026 and 03-030-027 using (a) *L. pentosus* and (b) *L. plantarum* species specific-primers of tuf gene. M: Marker (100 bp ladder); lane 1 (sample): (a) strain 03-030-026 and (b) strain 03-030-027; lane 2 (positive control): (a) *L. pentosus* BCRC 17972 and (b) *L. plantarum* BCRC 10069; lane 3 (negative control): (a) *L. plantarum* BCRC 10069 and (b) *L. pentosus* BCRC 17972; lane 4 (blank control): ddH2O.

meability by interacting with adenosine translocators and voltage-dependent ion channels for ADP/ATP exchange or interfering with oxidative phosphorylation during programed apoptosis (Marzo et al., 1998). The 3D structure of Bcl-2 was shown to have the ability to form channels on the mitochondrial membrane (Minn et al., 1997; Muchmore et al., 1996; Schendel et al., 1998). Bcl-2 regulates anti-apoptotic function of cells via post-transcriptional modifications (including phosphorylation, dimer formation, or proteolysis) (Cheng et al., 2001; Yin et al., 1994).

3.5 Biochemical and molecular identification of LAB strains

Fig. 5 demonstrates that strain 03-03-026 identified as *Lactobacillus* pentosus (93.6%). Strain 03-03-027 identified as *Lactobacillus* plantarum (99.2%) by API 50CHL. Molecular identification, via PCR, corroborated our biochemical findings, yielding the same results.

4 Conclusions

In this study, two strains, *Lactobacillus pentosus* 03-03-026 and *L. plantarum* 03-03-027, demonstrated good adsorption onto HCT-116 cells. When investigating these LAB strains in the context of inflammation, ultra-sonicated *S.* typhimurium co-cultured with the LAB strains in HCT-116 cells. We herein showed that LAB can inhibit IL-8 secretion. Assessing via Western blotting, the expression of phosphorylated p38 protein decreased after co-culturing of HCT-116 cells with *L. pentosus* 03-03-026 and *L. plantarum* 03-03-027, which also illustrated that these LAB strains can reduce inflammation. We also showed that co-culturing HCT-116 cells with *L. plantarum* 03-03-027 led to DNA fragmentation of the HCT-116 cells. In addition, after co-culturing HCT-116 cells with *L. pentasus* 03-03-026 and *L. plantarum* 03-03-027, Bcl-2 protein expression decreased, indicating that these LAB strains may promote apoptosis. Further studies are warranted to investigate the anti-tumour effects in the animal model.

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Antioxidant, Antimicrobial and Physicochemical Properties of Beef Sausages Enriched with an Aqueous Extract of Senduduk (*Melastoma malabathricum* L.) Leaf

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Abstract

The use of natural products in sausages has become a new trend for health reasons. A natural product that could be incorporated into sausages is an extract of the senduduk (*Melastoma malabathricum* L.) leaf. Senduduk is an abundant shrub herb in Indonesia. This kind of plant is mostly used as a traditional medical remedy and as an ingredient in some culinary recipes. This study was carried out to investigate the effect of an aqueous extract of senduduk leaf (SLE) on the antioxidant, antimicrobial and physicochemical properties of beef sausage. Four treatments were used: ingredients consisting of beef, vegetable oil, skim milk, tapioca, salt, phosphate, ice cubic, garlic, pepper, dan nutmeg as a Control; the Control ingredients plus 0.01% of butylated hydroxytoluene (BHT); the Control ingredients plus 0.83% of SLE (SLE-1), and the Control ingredients plus 1.1% of SLE (SLE-2). All ingredients of each formula were homogenously blended and the sausage mix was cooked. The addition of BHT and SLE affected the proximate composition, with the moisture content decreasing as the duration of chilled storage increased. The addition of SLE lowered the pH and a_w value and both tended to increase during chilled storage. SLE also enhanced the WHC of the sausages which increased in value during chilled storage. The addition of BHT and SLE could increase the antioxidant activity of the sausages as indicated by scavenging DPPH free radicals. SLE in sausages could inhibit microbial growth during chilled storage. It can be summarized that the addition of an aqueous extract of senduduk could improve the physicochemical, antioxidant and antimicrobial properties of beef sausages.

Keywords: Antioxidant; Antibacterial; Beef sausages; *Melastoma malabathricum*; Physicochemical

1 Introduction

The development of the sausage industry in Indonesia has a positive impact for the population by increasing the consumption of animal protein. From a nutritional perspective, sausage is rich in protein with a high biological value (Tran et al., 2020). However, sausages have limitations related to high-fat content and high-water activity (Boeira et al., 2020) which can undergo lipid oxidation and microbial contamination (Domínguez et al., 2019). Lipid oxidation and microbial con-

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tamination in the sausage can lead to deterioration in physical and sensorial properties (de Carvalho et al., 2020; Luong et al., 2020).
Several measures have been employed to reduce such deterioration and the most popular application is by use of synthetic agents. Unfortunately, these substances are associated with negative side effects on humans such as carcinogenic (Gultekin et al., 2015), triggering colorectal disease (Herrmann et al., 2015), intestinal and metabolic disorders, and also cardiovascular disease (Partridge et al., 2019). Application of plant extracts has also been extensively employed in meat products for health reasons (de Carvalho et al., 2020; Hung et al., 2016; Pateiro et al., 2021; Tran et al., 2020).
Plants are rich in polyphenols which play essential roles as antioxidants and antimicrobials. One of the potential plants to find greater use for food purposes is senduduk (*Melastoma malabathricum* L.). This plant is a shrub which is abundantly found in Indonesia and used for folk medicinal and food purposes (Susanti et al., 2008; Thatoi et al., 2008). A senduduk leaf extract (SLE) could act as a natural antioxidant and antimicrobial (Alwash et al., 2014; Wong et al., 2012; Zakaria et al., 2011), without causing any toxicity (Alnajar et al., 2012; Alwash et al., 2014; Kamsani et al., 2019).
For food purposes, the extraction process should use a solvent such as water which is not harmful to humans. An aqueous extract of senduduk leaf has antibacterial capability and antioxidant activity (Suharyanto et al., 2019). A SLE could improve the physicochemical properties of a beef sausage mix and replace the use of nitrite in the formulation (Suharyanto et al., 2020). This study aimed to investigate the effect of an extract of senduduk leaf on the physicochemical, antioxidant and antimicrobial properties of beef sausages.

2 Materials and Methods

2.1 Extract preparation

The senduduk leaves were cleaned of undesired materials and then air-dried for 5-6 h at 45 ^{o}C. The leaves were powdered into a 35 mesh. The extraction method was adapted from Doughari and Manzara (2008). Briefly, the powder (40 g) was macerated in distilled water (400 mL) in a 1000 mL Erlenmeyer flask for 24 h. The macerate was filtered using Whatman No. 1 filter paper and evaporated using a rotary evaporator (Heidolph, Antrieb-W-Mikro, Germany) at 40^{o}C. The viscous raw extract was freeze-dried (Snijders Scientific, LY5FME, the Netherlands). The extract of senduduk leaf (SLE) was stored at -25^{o}C until use.

2.2 Preparation of sausages

The Brahman cross round meat, free of connective and fat tissue, was cut into small pieces and then minced using a meat mincer. Ingredients used in the formulation of beef sausages are shown in Table 1. Four treatments were employed in the study: ingredients consisting of beef, vegetable oil, skim milk, tapioca, salt, phosphate, ice cubic, garlic, pepper and nutmeg as a Control; the Control plus 0.01% of BHT (BHT); the Control plus 0.83% of SLE (SLE-1) and the Control plus 1.1% of SLE (SLE-2).
The procedure used to prepare sausages was that of Arief et al. (2014), with slight modification. Briefly, all ingredients were blended homogeneously to form a mix for each treatment. The emulsified mix was filled into casings (food grade polyamide plastic with a diameter of 16 mm). The raw sausages were steamed at 65 ^{o}C for 45 min. The cooked sausages were stored at 4 ^{o}C and observed at 0, 6, 12 and 18 days of storage.

2.3 Proximate composition determination

Proximate composition was determined using AOAC (AOAC, 2005) methods on day 0 of storage. Moisture, crude protein and crude fat contents were determined by oven drying at 105 ^{o}C, the Kjeldahl method and the soxhlet method, respectively. The carbohydrate content was calculated by difference.

Table 1: Formulations of the sausage mixes.

Ingredients	Formula			
	Control	BHT	SLE-1	SLE-2
Beef (g)	500	500	500	500
Vegetable oil (g)	100	100	100	100
Skim milk (g)	30	30	30	30
Tapioca flour (g)	75	75	75	75
Cubic ice (g)	175	175	175	175
Salt (g)	15	15	15	15
Garlic (g)	8.75	8.75	8.75	8.75
Pepper (g)	1	1	1	1
Nutmeg (g)	2.5	2.5	2.5	2.5
Phosphate (g)	1.5	1.5	1.5	1.5
BHT (g)	**-**	**0.09 (0.01%)***	**-**	**-**
Extract (g)	**-**	**-**	**7.5 (0.83%)***	**10 (1.1%)***

* based on total mass of ingredients in the formulation (908.75 g)

2.4 pH value determination

The pH value was measured using the AOAC (AOAC, 2005) procedure. 10 g of crushed sausage was mixed into 100 mL of distilled water. The solution was filtered and then the pH of the filtrate was measured using a pH meter (Schott Instrument Lab 850).

2.5 Water activity determination

The water activity (a_w) was measured using the Lorenzo et al. (2014). The sufficiently crushed sausage was placed in the container and its a_w value was measured at 25 ^{o}C using a calibrated a_w-meter (Novasina Ms-1).

2.6 Water holding capacity determination

The water holding capacity (WHC) was determined using the Jung and Joo (2013) procedure, with a minor modification. 2.5 g of the crushed sausage was placed in a centrifugation tube, to which 10 mL of distilled water was added, and then incubated at 30 ^{o}C for 30 min. The supernatant was removed and the residual crushed sausage reincubated at 30 ^{o}C for 10 min. Finally, the remaining supernatant was removed. The WHC was calculated by the formula as shown below.

$$WHC(\%) = \frac{Weight\ of\ sample\ without\ supernatant}{Weight\ of\ sample\ with\ water\ added} \times 100 \quad (1)$$

2.7 Total phenolic content

Sample preparation was carried out according to the Sukisman et al. (2014) procedure by dissolving and homogenizing 1 g of crushed sausage in 5 mL of methanol for 24 h. The filtrate of the solution was used to determine the total phenolic content according to the Al-Saeedi and Hossain procedure (Al-Saeedi & Hossain, 2015), with a minor modification. 0.4 mL of the filtrate was mixed with 3 mL of 20% Folin-Ciocalteou solution (Merck KGaA, Germany) and left to stand for 5 min. Then, the mixture was reacted with 3 mL of 10% Na2CO3 and incubated for 60 min in the dark and at room temperature. The absorbance of the mixture was measured using a spectrophotometer (Agilent, UV-Vis 8453, USA) at 760 nm wavelength. An identical technique was employed with several standard gallic acid

concentrations (0-16 mg mL^{-1}). A linear regression equation of the gallic acid absorbance was used to calculate the total phenolic content of the sample and expressed in mg equivalent gallic acid [100 g] $^{-1}$ dry matter.

2.8 Antioxidant activity

The antioxidant activity was determined using the Mahmoudi et al. procedure (Mahmoudi et al., 2016). 0.2 ml of the prepared sample, according to the Sukisman et al. procedure (Sukisman et al., 2014), was reacted with 6 $\times 10^{-5}$ mol L^{-1} of 1.8 mL of DPPH solution (Sigma-Aldrich, D9132-1G, Germany), and then shaken gently for 20 s. The solution was left to stand in a dark place and at room temperature for 60 min. The absorbance of the solution was then measured using a spectrophotometer (Agilent, UV-Vis 8453, USA) at a wavelength of 517 nm. Standard butylated hydroxytoluene (BHT) (Himedia, GRM797-500G, India) solutions, at various serial dilutions (0.0-4.5 mg $[100\ mL]^{-1}$), were employed using the same technique.
Antioxidant activity was expressed by scavenging percentage and antioxidant capacity. The scavenging activity was calculated by the following formula:

$$Scavenging\ activity(\%) = \frac{Acontrol - Asample}{Acontrol} \times 100 \quad (2)$$

where Acontrol was the absorbance of the DPPH solution without sample and Asample was the absorbance of the sausage. The antioxidant capacity of the sample was calculated using the linear regression equation of BHT as a standard and expressed as mg equivalent BHT $[100]^{-1}$ g dry matter.

2.9 Thiobarbituric acid reactive substances (TBARS) assay

Malondialdehyde (MDA) was determined using the TBARS assay, according to the Turgut et al. method (Turgut et al., 2016). 5 g of crushed sausage was homogenized in 15 mL of distilled water and then centrifuged at 2000 × g for 15 min. One mL of the supernatant was reacted with 2 mL of 0.25 M HCl, containing 0.375% (w/v) Thiobarbituric acid (TBA) (Merck, KgaA, Germany) and 15% (w/v) Trichloroacetic acid (TCA) (Merck, KgaA, Germany), and then 3 mL of 2% Butylated Hydroxytoluene (BHT) was added. The mixture was vortexed and incubated at 100 C for 15 min. The mixture was cooled at room temperature and then centrifuged at 1000 × g for 10 min. A similar procedure was employed with the various concentrations (2×10^{-6} to 10×10^{-6}) of the 1,1,3,3-tetraethoxypropane (TEP) (Sigma-Alrdrich, Germany) standard. The absorbance of all samples and standard mixtures were measured using a spectrophotometer (Agilent, UV-Vis 8453, USA) at 531 nm wavelength. TBARS were calculated using the TEP standard curve and expressed as mg malondialdehyde (MDA) kg^{-1} of sausage.

2.10 Microbiological activity

Microbiological activity was determined by the pour method according to Arief et al. (2014). Aseptically, 25 g of the crushed sausage was homogenized in 225 mL of sterile buffered peptone water (Oxoid, UK). Serial dilutions of this suspension (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were then prepared. 1 ml was pipetted into a sterile Petri dish, for each series of the dilution and then 15-20 mL of plate count agar (Oxoid, UK) media was poured to determine the total plate count. In a similar way, in different Petri dishes, 15-20 of Eosin Methylene Blue Agar (Oxoid, UK) was poured to determine *Escherichia coli* and 15-20 of Xylose-Lyxine Deoxycholate Agar (Oxoid, UK) was poured to determine *Salmonella sp.* 100 ul of each dilution was pipetted into sterile Petri dishes and then 15-20 mL of with Baird Parker Agar (Oxoid, UK) was poured to determine *Staphylococcus aureus.* Once set, Petri dishes were incubated at 37 ^{o}C for 24-4 h. Then, the colonies formed were counted.

2.11 Statistical Analysis

A completely randomized experimental design was used and data were analyzed by one-way ANOVA. Tukey's multiple comparison test was used to determine if there were significant differences ($P<0.05$) between treatments.

3 Results and Discussion

3.1 Proximate composition

The proximate composition of the sausage is presented in Table 2. Addition of BHT and SLE up to 1.1% had no effect on the moisture content (P>0.05), however, the ash content of sausage, with added SLE, was higher (P<0.05) than for the Control and BHT sausages. SLE-2 and Control sausages contained similar fat and protein contents, and both were lower (P<0.05) compared to BHT and SLE-1 sausages. The BHT sausage had the lowest protein content and the SLE-1 sausage had the lowest carbohydrates content. The significant effect of SLE-1 and SLE-2 on some measures of proximate composition of sausage was most probably caused by the plant aqueous extracts.

3.2 Moisture content, pH, a_w and WHC

The addition of plant extract influences the proportion of ingredients in sausages. Therefore, it will affect the physical properties of sausages. The moisture content of sausages during cold storage is presented in Figure 1. There was no interaction (P>0.05) between the storage period and the formulation of sausage. The SLE-2 sausage had lower moisture content than the Control sausage (P<0.05). BHT and SLE-1 sausages were not significantly different from the Control. During cold storage, water vapor from the product surface migrates to the surroundings (El-Nashi et al., 2015). The lower moisture content of SLE-2 sausages was most possibly caused by the increased volume of solids so that the moisture content decreased.

Figure 2 shows that there was a significant interaction (P<0.05) between the chilled storage period and the sausage formulation on pH value. SLE-1 and SLE-2 sausages had a lower pH value (P<0.05) than the Control and BHT sausages. The low pH value of SLE-1 and SLE-2 sausages was most probably due to the SLE containing phenolic compounds (Fernandes et al., 2018; Suharyanto et al., 2019; Susanti et al., 2008; Wang et al., 2015; Wong et al., 2012). The decline in the pH value of SLE-1 and SLE-2 sausages was due to the phenolic compounds in the extract donating hydrogen so that the substance became acidic (Andarwulan & Faradilla, 2012).

The a_w values in this study showed an interaction between storage period and sausage formulation (P<0.05). In Figure 3, the SLE-1 and SLE-2 sausages had lower a_w values (P<0.05) compared to the Control. The a_w values increased on the 12th and 18th days of storage except for SLE-2 sausages. The low values of a_w in SLE-1 and SLE-2 sausages were thought to be due to the content of phenolic compounds in the extracts binding water molecules. Phenolic compounds contain many hydroxyl groups and can form hydrogen bonds with water molecules (Andarwulan & Faradilla, 2012) so that the presence of free water is reduced and results in a decreasing a_w value.

Water holding capacity (WHC) describes the ability of a matrix to bind water in the matrix or added water. The WHC during chilled storage is presented in Figure 4. On day 0, the WHC of SLE-1 and SLE-2 sausages was higher than the Control and BHT sausages. After 6 days of storage, the WHC of SLE-1 and SLE-2 sausages were lower than Control WHC and BHT sausages. SLE-2 sausages continued to degrade up to 18 days of storage. Although it decreased, the WHC value of SLE-2 sausages remained higher than the Control sausages until the 18^{th} day of storage.

3.3 Total phenolic content, radicals scavenging and antioxidant capacity

The total phenolic content of sausages is shown in Figure 5. The Control sausages contained the lowest phenolic content (P<0.05) when compared to sausages enriched with antioxidant agents (BHT, SLE-1, and SLE-2). The BHT sausage contained the highest total phenolic content, while SLE-1 and SLE-2 sausages had equivalent total phenolic contents. All sausages decreased in total phenolic content during storage (P<0.05). The total phenolic content on the 18^{th} day of storage declined by 12.40%, 8.50%, 9.14%

Table 2: Proximate composition of sausages enriched with an antioxidant agent on day 0 of storage

Treatments	Moisture	Ash	Fat	Protein	Carbohydrate
			%		
Control	63.66 ± 0.26^{a}	2.95 ± 0.04^{b}	5.96 ± 0.22^{b}	12.55 ± 0.11^{b}	14.88 ± 0.11^{a}
BHT	63.54 ± 0.34^{a}	2.97 ± 0.02^{b}	6.94 ± 0.03^{a}	11.88 ± 0.09^{c}	14.66 ± 0.40^{a}
SLE-1	63.45 ± 0.33^{a}	3.23 ± 0.02^{a}	7.26 ± 0.06^{a}	13.21 ± 0.21^{a}	12.85 ± 0.49^{b}
SLE-2	63.39 ± 0.18^{a}	3.27 ± 0.01^{a}	5.80 ± 0.14^{b}	12.49 ± 0.33^{b}	15.05 ± 0.64^{a}

Each value is expressed as mean ± standard deviation (n = 3). The different letter in the same column indicates significantly different ($P<0.05$).

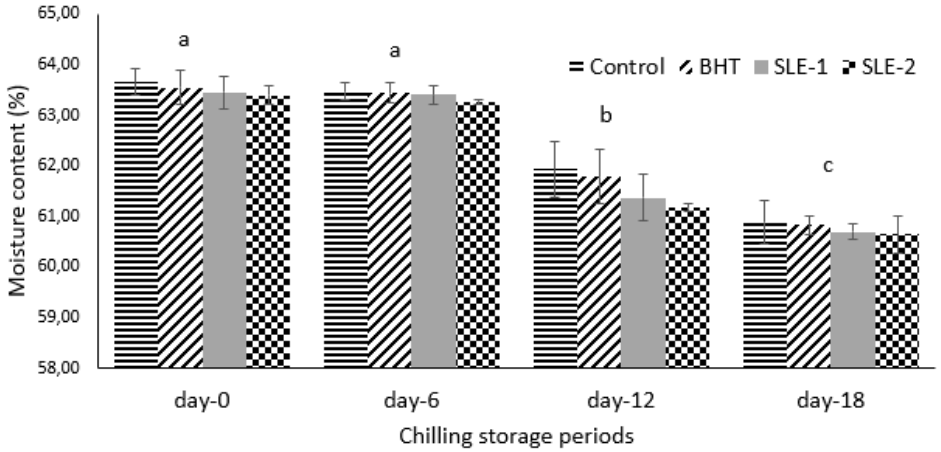

Figure 1: The moisture content of sausages enriched with an antioxidant agent during chilled storage (4 ± 1^{o}C).

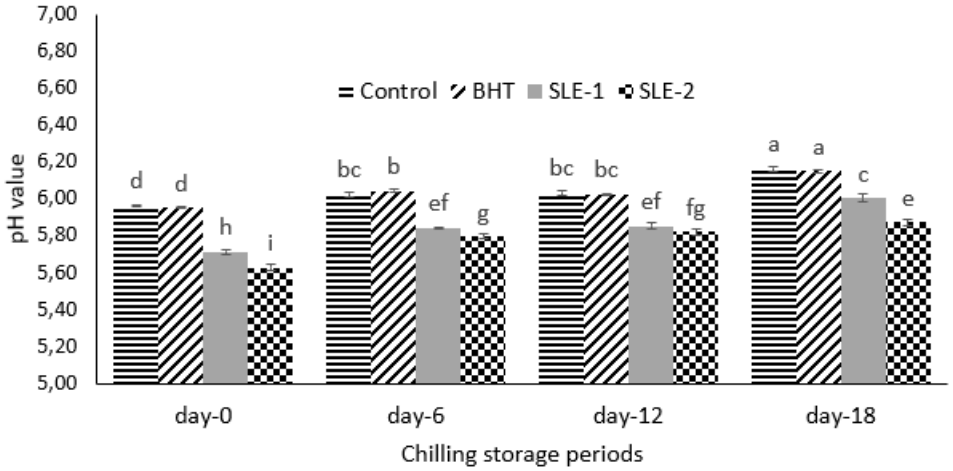

Figure 2: The pH value of sausages enriched with an antioxidant agent during chilled storage (4 ± 1^{o}C).

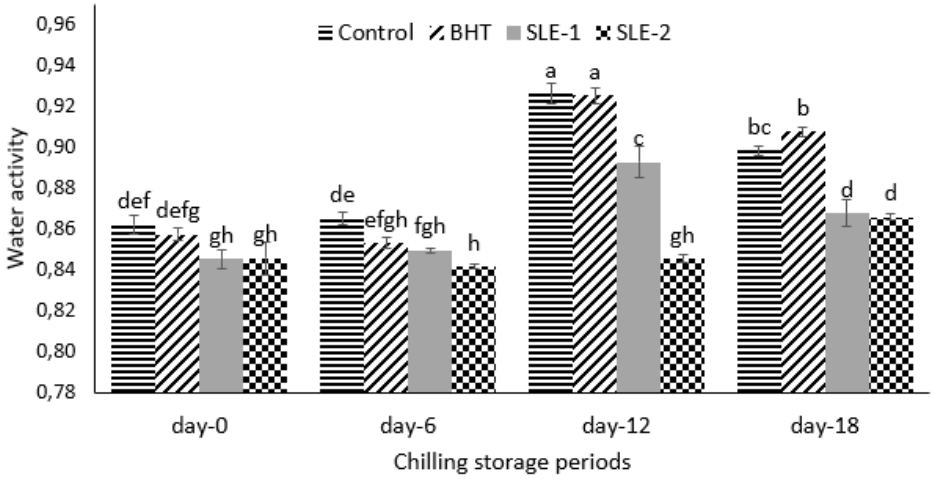

Figure 3: The water activity of sausages enriched with an antioxidant agent during chilled storage (4 ± 1^{o}C).

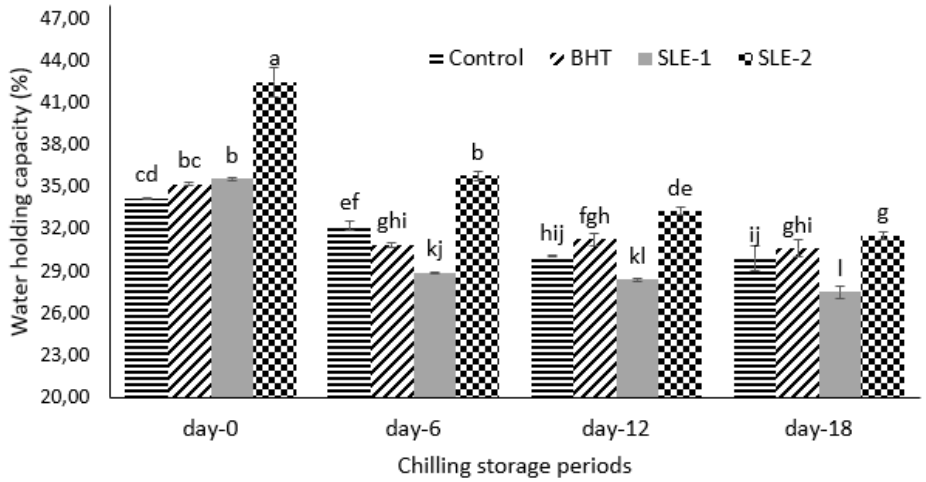

Figure 4: The water holding capacity of sausage enriched with an antioxidant agent during chilled storage (4 ± 1^{o}C).

and 9.05% for the Control, BHT, SLE-1 and SLE-2 sausages, respectively. The phenolic content in the Control sausage probably came from the spices. Spices such as garlic, pepper, nutmeg and others are rich in phenolic compounds (Suryati et al., 2014). The high content of total phenolic in SLE-1 and SLE-2 sausages might be caused by the addition of SLE. This is reasonable because the leaves contain a lot of phenolic compounds (Suharyanto et al., 2019; Susanti et al., 2008; Wong et al., 2012).

This research indicated that the total phenolic content of the sausage influences DPPH radicals scavenging and antioxidant capacity. Figures 6 and 7 show that the Control sausage had the lowest DPPH radical scavenging and antioxidant capacity ($P<0.05$), respectively. The SLE-1 and SLE-2 sausages had comparable DPPH radicals scavenging and antioxidant capacity to BHT sausages. All sausages underwent a decrease in their DPPH radical scavenging ability and antioxidant capacity during chilled storage. The decline was in line with the decrease in the total phenolic content of the sausages during storage. The phenolic compounds worked as antioxidants. A similar pattern of decline was also observed in the antioxidant capacity of sausages (Figure 7). The antioxidant capacity of all sausages decreased during chilled storage ($P<0.05$). Whilst the antioxidant capacity of Control sausages decreased in each storage period, the BHT, SLE-1 and SLE-2 sausages only decreased their antioxidant capacity on the 12^{th} and 18^{th} days of storage. Over 18 days of chilled storage, the antioxidant capacity of the Control, BHT, SLE-1 and SLE-2 sausages reduced by 43.37%, 11.76%, 12.06% and 10.11%, respectively. BHT, SLE-1 and SLE-2 sausages had equivalent antioxidant capacities except for the 6^{th} day of storage, whilst the BHT sausages had higher antioxidant capacities. The antioxidant capacity of the Control sausage was the lowest ($P<0.05$). This phenomenon is confirmed by the total phenolic content in each sausage.

The high percentage of DPPH scavenging and antioxidant capacity in BHT, SLE-1 and SLE-2 sausages was hypothesized to be due to the addition of antioxidant agents to the sausage formulation. BHT is a synthetic compound that contains a phenolic group and has an effective ability as an antioxidant. The SLE also contains phenolic compounds and plays an essential role as an antioxidant (Alwash et al., 2014; Suharyanto et al., 2019; Susanti et al., 2008; Wong et al., 2012).

3.4 Thiobarbituric acid reactive substances (TBARS)

TBARS value indicates the level of oxidation of a product. The lower the TBARS value of a sample, the lower oxidation of a product. Sausages enriched with antioxidant agents (BHT, SLE-1 and SLE-2) showed significantly lower TBARS values ($P<0.05$) than the Control. On day 0 of storage, BHT sausages had the lowest TBARS value. Yet, on days 6 and 12, it was not markedly different from SLE-2 sausages ($P>0.05$) but lower than SLE-1 sausages ($P<0.05$). However, on the 18th day of storage, these sausages had TBARS values that were not notably different. In general, all sausages underwent an increase in TBARS value. It indicates the accumulation of oxidation products in the sausages during storage. Although the TBARS value of the Control sausage was quite high, it was still below the detectable rancidity threshold of 5 mg MDA/kg (Insausti et al., 2001). The low TBARS values in SLE-1 and SLE-2 sausages indicates that the extract acted as an antioxidant (Alnajar et al., 2012; Alwash et al., 2014; Suharyanto et al., 2019; Zakaria et al., 2011). This ability was most likely contributed by the phenolic compounds of the extract (Jin et al., 2015; Kalem et al., 2017; Zhang et al., 2017).

Phenolic compounds are capable of scavenging DPPH radicals and have adequate antioxidant capacity so that they are able to inhibit oxidation characterized by low TBARS values. These capabilities are due to the phenolic compounds which have redox potential to absorb and neutralize free radicals, inhibit singlet oxygen and decompose peroxides (Kalem et al., 2017). This mechanism takes place by transferring the H atom from the OH group of the phenolic compounds to the peroxyl radical chain where the next reaction occurs with the resultant peroxyl (Bendary et al., 2013). Phenolic compounds can also donate hydrogen to react with reactive oxygen and nitrogen species in the termination reaction and play a role in

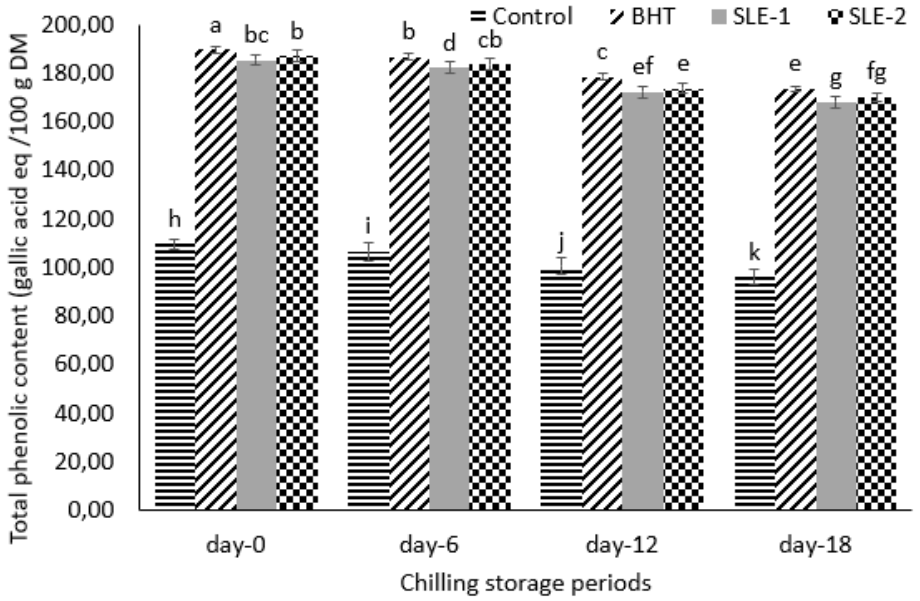

Figure 5: The total phenolic content of sausages enriched with an antioxidant agent during chilled storage (4 ± 1^oC).

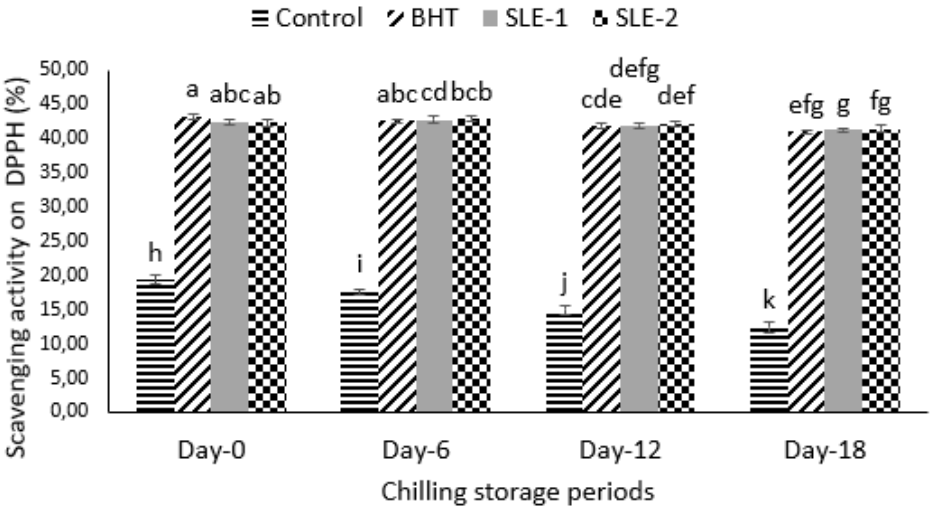

Figure 6: The scavenging activity on DPPH of sausages enriched with an antioxidant agent during chilled storage (4 ± 1^oC).

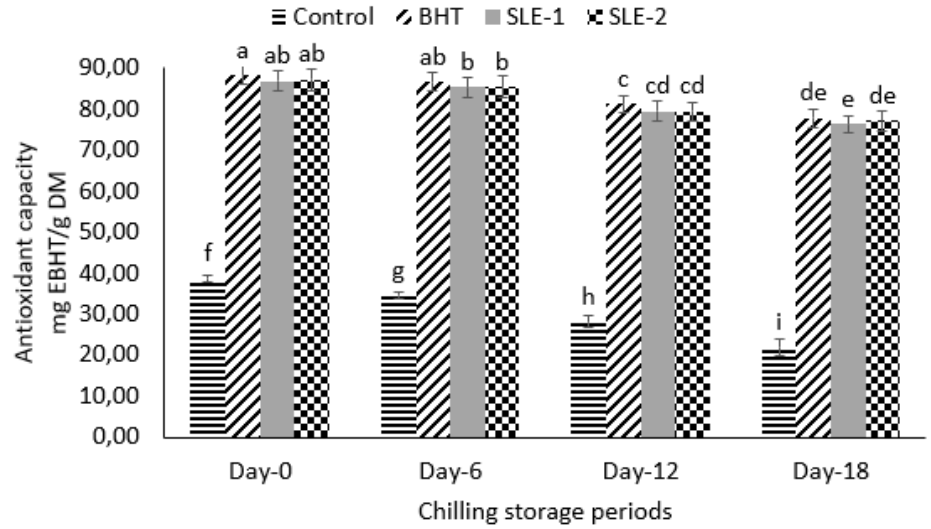

Figure 7: The antioxidant capacity of sausages enriched with an antioxidant agent during chilled storage (4 ± 1^oC).

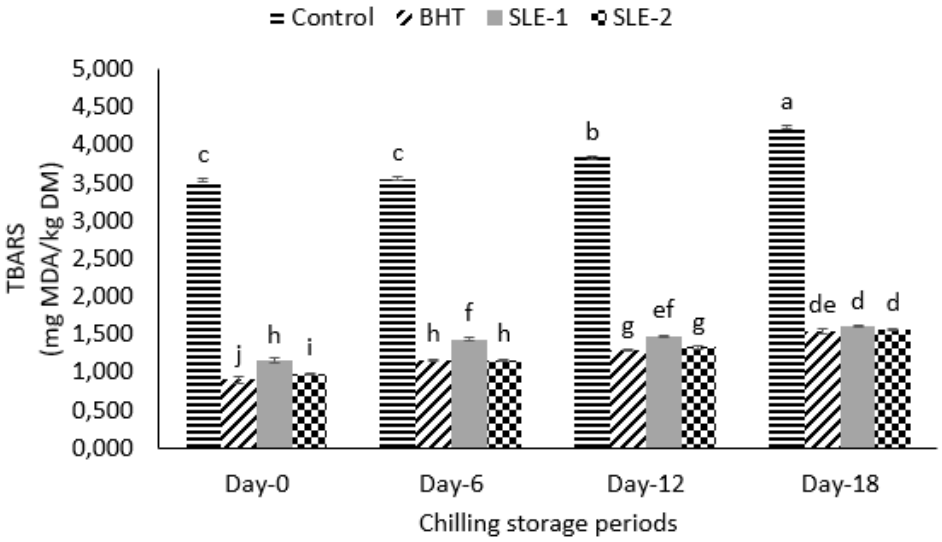

Figure 8: The TBARS of sausages enriched with an antioxidant agent during chilled storage (4 ± 1^oC).

Table 3: Bacterial growth in sausages enriched with an antioxidant agent during chilled storage (4 ± 1^{o}C).

Treatments	Day-0	Day-6	Day-12	Day-18
Total Plate Count (CFU g^{-1}):				
Control	nd	2.7×10^1	2.0×10^2	3.6×10^2
BHT	nd	$<10^1$	1.6×10^2	3.0×10^2
SLE-1	nd	nd	2.0×10^1	2.7×10^1
SLE-2	nd	nd	10^1	2.7×10^1
Staphylococcus aureus(CFU g^{-1}):				
Control	nd	nd	$<10^1$	2.7×10^1
BHT	nd	nd	$<10^1$	2.0×10^1
SLE-1	nd	nd	$<10^1$	2.0×10^1
SLE-2	nd	nd	$<10^1$	1.7×10^1
Salmonella (CFU g^{-1}):				
Control	nd	nd	nd	$<10^1$
BHT	nd	nd	nd	$<10^1$
SLE-1	nd	nd	nd	nd
SLE-2	nd	nd	nd	nd
E. coli (CFU g^{-1}):				
Control	nd	nd	nd	nd
BHT	nd	nd	nd	nd
SLE-1	nd	nd	nd	nd
SLE-2	nd	nd	nd	nd

CFU – colony forming unit, nd – not detected.

breaking the cycle of new radical formation. The radicals formed from the reaction are more stable than the initial radicals (Pereira et al., 2009).

3.5 Microbiological activity

Microbiological activity shows the extent of SLE's effect on the microbiological quality of sausages. The role of SLE as an antibacterial has been known through exploratory studies (Alnajar et al., 2012; Alwash et al., 2014; Wong et al., 2012). Bacterial growth in sausage enriched with an antioxidant agent during chilled storage (4 ± 1^{o}C) is presented in Table 3. Sausages without the addition of SLE (Control and BHT) grew bacteria (total plate count) on the 6th day of storage, while no microorganisms were detected in sausages with added SLE (SLE-1 and SLE-2). The longer the storage period, the higher the total plate count. On the 18^{th} day of storage, SLE-1 and SLE-2 sausages reached log 1 colonies but the Control and BHT sausages reached log 2 colonies.

On the 12^{th} day of storage, all sausages grew less than 1 log of *Staphylococcus* colonies. This bacterial colony developed up to the 18^{th} day of storage, with a population of about 1 log (Tables 3). *Salmonella sp.* bacteria colonies grew on the Control and BHT sausages on the 18^{th} day of storage with less than one log, while no *Salmonella sp* colonies were detected in SLE-1 and SLE-2 sausages until the end of the observation (Table 3). The results of this study also showed that *E. coli* bacteria were not detected in all sausages in each storage period.

The addition of 0.83% (SLE-1) and 1.1% (SLE-2) was able to inhibit the growth of several pathogenic bacteria in sausages until the 18^{th} day of chilled storage. This was most likely due to phenolic compounds contained in SLE (Susanti et al., 2008; Wong et al., 2012) which can act as antibacterial agent (Alnajar et al., 2012; Alwash

et al., 2014; Zakaria et al., 2011).
In general, all sausages meet the requirements of the Indonesian National Standard (SNI) except for the Control and BHT sausages where less than 10^1 *Salmonella* colonies were detected. Based on the SNI for sausages, the maximum total plate count is 1×10^5 CFU g^{-1}, the maximum *Staphylococcus* is 1×10^2 CFU g^{-1}, *Salmonella* must be negative, and the *E. coli* must be less than 3 MPN (most probable number) g^{-1} (BSN, 2015).

4 Conclusions

The addition of an extract of senduduk leaf up to 1.1% of the total mass of ingredients in the formulation improved the physicochemical properties of sausages, and inhibited oxidation and microbial growth in sausages until the 18th day of chilled storage. The ability to retard oxidation was equivalent to 0.01% BHT.

Acknowledgements

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Views of Food Stakeholders on Packaging Factors

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Abstract

Packaging has an enabling role in supply chains (SCs) as it facilitates a range of functions, specifically (i) marketing; (ii) logistics and SC management; (iii) food technology; and (iv) environmental protection. This study explores the appreciation towards certain food packaging factors and attributes by consumers and industrial experts for a range of food product types. Primary data was collected through a questionnaire in the Greek market and then analysed using a 1-way ANOVA and the Tukey test. The study findings reveal that food SC stakeholders mainly appreciate packaging attractiveness. Other attributes that promote environmental sustainability and facilitate logistics operations were of high importance as well.

Keywords: Food packaging factors; Food packaging attributes; Food supply chains; Food product types

1 Introduction

Packaging has an enabling role in supply chains (SCs) as it facilitates distribution and logistics operations from an end-to-end perspective whilst allowing the realisation of reverse product flows (Pålsson & Sandberg, 2020). Particularly for food SCs, packaging conditions and techniques are a key decision in planning operations to enable the hygienic and safe delivery of primarily perishable commodities (Tsolakis et al., 2014). Consumers greatly appreciate this latter function, especially in cases such as the coronavirus outbreak (Feber et al., 2020). Packaging selection has emerged as a critical parameter in the optimisation of perishable food production routing problems (i.e., joint optimisation of production, inventory, distribution, and routing operations) that propels economic and environmental benefits (Li et al., 2020). The business significance of food packaging is reflected upon the respective global market value of about US$305 billion (Statista, 2020), which accounts for about 65-70% of the total packaging sales (Brody, 2008).

In food SCs, packaging has a multi-functional role in terms of marketing, logistics and handling operations, mitigation of spoilage effects, and environmental protection (Konstantoglou et al., 2020a). In upstream SC operations, supplier selection for packaging materials is pivotal for ensuring low-cost sourcing (Kumar et al., 2011). Downstream the food SC, packaging acts as a communication medium of brand image and firms' social responsibility whilst having an influential role on consumers' choices (Marsh & Bugusu, 2007; Silayoi & Speece, 2007). To that end, several packaging attributes are utilised for com-

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munication purposes, such as colours, designs, shapes, symbols, and messages, to provide companies with a competitive advantage (Konstantoglou et al., 2020b). These attributes are further categorised into factors that influence the purchasing behaviour of the public (Konstantoglou et al., 2020b).
Notwithstanding the multi-disciplinarity of food packaging (Konstantoglou et al., 2020a), a number of research gaps are evident. First, extant studies tend to focus on certain food packaging function(s) and/or attribute(s), whilst considering either:

1. an overarching view of food as an end-product (Young et al., 2020);
2. specific food product categories such as dairy (Baruk & Iwanicka, 2016); or
3. a limited number of food types like indicative cornflakes and popcorn (Scarpi et al., 2019).

Second, to the best of our knowledge, no study considers the views of food SC consumers and industry executives for an extended range of packaging factors and attributes for a multitude of product types. Third, consumers' and managers' views about packaging functionalities and attributes for specific food types remain poorly understood (Konstantoglou et al., 2020b). Owing to the ongoing advancements in packaging technology and reconfigurations in the food SC landscape (Han et al., 2018; Tsolakis et al., 2014), food SC stakeholders' views over packaging need also to be continuously monitored to capture emerging market requirements and business opportunities.
This research aims to shed light on the contemporary perceptions of consumers and industrialists about the appreciated packaging factors and attributes for specific food product types, addressing the following research questions:

Research Question #1 What are the key packaging factors that are appreciated by consumers and industry executives concerning specific food product types?

Research Question #2 How do consumers and industry executives value the attributes of packaging for particular food product types?

It is critical to answer the above research questions since food packaging is a key operational echelon in food SCs (FoodDrink Europe, 2019), with implications for both processors and consumers. In order to address Research Question #1, a literature overview and bibliometric analysis were conducted to map the relevance of key terms in the packaging domain that are dominant in the respective functionalities. Thereafter, a specifically designed primary survey tool to gather primary data from consumers and managers to tackle Research Question #2. The primary data was analysed using 1-way ANOVA and the Tukey test. The synthesis of the literature output and the survey findings reveal future research pathways.
Our research contributes to the food SC management field by contemporality exploring the views of consumers and industry executives on particular packaging factors and attributes for a range of food product types. To the best of our knowledge, this is the first research to embrace these multi-faceted aspects with regard to food packaging within an SC context.
The remainder of this paper is structured as follows. Section 2 describes the materials and methods relevant to this research by initially defining the literature search and bibliometric analysis process. The survey design and methodology used to gather data and analyse SC stakeholders' views on packaging factors and attributes for particular food product types are described as well. Section 3 presents the literature background on food packaging functionalities, while Section 4 discusses the primary research findings. A discussion of the results is provided in Section 5, while conclusions, limitations, and recommendations for future research are discussed in the final Section 6.

2 Materials and Methods

This research is expected to contribute to the food SC management domain via exploring diverse stakeholders' views about the significance of packaging factors and attributes for specific products; therefore, the object of scrutiny has to

be an analysis of the relevant literature (Tranfield et al., 2003) along with a questionnaire-based survey (Gideon, 2012, p.91-93). The research perspective, the literature overview and the questionnaire-based survey method underpinning this study are detailed in the subsections that follow.

2.1 Research perspective

To investigate the views of food SC stakeholders on packaging factors and attributes for specific product types, it is important to identify the constructs. This research adopts the view of Konstantoglou et al. (2020a) about the contributing role of food packaging with regard to the functional areas of

1. marketing;
2. logistics and SC management;
3. food technology; and
4. environmental protection.

The latter functional areas support packaging multi-disciplinarity and multi-functionality and shall be considered in food manufacturing and retailing. This view is also in alignment with (Rundh, 2013, p.1548) who quotes that: "... new customer needs have led to a consideration of new requirements for the design of a package and a development process involving the logistic, commercial and environmental functions of packaging".
Based on these four functional roles, and in alignment to the findings of Konstantoglou et al. (2020b), this research recognizes seven packaging factors, including:

1. informational content;
2. content protection and recognition;
3. smart functioning;
4. geometry;
5. environmentally friendliness;
6. endurance; and
7. coloration.

The latter denote specific attributes that directly appeal to consumers and industry executives. Figure 1 depicts the hierarchical structure of the considered food packaging constructs in this research.

2.2 Literature overview and bibliometric analysis

Literature searches were performed to gather relevant articles and conduct bibliometric analyses per each of the four identified food packaging functions. The bibliometric analyses helped to develop a holistic understanding of food packaging and functionalities by charting the knowledge structure and evolution of the research field (Danvila-del-Valle et al., 2019).
Provided that this research investigates food packaging factors and attributes across the functions of marketing, logistics and SC management, food technology, and environmental protection, four different literature searches with corresponding keywords (Table A1 in Appendix I) were conducted. The literature search terms were derived from a preliminary analysis of the literature on food packaging (e.g., Büsser and Jungbluth (2009) and Molina-Besch and Pålsson (2020). The literature searches were performed only via using the Scopus database owing to its recognition by the academic community and the reliability of the included academic outlets (Caviggioli & Ughetto, 2019). Ultimately, by 2nd May 2021, the four individual searches led to the identification of a total of 1,579 articles written in English.
In addition, an exploration how 'packaging' relates to each of the topics of 'marketing', 'logistics and SC management', 'food technology', and 'environmental protection' was conducted. Using the literature search outputs (as outlined above), we extracted the unstructured part of the publications' metadata (article title, paper abstract) and created a topic model using the co-occurrence relationship of the words in the abstracts of the retrieved articles and the semantic distance between the most frequent terms (the stop-words were excluded from the analysis). Even if some words are rather generic such as group, participant, sample, etc. we kept them

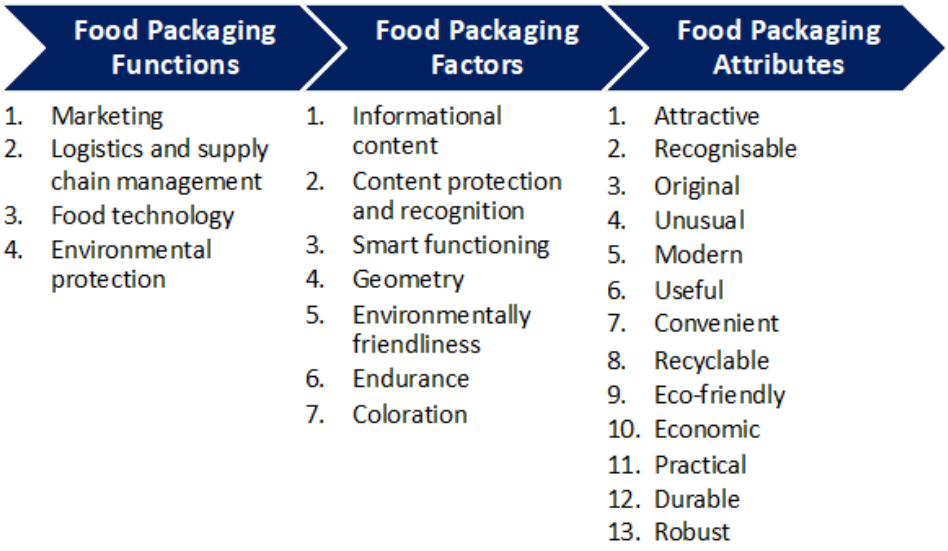

Figure 1: Food packaging functional roles, factors and attributes.

to capture the quantitative approaches of the research papers that are used in order to examine the relationships between the examined disciplines. The ratio of raw frequency counts over total counts was calculated to identify the underlining themes (i.e., clusters of terms) per every food packaging function.

2.3 Primary data collection

To evaluate the consumers' and industrial experts' views on food packaging, a survey to collect primary data was designed. The questionnaire was originally written in Greek and is inserted in Annex II (translated in English). The questionnaire is inserted in Appendix II. First, for consumers, the sampling locations were the stores of large retail chains in Northern Greece where the questionnaire was delivered face-to-face. To target managers in the food industry, an invitation was sent to the executive staff of 180 Greek companies that produce packaged food, asking them to complete the corresponding online questionnaire. These managers were in marketing/sales or logistics/supply chain departments, as well as, as food technologists or responsible for the environmental actions in their companies.

The survey involved 188 consumers and 123 industry executives who were asked to rate the significance of seven packaging factors and thirteen packaging attributes. Nineteen different food types were rated. The instructions given to consumers were to choose the products they usually purchase for themselves and/or their families. Industry experts were asked to rate products for which they had relevant knowledge; the informants were not employed in the manufacturing companies of the products for which characterization was provided.

3 Results and Discussion

Packaging supports multi-functional areas in food SCs (Hellström & Saghir, 2007), with Marsh and Bugusu (2007) denoting that "the goal of food packaging is to contain food in a cost-effective way that meets industry requirements and consumer desires, maintains food safety and minimizes environmental impact", thus highlighting the critical role of packaging in farm-to-fork operations. First, packaging helps promote food brands via displaying and communicating tangibly the brands its history/values (Ampuero

& Vila, 2006). A challenging fact is that packaging has to induce consumers to purchase particular products over alternative ones within a very limited amount of time (e.g., in the range of seconds). Second, Paine (1981) defines packaging as a system for preparing goods for their efficient transport, distribution, storage, and retail for the safe delivery of products to consumers at the lowest possible cost.
Third, food packaging is used to preserve and extend the shelf life of food products, e.g., in terms of nutritional value, appearance, and freshness, which is highly appreciated by consumers (Giusti et al., 2008). Fourth, the recognition of the growing impact of production and consumption on the environment has led to the development of certain packaging production methods that are environmentally friendly whilst informing consumers about packaging circularity via reusing and recycling (Yokokawa et al., 2021). The following subsections focus on the main considered functionalities of food packaging, namely:

1. marketing;
2. logistics and SC management;
3. food technology; and
4. environmental protection (Konstantoglou et al., 2020a).

3.1 Marketing

Packaging has multiple roles and affects almost all parts of a business (Denison & Cawthray, 1999). The role of packaging is not merely limited to protecting the enclosed products but has a function as a branding and advertising medium (Silayoi & Speece, 2004). Consumers are generally attracted by appealing packaging, which is in alignment with brand identity and consumers' personality (Underwood, 2003). In particular, packaging has a critical role in assisting corporations to effectively communicate a distinct food product proposition and attract the attention of consumers (Coles et al., 2003). Overall, food packaging is recognized to appeal to consumers' purchasing decisions (Koutsimanis et al., 2012), and relates to five major marketing implications, namely:

1. Increases product perception via motivating consumers' senses,
2. Attracts the attention of consumers,
3. Affects the purchasing behaviour of consumers,
4. Influences the decision to purchase certain products, and
5. Advertises and promotes the product.

The marketing functionality of packaging is appreciated due to the structure of the food retailing market that is characterised by the increased number of self-service stores, thus rendering packaging a significant medium to promote corporate image (Becker & Remington, 2011). The function of packaging in terms of marketing can be traced to the claim of Meyers and Gerstman (2004, p.40) who stated: "We select products by the perception of what we see and read on the package". To a greater extent, based on Muratoglu, Vice President of Marketing and Product Management at Tetra Pack Inc., "... packaging can serve as a point of differentiation and generate further value for consumers" (Furhman, 2011). Notably, Rundh (2013) recognised the marketing role of packaging as a more vital function to food protection.
Marker leaders have managed to create such recognizable packages, which are now an integral part of their corporate brand and communication strategy (Ares & Deliza, 2010). Owing to the influential role of packaging over children, research suggests that institutional directives shall focus on regulating child-appealing marketing of packaging to mitigate the risk of diet-related chronic diseases (Mulligan et al., 2020). Figure 2 illustrates the bibliometric map to understand the relevance of the terms 'packaging' and 'marketing' to other concepts in the literature. Findings confirm that Health and nutrition are two interrelated concepts that receive constant attention from the consumers, and the quality of a food product is inextricably linked to the quality of its packaging.

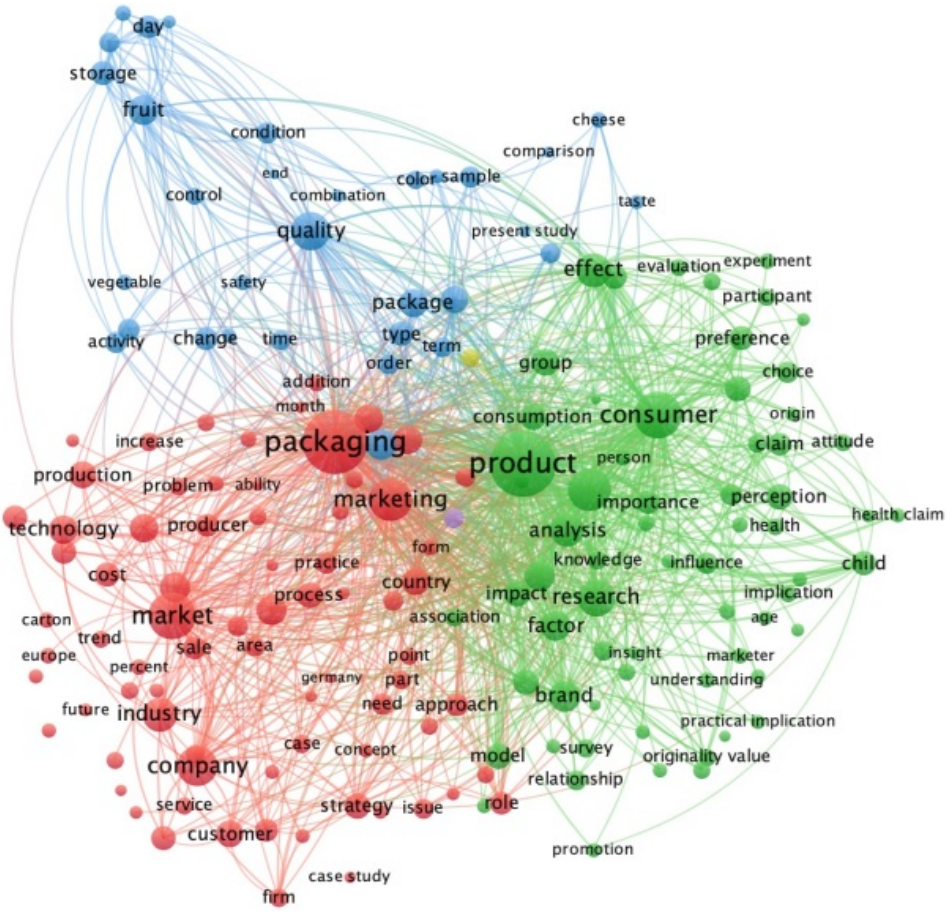

Figure 2: Network map illustrating the relations between the terms 'packaging' and 'marketing' in food supply chains.

3.2 Logistics and Supply Chain Management

Packaging is considered to be one of the most important means for material storage and management (Lambert et al., 1998). According to Sara (1990), the packaging is another subsystem of a company's logistics, while it should be considered as a medium that facilitates customer service and is closely linked to a product per se. The efficiency of product flows across a supply chain depends, among others, on packaging (Hellström & Saghir, 2007). Many studies in the literature examine how packaging supports logistics processes and food traceability in the food SC (Ahmed et al., 2005; Rundh, 2009).

In terms of food SCs, packaging must be properly designed to protect the enclosed goods during logistics operations (e.g., transportation, warehousing) whilst preserving the intrinsic food quality attributes (Lemaire & Limbourg, 2019). Packaging also affects the cost of logistics. Indicatively, Bowersox and Closs (1996) and Mohite and Chandel (2020) identified the business areas where packaging positively affects costs, including

1. inventory management (control) – the accuracy of the inventory process is positively affected by the proper selection and use of

packaging;

2. speed, accuracy and efficiency – three operational constituents that affect product identification the ease of handling;
3. cost management – facilitating handling operations while at the same time the operations are accelerated;
4. transport and storage costs –packaging must meet the different transport and storage needs in order to reduce costs and facilitate the whole process; and
5. customer service – customer satisfaction depends on the packaging to achieve quality control during distribution, providing useful information on handling, storage, ingredients, etc. and compliance with regulations (such as environmental, food, etc.).

Figure 3 depicts the bibliometric map of the relevance of the terms 'packaging' and 'logistics' to other concepts in the pertinent literature. Once again, the findings support packaging in terms of the protection and safety of products.

3.3 Food Technology

During distribution, food quality may deteriorate across biological and chemical attributes; storage time and temperature are determinant factors of food quality (Labuza & Breene, 1989). Therefore, in addition to enabling efficient distribution, sales and consumption, packaging prevents the deterioration of food and beverages' quality that occurs due to environmental effects (Han, 2014). Furthermore, an important concept for food technology is the nutrition label as it enables communication between the food producer and consumers (van Kleef et al., 2008). In every packaged food product, the nutrition label must be indicated in order to provide the consumer with useful information about

1. nutritional information such as protein content, fat, calorific value, and other nutrients;
2. information required by law (e.g., ingredients, weight/volume, storage conditions, shelf life); and
3. information not required by law.

Labels can help promote options for a proper, healthy and balanced diet (Agarwal et al., 2008). The bibliometric map of the relevance of the terms 'packaging' and 'food technology' to other concepts in the relevant literature is demonstrated in Figure 4. The findings focused on the safety and quality of the products and the information provided by the packaging, and less so on the aesthetic elements. The findings of this study show the acquisition of the environmentally friendly awareness of consumers.

3.4 Environmental protection

According to the US Environmental Protection Agency (2020), packaging materials consist of circa 28% of all municipal solid waste. As food is the only product that is consumed frequently daily, good practices for the disposal and management of food packaging waste are needed (e.g., plastic and glass recycling, composting). The monitoring of the environmental footprint of food along with the design and development of environmentally friendly packaging and labellin are other important initiatives for stakeholders (Leire & Thidell, 2005; Teisl et al., 2002). For example, the use of recyclable plastic containers in food catering chains had been demonstrated through LCA analysis to lead to reduced environmental impact in terms of CO_2-eq emissions (Accorsi et al., 2014). Recently, the use of natural leaf-type packaging material for local food products has been demonstrated to ensure environmental and health advantages, compared to synthetic packaging material, with further socio-economic implications in developing countries (Ezeudu et al., 2021).
Environmental labelling, defined as a visual communication tool that indicates products, services, and companies that operate according to specific environmental standards and criteria (Follows & Jobber, 2000; Taufique et al., 2014), has a decisive influential role in purchasing and consumption patterns (Banyte et al., 2010). Therefore, environmental labelling (or eco-labelling) is a modern environmental policy tool that stresses the role of information on the impact to the environment of production, distribution, consump-

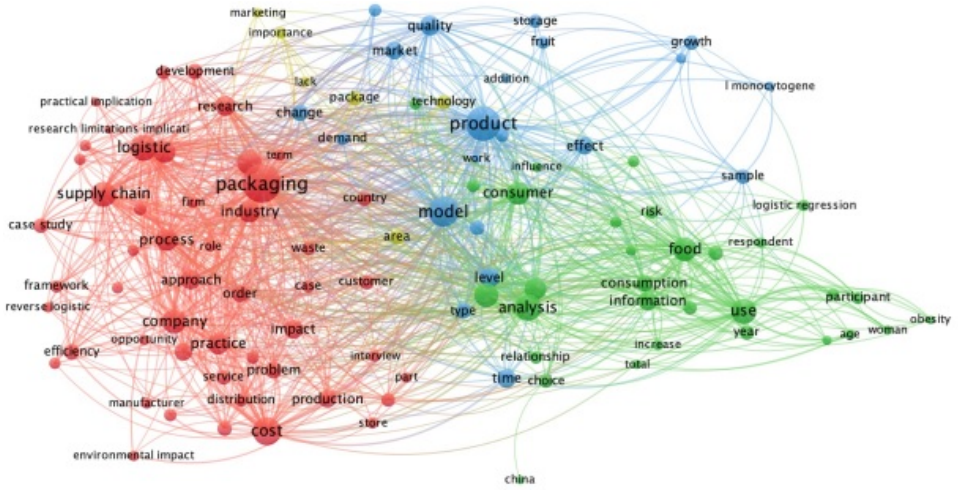

Figure 3: Network map illustrating the relations between the terms 'packaging' and 'logistics' in food supply chains.

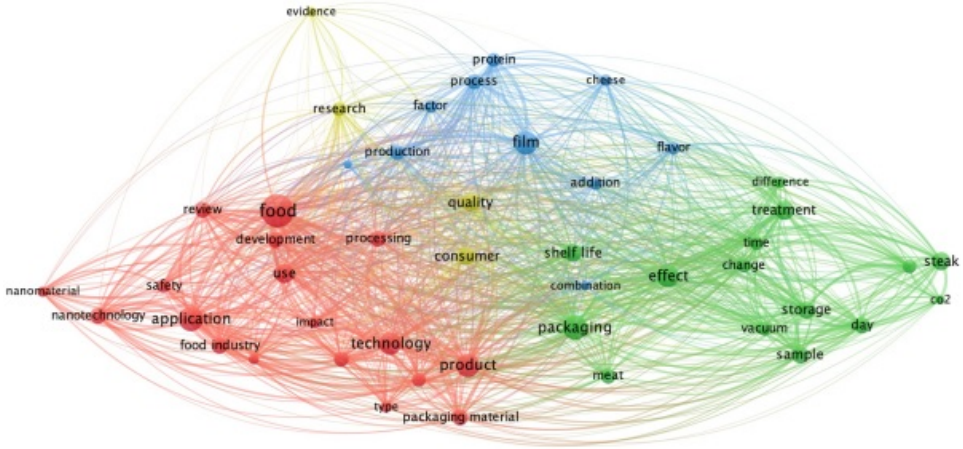

Figure 4: Network map illustrating the relations between the terms 'packaging' and 'food technology' in food supply chains.

tion and disposal of products (Jordan et al., 2003). At the consumer's end, environmental labels help distinguish "green" from conventional food products, thus influencing consumer behaviour in terms of preferences, purchase intention, willingness to pay, future redemption patterns and the word-of-mouth dissemination of relevant information (Brécard et al., 2009; Chen & Chang, 2012). Figure 5 shows the bibliometric map of the relevance of the terms 'packaging' and 'environmental protection' to other concepts in the literature.

4 Conclusions

The views of the informants with regard to packaging factors and packaging attributes, per the product type, are inserted in the sub-sections that follow.

4.1 Packaging Factors

In general, for all product types, consumers considered the factors of 'Environmental Friendliness' and 'Geometry' as important by assigning above-average values (Table 1). Notably, the gathered responses demonstrate the environmental sensitivity of consumers. On the opposite side, 'Smart Functioning' is regarded by consumers as the least significant packaging factor for all product types. Interestingly, cold cuts comprise a product type where the functions of 'Information Content', 'Content Protection & Recognition' and 'Endurance' are highly regarded by consumers, probably due to the elevated retail price and nutritional significance. Consumers also highly rated the packaging endurance of canned food and are interested in information and endurance of packaging for legumes and salad dressings.
Industry executives tend to appreciate packaging factors that enable logistics operations such as 'Endurance' and 'Geometry' (Table 2). The informative character of packaging is also appreciated. In alignment with consumers, industry executives regard 'Smart Functioning' as the packaging factor with the least significance for all product types. However, contrary to consumers, the environmental sustainability of packaging is of low concern for industrialists.

4.2 Packaging attributes

In terms of packaging attributes, consumers highly appreciate recyclability for all products studied, except for spices (Table 3). Following that, cost-effectiveness along with practicality and convenience are packaging attributes that consumers value. Interestingly, originality and robustness are the least appreciated attributes for all product types, except for spices.
The results highlight the diversity of consumers' views on packaging attributes and product types. Recyclability, economic, practicality, eco-friendliness, and convenience are attributes that are highly valued for fast-moving products such as dairy, salad-vegetables and nuts. Consumer concerns on premium and long shelf-life products, such as spices, are related to packaging characteristics associated with product safety and security, branding and uniqueness. The latter is demonstrated via the high appreciation of packaging solutions that are recognisable, durable, modern and attractive.
Industry executives do not seem to demonstrate any particular appreciation with regard to certain packaging attributes for product types (Table 4). Notable exceptions include chocolates, soft drinks and snacks, which are expected to have recognisable and attractive attributes to entice consumers' purchasing interest and increase sales. In addition, convenience and practicality are valued for cold cuts and legumes.

4.3 Comparative views

To identify any differences in the perceptions of consumers and industry executives with regard to the different factors of food packaging, a 1-way ANOVA at 99% confidence interval analysis was performed to compare the mean values for every food product type. In case that the differences in the means were considered, the data was analysed by the Tukey test ($p < 0.001$) to determine if the relationship between the data sets is statistically significant. The findings are summarised in Table 5.

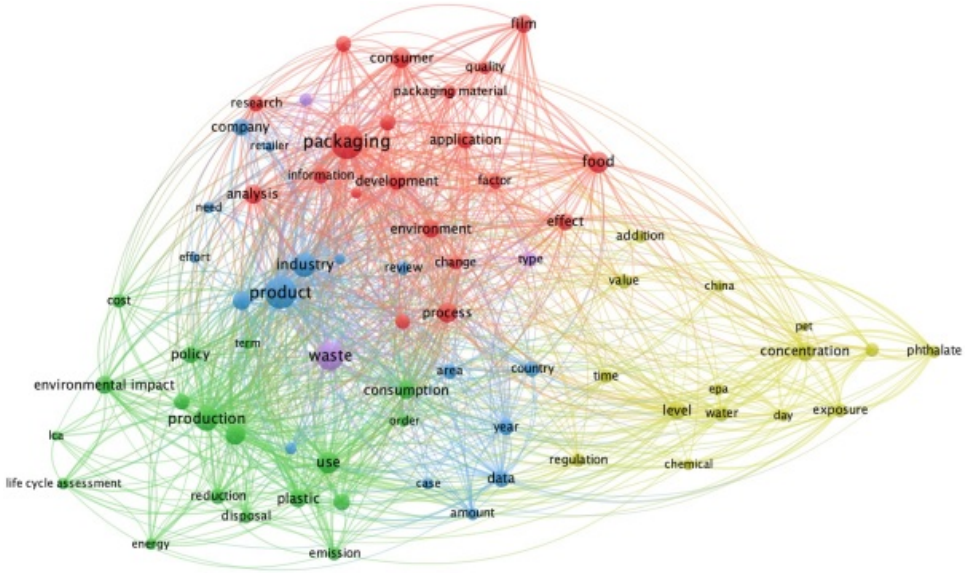

Figure 5: Network map illustrating the relations between the terms 'packaging' and 'environmental protection' in food supply chains.

Table 1: Consumers' appreciation over food packaging factors per product type.

Food Product Type (N)	Informational Content	Content Protection & Recognition	Smart Functioning	Geometry	Environmental Friendliness	Endurance	Coloration
• Alcoholic Beverages (10)	3.1 (0.4)	2.3 (0.3)	2.4 (0.4)	4.1 (0.9)	4.4 (0.3)	2.0 (0.6)	2.8 (0.5)
• Canned Food (10)	2.4 (0.6)	3.0 (0.6)	2.0 (0.7)	3.9 (1.0)	4.0 (0.6)	4.2 (0.7)	2.8 (0.5)
• Cereals (10)	1.5 (0.2)	1.5 (0.2)	2.2 (0.9)	5.0 (0.0)	3.6 (0.5)	2.9 (0.2)	3.1 (0.5)
• Cheese (10)	2.4 (0.5)	2.6 (0.7)	2.3 (0.9)	3.5 (0.9)	3.1 (1.0)	3.2 (0.6)	2.4 (0.4)
• Coffee (10)	3.2 (0.3)	3.2 (0.2)	1.6 (0.7)	4.6 (0.3)	3.8 (0.7)	2.4 (0.9)	2.6 (0.3)
• Cold Cuts (10)	4.1 (0.2)	3.7 (0.2)	2.4 (0.2)	5.0 (0.0)	3.3 (0.5)	4.3 (0.0)	1.7 (0.1)
• Dairy (10)	1.9 (0.4)	1.5 (0.1)	2.3 (0.4)	3.9 (0.4)	4.7 (0.0)	1.6 (0.3)	2.2 (0.2)
• Flour (10)	3.2 (0.2)	3.0 (0.2)	2.0 (0.5)	3.0 (0.0)	4.5 (0.2)	1.9 (0.8)	2.5 (0.4)
• Juices (10)	2.9 (1.1)	2.8 (0.8)	2.0 (1.0)	2.9 (1.1)	4.0 (1.1)	3.1 (1.3)	2.6 (0.7)
• Legumes (10)	3.4 (0.6)	3.7 (0.5)	2.9 (1.1)	3.5 (0.9)	3.7 (0.6)	3.4 (0.6)	3.2 (0.8)
• Meat (10)	2.5 (0.3)	3.3 (0.2)	1.6 (0.2)	3.3 (0.9)	2.5 (0.2)	3.4 (0.4)	2.5 (0.5)
• Nuts (10)	3.2 (0.0)	2.7 (0.0)	1.0 (0.0)	3.0 (0.0)	3.0 (0.0)	4.3 (0.0)	2.8 (0.2)
• Pasta (10)	3.0 (0.6)	2.3 (0.3)	1.0 (0.0)	3.8 (0.4)	1.2 (0.4)	2.9 (0.7)	2.1 (0.5)
• Salad Ointments (10)	3.6 (0.4)	3.2 (0.6)	2.0 (1.4)	3.6 (0.9)	3.4 (0.8)	3.3 (0.6)	2.7 (0.5)
• Salads – Vegetables (10)	2.3 (0.4)	2.5 (0.3)	1.0 (0.0)	2.5 (0.4)	5.0 (0.0)	2.0 (0.2)	2.4 (0.5)
• Snacks (8)	2.4 (0.4)	2.3 (0.3)	1.5 (0.6)	4.0 (1.3)	2.3 (0.9)	3.0 (1.0)	3.0 (0.7)
• Soft Drinks (10)	1.7 (0.4)	1.8 (0.5)	1.3 (0.4)	3.4 (0.7)	3.9 (0.6)	2.0 (0.8)	2.1 (0.4)
• Spices (10)	2.4 (0.3)	3.2 (0.2)	1.4 (0.2)	2.6 (0.4)	5.0 (0.0)	3.3 (0.2)	2.5 (0.3)
• Tea (10)	3.2 (0.4)	2.9 (0.4)	1.5 (0.2)	3.6 (0.4)	4.1 (0.6)	1.9 (0.5)	2.6 (0.4)

Table 2: Industry executives' appreciation over food packaging factors per product type.

Food Product Type (N)	Informational Content	Content Protection & Recognition	Smart Functioning	Geometry	Environmental Friendliness	Endurance	Coloration
• Bakery Products (3)	3.4 (0.3)	2.8 (0.1)	1.0 (0.0)	3.7 (1.2)	2.4 (0.5)	3.6 (0.5)	3.0 (0.4)
• Canned Food (10)	3.1 (0.5)	3.3 (0.4)	1.0 (0.0)	3.7 (0.0)	2.4 (0.1)	4.7 (0.4)	2.2 (0.4)
• Cereals (12)	3.0 (0.7)	2.5 (0.4)	1.0 (0.0)	3.2 (0.4)	3.1 (1.0)	3.0 (0.8)	2.5 (0.5)
• Cheese (1)	2.2 (0.0)	3.2 (0.0)	0.5 (0.0)	5.0 (0.0)	1.0 (0.0)	4.7 (0.0)	2.5 (0.0)
• Chocolatery (12)	3.2 (0.4)	3.1 (0.6)	1.0 (0.1)	4.2 (1.1)	3.1 (0.6)	3.6 (0.8)	2.9 (0.5)
• Coffee (14)	2.5 (0.7)	2.9 (0.5)	1.0 (0.0)	3.5 (0.9)	3.4 (1.1)	4.1 (0.6)	2.2 (0.5)
• Cold Cuts (2)	3.6 (0.3)	3.4 (0.1)	1.0 (0.0)	5.0 (0.0)	2.8 (0.7)	4.3 (0.5)	2.8 (0.4)
• Confectionery (3)	2.8 (0.8)	3.0 (0.2)	1.0 (0.0)	2.6 (1.0)	3.0 (0.7)	4.4 (0.5)	2.3 (0.8)
• Dairy (14)	3.3 (0.4)	2.7 (0.4)	1.0 (0.0)	2.9 (0.8)	2.9 (0.9)	3.9 (0.7)	2.7 (0.6)
• Jam – Honey (1)	2.3 (0.0)	3.2 (0.0)	1.0 (0.0)	4.3 (0.0)	3.3 (0.0)	4.3 (0.0)	3.0 (0.0)
• Legumes (3)	3.5 (0.4)	2.5 (0.3)	1.0 (0.0)	3.7 (1.2)	2.2 (0.2)	4.0 (0.6)	2.6 (0.6)
• Pasta (13)	3.7 (0.5)	3.1 (0.6)	1.0 (0.0)	4.4 (0.9)	2.8 (0.9)	3.3 (0.8)	2.6 (0.6)
• Salad Ointments (3)	3.0 (0.2)	3.1 (0.3)	1.0 (0.0)	3.0 (1.2)	2.4 (0.2)	4.4 (0.2)	3.3 (0.4)
• Salads – Vegetables (3)	2.3 (1.3)	2.5 (0.3)	1.0 (0.0)	2.8 (0.8)	1.8 (1.1)	2.0 (1.5)	2.1 (0.1)
• Snacks (12)	2.9 (0.4)	2.6 (0.4)	1.2 (0.6)	2.6 (0.7)	2.4 (0.9)	3.8 (0.8)	2.6 (0.5)
• Soft drinks (2)	2.5 (0.1)	2.8 (0.1)	1.0 (0.0)	2.0 (0.5)	2.0 (0.0)	4.5 (0.2)	2.8 (1.1)
• Spices (6)	3.0 (0.4)	2.8 (0.2)	1.0 (0.0)	2.4 (0.5)	2.7 (1.0)	4.4 (0.7)	2.5 (0.4)
• Tea (1)	3.9 (0.0)	3.0 (0.0)	1.0 (0.0)	3.7 (0.0)	4.0 (0.0)	4.7 (0.0)	2.8 (0.0)
• Wine (8)	2.7 (0.7)	2.8 (0.2)	1.0 (0.0)	2.8 (0.9)	3.4 (1.0)	4.3 (0.3)	2.8 (0.7)

Table 3: Consumers' views on packaging attributes per food product type.

Food Product Type	Attractive	Recognisable	Original	Unusual	Modern	Useful	Convenient	Recyclable	Eco-friendly	Economic	Practical	Durable	Robust
• Alcoholic Beverages	10%	10%	10%	0%	20%	60%	50%	70%	40%	30%	30%	20%	10%
• Canned Food	40%	0%	0%	10%	20%	60%	60%	50%	50%	50%	70%	50%	30%
• Cereals	40%	50%	0%	0%	0%	60%	30%	100%	100%	20%	60%	0%	0%
• Coffee	100%	20%	0%	60%	0%	100%	10%	0%	0%	10%	100%	10%	0%
• Cold Cuts	30%	80%	40%	30%	50%	40%	40%	60%	30%	30%	30%	10%	0%
• Dairy	0%	30%	40%	0%	0%	100%	100%	100%	100%	100%	100%	100%	0%
• Flour	0%	50%	40%	0%	40%	0%	0%	100%	100%	0%	0%	50%	0%
• Juices	20%	50%	0%	10%	0%	40%	60%	40%	40%	70%	60%	40%	20%
• Legumes	40%	20%	30%	60%	20%	40%	50%	40%	40%	10%	10%	20%	30%
• Meat	60%	60%	0%	0%	60%	10%	10%	80%	0%	70%	10%	10%	0%
• Nuts	0%	0%	0%	0%	0%	100%	100%	100%	100%	100%	100%	100%	0%
• Pasta	0%	80%	0%	0%	0%	0%	0%	100%	0%	0%	10%	0%	0%
• Salads Ointments	70%	60%	20%	20%	10%	70%	50%	80%	0%	50%	70%	10%	0%
• Salads – Vegetables	0%	0%	0%	0%	0%	0%	100%	100%	100%	100%	100%	0%	0%
• Snack	38%	25%	0%	0%	0%	50%	63%	75%	13%	75%	38%	13%	0%
• Soft Drinks	80%	70%	20%	0%	30%	20%	60%	40%	80%	90%	50%	40%	0%
• Spices	100%	100%	100%	100%	100%	0%	0%	0%	0%	100%	0%	100%	100%

Table 4: Industry experts' views on packaging attributes per food product type.

Food Product Type	Attractive	Recognisable	Original	Unusual	Modern	Useful	Convenient	Recyclable	Eco-friendly	Economic	Practical	Durable	Robust
• Canned Food	10%	10%	10%	10%	0%	60%	50%	0%	0%	50%	70%	90%	60%
• Cereals	75%	58%	0%	8%	42%	58%	8%	67%	17%	8%	33%	0%	0%
• Chocolatery	100%	100%	8%	8%	67%	33%	17%	33%	8%	0%	58%	8%	0%
• Coffee	86%	57%	7%	7%	64%	43%	14%	50%	0%	14%	7%	43%	7%
• Cold Cuts	0%	0%	0%	0%	0%	100%	100%	0%	0%	0%	100%	100%	0%
• Dairy	71%	29%	0%	0%	21%	43%	29%	57%	0%	7%	7%	36%	21%
• Legumes	33%	0%	0%	0%	0%	67%	100%	0%	0%	0%	100%	67%	0%
• Pasta	69%	23%	38%	38%	46%	38%	23%	62%	31%	8%	8%	0%	0%
• Salad Ointments	33%	33%	0%	0%	0%	0%	67%	67%	0%	0%	0%	0%	0%
• Salads – Vegetables	33%	33%	0%	0%	0%	100%	0%	33%	0%	0%	0%	0%	0%
• Snack	92%	50%	25%	0%	50%	42%	42%	8%	0%	0%	17%	17%	0%
• Soft Drinks	50%	100%	0%	0%	0%	50%	0%	50%	0%	0%	50%	0%	0%
• Spices	67%	50%	0%	0%	0%	50%	17%	33%	17%	17%	17%	33%	17%
• Wine	50%	25%	38%	0%	50%	25%	0%	25%	25%	25%	25%	25%	13%

Table 5: Comparative views of food stakeholders on packaging factors.

Informational Content		
• Consumers	($p < 0.001$)	Cereals (1.5) < Salads – Vegetables (2.3) < Alcoholic Beverages (3.1) < Cold Cuts (4.1)
• Industry executives	($p < 0.001$)	Salads – Vegetables (2.3) < Pasta (3.7)
Content Protection & Recognition		
• Consumers	($p < 0.001$)	Dairy (1.5) && Cereals (1.5) < Alcoholic Beverages (2.3) < Tea (2.9) < Legumes (3.7) && Cold Cuts (3.7)
• Industry executives	($p = 0.001$)	No clustering is detected by the Tukey test
Smart Functioning		
• Consumers	($p < 0.001$)	Pasta (1.0) && Salads – Vegetables (1.0) && Nuts (1.0) < Legumes (2.9)
• Industry executives	($p = 0.856$)	No clustering is detected by the Tukey test
Geometry		
• Consumers	($p < 0.001$)	Salads – Vegetables (2.5) < Pasta (3.8) && Canned Food (3.9) && Dairy Products (3.9) && Snacks (4.0) < Cereals (5.0) && Cold Cuts (5.0)
• Industry executives	($p < 0.001$)	Soft Drinks (2.0) < Spices (2.4) < Cold Cuts (5.0)
Environmental Friendliness		
• Consumers	($p < 0.001$)	Pasta (1.2) < Snacks (2.3) < Cheese (3.1) < Juices (4.0) && Canned Food (4.0) && Tea (4.1) < Spices (5.0) && Salads – Vegetables (5.0)
• Industry executives	($p = 0.024$)	No clustering is detected by the Tukey test
Endurance		
• Consumers	($p < 0.001$)	Dairy (1.6) && Flour (1.9) && Tea (1.9) && Soft Drinks (2.0) && Alcoholic Beverages (2.0) && Salads – Vegetables (2.0) < Cereals (2.9) && Pasta (2.9) && Snacks (3.0) && Juices (3.1) && Spices (3.3) && Salad Ointments (3.3) && Meat (3.4) < Canned Food (4.2) && Cold Cuts (4.3) && Nuts (4.3)
• Industry executives	($p < 0.001$)	Salads – Vegetables (2.0) < Canned Food (4.7)
Coloration		
• Consumers	($p < 0.001$)	Cold Cuts (1.7) < Cheese (2.4) < Spices (2.5) && Flour (2.5) && Meat (2.5) < Juices (2.6) && Coffee (2.6) && Tea (2.6) < Salad Ointments (2.7) < Legumes (3.2)
• Industry executives	($p = 0.046$)	No clustering is detected by the Tukey test

5 Discussion

A discussion of the survey findings, related to food packaging attributes, and highlighting the stakeholders' views follows, to inform subsequent strategies.

5.1 Consumers

The packaging attributes that are highly appreciated by consumers are inserted in Table 6.
The consumers' survey results indicate that two food categories can be identified, regarding significant packaging attributes:

1. food product types where the packaging shall be 'Recognisable' and 'Attractive', and
2. food product types where the packaging has to be 'Eco-friendly'.

In the first food category, the food product types include cold cuts, spices, pasta, and coffee. This finding is in line with the research of Prieto-Castillo et al. (2015), who found that the most common cause of the consumers' agnosticism about food packaging is attributed to the lack of time availability. However, the attractiveness of packaging is determinant in stimulating the attention of these consumers. Furthermore, Kacen et al. (2012) identified that the buying process of grocery shoppers follows three stages in which, based on the theory of impulsive buying behaviour, the attraction of a stimulus leads to a desire that is ultimately manifested in a purchasing choice.
The two consumers' characteristics at the time of purchase are impulsiveness and the mental mood of the consumer. Throughout the process of food product purchase to disposal, consumers' interactions with packaging consist of the following steps: immediate understanding of the packaging and product use, opening (where and how), correct and accurate distribution, re-closure and ease of management (Fuente et al., 2015). In the latter study, the proposed conceptual model incorporates four usability constituents that include user, package, content, user's effort. Furthermore, perceptual, cognitive and kinaesthetic elements are identified as important for the design of the optimal packaging.

In the second food category, the following food product types can be classified: flour, soft drinks, dairy, cereals, meat, nuts, salad dressings, salads – vegetables, and snacks. The appearance of packaging (e.g., shapes, images, colours) attracts consumers' interest and affects their purchasing choices (Mohite et al., 2020). Other practical aspects such as the ability to re-use packaging, easiness in opening-closing the package, packaging quality and the capability to recycle can also incentivise consumers' buying preferences. Respondents consider highly packaging that indicated the incorporation of environmentally friendly practices and the manufacturing from eco-friendly materials. Notably, two out of three consumers appreciated highly recyclable packaging. This observation demonstrates consumers' ecological consciousness, and it could be used to inform corporate social responsibility agendas and direct research and investments towards eco-packaging.

5.2 Industry Executives

The packaging attributes that are highly appreciated by industry experts are inserted in Table 7.
Industry executives recognise that a food package shall be 'Attractive'; this is regarded as the most important attribute. Industrialists further apprehend the facilitating role of packaging in handling and logistics operations for the effective use of both storage and secondary packaging, whilst addressing problems with the freight weight (Stock & Lambert, 2001).

6 Conclusions

Packaging is related to all activities across an SC (Regattieri & Santarelli, 2013) as it determines the unit load by creating a "common business language" along with the supply network. Logistics operators of the products in circulation recognise and interpret in the same way specific elements (e.g., marking, quantities, and barcode).
Specifically, the food industry has made great progress in the packaging domain, with a range of smart innovations emerging during the last

Table 6: The attributes that consumers appreciate in packaging.

Food Product Type		**Packaging Attributes**												
		Attractive	**Recognisable**	**Original**	**Unusual**	**Modern**	**Useful**	**Convenient**	**Recyclable**	**Eco-friendly**	**Economic**	**Practical**	**Durable**	**Robust**
• Cereals	:								•	•				
• Coffee	:	•					•					•		
• Cold Cuts	:		•											
• Dairy	:						•	•	•	•	•	•	•	
• Flour	:								•	•				
• Meat	:								•					
• Nuts	:						•	•		•	•	•	•	
• Pasta	:		•						•					
• Salad Ointments	:								•					
• Salads – Vegetables	:							•	•	•	•	•		
• Snacks	:								•		•			
• Soft Drinks	:	•								•	•			
• Spices	:	•	•	•	•	•					•		•	•

Table 7: The attributes that consumers appreciate in packaging.

Food Product Type		**Packaging Attributes**												
		Attractive	**Recognisable**	**Original**	**Unusual**	**Modern**	**Useful**	**Convenient**	**Recyclable**	**Eco-friendly**	**Economic**	**Practical**	**Durable**	**Robust**
• Canned Food	:												•	
• Cereals	:	•												
• Chocolate	:	•	•											
• Coffee	:	•												
• Cold Cuts	:						•	•				•	•	
• Dairy	:	•												
• Legumes	:							•				•		
• Salads – Vegetables	:						•							
• Snacks	:	•												
• Soft Drinks	:		•											

decade, thus leading to improved food quality and safety. Notably, the majority of these innovations stem from the changing preferences of consumers (Kour et al., 2013) such as the adoption of western dietary norms across the globe along with elevated awareness about food quality and nutrients. Previous research has identified the multi-functional nature of packaging in the food industry. In addition, according to the extant research, food packaging in SCs can only be approached holistically, i.e., emphasising on its multi-disciplinary and multi-functional character (Konstantoglou et al., 2020a, 2020b).

The effect of packaging on SCs mainly relates to the provision of information, product standardisation and protection. Indicatively, Coles et al. (2003) emphasise that food packaging design shall consider factors such as promotion, safety, environmental impact, and waste management of material throughout the life cycle of food. Remarkably, the extant body of research reveals a divide between consumers' perceptions and industrial experts' focus on the functionality and attributes of packaging in the food sector (Zeng et al., 2021). To a greater extent, this research investigated consumers' and industrial experts' appreciation towards certain food packaging factors and attributes, for a range of food product types. A common attribute that is appreciated by these stakeholders refers to attractiveness. Other attributes that relate to environmental sustainability and facilitation of logistics operations are also esteemed. Notably, consumers value more packaging attributes compared to industrial executives, thus indicating the limited scope of organisations with regard to the market and business potential of packaging.

Considering the scope of this research, a few limitations can be identified. Firstly, a small number of consumers and industry experts were surveyed within Greece, thus the generalisability of the results is not obtainable. Secondly, possibly certain factors and attributes could have not to be included in this study.

Amidst the Internet of Things era, digital technologies have expanded the portfolio of packaging functionalities. Therefore, future research could focus on how novel smart packaging solutions can act as enablers of product traceability through near real-time data and information monitoring, thus leading to consumers' trust, improved scheduling of logistics operations, and reduced food losses and waste (Chen et al., 2020).

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Appendix

Appendix I. Literature Search

Table A1. Literature search terms.

Packaging Functional Area	Search Keywords	Results
1. Marketing	"packaging" AND "marketing"	1598
2. Logistics and Supply Chain Management	"packaging" AND "logistics"	382
3. Food Technology	"packaging" AND "food technology"	218
4. Environmental Protection	"packaging" AND "environmental protection"	331

Search Query: "packaging" and "marketing

TITLE-ABS-KEY ("packaging" AND "marketing") AND (LIMIT-TO (SUBJAREA , "BUSI") OR LIMIT-TO (SUBJAREA , "AGRI") OR LIMIT-TO (SUBJAREA , "DECI")) AND (LIMIT-TO (PUBYEAR , 2021) OR LIMIT-TO (PUBYEAR , 2020) OR LIMIT-TO (PUBYEAR , 2019) OR LIMIT-TO (PUBYEAR , 2018) OR LIMIT-TO (PUBYEAR , 2017) OR LIMIT-TO (PUBYEAR , 2016) OR LIMIT-TO (PUBYEAR , 2015) OR LIMIT-TO (PUBYEAR , 2014) OR LIMIT-TO (PUBYEAR , 2013) OR LIMIT-TO (PUBYEAR , 2012) OR LIMIT-TO (PUBYEAR , 2011) OR LIMIT-TO (PUBYEAR , 2010) OR LIMIT-TO (PUBYEAR , 2009) OR LIMIT-TO (PUBYEAR , 2008) OR LIMIT-TO (PUBYEAR , 2007) OR LIMIT-TO (PUBYEAR , 2006) OR LIMIT-TO (PUBYEAR , 2005) OR LIMIT-TO (PUBYEAR , 2004) OR LIMIT-TO (PUBYEAR , 2003) OR LIMIT-TO (PUBYEAR , 2002) OR LIMIT-TO (PUBYEAR , 2001) OR LIMIT-TO (PUBYEAR , 2000) OR LIMIT-TO (PUBYEAR , 1999)) AND (LIMIT-TO (DOCTYPE , "ar") OR LIMIT-TO (DOCTYPE , "re")) AND (LIMIT-TO (LANGUAGE , "English"))

Search Query: "packaging" and "logistics"

TITLE-ABS-KEY ("packaging" AND "logistics") AND (LIMIT-TO (SUBJAREA , "AGRI") OR LIMIT-TO (SUBJAREA , "BUSI") OR LIMIT-TO (SUBJAREA , "DECI")) AND (LIMIT-TO (PUBYEAR , 2021) OR LIMIT-TO (PUBYEAR , 2020) OR LIMIT-TO (PUBYEAR , 2019) OR LIMIT-TO (PUBYEAR , 2018) OR LIMIT-TO (PUBYEAR , 2017) OR LIMIT-TO (PUBYEAR , 2016) OR LIMIT-TO (PUBYEAR , 2015) OR LIMIT-TO (PUBYEAR , 2014) OR LIMIT-TO (PUBYEAR , 2013) OR LIMIT-TO (PUBYEAR , 2012) OR LIMIT-TO (PUBYEAR , 2011) OR LIMIT-TO (PUBYEAR , 2010) OR LIMIT-TO (PUBYEAR , 2009) OR LIMIT-TO (PUBYEAR , 2008) OR LIMIT-TO (PUBYEAR , 2007) OR LIMIT-TO (PUBYEAR , 2006) OR LIMIT-TO (PUBYEAR , 2005) OR LIMIT-TO (PUBYEAR , 2004) OR LIMIT-TO (PUBYEAR , 2003) OR LIMIT-TO (PUBYEAR , 2002) OR LIMIT-TO (PUBYEAR , 2001) OR LIMIT-TO (PUBYEAR , 2000)) AND (LIMIT-TO (DOCTYPE , "ar") OR LIMIT-TO (DOCTYPE , "re")) AND (LIMIT-TO (LANGUAGE , "English"))

Search Query: “packaging” and “food technology”

TITLE-ABS-KEY ("packaging" AND "food technology") AND (LIMIT-TO (SUBJAREA , "AGRI") OR LIMIT-TO (SUBJAREA , "BUSI") OR LIMIT-TO (SUBJAREA , "DECI")) AND (LIMIT-TO (PUBYEAR , 2021) OR LIMIT-TO (PUBYEAR , 2020) OR LIMIT-TO (PUBYEAR , 2019) OR LIMIT-TO (PUBYEAR , 2018) OR LIMIT-TO (PUBYEAR , 2017) OR LIMIT-TO (PUBYEAR , 2016) OR LIMIT-TO (PUBYEAR , 2015) OR LIMIT-TO (PUBYEAR , 2014) OR LIMIT-TO (PUBYEAR , 2013) OR LIMIT-TO (PUBYEAR , 2012) OR LIMIT-TO (PUBYEAR , 2011) OR LIMIT-TO (PUBYEAR , 2010) OR LIMIT-TO (PUBYEAR , 2009) OR LIMIT-TO (PUBYEAR , 2008) OR LIMIT-TO (PUBYEAR , 2007) OR LIMIT-TO (PUBYEAR , 2006) OR LIMIT-TO (PUBYEAR , 2005) OR LIMIT-TO (PUBYEAR , 2004) OR LIMIT-TO (PUBYEAR , 2003) OR LIMIT-TO (PUBYEAR , 2002) OR LIMIT-TO (PUBYEAR , 2001) OR LIMIT-TO (PUBYEAR , 2000)) AND (LIMIT-TO (DOCTYPE , "ar") OR LIMIT-TO (DOCTYPE , "re")) AND (LIMIT-TO (LANGUAGE , "English"))

Search Query: “packaging” and “environmental protection”

TITLE-ABS-KEY ("packaging" AND "environmental protection") AND (LIMIT-TO (SUBJAREA , "BUSI") OR LIMIT-TO (SUBJAREA , "AGRI") OR LIMIT-TO (SUBJAREA , "ENVI") OR LIMIT-TO (SUBJAREA , "DECI")) AND (LIMIT-TO (PUBYEAR , 2021) OR LIMIT-TO (PUBYEAR , 2020) OR LIMIT-TO (PUBYEAR , 2018) OR LIMIT-TO (PUBYEAR , 2017) OR LIMIT-TO (PUBYEAR , 2016) OR LIMIT-TO (PUBYEAR , 2015) OR LIMIT-TO (PUBYEAR , 2014) OR LIMIT-TO (PUBYEAR , 2013) OR LIMIT-TO (PUBYEAR , 2012) OR LIMIT-TO (PUBYEAR , 2011) OR LIMIT-TO (PUBYEAR , 2010) OR LIMIT-TO (PUBYEAR , 2009) OR LIMIT-TO (PUBYEAR , 2008) OR LIMIT-TO (PUBYEAR , 2007) OR LIMIT-TO (PUBYEAR , 2006) OR LIMIT-TO (PUBYEAR , 2005) OR LIMIT-TO (PUBYEAR , 2004) OR LIMIT-TO (PUBYEAR , 2003) OR LIMIT-TO (PUBYEAR , 2002) OR LIMIT-TO (PUBYEAR , 2001) OR LIMIT-TO (PUBYEAR , 2000) OR LIMIT-TO (PUBYEAR , 1999)) AND (LIMIT-TO (DOCTYPE , "ar") OR LIMIT-TO (DOCTYPE , "re")) AND (LIMIT-TO (LANGUAGE , "English"))

Appendix II. Questionnaire

QUESTIONNAIRE

The purpose of the questionnaire is to identify the packaging elements and attributes of selected foods products that can be found on the shelves of retail stores (packaging units – primary packaging) and on the warehouse (carton boxes – secondary packaging). The questionnaire consists of three (3) parts. Responses to all fields are mandatory.

Part A: Food Type of purchased SKUs

In which food product type does the considered SKU belongs?

Food Product Type	Food Product Type
Alcoholic Beverages	Meat
Canned Food	Nuts
Cereals	Pasta
Cheese	Pastries
Chocolatery	Salad Ointments
Coffee	Salads – Vegetables
Cold Cuts	Snacks
Confectionery	Soft Drinks
Dairy	Spices
Flour	Tea
Jam – Honey	Wine
Juices	Other (please indicate)
Legumes	

Indicate the **name** of the product and its **manufacturer**.

Name	:
Manufacturer	:

Part B: Degree of Conformity of Packaging Elements

To what extent does the packaging of the considered SKU meet the following conditions:
(Note with "PU" the desired value for a packaging unit and with "CA" for a carton)

Packaging Element	Not Important	Less Important	50-50	Important	Very Important
1. Have bright colours					
2. Be monochrome					
3. Have white (background)					
4. Contain blanks					
5. Include image					

Packaging Element	Not Important	Less Important	50-50	Important	Very Important
6. Mention nutritional information					
7. Provide information on the production techniques of the product					
8. Include quality certification labelling					
9. Include signs indicating compliance with environmental practices					
10. Include information that helps product tracking					
11. Indicate traceability elements (barcode, QR-code)					
12. Mark for the use of flammable or other hazardous materials					
13. Indicate suggested product consumption ways					
14. Have a size marginally larger than the product					
15. Have a volume marginally larger than the product					
16. Have a base that follows known geometric shapes (cube, cylinder, etc.)					
17. Manufactured from durable materials					
18. Be waterproof					
19. Withstand mechanical stress					
20. Withstand wear and corrosion					
21. Protect the product from theft					
22. Protect the product from moisture					
23. Easily placeable on the shelf					
24. Not exposing the product to sunlight					
25. Allow visual contact with part of the product					

Packaging Element	Not Important	Less Important	50-50	Important	Very Important
26. Have a lightweight					
27. Be made of materials that give prestige to the product					
28. Be made of environmentally friendly materials					
29. Be made of materials that can be reused					
30. Be easily transported					
31. Allow the packaging of the product in larger groups (cartons, etc.)					
32. Be cheap					
33. Suggest recipes for the containing product					
34. Be resistant to cooking, baking, etc. (ready to cook)					
35. Indicate country of origin of primary and secondary materials					
36. Show the temperature of the product at any time					
37. Increase the shelf life of the containing product					
38. Be "smart"					
39. Indicate if a product is of a protected designation of origin					
40. The shape of the package should describe the contents					
41. Do not expose the product to outside temperature					
42. Not allow odours to leak					
43. Be made of recycled materials					

Part C. Packaging Attributes

If you were to describe the packaging attributes, which of the following adjectives would you use:
(Note: Mark the desired value with an "X" – Indicate up to 5 answers for each case)

Attribute	Attribute
Attractive	Recyclable
Recognisable	Eco-friendly
Original	Economic
Unusual	Practical
Modern	Durable
Useful	Robust
Convenient	Other (please indicate)

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Development of High-Fiber, Low Fat Chicken Nuggets

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Abstract

Dietary fiber intake is significantly below the recommended daily allowances worldwide, making fortification of foods with dietary fiber a vital strategy. Simultaneously, there is a trend towards increased consumption of processed meat products containing substantial amounts of fat, making processed meat products an excellent vehicle to deliver fiber. In this study, the effects of adding four types of dietary fiber (Resistant Starch (RS), Polydextrose (POD), Fructooligosaccharides (FOS) and Galactooligosaccharides (GOS)) to chicken nuggets were investigated. Fibers were added at three levels (5, 10 and 15%) to replace 33.33, 66.66, and 100%, respectively, of the chicken skin. The difference between the removed quantity of chicken skin and added fiber was compensated with water. Chicken nuggets were evaluated by measuring color, texture, proximate composition, yield and consumers' acceptability. Results indicated that replacement of the chicken skin entirely with dietary fiber is possible without negatively affecting the final product quality.

Keywords: Nuggets; Resistant starch; Polydextrose; Fructooligosaccharides; Galactooligosaccharides; Texture; Color

1 Introduction

Meat and processed meat products occupy a significant proportion of food consumed daily (Felisberto et al., 2015). One of these products is chicken nuggets, which are acceptable to adults and children. Chicken nuggets are considered battered meat products which are produced from comminuted chicken meat, with the addition of other ingredients to extend the product and consequently reduce its cost. Among these ingredients are chicken skin, starch and soy proteins. In addition to its content of valuable nutrients like other meat products, this product is available in a partially pre-cooked form, which makes it a convenient food. However, these products are considered a source of saturated fat and cholesterol, and a poor source of dietary fiber, which make these products a risk factor for coronary heart disease (CHD), obesity, diabetes (Stender et al., 2007), cancer (Bolger et al., 2017) and cardiovascular diseases (Lairon et al., 2005). It has been estimated that more than 80 million people have been affected by coronary heart disease, stroke and hypertension. These diseases are the primary causes of morbidity and mortality in the United States (Rosamond et al., 2008). However, in Jordan, the statistical data showed that 30% of the population were overweight, 36% obese and 20.6% suffered from hypertension (Takruri & Alkurd, 2014). The weight of scientific evidence about the association between the consumption of processed meat products and some diseases pushed the International Agency for Research on Cancer to declare that processed meat is carcinogenic to humans (Shan et al., 2017).
There are two ways to solve this problem: the first way is making health campaigns to edu-

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cate consumers about the health risks associated with these products, and the second way is reformulating these products to decrease the amount of fat and increase the amount of dietary fiber which converts these products to healthy products "functional food" (Shan et al., 2017). Each way is complementary to the other one. In other words, health campaigns alone will not reduce these products' consumption to a great extent. It succeeded in raising consumer awareness about the health risk without giving the "healthy" alternative to these products. The consumer will still choose these products for many reasons such as taste, texture, and easy-to-create and serve features (Polizer et al., 2015). Knowing that dietary fiber intake is significantly below the recommended daily allowances throughout the world makes fortifying foods with dietary fiber a vital strategy to bridge the gap in dietary fiber consumption and convert the processed meat product into functional food (Sathu et al., 2017). Dietary fiber addition could fulfill multiple roles, such as having positive health effects after consumption, and at the same time, they may have some functional properties that modify the sensory properties of the developed product. One of the essential functional properties is their ability to perform as a fat replacer (Mehta et al., 2015; Yadav et al., 2018). This property is critical because previous studies indicated that eliminating or reducing the amount of fat during the formulation of processed meat products negatively affected the sensory properties and yield (Mallika et al., 2009). Formulating processed meat products with dietary fiber is a relatively new concept in manufacturing functional processed meat products (Polizer et al., 2015). Significant work has been performed in this field, but still, there is a need to explore the functionality and consumer acceptability of isolated and synthetic fiber in new food products (Bolger et al., 2017).

The definition of dietary fiber was recently reviewed several times as the number of digestion-resistant materials increased significantly, either in the isolated or synthetic form. The new definition includes any substances that behave like fiber regardless of the method used in manufacturing them if they exhibit positive physiological benefits (Bruno-Barcena & Azcarate-Peril, 2015; Raigond et al., 2015; Veena et al., 2016). Using commercially available isolated or synthetic dietary fiber has several advantages: cost reduction and consistent quality (Ibrahim, 2018). This study aimed to produce low-fat and high-fiber chicken nuggets by adding dietary fiber and, at the same time, reducing chicken skin in varying proportions.

2 Materials and Methods

2.1 Ingredients

The following ingredients were used in this study: frozen deboned skinless broiler chicken breast (21.24% protein, 2.28% fat, 74.07% moisture as tested by Foodscan meat analyzer) and chicken skin (10.80% protein, 34.19% fat, 53.87% moisture as tested by Foodscan meat analyzer) obtained from the national poultry company slaughterhouse (Al Karak-Jordan); Soya protein concentrate (70% protein concentration; Arcon SJ, USA); Sodium triphosphate (Anhul Kemfood international CO.LTD, China), Corn starch (Trakya, Turkey), Refined salt (Amra, Jordan), Spices (Alcest, China); and RS (48% Dietary fiber, Germany), POD (>90% Dietary fiber. Tailijie, China), FOS (92.5% Dietary fibers, USA), and GOS (43.2% Dietary fiber, USA).

2.2 The basic formula used in the production of chicken nuggets

A commercial chicken nuggets recipe was adapted from one of the meat producers in the local market (National Poultry Company, meat processing plant, Al Karak-Jordan). The formula had the following composition: deboned skinless broiler chicken breast (40.0%), chicken skin (30.0%), water (18.0%), soya 70% (0.60%), sodium triphosphate (0.03%), corn starch (0.20 %), salt (0.10%) and spices (11.07%).

2.3 Development of high fiber low-fat chicken nuggets

Thirteen chicken nuggets formulas were used in this experiment (Table 1). One formula was the original formula (Control) described previously

(section 2.2). The other formulas were adapted from the basic recipe (Control) using four dietary fibers (RS, POD, FOS, and GOS). Each fiber was added to chicken nuggets formulas with three levels: 5, 10, and 15% to replace 33.33, 66.66, and 100% of the chicken skin. The difference between the removed quantity of chicken skin and added fiber was compensated with water.

2.4 Chicken nuggets processing steps

Figure 1 shows the steps used to prepare chicken nuggets. The first step was to weigh all the required ingredients using top loading balance (Mettler Toledo, ICS226-QA15FCL Max=15kg, China). The frozen deboned skinless broiler chicken breast (-7 to -9 ^{o}C) and frozen chicken skin (-7 to -9 ^{o}C) were ground using a commercial frozen meat cutter (Auto-grind machine, CFS, Denmark) equipped with a 20 mm grinding plate.

After grinding, the meat and skin temperature ranged between -4 and -6 ^{o}C. Next, the meat and skin were minced through a meat mincer (K&G WATTER, 419/E130, Germany) equipped with a 3 mm mince plate. The weighed non-meat ingredients (including dietary fiber) were added and mixed with meat and skin - if it was included in the formula- manually for 1 minute to obtain a uniform mixture before freezing.

After mincing, the meat temperature ranged between 0 and -2 ^{o}C. Minced meat and skin were placed in a shock freezer (-20 ^{o}C) for 30 minutes until the meat's temperature reached -5 to -6 ^{o}C, which is the optimum temperature for the nugget pieces to form. A circular Teflon mold (2 cm diameter and 1 cm thickness) was used to form chicken nugget pieces. A specific amount of meat mixture (35 grams) was placed in the mold manually and pressed to ensure no air spaces were left in the nugget pieces.

Formed nugget pieces were immersed in the batter (Super batter W, Jada'l, Jordan). Batter temperature ranged between 0-2 ^{o}C, and battered pieces were manually breaded before flash-frying. The nugget pieces were flash-fried using a commercial fryer (CFS, Model 1627, Denmark) at 184 to 188 ^{o}C for 25 seconds.

Nugget pieces were then frozen using a spiral freezer (Jack stone, freezing system LFD, model 100075, USA) at -25 to -27 ^{o}C for 1 hour and a half. The core temperature of the product reached -15 to -18 ^{o}C. Finally, frozen nuggets were stored in a deep freezer room at -16 to –18 ^{o}C for six days before evaluation.

2.5 Cooking of Chicken Nuggets

Frozen nugget pieces were removed from the freezer and fried directly using a continuous commercial fryer (CFS, Model 1627, Denmark). The frying temperature ranged from 184 to 188 ^{o}C for 180 seconds. After that, nuggets pieces were strained to remove oil, cooled and packed in plastic bags for further evaluation.

2.6 Color Evaluation

The color of cooked samples was measured after removing the breading layer using a non-contact spectrophotometer (X-rite VS-450, UK) equipped with Oncolor software (CyberSoft, UK). The CIE Lab color values and color difference were calculated where: L^* represents the reflection of light; a^* values represent the red/green colors (+ values for red color and – values for green color); b^* values represent yellow/blue color (+ values for yellow color and – values for blue color). Five nugget pieces were tested for each treatment, and the results were averaged for statistical analysis (Akesowan, 2016).

2.7 Texture Profile Analysis (TPA)

TPA was performed using a texture analyzer (TVT 6700, Perten, Sweden), previously calibrated with a standard weight of 2 kg and using a load cell of 5 kg and a 40mm diameter cylinder probe. Four cooked chicken nugget pieces were tested from each treatment. Circular samples (2 x 1 cm) were cut from nugget pieces and tested using the following profile: sample compression=50%, starting distance from the sample=5 mm, initial speed=2mm/s, test speed=2 mm/s and the trigger force=10g. Figure 2 shows

Table 1: Formulas developed to study the effects of type and level of added dietary fiber on the quality of chicken nuggets

Ingredient	Control	Treatment*		
	Without Fiber	Fiber 5%	Fiber 10%	Fiber 15%
Frozen chicken breast	40	40	40	40
Soya 70%	0.60	0.60	0.60	0.60
Sodium triphosphate	0.03	0.03	0.03	0.03
Corn starch	0.20	0.20	0.20	0.20
Salt	0.10	0.10	0.10	0.10
Spices	11.07	11.07	11.07	11.07
Frozen chicken skin	30.0	20.0	10.0	0.00
Water	18.0	23.0	28.0	33.0
Fiber	0.00	5.00	10.0	15.0
Total	100%	100%	100%	100%

*Four types of fibers were used

a typical TPA curve, from which the following parameters were calculated: firmness, cohesiveness, chewiness and resilience (Bonato et al., 2016). Table 2 summarizes how each parameter was tested.

2.8 Shear Test

The shear test was performed using a texture analyzer (TVT 6700, Perten, Sweden) equipped with a 30 mm knife blade probe and a 5 kg load cell. Test profile was starting distance from sample=5 mm, sample compression=30mm, initial speed=2 mm/s and trigger force=5 g. Four cooked chicken nugget pieces were used as-is for the test. The software (TexCal, Perten, Australia) drew the time-force curve from which two parameters were calculated: cutting force and the work of cutting (Bonato et al., 2016).
Figure 3 shows a typical shear test curve where the cutting force is the maximum peak force (g), and the work of cutting is the total area under the curve (J).

2.9 pH

A five grams sample of chicken nuggets, taken before the battering step, was homogenized with 45 mL distilled water using a blender. Then, pH values were determined using a portable pH meter (Cyberscan 510, Singapore) (Polizer et al., 2015).

2.10 Batter Pickup

Batter pickup was recorded after the battering and breading step was carried out through the following equation (Kilincceker & Kurt, 2018).

$$\text{Batter Pickup}_{\text{ after breading}}(\%) = (W_2/W_1) \times 100 \quad (1)$$

where:
W_1 = weight of the sample before the battering and breading step.
W_2 = weight of the sample after the battering and breading step.

2.11 Cooking Yield

The yield was recorded after the final cooking step was carried out through the following equation (Kilincceker & Kurt, 2018).

$$\text{Yield}_{\text{ after cooking}}(\%) = (W_3/W_4) \times 100 \quad (2)$$

where:
W_4 = weight of the sample after the flash-frying step.
W_3 = weight of the sample after the final cooking step.

Table 2: Parameters measured using multiple/ double cycle test

Parameter	Definition	Formula	Unit
Firmness	The maximum force recorded during the first compression/extension cycle	F_A	g
Cohesiveness	The total area (work) of the second compression/extension cycle, divided by the total area (work) of the first compression/ extension cycle.	$\frac{A2}{A1}$	-
Chewiness	The product of Force A, cohesiveness, and springiness.		g
Resilience	The retract area in the first cycle divided by the compression area in the first cycle		-

2.12 Proximate Composition

Moisture, Protein, Fat and Ash of the final product were determined according to the Association of Official Agricultural Chemists (AOAC, 2002) and International Organization for Standardization ISO (1973) procedures: Moisture% (AOAC 950.46), Protein% (AOAC 981.10), Ash% (AOAC 920.153) and Total Fat% (ISO 1443-1973).

2.13 Sensory Evaluation

Based on the results of the previous tests, three treatments were selected for sensory evaluation. Thirty untrained panelists were recruited from a meat production plant (National Poultry Company, Al-Karak). Panelists were asked to evaluate the samples and record the results on the sensory evaluation form. A 9-point hedonic scale was used to evaluate the samples, where one denotes dislike extremely and nine denotes like extremely (Dethmers et al., 1981). Three sensory parameters (color, taste and texture) were evaluated for each sample. To avoid bias, each treatments (RS, GOS, and control) were coded with randomly selected 3-digit numbers. Before serving, chicken nuggets were warmed in a microwave oven for 30 seconds (Gedikoglu, 2015).

2.14 Statistical Analysis

For the multi-factor experiment (the type of dietary fiber and the level of addition), a completely randomized design (CRD)-factorial design (4x3) with two replicates was used to analyze the data using a statistical analysis system (SAS-University edition, SAS Institute Inc., Cary, NC, USA). Means' separation for the main effects and interaction effect was performed using a Duncan's multiple range test ($P< 0.05$).
For one factor experiments (sensory analysis and proximate composition), CRD design with three replicates was used to analyze the data using the SAS system (SAS-University edition, SAS Institute Inc., Cary, NC, USA). Means' separation was performed using a Duncan's multiple range test ($P< 0.05$).

3 Results

In the following presentation of the factorial experiment results, only the results of the significant interaction effect are presented. When the interaction effect between the types of fibers and the addition level was not significant, the significant main effects will be presented.

3.1 Color Evaluation

L^* values of chicken nuggets were significantly ($P<0.05$) affected by the level of dietary fiber and the interaction between the main effects. However, the type of dietary fiber did not significantly affect it ($P\geq 0.05$). Therefore, only the results of the interaction effect are presented in Figure 4. Only RS dietary fiber, added at different levels, significantly ($P< 0.05$) affected the L^* values.

However, all levels of RS used did not differ significantly from the control treatment.
a^* values of chicken nuggets were significantly ($P<0.05$) affected by the type of dietary fiber only (RS, POD, FOS, and GOS). The effect of the type of dietary fiber on a^* value is presented in Table 3. The use of RS dietary fiber significantly ($P<0.05$) decreased the a* values as compared to control and other treatments. No significant effects were observed for other dietary fibers.
b^* values of chicken nuggets were significantly ($P<0.05$) affected by the type of dietary fiber only. Table 3 summarizes the effect of the type of dietary fiber on b^* value. The effect of the type of dietary fiber on b^* values showed the same pattern as the effect of the type of dietary fiber on a^* values. Only RS dietary fiber reduced the b^* value significantly ($P< 0.05$) compared to control, and there were no significant effects of other dietary fibers.
ΔE^*ab values of chicken nuggets were significantly ($P<0.05$) affected by the type of dietary only. The highest total color difference values were for RS dietary fiber, which differs significantly ($P< 0.05$) from other treatments, as shown in Table 3. Other dietary fibers did not significantly ($P\geq 0.05$) affect the total color difference values compared to the control treatment.

3.2 Texture Profile Analysis (TPA)

Firmness values were significantly ($P<0.05$) affected by the type of dietary fiber and the interaction effect. The effects of type and level of added dietary fiber are summarized in Figure 5. Firmness values were not significantly affected ($P\geq 0.05$) by RS, FOS or GOS in all used levels. Using POD, with a level of addition above 5%, significantly ($P<0.05$) reduced the firmness values compared to the control treatment.
Cohesiveness values were significantly ($P<0.05$) affected by the main effects (dietary fiber and addition levels) with no interaction effect (Table 4 and 5, respectively). POD dietary fiber significantly ($P<0.05$) reduced the cohesiveness. Other fibers did not significantly affect cohesiveness compared to the control (Table 4). The addition of dietary fiber up to 10% did not affect the cohesiveness significantly ($P\geq 0.05$). At a 15% addition level, the cohesiveness decreased significantly ($P<0.05$) (Table 5).
Chewiness values were significantly ($P<0.05$) affected by the main and interaction effects. Figure 6 shows the effects of type and level of added dietary fiber on chewiness values. The addition of RS dietary fiber at different levels did not affect the chewiness values compared to the control treatment. However, there was a significant ($P<0.05$) reduction in the chewiness for RS at a 15% addition level compared to other levels. Other dietary fibers significantly ($P<0.05$) reduced the chewiness values compared to the control treatment, where the chewiness decreased with increasing the addition levels to varying degrees.
Resilience was significantly affected by the main effect, with no significant interaction effect. Table 4 shows the effect of type of dietary fiber on resilience. The addition of RS or FOS did not affect the resilience values significantly ($P<0.05$) compared to the control, and GOS reduced the resilience values significantly ($P<0.05$) compared to the control. POD had the lowest resilience value that differed from other treatments significantly ($P<0.05$). The effect of the addition level on resilience is shown in Table 5 Up to 5%, resilience did not change significantly ($P\geq 0.05$) compared to control. At a 10% addition level or higher, the resilience value decreased significantly ($P<0.05$) compared to the control treatment.

3.3 Shear Test

Firmness was affected by the main effects, with no interaction effect. Table 6 shows the effect of type of dietary fiber on firmness values. The highest firmness values were for RS, which differ significantly ($P<0.05$) from other treatments. POD had the lowest firmness values that significantly ($P<0.05$) differed from other treatments except for FOS. The effect of the addition level is shown in Table 7. A 5% addition level significantly ($P<0.05$) increased the firmness values compared to the control treatment. Nuggets

Table 3: Effect of the type of dietary fiber on the color values of chicken nuggets[1]

Type of Fat Replacer	Color Values						
	L^*	a^*	b^*	ΔL	Δa	Δb	ΔE^*ab
Control	77.47 ± 0.18^a	2.83 ± 0.78^a	21.69 ± 0.20^a	77.47 ± 0.00^a	00.00 ± 0.00^a	00.00 ± 0.00^a	00.00 ± 0.00^b
RS	73.21 ± 8.74^a	1.44 ± 0.36^b	19.02 ± 1.80^b	-04.25 ± 8.74^b	-01.37 ± 0.33^b	-02.66 ± 1.80^b	46.86 ± 47.83^a
POD	74.10 ± 2.99^a	2.50 ± 0.57^a	21.24 ± 1.32^a	-03.36 ± 2.99^b	-00.32 ± 0.57^a	-00.44 ± 1.32^a	10.40 ± 12.13^{ab}
FOS	74.06 ± 2.47^a	2.26 ± 0.34^a	21.78 ± 0.59^a	-03.40 ± 2.47^b	-00.56 ± 0.34^a	00.09 ± 0.59^a	08.72 ± 8.20^{ab}
GOS	77.51 ± 1.11^a	2.22 ± 0.45^a	21.84 ± 1.24^a	00.04 ± 1.11^b	-00.38 ± 0.68^a	00.15 ± 1.24^a	01.44 ± 1.12^b

[1] All values are mean ± standard deviation; Values followed by the same letter in the same column are not significantly different (P≥0.05) according to Duncan's multiple range test.

Table 4: Effect of the type of dietary fiber on the TPA of chicken nuggets[1]

Type of Fat Replacer	TPA			
	Firmness (g)	Cohesiveness	Chewiness (g)	Resilience
Control	3906.25 ± 45.43^b	0.50 ± 0.00^a	1972.83 ± 65.99^a	0.23 ± 0.00^a
RS	4463.48 ± 622.09^a	0.47 ± 0.15^a	2072.04 ± 510.16^a	0.19 ± 0.02^{ab}
POD	2230.88 ± 993.00^d	0.36 ± 0.05^b	840.48 ± 543.41^c	0.14 ± 0.05^c
FOS	2446.39 ± 107.85^d	0.47 ± 0.07^a	1152.61 ± 213.95^b	0.20 ± 0.04^{ab}
GOS	3076.22 ± 228.55^c	0.44 ± 0.04^{ab}	1369.72 ± 160.44^b	0.18 ± 0.03^b

[1] All values are mean ± standard deviation; Values followed by the same letter in the same column are not significantly different (P≥0.05) according to Duncan's multiple range test.

Table 5: Effect of the level of dietary fiber on the TPA of chicken nuggets[1]

Level of Fat Replacer	TPA			
	Firmness (g)	Cohesiveness	Chewiness (g)	Resilience
Control	3906.25 ± 48.43^a	0.50 ± 0.00^a	1972.83 ± 65.99^a	0.23 ± 0.00^a
5%	3204.27 ± 648.33^b	0.51 ± 0.08^a	1661.17 ± 474.20^b	0.22 ± 0.02^a
10%	3046.73 ± 1216.62^b	0.44 ± 0.08^a	1368.05 ± 744.32^c	0.17 ± 0.04^b
15%	2911.76 ± 1295.11^b	0.36 ± 0.06^b	1046.91 ± 395.14^d	0.15 ± 0.03^b

[1] All values are mean ± standard deviation; Values followed by the same letter in the same column are not significantly different (P≥0.05) according to Duncan's multiple range test.

with a 10% addition level did not differ significantly (P≥0.05) from the control treatment but it was significantly (P<0.05) lower than the 5% addition level. Increasing the addition level to 15% decreased the firmness value significantly (P<0.05) compared to other treatments.

The cutting work was affected by the main effects, with no significant interaction. The effect of type of dietary fiber on cutting work is shown in Table 6. RS dietary fiber had the highest work of cutting values that differ significantly (P<0.05) from other treatments, whereas POD dietary fiber had the lowest cutting work that was significantly (P<0.05) different from other treatments except for GOS. FOS and GOS were not significantly (P≥0.05) different from the control treatment. Table 7 shows the effect of addition level on the work of cutting values. Addition level up to 10% did not significantly (P≥0.05) affect cutting work compared to the control treatment. At a 15% addition level, the work of cutting values decreased significantly (P<0.05).

3.4 pH

pH values were significantly (P<0.05) affected by the addition level and the interaction effect with no significant effect by the type of dietary fiber (Table 8). Figure 7 shows the interaction effect between the type of dietary fiber and the addition level on pH values. There are significant differences between some treatments; however, all treatments did not significantly (P≥0.05) differ from the control treatment.

3.5 Yield

Yield values after breading (batter pickup) were significantly (P<0.05) affected by the type of dietary fiber only. Table 8 shows the effect of type of dietary fiber on yield after the battering step. The addition of RS dietary fiber did not significantly (P≥0.05) affect the yield values compared to the control treatment, whereas other treatments increased the yield values significantly (P<0.05) compared to the control treatment, with no significant differences between them.

Yield values after cooking were significantly (P<0.05) affected by the type of fiber only. Table 8 shows the effect of type of dietary fiber on yield after the final cooking step. Only POD dietary fiber significantly (P<0.05) increased the yield values compared to the control treatment, whereas other treatments did not significantly (P≥0.05) affect the yield values compared to the control treatment.

3.6 Sensory Evaluation

Sensory evaluation scores for the three treatments (RS, GOS and control) were not significantly (P≥0.05) differed (Table 10).

3.7 Proximate Composition

Moisture, ash, protein and fat were significantly (P<0.05) affected by GOS addition. Table 9 shows samples formulated with 15% GOS had significantly (P≤0.05) lower moisture (40.53%), ash (2.91%), fat (10.21%) and protein (13.77%) compared to the control treatment (44.07%, 3.84%, 14.85% and 14.72% respectively).

4 Discussion

4.1 Formulation of chicken nuggets

Dietary fibers were added to increase the level of dietary fiber in the final product and, at the same time, to compensate for negative sensory attributes resulting from chicken skin removal (Mehta et al., 2015). For this purpose, four commercially available dietary fibers were used: RS, POD, FOS and GOS. These fibers were recently declared to meet dietary fiber's new definition by the Center for Food Safety and Applied Nutrition (2018), and consequently, there is little scientific work on their use in nuggets' production (Felisberto et al., 2015). The use of fiber in an isolated and purified form offers several advantages: consistent quality, low cost, availability, and better functional and health properties (Ibrahim, 2018). Most of the work done to enhance fiber content in chicken nuggets was performed by adding fruit and vegetable flour or waste by-products. However, these natural sources of dietary fiber were

Table 6: Effect of the type of dietary fiber on the shear test of chicken nuggets[1]

	Shear Test	
Type of Fat Replacer	Firmness (g)	Work of Cutting (J)
Control	1679.72 ± 180.06^{b}	23078.17 ± 2757.68^{b}
RS	2715.89 ± 620.67^{a}	32987.11 ± 8983.25^{a}
POD	1167.52 ± 481.48^{c}	14612.69 ± 6070.01^{c}
FOS	1456.25 ± 309.10^{bc}	20902 ± 3550.92^{b}
GOS	1511.49 ± 176.84^{b}	18817 ± 2430.35^{bc}

[1] All values are mean ± standard deviation; Values followed by the same letter in the same column are not significantly different (P≥0.05) according to Duncan's multiple range test.

Table 7: Effect of the level of dietary fiber on the shear test of chicken nuggets[1]

	Shear Test	
Level of Fat Replacer	Cutting Firmness (g)	Work of Cutting
Control	1679.72 ± 180.06^{b}	23078.17 ± 2757.68^{a}
5%	2100.54 ± 753.24^{a}	26456.11 ± 9250.23^{a}
10%	1749.83 ± 689.09^{b}	22340.74 ± 8447.18^{a}
15%	1287.99 ± 563.87^{c}	16692.33 ± 6723.60^{b}

[1] All values are mean ± standard deviation; Values followed by the same letter in the same column are not significantly different (P≥0.05) according to Duncan's multiple range test.

Table 8: Effect of the type of dietary fiber on the batter pickup, pH and cooking yield values of chicken nuggets[1]

	Batter pickup, pH, Cooking yield		
Type of Fat Replacer	Batter Pickup	pH	Cooking Yield
Control	123.60 ± 0.00^{b}	6.12 ± 0.00^{a}	89.10 ± 0.00^{b}
RS	123.18 ± 2.00^{b}	6.15 ± 0.07^{a}	92.68 ± 1.11^{ab}
POD	131.06 ± 7.20^{a}	6.17 ± 0.09^{a}	94.55 ± 2.41^{a}
FOS	133.15 ± 5.21^{a}	6.09 ± 0.04^{a}	89.36 ± 3.70^{b}
GOS	133.91 ± 1.46^{a}	6.12 ± 0.08^{a}	91.08 ± 1.43^{ab}

[1] All values are mean ± standard deviation; Values followed by the same letter in the same column are not significantly different (P≥0.05) according to Duncan's multiple range test.

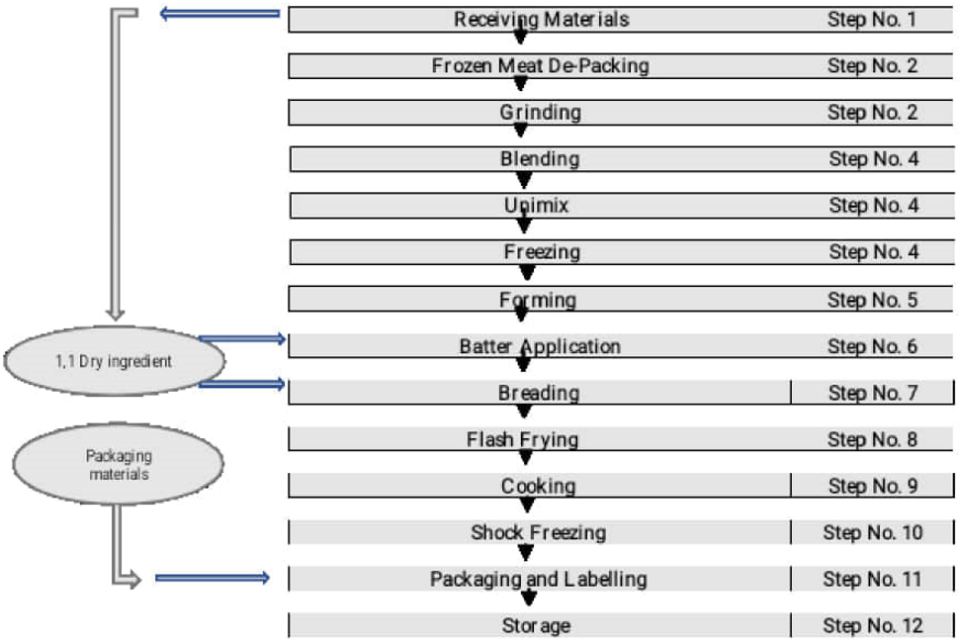

Figure 1: Flow chart for production of chicken nuggets

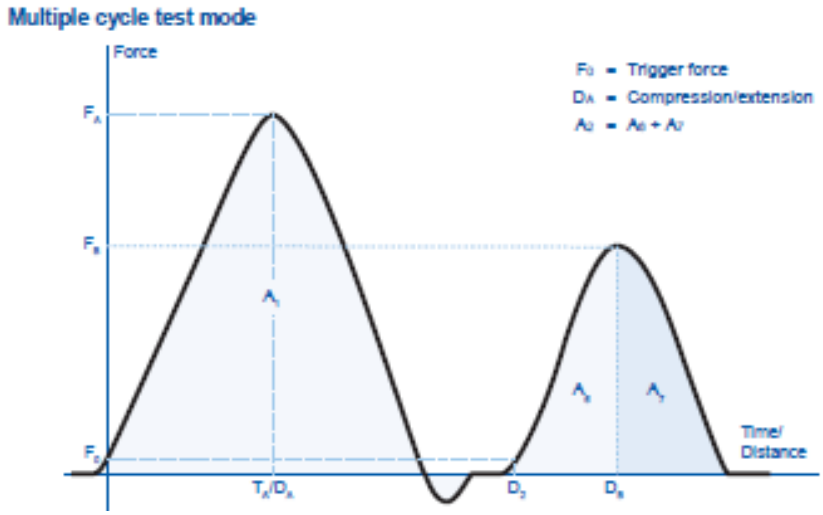

Figure 2: A typical TPA curve

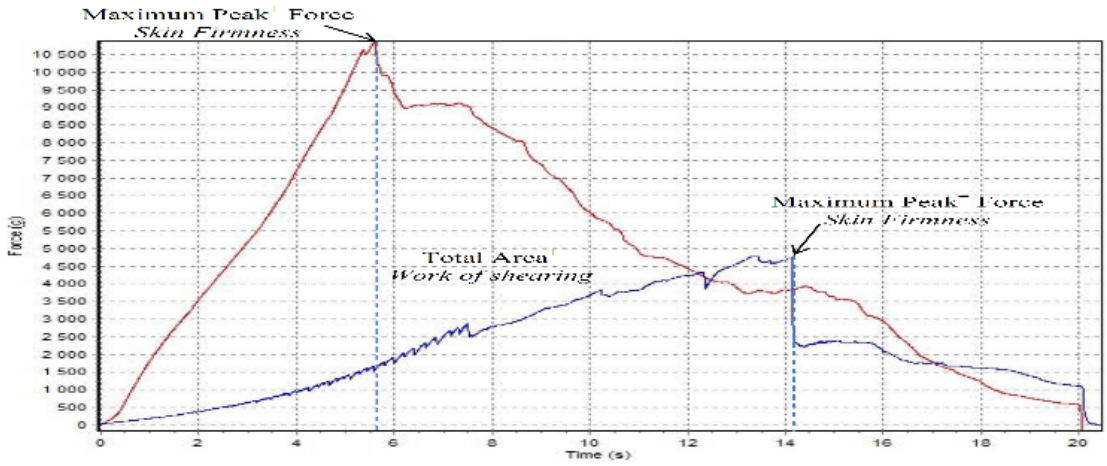

Figure 3: A typical shear test curve

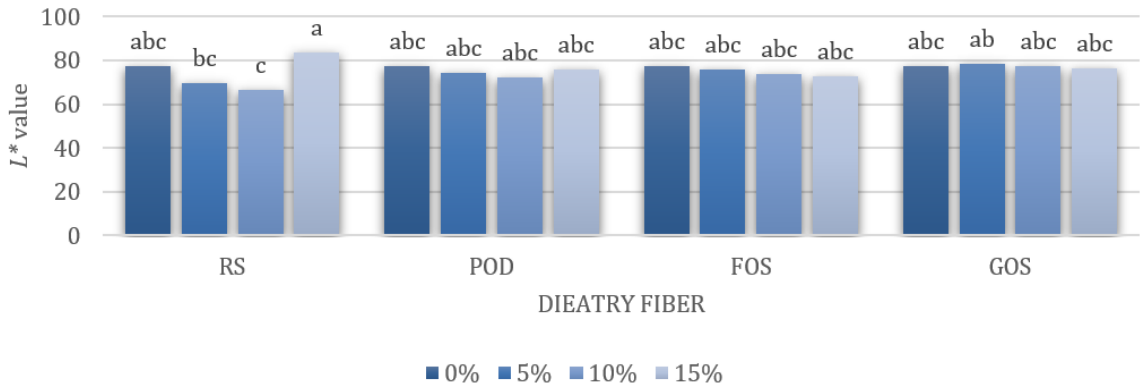

Figure 4: Effects of type and level of added dietary fiber on the L^* value of chicken nuggets

Table 9: Proximate composition of chicken nuggets formulated with 15% GOS and control[1]

	Proximate Composition			
Treatment	Moisture	Ash	Fat	Protein
Control	44.07 ± 0.04^a	03.84 ± 0.10^a	14.85 ± 0.16^a	14.72 ± 0.26^a
GOS 15%	40.53 ± 0.67^b	02.91 ± 0.04^b	10.21 ± 0.29^b	13.77 ± 0.02^b

[1] All values are mean ± standard deviation; Values followed by the same letter in the same column are not significantly different (P≥0.05) according to Duncan's multiple range test.

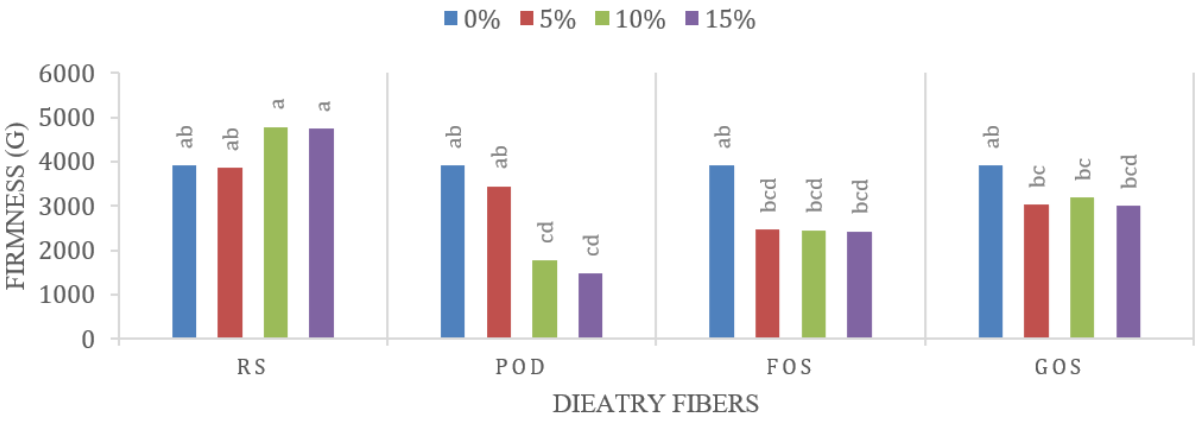

Figure 5: Effects of type and level of added dietary fiber on the firmness value of chicken nuggets

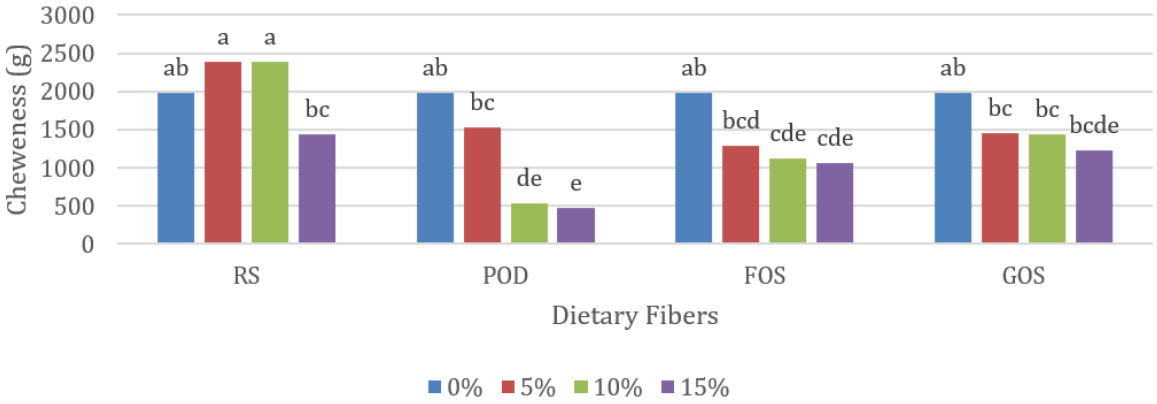

Figure 6: Effects of type and level of added dietary fiber on the chewiness value of chicken nuggets

Table 10: Sensory evaluation of chicken nuggets formulated with 15% RS or GOS and control[1]

	Sensory Evaluation		
Treatment	Color	Taste	Texture
Control	7.13 ± 0.73^a	7.00 ± 0.87^a	7.30 ± 3.34^a
RS*15%	7.10 ± 0.71^a	7.13 ± 0.77^a	7.13 ± 1.53^a
GOS*15%	7.23 ± 0.67^a	7.16 ± 0.74^a	7.30 ± 0.62^a

[1] All values are mean ± standard deviation; Values followed by the same letter in the same column are not significantly different ($P \geq 0.05$) according to Duncan's multiple range test.

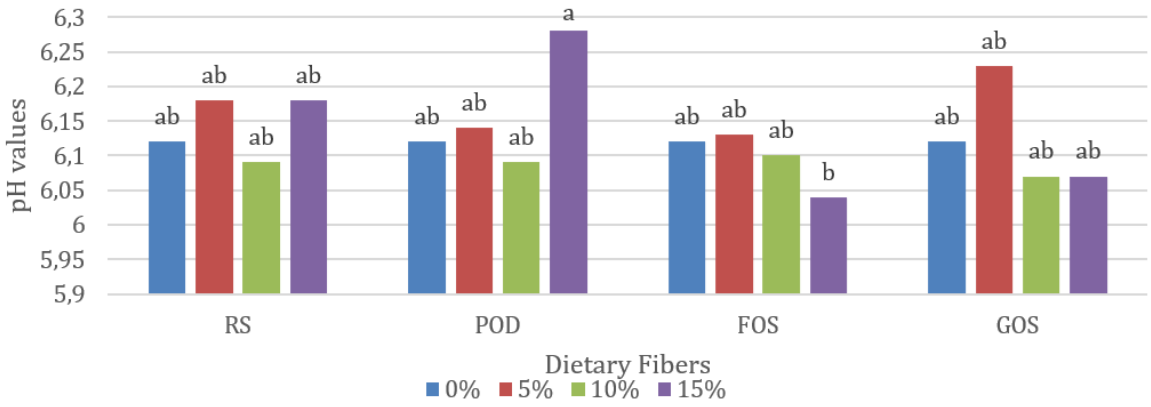

Figure 7: Effects of type and level of added dietary fiber on the pH value of chicken nuggets

found to affect final product sensory attributes (Ibrahim, 2018; Pérez-Chabela & Hernández-Alcántara, 2018). To the best of our knowledge, the fibers used in this study were not tested before in chicken nuggets' production.

The chicken nugget recipe was produced based on a local company bill of material (BOM) (Table 1). Different studies used different proportions of chicken breast and skin: 70% of chicken meat and 20% of skin (Polizer et al., 2015), 100% of chicken meat (Nath et al., 2016), 91% of chicken meat and 3.5% of chicken skin (Akesowan, 2016), 70% of chicken breast and 10% of chicken skin (Kim et al., 2015), and 70% of chicken breast and 10 of skin (Fang, 2015). Perhaps cost is the main factor affecting the proportion of different components. In the previous studies, fibers were added in different proportions ranging from 2 to 10% (Akesowan, 2016; Fang, 2015; Kim et al., 2015; Polizer et al., 2015; Taşbaş et al., 2016).

Due to consumers' low fiber intake and the low fiber content of processed products like chicken nuggets, the fibers mentioned previously have been incorporated into the chicken nuggets in this study. So, the present study relied on these materials due to their new declaration by the FDA to meet the definition of dietary fiber (Center for Food Safety and Applied Nutrition, 2018), consistent quality and low cost (Felisberto et al., 2015).

Some earlier research reported the use of different types of fiber from different sources, such as flours processed from fermented cowpeas and fermented partially defatted peanuts (Prinyawiwatkul et al., 1997), roasted pea flour (Singh et al., 2008), pea hull flour, gram hull flour, bottle gourd and apple pulp (Verma et al., 2010, 2019). However, none of these previous studies used commercial dietary fiber products with high purity of specific types of soluble fiber like RS, POD, FOS and GOS, which could provide more health benefits and better sensory properties.

4.2 Instrumental Color Analysis

Consumer acceptance is commonly dependent on the product's color (Fang, 2015). Color analysis is a very important parameter to investigate since many studies rely on it to evaluate the quality of low-fat food products (Cáceres et al., 2004; Jimenez-Colmenero et al., 2010). In this study, the CIE Lab color values (L^*, a^*, b^*, and ΔE^*ab) were determined for samples of chicken nuggets (Figures 4 and Table 3). Results indicated that all fibers used (RS, POD, FOS and GOS) did not affect the color values L^*, a^*, b^* significantly ($P \geq 0.05$) compared to the control treatment regardless of the addition level used except RS fiber, which reduced the a^* and b^* values significantly ($P < 0.05$) compared to control and other treatments. As a result, the highest ΔE^*ab was for RS (Table 3).

There was no agreement in the literature about the effects of fiber addition on final product color. Alves et al. (2016) found that the replacement of pork back fat (up to 60% of reduction) for pork skin and green banana flour gel (PSGBF) in bologna-type sausage did not affect the color parameters. Bis-Souza et al. (2018) reported a reduction in L^* value in a low-fat beef burger with added FOS and dietary inulin fiber at 3 and 6% levels. The addition of chickpea flour to chicken nuggets increased a^* and b^* values (Sharima-Abdullah et al., 2018). Kilincceker and Yilmaz (2016) found that adding wheat and apple fibers increased the a^* values, and pea fiber increased the b^* values of fried chicken meatballs. It has been suggested that the natural color of fiber, source of fiber (Kilincceker & Yilmaz, 2016), the addition level (Kilincceker & Kurt, 2018) and type of meat (Mittal & Barbut, 1994) affect the final product color.

Although the instrumental color evaluation in our study showed color differences between samples with added RS and other samples, these differences were undetectable by panelists who evaluated the color (Table 10), where no significant differences were found between different treatments' color scores. This result was in agreement with previous studies (Fang, 2015; Polizer et al., 2015).

4.3 Instrumental Texture Analysis

Many studies determined the final product's texture due to its importance to customers and most of the studies evaluated the texture using TPA. From the results of this study, it can be concluded that POD fiber addition had the highest impact on nuggets' texture compared to other fibers. POD significantly ($P \leq 0.05$) reduced the values of all test parameters tested in this study more than the control treatment. Interestingly, other fibers gave comparable results compared to the control treatment with varying degrees. Generally, TPA parameters were not affected by the other three fibers (RS, FOS, GOS) except in a few cases. For instance, cohesiveness and resilience values reduced significantly ($P<0.05$) when the addition level was above 10%. Additionally, FOS significantly ($P<0.05$) reduced chewiness when the addition level was above 5%. It was not easy to compare our results with what has been published due to the differences in nugget formulas, type of fiber, fiber source, addition levels the target of addition and processing steps. It is worth mentioning again that fibers were added in this study to achieve two goals: increasing the fiber content in the final product and replacing chicken skin. The changes in the TPA parameters resulting from fiber addition to chicken nuggets were reported in several pieces of literature. Verma et al. (2015) reported a decrease in chicken nuggets' firmness values when the percentage of pea hall fiber increased from 8% to 12%. However, cohesiveness and chewiness increased when meat substitution with pea hall flour was greater than 8%. (Wan Rosli et al., 2011) found a decrease in hardness, cohesiveness and chewiness of chicken patty formulated with an oyster mushroom when the addition level of mushroom was 25% and 50%, to replace chicken meat. Alves et al. (2016) observed a decrease in hardness at 80% substitution of pork back fat by PSGBF gel, a decrease in cohesiveness at 100% substitution of pork back fat by PSGBF gel and a decrease in chewiness at 80% substitution of pork back fat by PSGBF gel when green banana flour was used as a fat replacer in bologna type sausage. Akesowan (2016) noticed that firmness was increased, with increasing the konjac flour/xanthan gum (KF/XG) mixture of the produced chicken nuggets, when the shiitake powder (SP) was maintained at 1–2 %.

This study performed two texture measuring tests: TPA (Table 4 and 5, and Figures 5 and 6) discussed above, and the shear force test (Table 6 and 7). Both tests measure firmness using different probes and test conditions. Comparing the results of both tests, it can be concluded that the shear test was more sensitive to change in the type and level of dietary fiber. According to the shear test, RS and GOS addition up to 15% did not significantly ($P \geq 0.05$) affect the firmness values compared to the control treatment. Whereas, for POD and FOS, the change in firmness value became significant ($P<0.05$) above the 5% addition level. The decrease in shear force was reported in previous studies involving the addition of fiber to chicken nuggets.

A more tender texture was found when dietary fiber was incorporated in the core portion of the chicken nuggets (Verma et al., 2010). A slight decrease in shear force value, with an increase in dietary fiber incorporation, was also reported by Das et al. (2006) and Atughonu et al. (1998). Soher et al. (2013) found that the shear force value increased in a chicken burger formulated with carrot pomace.

4.4 Yield

Many studies reported different yield types such as batter pickup%, par-fry yield%, cook loss%, freeze loss%, and total yield% (Fang, 2015). In this study, the yield was measured using two methods: After the breading step (Batter Pickup) and after the final cooking step (Yield). After the breading step, POD, FOS and GOS significantly ($P<0.05$) increased the yield compared to the control by 7.46%, 9.55% and 10.31%, respectively (Table 8), Whereas RS treatments did not differ significantly ($P>0.05$) from the control. Little work was found in literature about the effect of adding fiber to nuggets on batter pickup. For instance, Fang (2015) calculated the percentage of batter pickup of soya chicken nuggets treated by functional fibers (70% isomalto-oligosaccharide (IMO) and 30% hydroxypropyl methylcellulose (HPMC)), and it was found that the percentage of batter pickup did not significantly differ from the control, in contrast to our results. It is unknown how fiber addition to chicken nuggets affects batter pickup, but it seems that fiber affects the chicken nuggets' adhesive properties (Fang, 2015).
After cooking yield, POD significantly ($P\leq 0.05$) increased the yield by 5.45%, 5.19%, 3.47% and 1.87% as compared to the control, FOS, GOS and RS, respectively (Table 8). To the best of our knowledge, the fibers used in this study to formulate chicken nuggets were not previously investigated. Several studies reported an increase in yield but the fibers used in this study increased the yield to higher values. Ammar (2017) found that chicken nuggets which incorporated the natural fiber sources, orange albedo and eggplant pulp, significantly enhanced the yield% by 3.73% and 3.53%, respectively. Adding bajra flour to chicken nuggets at two levels, 10% and 20%, increased the yield by 1.27% and 2.17%, respectively (Para & Ganguly, 2015). Sathu et al. (2017) also observed a significant increase in cooking yield (1.78% - 2.89%) in chicken nuggets with added oats. In contrast, Polizer et al. (2015) reported no differences in chicken nuggets' cooking yield formulated with added pea fiber.

4.5 pH

The quality attributes such as texture and color are correlated with pH value (Sharima-Abdullah et al., 2018). The pH values in this study were measured after the chicken nuggets' formulation step. The results indicated that the pH value was not affected by the type of fiber and addition level compared to the control treatment (Figure 7 and Table 8).
No previous studies were conducted to investigate addition of the dietary fibers used in this study on chicken nuggets' pH values after the formulation step. Reviewing the previous scientific works performed on dietary fiber from different plant sources in chicken nuggets indicated no agreement among these studies about the effect of adding these fibers on chicken nuggets' pH after the formulation step. Some previous works, involving the addition of banana flour and soybean skin (Kumar et al., 2017) and citrus fibers (Gedikoglu, 2015), agreed with our results. On the contrary, studies involving the addition of pea fibers (Polizer et al., 2015), flaxseed flour (Bilek and Turhan, 2009) and whey powder (Serdaroğlu, 2006) reported pH changes compared to the control. The changes in pH values were attributed to the pH of the dietary fiber plant source (Mehta et al., 2015; Verma et al., 2016) and to the addition level (Verma et al., 2016). It should be mentioned that the absence of pH changes in chicken nuggets after the formulation step in our study does not mean that the final product from different treatments will have the same pH value. Ammar (2017) reported that the finished product's pH value was higher than raw meat, and this increase in pH value might be due to the release of alkali compounds from amino acids upon cooking (Choe et al., 2013; Gedikoglu, 2015; Kim et al., 2010).

4.6 Sensory Evaluation

Sensory evaluation was carried out to study consumers' acceptability and satisfaction with the 15% RS and 15% GOS (were selected for their minor effects on nuggets' physical properties) chicken nugget treatments compared to the control (Table 10). The results showed no significant ($P \leq 0.05$) difference between the dietary fiber used (RS and GOS) compared to the control, although there were significant differences between treatments regarding instrumental texture and color measurements. This result agrees with the work of Polizer et al. (2015) who reported that partial replacement of meat (10%) or fat (10%) with pea fiber (2%) and water did not change product acceptance by consumers. Additionally, it has been found that incorporating bajra flour into chicken nuggets at the addition level of 10-20% did not impact the final product's sensory properties despite differences in instrumental texture measurements (Para & Ganguly, 2015). In contrast, Sathu et al. (2017) found that lupin flour at a 4.0 % level adversely affected chicken nuggets' appearance, color, flavor and overall acceptability. Akesowan (2016) observed that shiitake powder (SP) affected chicken nuggets' sensory properties where an increase in the addition level from 1 to 2.5% affected the color score. Simultaneously, the taste score was affected by the mixture of SP and the konjac flour/xanthan gum (KF/XG) mixture.

4.7 Proximate Composition

Nuggets formulated with 15% GOS was selected from other types of dietary fibers to compare their composition with the control nuggets. GOS was selected due to its minor effects on the nuggets' physical properties. The results of this study demonstrated that moisture, ash, fat and protein content of chicken nuggets formulated with GOS were significantly ($P < 0.05$) lower than the control treatment (Table 9). One of this study's objectives was to decrease chicken nuggets' fat content by replacing chicken skin with dietary fiber. Complete replacement of chicken skin with GOS reduced the fat content from 14.84 to 10.21% (31.24% fat reduction). Several studies investigated changes in the proximate composition resulting from the addition of dietary fiber to replace fat in the processing of chicken nuggets. Akesowan (2016) reported a 17.6% reduction of fat in chicken nuggets which initially contained 3.5% using konjac flour/xanthan gum (KF/XG) mixture (0.2–1.5 %) and shiitake powder (SP) (1–4 %). Verma et al. (2015) observed that moisture, ash and protein content were statistically reduced in chicken nuggets which incorporated 8-12% pea hall flour. Kim et al. (2015) observed that the protein, fat and ash content were significantly reduced in chicken nuggets which contained mixtures of chicken skin and fiber at four levels (2.5, 5.0, 7.5 and 10%) of addition. The fat content was 11.61% in the control and was reduced by 44.87% in the chicken nugget treatment at 10% addition level. Sharima-Abdullah et al. (2018) reported that chicken nuggets with added chickpea flour and textured vegetable protein had no differences in % ash content, an increased % protein content and a reduced-fat content from 7.50% to 3.83% (48.39% reduction) compared to the control. Polizer et al. (2015) reported no significant differences in the % ash content, while the % moisture content was increased and the % fat content decreased from 14.32% to 10.66% (25.55% reduction). These changes resulted from adding fiber to create a reduced fat treatment. The variation in % fat reduction in previous studies may be related to the initial fat content and fat replacement levels in the formulations.

5 Conclusions

Total replacement of chicken skin with dietary fibers (RS or FOS or GOS) affects the final product's texture, color, yield and proximate composition. However, sensory acceptability was not affected. All fibers used (RS, POD, FOS and GOS) did not affect the color values L^*, a^*, b^* significantly compared to the control treatment regardless of the addition level used except RS fiber, which reduced the a^* and b^* values significantly compared to control.

POD significantly reduced the values of all test parameters used to study texture in this study compared to the control treatment. Generally,

TPA parameters were not affected by the other three fibers (RS, FOS and GOS) except in a few cases. For instance, cohesiveness and resilience values reduced significantly when the addition level was above 10%. Additionally, FOS significantly reduced chewiness when the addition level was above 5%. Moisture, ash, fat and protein content of chicken nuggets formulated with GOS were significantly lower than the control treatment.

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The Effects of Processing Methods on the Quality of Arabica Kintamani Green Beans

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Abstract

The coffee of Arabica Kintamani is one of the most popular coffees in the world due to its specific taste. The quality of coffee beans depends on the post-harvest and processing method. Dry processing and wet processing are the most popular methods used and each process produces different quality coffee beans. The objective of this research was to study and analyze various processing methods of coffee beans and to determine the best processing method to apply by the farmers and processors. This research consisted of three processing methods for the coffee namely dried processing (natural); wet processing; and semi-wet processing (honey). The research used a randomized complete design with one factor and five replications. The study showed that dry processing (natural) produced good quality coffee beans compared with wet or semi-wet processing, with significantly higher polyphenols content of 40.80 ± 0.053 mg GAE g^{-1}, approximately the same caffeine content (1.19 ± 0.016 %), significantly higher antioxidant activity (% DPPH) 89.53 ± 0.229 % with an EC_{50} equal to 102.44 ± 0.130 mg L^{-1}, similar lightness 13.63 ± 8.281 and a significantly lower moisture content of 7.54 ± 0.474 %. This indicated that dry processing could be used as an alternative processing method by farmers and processors due to it being easier, cheaper, with more efficient water use as well as giving a product contained the highest levels of polyphenols and antioxidant activity that are good for human health.

Keywords: Arabica coffee; Processing; Quality; Kintamani

1 Introduction

Coffee is one of the most traded commodities and popular drinks nowadays as well as the most widely consumed and traded beverage in the world after water (Jeon et al., 2019). Indonesia's position is considered quite strategic in the international coffee world because Indonesia is the fourth largest coffee exporting country after Brazil, Vietnam, and Colombia (International Coffee Organization, 2019). Coffee contains significant amounts of phenolic compounds such as chlorogenic and hydroxycinnamic acids and antioxidants including caffeine, melanoidins, and other Maillard reaction products and volatile compounds (Kwak et al., 2018). Traditionally, green beans are produced in two ways, namely the wet processing method and the dry method (Sulistyaningtyas, 2017). The physical quality, coffee taste, and chemical composition of cof-

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fee beans are determined by the cultivar, the environmental condition, agricultural management, (Ahmed et al., 2021; Happyana et al., 2021), processing, fermentation, and roasting process (Azuan et al., 2020; Tarigan & Towaha, 2017). According to Rodriguez et al. (2020) and Duguma and Chewaka (2019), post-harvest processing is to be a key factor contributing to the high quality of coffee. One of the stages of the primary processing process that greatly determines the quality of coffee taste is fermentation. The functionality of food products can be increased by fermentation. The phenolic compounds and antioxidant activity of green beans also can be increased by fermentation (Kwak et al., 2018). During the fermentation process, several precursor compounds are formed, namely organic acids, amino acids, and reducing sugars (Hatiningsih et al., 2018). The type and amount of flavour compounds formed in the roasting process are highly dependent on the variation of precursor compounds (Samantha & Almalik, 2019). The fermentation of coffee is known as coffee cherry fermentation and effectively removes the mucilage layer before the drying process to obtain green coffee beans. Therefore, the primary objective of coffee cherry fermentation is to improve the ease of obtaining green coffee beans rather than to increase the functionality of the coffee beans. Green coffee beans can gain higher functionality with additional processing steps such as soaking in fruit extracts and fermentation (Hatiningsih et al., 2018). As the fermentation of tea products increases their antioxidant activity and the number of phenolic compounds, the antioxidant activity and phenolic compounds in green coffee beans could also be increased by fermentation (Kwak et al., 2018). According to Mangku et al. (2019), increased chlorogenic acid concentration during dried fermentation is due to the higher temperature and the longer fermentation time. The fermentation process carried out at a temperature of 40 ± 1 ^{o}C for 20 hours is better than at temperatures of 20 ± 1 ^{o}C and 30 ± 1 ^{o}C (Mangku et al., 2019). The objective of this research was to assess the effect of different processing and drying methods on the physicochemical quality of green beans.

2 Materials and Methods

2.1 Materials

The material used in this study was the Arabica coffee cherry of the "Sigararutang" variety that grows 1200 m above sea level. The coffee cherries were harvested from farmers at "UPP. Catur Paramitha", Catur Village, Kintamani District, Bangli. The maturity level of coffee cherries was optimally ripe with a red skin color of 95 %.
The specialist equipment used in coffee processing included pulper machine Type Horja, huller MPK 2500, Starcom Coffee Grinder model SCG-017, moisture tester Wile 55, aluminum pan (size: l x w x h: 40 x 20 x 5 cm), and aluminum foil packaging size 12 x 22 mm, etc. Instruments for chemical and physical analysis included Colorimeter CS-280, Soxhlet fat extractor, Memmert incubator, and UV-Vis double beam spectrophotometer Libra S 60.

2.2 Experimental design

This research used a complete randomized design that consisted of three treatments, namely: dried processing (natural process); semi-wet processing (honey process); and wet processing. This research was replicated three times thus giving nine sample units.

2.3 Processing of green beans with the dried processing (natural processing)

The drying process is the simplest method that is usually used by most farmers in the village and also the low-cost production is the other reason although the time used for drying is longer than the other methods. The process for producing green beans began with the sorting of coffee cherries manually to get those with optimum maturity (the red color minimum 95 %) and then continued to sort using clean water in a plastic basket to obtain superior coffee cherries. After sorting the coffee cherries (1000 g) were sun-dried for 45 days to get to a moisture content of 12.5 %. The temperature of the drying process was around 22-27 ^{o}C depending on the weather and

ambient temperature. After the drying process was finished, the dried skin layer of the cherries was removed using a huller machine type MPK 2500. The green beans were used to analyze the physical-chemical quality.

2.4 Processing of green beans with the wet processing (full wash)

Full wash is wet processing that is usually done by farmers to produce coffee beans with higher acidity and lower bitterness. This process needs a fermentation step and is more difficult to apply by the farmers due to most arabica coffee in Bali being processed with dry processing. The fermentation process will give the coffee beans a good aroma, flavour, and higher quality than the dried processing. The wet processing consisted of harvest at an optimum maturity with a red colour minimum 95 %, with manual sorting. To get superior coffee cherries, they were soaked in clean water so that the superior cherries floated, leaving the inferior ones at the bottom. 1000 g superior coffee cherries were then pulped, and from this process we obtained 500 g coffee beans that continued on to be washed and then dry-fermented. The fermentation was conducted by putting the coffee beans in an incubator with a set temperature of 40 ± 1 ^{o}C for 20 hours; after the fermentation, the coffee beans were washed to remove the mucilage layer. The cleaned coffee beans were dried in the sun for 14 days to reach 12.5 % of moisture content. The length of the drying process was affected by the temperature, relative humidity, and environmental conditions.

2.5 Processing of green beans with semi-wet processing (honey processing)

The coffee cherries were harvested with a red colour minimum of 95 % then sorted manually and then with water in basket plastic to get superior coffee cherries. About 1000 g of coffee cherries were pulped with a pulper machine type Horja to remove the outer skin and pulp from the beans. The coffee beans were then dried using sun drying for 30 days to 12.5 % moisture. The moisture content was measured every week using a moisture tester. After the coffee beans had reached a moisture content of 12.5 %, the hard skin was removed to produce coffee beans using a huller machine. The temperature for drying the coffee beans fluctuated and was not stable, being between 22-27 ^{o}C depending on the ambient temperature. After drying and hulling, the coffee beans were used for the analysis of physical-chemical quality.

2.6 Physicochemical analysis procedure of the green beans

The physicochemical quality of green coffee beans was evaluated through moisture content, caffeine, polyphenols, degree of lightness, and antioxidant activity (% DPPH).

Moisture content

The moisture content of coffee beans was analyzed using the gravimetric method (Kyaw et al., 2020). Firstly, the dish and its lid were dried in the oven at 105 ^{o}C for 3 h and then transferred to the desiccator to cool. The dish and lid were weighed after cooling. Secondly, 3 g of the coffee sample was weighed and placed in the dish. The dishes with the samples were placed in the oven and dried at 105 ^{o}C for 3 h. After drying, the dish, partially covered with a lid, was transferred to the desiccator to cool. The dish and sample were re-weighed after cooling, and the moisture content of the samples was calculated by Equation 1.

$$\%Moisture = \frac{W1 - W2}{W1} \times 100 \qquad (1)$$

Where W1 = weight of the sample before drying (g) W2 = weight of the sample after drying (g)

Caffeine content

The caffeine content of coffee beans was determined by a spectrophotometric method (Shao & Zhang, 2019). Exactly 2 g of coffee beans powder sample was weighed. 20 mL of distilled water was added to the sample which was then boiled for 10 mins. A total of 2 g of sodium carbonate

was added to each sample to precipitate tannins. Samples were filtered and filtrates were concentrated to 5 mL by evaporation and transferred to a separation funnel. Caffeine was extracted by adding 5 mL of chloroform to the separation funnel with shaking for a few minutes. The lower caffeine-containing layer was separated and analyzed for caffeine content with a UV/Vis spectrophotometer as follows. A sample of the extract (0.1 mL) was mixed with 10 mL of chloroform and placed in a quartz cuvette. The wavelength at which caffeine absorbs maximum was determined by scanning the range of 190-400 nm. The wavelength at which caffeine absorbed maximumally was found to be 274 nm. Absorbance was measured at 274 nm. A standard curve was made with increasing concentrations of caffeine (0,2,4,6,8,10, and 12 ppm). The concentration of caffeine c (μg/mL) was calculated from the absorbance of the sample by reference to the standard curve.

Polyphenol content

The polyphenol content of coffee beans was determined spectrophotometrically using the Folin-Ciocalteu method with some modification (Mehari et al., 2021). Before determination, a 100 μL portion of the soluble polyphenol extract was diluted to 1.6 mL by adding distilled water, whereas the extract corresponding to the cell wall-bound polyphenols was used directly for analysis. A 10 μL aliquot of the extract was mixed with 100 μL of Folin-Ciocalteu reagent, which had been diluted by a factor of 10 with distilled water, and kept for 5 min. Subsequently, 100 μL of 10 % Na_2CO_3 was added to the mixture and incubated for 90 min at room temperature in the dark. The absorbance of the resulting mixture was measured at 760 nm against a blank sample, comprising 10 μL of the extraction solvent treated with all the reagents and incubated for the same period, under identical conditions to the samples. Each sample was extracted in triplicate and each of these extracts was submitted to the Folin-Ciocalteu assay in triplicate, thus a total of nine measurements corresponded to each sample. Results were expressed as average values, together with the associated standard deviations. The analysis was performed by using 96-microwell reaction plates and an absorbance microplate reader (Spectra max 190, China). A total of 9 different samples together with six blank samples were analyzed on a single plate. Gallic acid was used as the reference standard. For this, a stock solution of gallic acid (500 mg L^{-1}) was prepared by dissolving 50 mg of gallic acid powder in 100 mL of 5 % aqueous methanol. A series of standard solutions were then prepared by appropriate dilution from the stock solution with distilled water. A calibration curve was constructed in the range of 10 - 100 mg L^{-1} of gallic acid after treatment with the Folin-Ciocalteu reagents as described. The regression coefficient (R^2) of the calibration equation was 0.998. The results from the analyses of the samples were expressed as milligrams of gallic acid equivalents per gram (mg GAE g^{-1}) of dry mass.

Lightness (L*)

The colour, mainly the degree of lightness, of coffee beans was analyzed using colorimeter type PCE-CSM 1, represented in the coordinates L*, a*, and b* (Commission Internationale de l'Éclairage, CIE). Coffee beans (2 g) were put into a small Petri dish for measurement. Lightness (L*) value is 0 - 100 (black-white); redness to green (a*) value is +100 - (-100); yellow to blue (b*) value is -100 - (-100) (Kwak et al., 2018).

Antioxidant activity

The antioxidant activity of coffee beans was analyzed according to the DPPH method (Avila et al., 2018; Kurang & Kamengon, 2021). Standard DPPH solution (6x10-5 M) was made by dissolving. 1.182 mg of DPPH in 50 mL of methanol. 500 ppm test solution was made and diluted to 100 ppm, 50 ppm, 25 ppm, and 12.5 ppm. The test solution (33.33 μL) was pipetted into a tube protected from light, and then 1 mL of DPPH was added. The solution mixture was stirred by using a vortex mixer for 10 seconds or until homogeneous. Next, the solution was incubated at 30 ^{o}C for 30 minutes. The DPPH radical solution changed its colour from purple to pale yellow during the reduction process by antioxidants. The decrease in absorbance was measured by a UV-

Vis spectrophotometer at a wavelength of 515 nm (As). The blank solution consisted of methanol (1 mL) and DPPH (33.33 μL). A sample of this solution (up to 1 mL) was measured at the same wavelength (Ab). Ascorbic acid was used as a positive control. The treatment was repeated three times. The percentage of reduced DPPH (% DPPH) was calculated using Equation 2.

$$\%DPPH = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (2)$$

A curve was drawn of the antioxidant capacity of the extract against its concentration. Linear regression of the data gave the regression equation that was used to calculate the EC_{50} (Faria et al., 2022). All the analyses were compared with the control extract that was taken from coffee beans without using heat and the extraction was done at room temperature (26 ^{o}C). The free radical scavenging activity was expressed as the concentration required to inhibit 50 % of free radicals (EC_{50}). To obtain the EC_{50} values (concentration of extract necessary to reduce 50 % of the DPPH radical) of the extracts, the antioxidant activity in different concentrations was calculated using Equation 3.

$$EC_{50} = \frac{\text{Concentration of sample (mg/mL)}}{\text{\% reduce of DPPH of the sample}} \times 50\% \quad (3)$$

2.7 Statistical Analysis

The experiments were replicated three times and the data obtained from the physical-chemical analysis was submitted to Analysis of Variance (ANOVA) with a confidence level of 95 %. The analysis was continued using the t-test to determine if the different processing methods were significantly different.

3 Results and Discussion

The result of the coffee beans after processing with different methods is shown in Figure 1 and the analysis of coffee beans from various processing in this study showed in Table 1 below.

3.1 Lightness (L*)

The lightness is one of the parameters of the coffee beans used as a quality attribute. The increasing lightness value means that the coffee beans look clear and bright, while the lower value of lightness indicates the green coffee beans look dark in colour. The dried process produced a higher lightness value (L*) of 13.63 ± 8.281 and the lowest L* value was produced by the semi-wet process (9.75 ± 4.850). However the lightness of the coffee beans was not significantly different (Table 1). The study showed that the colour of coffee beans in the dried process was brighter, whereas both the wet processing and the semi-wet processing produced a darker colour (Figure 2). The difference in the lightness level was probably due to the phenol content in the wet processing and semi-wet processing being higher and during drying the phenols would oxidise thus making the colour of the coffee beans darker. According to Rodriguez et al. (2020), the semi-dry processing method presented higher values of lightness than the wet processing method; this is because the beans had dried with adhering mucilage, enhancing the values of the colour coordinates; these differences were therefore attributable to the processing method.

3.2 Moisture Content

Moisture content is another attribute that is used for indicating the quality of the coffee beans and contributes to the growth of molds as well as the formation of the aroma and flavour of coffee products. According to the Indonesian National Standard (SNI-01-2907-2008) (Badan Standar Nasional [BSN], 2008), the maximum moisture content of coffee beans is 12.5 %. This study showed that the moisture content of coffee beans from all processing methods was lower than the standard 7.54 ± 0.474 % to 8.71± 0.119 %, indicating the coffee beans to have fulfilled the Indonesia National Standard (SNI) (Figure 3). This means that moisture content of 12.5 % or lower will prevent the growth of mold, increase the shelf-life of the coffee beans as well as produce good quality coffee products.
The dry processing had a lower moisture content of 7.54 ± 0.474 % and the wet processing gave higher moisture content of 8.71 ± 0.119 % and the semi-wet processing was 8.64 ± 0.053 %. Both the dried and wet processing showed sig-

Figure 1: a) Coffee beans with natural processing; b) Coffee beans with wet processing; c) Coffee beans with honey processing

Table 1: Physicochemical characteristics of coffee beans in various processing methods

Processing Methods	Lightness (L*)	Moisture content (%)	Polyphenols (mg GAE g-1)	Caffeine (%)	Antioxidant activity (% DPPH)
Dry processing (natural)	13.63 ± 8.281^{a}	7.54 ± 0.474^{b}	40.80 ± 0.053^{a}	1.19 ± 0.016^{ab}	89.53 ± 0.229^{a}
Wet processing (fullwash)	11.47 ± 6.654^{a}	8.71 ± 0.119^{a}	$36{,}20 \pm 0.015^{b}$	1.13 ± 0.003^{b}	87.32 ± 0.153^{b}
Semi-wet processing (honey)	9.75 ± 4.850^{a}	8.64 ± 0.053^{a}	24.10 ± 0.017^{c}	1.26 ± 0.008^{a}	86.70 ± 0.153^{c}

Meanse ± standard deviation with different superscript letters in the same column were significantly different ($p < 0.05$).

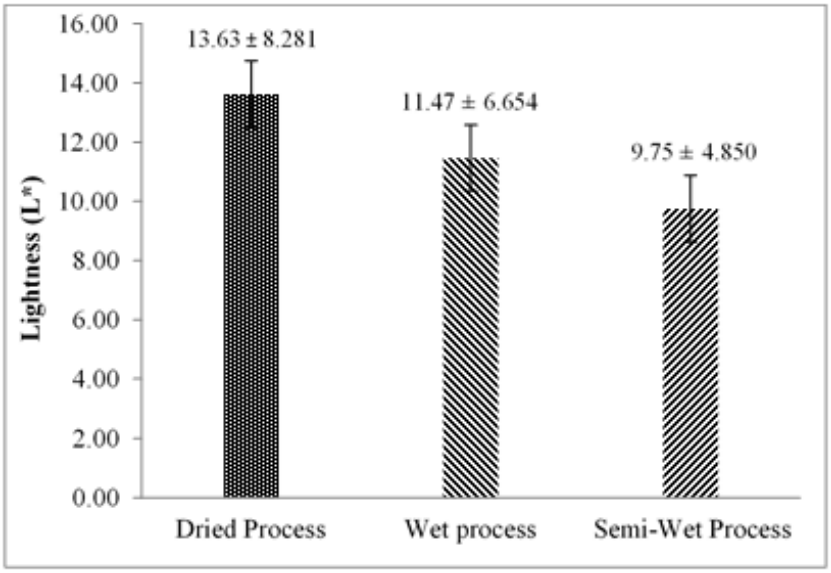

Figure 2: The Lightness (L*) of coffee beans in various processing method

nificant differences ($p < 0.05$) (Table 1). In line with previous research (Tadesse et al., 2016), the moisture content of the coffee processed in the wet method was higher than in the dry methods are 11.20 % and 10.80 % respectively. According to Tadesse et al. (2016), moisture content higher than 12.5 % will accelerate the growth of mold that can decrease the quality of coffee beans, therefore, can reduce self-life.

3.3 Polyphenols content

Chlorogenic acids (CGA) are the main phenolic compounds found in coffee beans. CGA have an important role in determining the quality of coffee beans and beverage taste, and aroma and are a key contributor to the radical scavenger activity of coffee brews (Awwad et al., 2021). The result of studied showed that the polyphenols content of the coffee beans for all process methods was 24.10 ± 0.017 mg GAE g^{-1} to 40.80 ± 0.053 mg GAE g^{-1} and between the treatments process showed significant differences ($p < 0.05$) (Table 1). The higher polyphenols content of 40.80 ± 0.053 mg GAE g^{-1} was given by the dried method and followed by the wet process of 36.20 ± 0.015 mg GAE g^{-1} and the lowest polyphenols content was 24.10 ± 0.017 mg GAE g^{-1} was produced by the semi-wet process method (Figure 4). The lower polyphenols content of coffee beans with the wet process and semi-wet process was due to fermentation during the process that caused the breakdown of phenol compounds such as chlorogenic acid to smaller compounds that reduced the concentration of phenol content in the coffee beans.
According to Mangku et al. (2019), the chlorogenic acid content can be increased by the wet process method with control of the temperature and time of the fermentation process. The chlorogenic acid content of the arabica coffee beans Kintamani was between 4.69 to 11.21 %. The increasing fermentation process would probably decrease phenol content, therefore the fermentation period has to control in optimum conditions.

3.4 Caffeine

Rosita et al. (2016), states that caffeine is one of the important indicator qualities of coffee. Caffeine and chlorogenic acid are the main compounds in coffee beans and both compounds have antioxidant activities (Affonso et al., 2016). The caffeine content of coffee beans due to various processing methods was 1.13 ± 0.003 % to 1.26 ± 0.008 %. There was a significant difference ($p < 0.05$) between caffeine content in producing both wet processing and semi-wet processing (Table 1). The higher caffeine content of 1.26 ± 0.008 % was given by the semi-wet processing followed by the dried processing at 1.19 ± 0.016 % and the lower caffeine content of 1.13 ± 0.003 % was given by the wet processing (Figure 5). Arabica coffee had twice the antioxidant activity of a cup of green and black tea (Kwak et al., 2018).
The wet processing provides good conditions for microorganisms to grow during the fermentation, which then decreases the caffeine content due to the degradation of caffeine compounds and dissolution in the surrounding water. Increasing the fermentation time tends to decrease the caffeine content of arabica coffee beans. In fermentation of up to 20 hours the caffeine content of coffee beans is still relatively high then it tends to decrease after fermentation for 30 and 40 hours (Mangku et al., 2019).

3.5 Antioxidant activity

The bioactive compounds in coffee give benefit to human health; chlorogenic acid is a phenolic compound, which with caffeine, can provide physiological effects on the human body. Affonso et al. (2016) found that a certain amount of available caffeine compounds in coffee beans is needed because it is a bioactive compound and is an antioxidant that can provide physiological effects on the human body. The main compounds found in coffee beans are caffeine and chlorogenic acid, and caffeine is known to have antioxidant properties. The antioxidant activity is affected by the caffeine and phenol content of the coffee beans. This study found that the antioxidant activity of coffee beans that were produced by three dif-

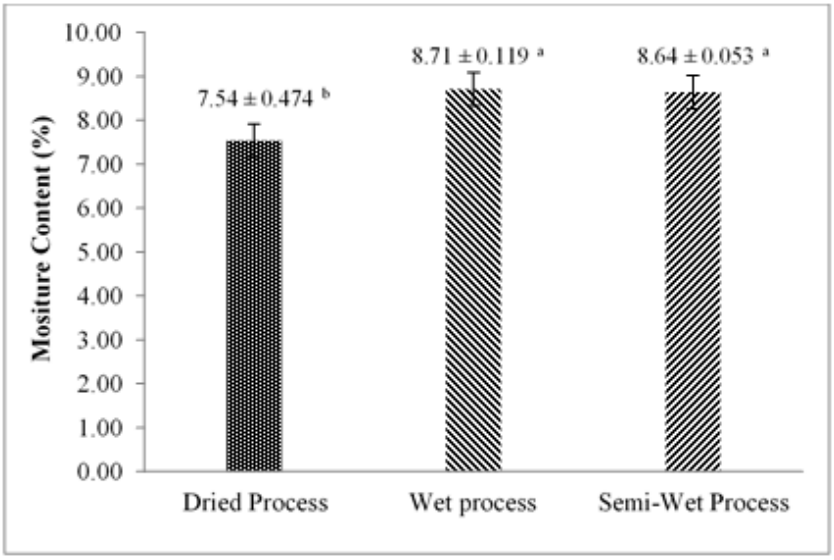

Figure 3: Moisture content (%) of coffee beans from the various processing methods. Means with different superscripts were significantly different ($p < 0.05$).

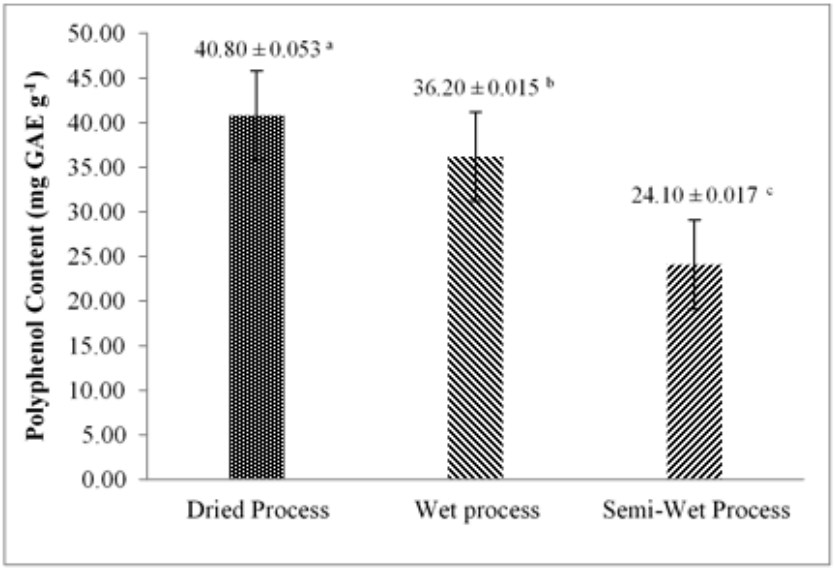

Figure 4: Polyphenols content (mg GAE g^{-1}) of coffee beans in various processing methods. Means with different superscripts were significantly different ($p < 0.05$).

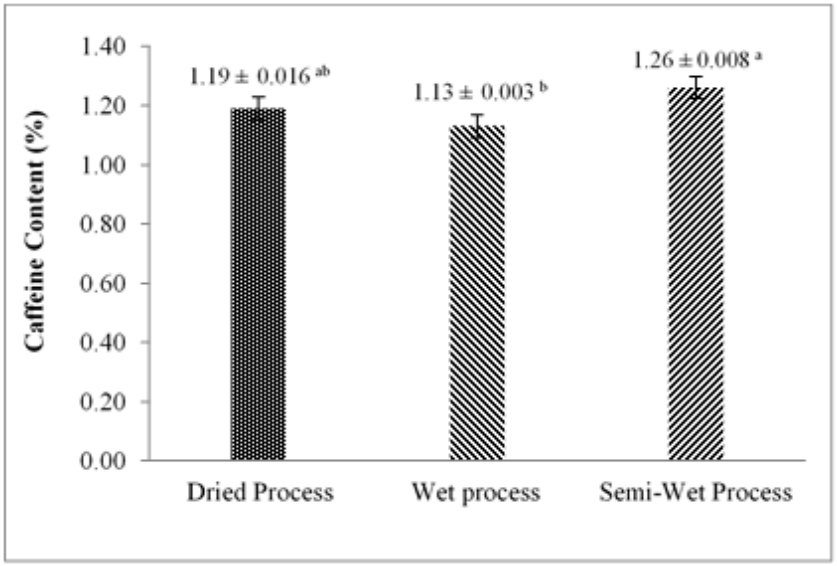

Figure 5: Caffeine content (%) of coffee beans in various processing methods. Means with different subscripts were significantly different ($p < 0.05$)

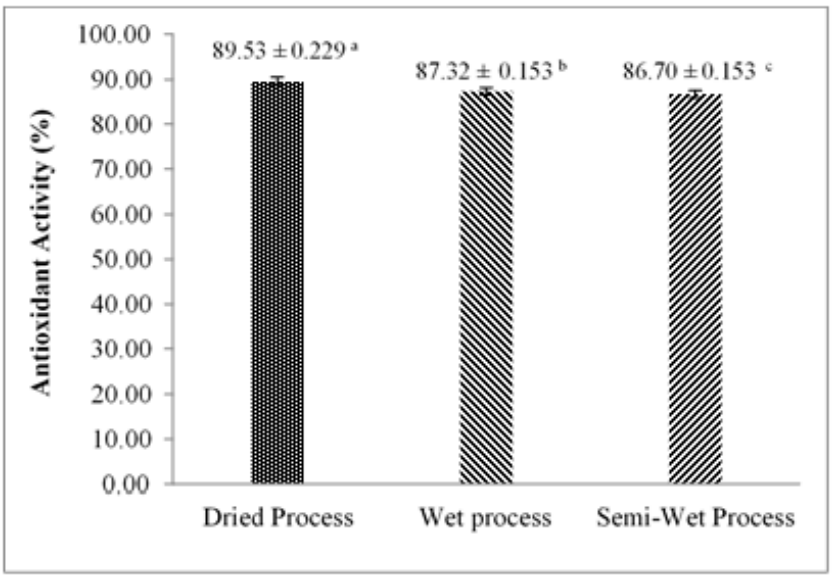

Figure 6: Antioxidant activity (% DPPH) of coffee beans in various processing methods. Means with different superscripts were significantly different ($p < 0.05$).

ferent processing was between 86.70 ± 0.153 % to 89.53 ± 0.229 % and the value of antioxidant activity for all processing methods gave significant differences ($p < 0.05$) (Table 1). The highest antioxidant activity of 89.53 ± 0.229 % with an EC_{50} equal to 102.44 ± 0.130 mg L^{-1} was given by the dried processing (Figure 6) followed by the wet processing with 87.32 ± 0.153 % and an EC_{50} equal to 108.00 ± 0.188 mg L^{-1} and the lowest antioxidant activity was given by the semi-wet processing at 86.70 ± 0.153 % with an EC_{50} equal to 111.69 ± 0.35 mg L^{-1}. The higher antioxidant activity for the dried processing was due to a higher content of phenolic compounds. The dried processing had a higher polyphenol content of 40.80 ± 0.053 % than the wet processing and semi-wet processing (Table 1). On the other hand, the caffeine content in coffee beans also contributed to increasing the antioxidant activity. Online research conducted by Kwak et al. (2018) and Mangku et al. (2019) found that the availability of caffeine and phenolic compounds in coffee beans is needed up to a certain level due to both compounds being bioactive and antioxidants that can provide physiological effects on the human body.

4 Conclusions

The different processing methods of the coffee did not affect the degree of lightness but did affect the moisture content, caffeine, polyphenol content, and antioxidant activity of the coffee beans. Dry or natural processing can be used as alternative coffee processing due to it giving higher polyphenol content (40.80 ± 0.053 mg GAE g^{-1}), caffeine content (1.19 ± 0.016 %), antioxidant activity (89.53 ± 0.229 % DPPH with an EC_{50} equal to 102.44 ± 0.130 mg L^{-1}), more lightness, and having the lowest moisture content of 7.54 ± 0.474 %. In addition, this method is easier, cheaper for farmers or processors, and is more efficient of water use.

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Drinking Coffee May Reduce Chances of Developing Alzheimer's Disease: Systematic Literature Review and Meta-Analysis

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Abstract

Coffee is a popular beverage, and it contains caffeine, a psychoactive substance. Consuming coffee may reduce the risk of developing Alzheimer's disease (AD). However, the association between the reduced risk of developing AD and the consumption of coffee is controversial. Therefore, we conducted a systematic literature review and quantitative synthesis meta-analysis that included dose-response analysis on the relationship between the consumption of coffee and the risk of developing AD. Based on PRISMA guidelines, we analysed standard databases of journals published between January 1999 and May 2020. We included the two population-based cohort studies and one case-control study. All studies included looked at the association between consuming many cups of coffee, the amount of coffee consumed in milligrams per day and the risk of developing AD. The systematic literature review and meta-analysis had 1670 participants with follow-up years that ranged from 5 to 21. The consumption of moderate or 3-5 cups per day reduces the risk of developing AD. The pooled relative risk and 95% confidence interval of the 3 included studies were 0.63 (0.3, 1.54). Dose-response curve analysis appears to be U-shaped. The results of the forest plot showed that there is low heterogeneity between the studies. Plotting the funnel plot and the Galbraith plot demonstrated publication bias of the three included studies. More prospective and long-term studies have to be conducted in other countries to determine the exact risk of developing AD.

Keywords: Coffee; Caffeine; Alzheimer's disease; Dementia; Cognitive

1 Introduction

The aging population worldwide is the most critical driver of the increase in age-related disorders such as Alzheimer's disease (AD), dementia and other late-life cognitive disorders (Panza et al., 2015). Dementia disorders affect 6.4% of European people older than 65 years old, with AD being the most common cause of dementia (Beydoun et al., 2014). More than 40% of 85-year olds and older develop AD, compared to less than 1% by those less than 60 years old (Beydoun et al., 2014). AD is a neurodegenerative disorder, and it is considered an emotional and economic burden to the individual affected by AD and the families of the individual affected by this disease (Rosso et al., 2008). AD begins with loss in mem-

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ory and other cognitive skills (Winblad et al., 2007). AD significantly interrupts the structure and function of a normal brain (Korolev, 2014). The rate of AD progression is due to both environmental and genetic risk factors (Marques et al., 2011).

Coffee is a ubiquitous beverage consumed by many people. Studies have shown that coffee is able to protect from neurodegenerative disorders, it has pharmacological properties and it can also regulate the neurotransmitter and the receptor systems (Barranco Quintana et al., 2007).

Caffeine in coffee is a psychoactive drug and acts as an adenosine receptor antagonist (Cappelletti et al., 2015; Marques et al., 2011). Caffeine reduces the risk of developing AD as it does not allow the amyloid- β-peptide (Aβ-P) to accumulate inside and around the cerebral blood vessels of the brain (Cappelletti et al., 2015). It helps reverse the cognitive dysfunctional skills and helps to decline the brain Aβ-P levels in transgenic mice diagnosed with AD. The consumption of 3-5 cups of coffee in a day during midlife reduces the risk of developing AD by 65% in late life. It has been reported to protect the rabbit hippocampus against oxidative stress the and the rise in the function of mitochondria that includes blocking melatonin signalling. Men who consume more caffeine are less likely to develop AD-related lesions than men who consume less caffeine (Cappelletti et al., 2015). Consumption of coffee can improve cognition in older people and it may also have neuroprotective effects against AD (Rosso et al., 2008). When caffeine is consumed daily, it blocks and deactivates the adenosine receptors, blocking cell response and can cause reduced risk of developing AD (Flaten et al., 2014). The role of the adenosine receptors is to control the transmission of the synapse and plasticity.

Epidemiological and experimental studies show that the caffeine component in coffee, when administered in the human body, has beneficial effects against some neurological disorders such as stroke, Parkinson's disease, AD, dementia and amyotrophic lateral sclerosis (Panza et al., 2015). Caffeine affects the cardiovascular system by increasing the heart rate and heart conductivity, and affects the central nervous system by improving cognitive skills and causes increased alertness and wakefulness (Cappelletti et al., 2015). Cross-sectional studies show that consumption of coffee in the younger generation and the older generation is related to better cognitive skills (Arendash & Cao, 2010). The inhibition of Aβ-P production and cognitive improvements is not only in rabbits but also in brain of rats or mice (Panza et al., 2015). The data of many epidemiological studies such as the Finland Italy Netherlands Elderly study (FINE study), the Three-City Study and the Canadian Study of Health and Aging have shown that the consumption of coffee can help to slow down the progression of AD and also reduce the risk of developing AD (Flaten et al., 2014).

Since there is less pharmacological treatment available in the medical field to treat neurodegenerative diseases, it is essential to identify environmental factors such as lifestyle factors to prevent such neurodegenerative diseases. Dietary factors are often related to reduced or more risk of cognitive disorders (Wu et al., 2017). The consumption of coffee is inversely related to the risk of developing AD (Yenisetti & Muralidhara, 2016). AD patients consumed less caffeine in the 20 years compared to controls (Araújo et al., 2015). There is no cure for AD (Xu et al., 2015). Since there is no cure for AD hence, it may contribute to being a neuroprotective factor, reduce the risk of developing AD with fewer side effects (Rosso et al., 2008; Xu et al., 2015).

Therefore, we conducted a systematic literature review and quantitative synthesis meta-analysis to pool the evidence and summarize the three selected studies (1 case-control and 2 population-based cohort studies). These studies looked at the association between consumption of coffee and AD. We also carried out a meta-analysis to detect heterogeneity of the 3 included studies concerning consumption of coffee and AD using the forest plot. Furthermore, we also plotted a dose-response curve analysis to critically evaluate the dose-response patterns of the relationship between consuming coffee and the risk of developing AD. We evaluated publication bias by analysing the funnel plot and the Galbraith plot.

2 Methods

The standard protocol criteria that were followed for conducting and reporting this systematic literature review and meta-analysis was the "Preferred Reporting Items for Systematic Reviews and Meta-Analyses" PRISMA protocol. The literature search strategy and selection process were conducted by using five databases namely, PubMed, EBSCOhost, Ovid, ScienceDirect and SpringerLink. The literature search of the articles were those published between January 1999 and May 2020 that dealt with coffee consumption and the incidence of Alzheimer's disease. The mesh terms which were utilized to write up this systematic literature review and meta-analysis were "coffee", "caffeine", "caffeinated", "decaffeinated", "dementia", "Alzheimer disease", and "cognitive".
The literature search was used to identify the mesh terms the combination risk and the outcomes of interest: coffee AND Alzheimer's disease, coffee OR caffeine OR caffeinated OR decaffeinated AND Alzheimer's disease, coffee AND dementia, coffee OR caffeine OR caffeinated OR decaffeinated AND dementia, coffee AND cognitive, coffee OR caffeine OR caffeinated OR decaffeinated AND cognitive. The PRISMA flowchart in Figure 1 shows the workflow and the detailed literature search strategy and selection process for the eligible articles. The full-text articles were grouped into systematic literature reviews and meta-analysis, clinical trial study designs, newsletters, poster presentations and a case report.

2.1 Selection Criteria

When duplicate study design articles were detected in more than one database or more than one article, the article was eliminated. The types of articles that were excluded were laboratory studies, no appropriate outcome, articles about Parkinson disease, no coffee consumption intervention and articles written in a different language. The PICOS (participants, intervention, comparison, outcome and study design) methodology was used to conduct the inclusion criteria and selection of the eligible articles. Only studies that met the following inclusion criteria were included to write this systematic literature review and meta-analysis: (i) articles or papers which were published from 1999; (ii) the main participants who were involved in the study were those who had AD or who have signs and symptoms of the disease; (iii) the intervention exposure was coffee consumption; (iv) the comparison was no treatment or placebo; (v) the outcomes were changes in Alzheimer's signs and symptoms, prevention or treatment; (vi) the study conducted was a proper clinical trial study design.

Quality Assessment

The relative risks (RRs) were utilized to measure the effect size of the articles which, were included in the meta-analysis (Table 1). The data collected were categorized into 0 cups of coffee consumed per day, 0-2 cups of coffee consumed per day, >1 cup of coffee consumed per day, 3-5 cups of coffee consumed per day and >5 cups of coffee consumed per day. The data extraction of the eligible studies of interest was included in the study characteristics table. Meta-analysis graphs such as the forest plot, the dose-response curve, the funnel plot and the Galbraith plot were plotted for the 3 included articles using Microsoft Excel software. Egger test was used to detect for publication bias of the 3 included articles.

3 Results and Discussion

The characteristics of the study design were shown in Tables 2 and 3 according to the inclusion criteria present in this study. The year of publication of these 3 included articles ranged from 2002 to 2009. The number of years of follow-up of the duration of these 3 included articles ranged from 5 to 21 years. All the 3 included articles included both male and female participants. The first study was conducted in Finland, the second in Canada and the other study was in Portugal. The type of study design of the 2 included articles which were conducted in Finland and Canada were the population-based cohort studies and the type of study design of the 1 article which was conducted in Portugal was the case-control study. The baseline age of

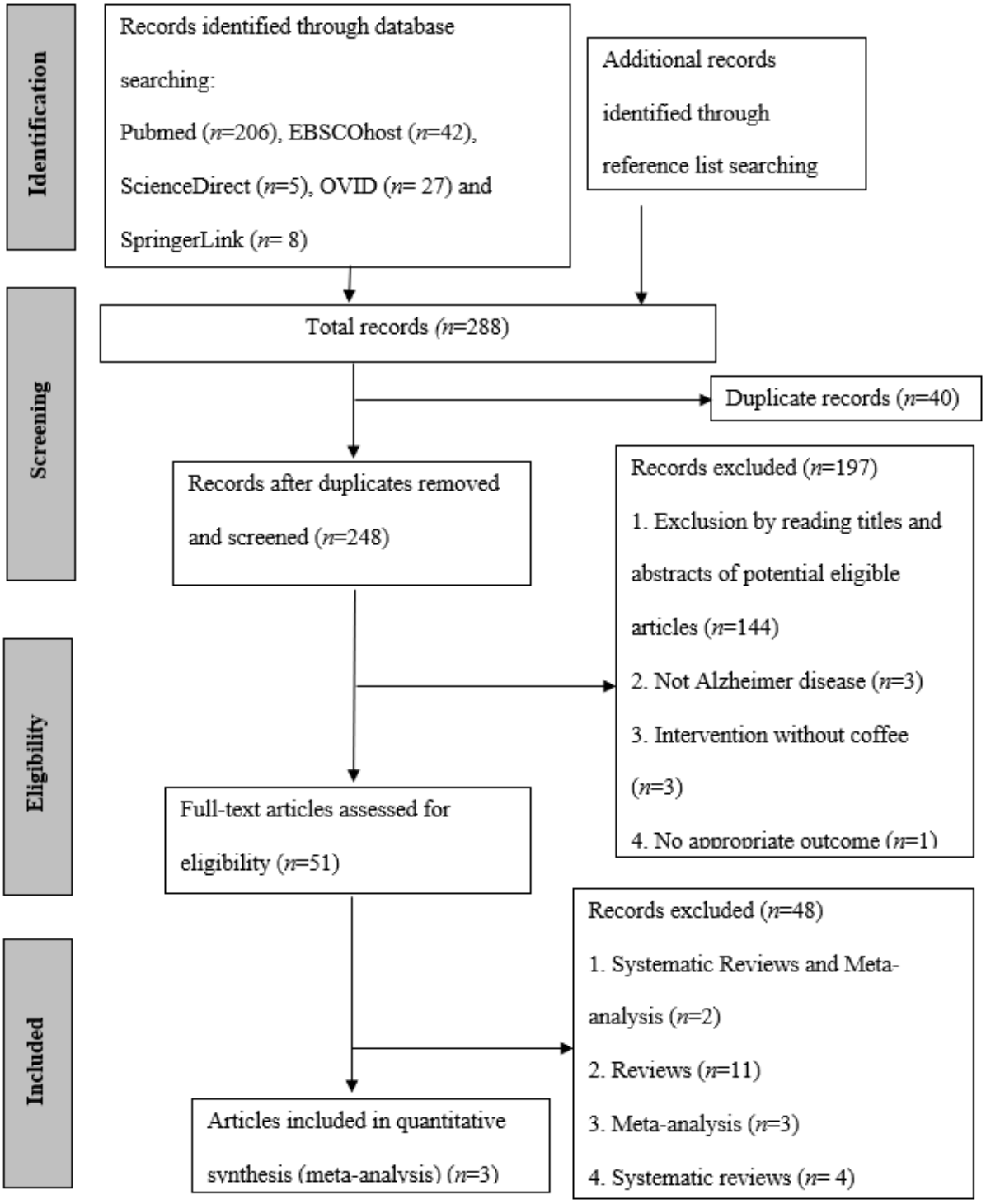

Figure 1: Literature search strategy and selection process, PRISMA flowchart.

Table 1: The Newcastle-Ottawa Scale used to grade the quality of each study (Wells et al., 2000) (maximum = 9 stars)

First author, year	Selection	Comparability	Outcome	Total
(Eskelinen et al., 2009)	**	**	**	******
(Lindsay et al., 2002)	***	**	*	******
(Maia & de Mendonca, 2002)	***	**	***	********

Footnote: Symbols indicate the star rating according to the Newcastle-Ottawa Scale. Good quality: 3 or 4 stars in selection domain AND 1 or 2 stars in comparability domain AND 2 or 3 stars in outcome/exposure domain. Fair quality: 2 stars in selection domain AND 1 or 2 stars in comparability domain AND 2 or 3 stars in outcome/exposure domain. Poor quality: 0 or 1 star in selection domain OR 0 stars in comparability domain OR 0 or 1 stars in outcome/exposure domain.

the participants were 50 years or above in all the 3 included articles. The participant sample size ranged from 146 to 4615 for a total number of 6170 participants. The method of assessment for coffee intake for all 3 included articles was a self-administered questionnaire. The range of coffee intake was from 0 to greater than 5 cups per day (Table 2).

This systematic literature review and quantitative synthesis meta-analysis were conducted on 3 included articles that involved a total sample size of 6170 participants and a total number of 296 cases diagnosed with AD. According to the meta-analysis forest plot results, there was low heterogeneity of all the 3 included studies. Consumption of moderate or 3-5 cups of coffee in a day was inversely associated with the occurrence of AD. The consumption of low or 1-2 cups of coffee in a day was not significantly related to AD. A non-linear association was observed between AD and coffee consumption and a U-shaped dose-response curve was obtained. According to the dose-response curve results, moderate, or 3-5 cups of coffee consumed in a day was associated with a reduced risk of developing AD. Publication bias for the 3 included were analysed with Egger's test and were inspected visually after plotting the funnel plot and Galbraith plot. The funnel plot was asymmetrical showing uneven distribution of the selected 3 studies and in the Galbraith plot 2 studies were scattered away from the line of origin (Table 3).

3.1 Coffee consumption and the risk of Alzheimer's disease

The relative risks in Eskelinen's study with 3-5 cups and with more than 5 cups are: 0.42 (0.12, 1.46) and 1.01 (0.33, 3.09) respectively (Figure 2) (Eskelinen et al., 2009). The box represents the relative risk with corresponding confidence intervals. The left-hand panel shows the study ID, the last name of the first author and the year of publication of every included article. The right-hand panel shows the relative risk and 95% confidence intervals (lower and upper) of every included article. The right side of the graph favours the control group and the left side of the graph favours the experimental group. The x-axis is labelled as Relative Risk (95% CIs) and the y-axis is labelled as the line of no effect.

The results of the quantitative synthesis meta-analysis of the 3 included studies have shown that consuming coffee was non-significantly associated with the risk of developing AD and these results coincide with other studies. The summary risk estimates of these 3 studies between AD and coffee is 0.63 (0.3, 1.54) and this value is also quite close to other systematic literature reviews and meta-analyses conducted. The first meta-analysis which was conducted on 4 studies reported that consuming coffee was inversely related to the risk of developing AD with a summary risk estimate of 0.73 (95% CI: 0.58, 0.92) (Panza et al., 2015). A 2007 quantitative review that was conducted on 4 studies, 2 control studies

Table 2: The study design characteristics of the 3 included articles

The first author (year)	Country (years)	Follow-up (minimum-maximum)	Gender	Age Range of Participants	Study design	No.
(Eskelinen et al., 2009)	Finland	21	Both	65-79	population-based cohort	1409
(Lindsay et al., 2002)	Canada	5	Both	≥ 65	population-based cohort	4615
(Maia & de Mendonca, 2002)	Portugal	20	Both	50 - >75	case-control	146

and 2 case-cohort studies to determine the association between coffee consumption and AD had a summary risk estimate of 0.70 (0.55, 0.90) and reduces the risk of AD by 30% (Barranco Quintana et al., 2007). There was a systematic literature review and meta-analysis that was conducted on 11 studies to determine the association between coffee consumption and the risk of developing AD and reported that consuming more than 1 cup of coffee in a day was inversely associated with AD, the summary risk estimate 1.02 (0.95, 1.08) (Liu et al., 2016). Another systematic literature review and meta-analysis that was conducted on 11 studies reported that drinking coffee or caffeine intake was inversely associated with the risk of developing AD. The summary relative risk and 95% confidence interval were 0.84 (0.72, 0.99) (Santos et al., 2010). A meta-analysis conducted on 20 observational studies showed that the relative risk and confidence interval of consuming coffee on cognitive decline was 0.82 (0.67, 1.01) (Kim et al., 2015). A meta-analysis conducted on 3 observational studies has also shown that consuming coffee plays a protective role from the risk of getting other cognitive disorders (Wu et al., 2017). Honolulu–Asian Aging Study did not determine any relationship between the intake of caffeine and the risk of developing dementia. The autopsy on patients who consumed a high amount of coffee (>277.5 mg/day) did not have any type of pathological lesions that were related to Alzheimer's disease such as microvascular ischemic lesions, cortical Lewy bodies, hippocampal sclerosis, or generalized atrophy (Paganini-Hill et al., 2016). In this study, among the 3494 men, 418 of them were deceased and were used for autopsy purposes (Gelber et al., 2011).

3.2 Dose-response curve analysis

The relative risk of all studies with cups of coffee consumed is 1 (Figure 3). The relative risk is higher when consuming 1-2 cups of coffee per day than when consuming 3-5 cups of coffee per day. The relative risk is higher when consuming >5 cups of coffee per day than when consuming 3-5 cups of coffee per day. A non-linear association is observed and the dose-response curve is U-shaped.

According to the U-shaped dose-response curve results (Figure 3), a moderate consumption of 3-5 cups of coffee in a day is associated with a reduced risk of developing AD than when consuming 1-2 or >5 cups of coffee. This dose-response result matches the results of several epidemiological studies. One study by Andersen et al. (2006), suggested that the post-menopausal women who consumed a moderate or 3-5 cups of coffee in a day had a 30% reduced risk of developing an inflammatory disease such as AD with a summary risk estimate of 0.67 (95% CI: 0.50, 0.90) (Andersen et al., 2006). Also, a review conducted in 2010 reported that consuming 3-5 cups of coffee in a day reduces the risk of AD by 64% (Wierzejska, 2017). A cohort study conducted in 3 European countries reported that the consumption of 3-5 cups of coffee in a day reduces the risk of dementia (Wierzejska, 2017). The Women's Antioxidant Cardiovascular Study reported that drinking 4 cups of coffee a day was associated with improved cognitive maintenance during the 5-year follow up (Panza et al., 2015). The FINE study was conducted on old European men and the study suggested that consuming 3 cups of coffee per day showed the least decline in cognitive skills after a J-shaped dose-response curve was obtained (van Gelder et al., 2007). Ritchie et al. mentioned in the three city study that women who consumed more than 3 cups of coffee a day

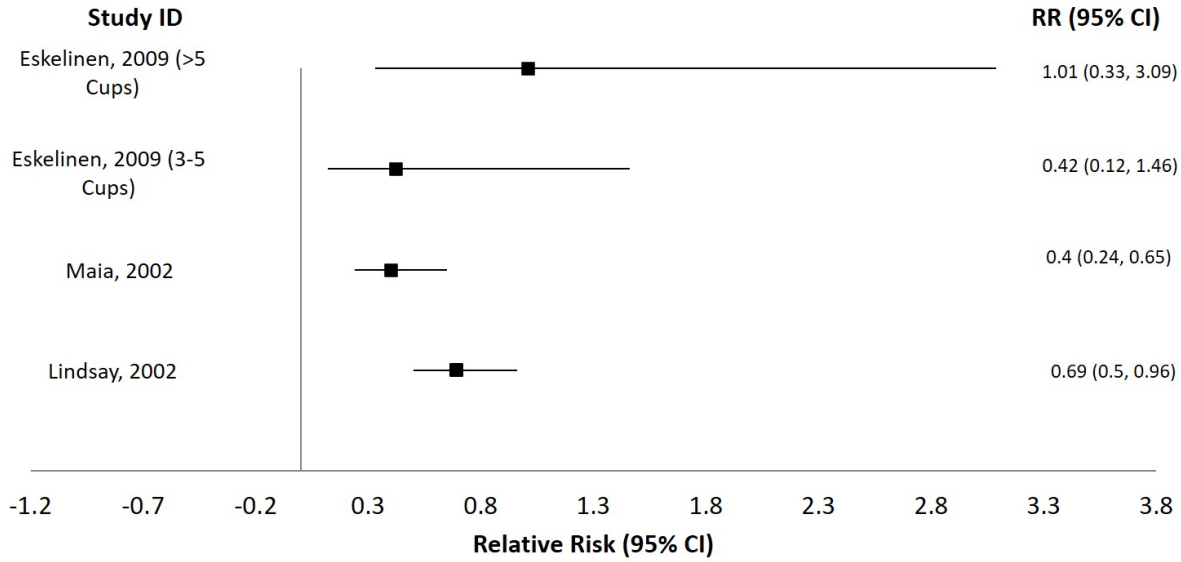

Figure 2: Meta-analysis forest plot for the relationship of coffee consumption with the risk of developing Alzheimer's disease.

had improved verbal retrieval and visuospatial memory (Ritchie et al., 2007). Paganini-Hill et al. reported in the 90+ study of elderly people that consuming 200+ milligrams of caffeine per day reduces the risk of developing dementia or AD by 34% (Paganini-Hill et al., 2016).

There are also experimental studies that prove that moderate consumption or 3-5 cups of coffee consumed in a day is related to a reduced risk of developing AD. In an experimental study, when mice diagnosed with AD were fed with caffeine that was added to drinking water, caffeine caused a decline in the levels of blood Aβ-P in the mice (Arendash & Cao, 2010). This experimental study results also match with the U-shaped dose-response curve results, which shows that a moderate number of 3-5 cups of coffee consumption per day contributes to protection against AD. There are some case-control, cross-sectional and population-based studies that were conducted to determine the long-term effects of caffeine and these studies suggest that caffeine has a protective contribution to dementia or AD (Panza et al., 2015). These study results are also similar to the results of this systematic literature review and meta-analysis studies. Five prospective studies suggested that consuming a moderate number or 3-4 cups of coffee a day (>300mg) is associated with a decreased risk of developing AD or dementia (Carman et al., 2014). The other prospective study that was conducted on 7000 elderly people who had an average age of 74 showed that drinking 3 or more cups of coffee in a day was related to improved verbal retrieval and improved visuospatial test scores (Kromhout et al., 2014).

3.3 Publication bias

The standard errors (z) for Eskelinen (3-5 Cups), Lindsay and Maia are 0.01, 0.08 and 0.03 respectively (Figure 4). Every blue dot in the figure is represented by the study ID (the last name of the first author and the year published). The grey dot represents the combined effect size and the orange dot represents the adjusted combined effect size. The grey and orange dots have corresponding confidence intervals. The funnel plot is inverted. The y-axis is labelled as standard error (z) and the x-axis is labelled as correlation (z). The inverse standard errors for (3-5 Cups) Es-

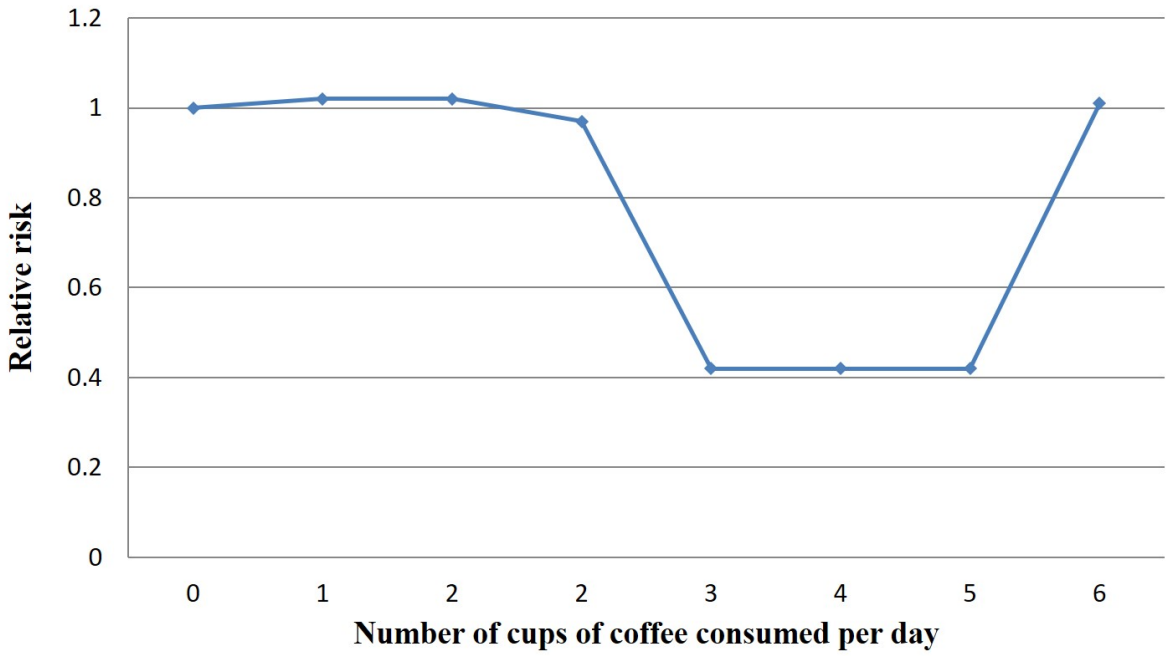

Figure 3: Dose-response curve relationship of coffee consumption (number of cups per day) with the risk of developing Alzheimer's disease.

kelinen, Lindsay and Maia are 67.91, 11.96 and 37.50 respectively (Figure 5). Every blue dot in the figure is represented by the study ID (the last name of the first author and the year published). The y-axis is labelled as z-score and the x-axis is labelled as inverse standard error. The darker line is the line of origin. The light lines are the 95% confidence intervals. Maia and de Mendonca (2002) is within the light line (Maia & de Mendonca, 2002). However, Eskelinen et al. (2009) and Lindsay et al. (2002) are away from the light lines.

3.4 Clinical and Animal Experimental Studies Correlation

The results of the included studies can also be justified by certain theoretical and practical facts, evidence such as experimental studies, bioactive components that can be found in coffee and its properties, epidemiological studies and genetic studies. The laboratory experimental studies on transgenic mice showed that caffeine and the other bioactive components that are present in coffee possesses neuroprotection on cognitive dementia and cognitive decline (Liu et al., 2016). Numerous bioactive components can be found in coffee which, include caffeine, polyphenols like chlorogenic acids, ferulic acid and caffeic acid. These bioactive components have antioxidant properties (Cropley et al., 2012). The experimental studies have shown that caffeine has effects on the rise in alertness and it also reduces extreme tiredness or fatigue (Cropley et al., 2012). One of the studies suggested that the individuals who consumed 1 to 2 cups of coffee in a day have mild cognitive impairment with increased levels of caffeine and had lesser progress to dementia when compared with the individuals who had decreased levels of caffeine in the blood (Cao et al., 2012). When experiments were conducted on animal models, there was proof that caffeine has neu-

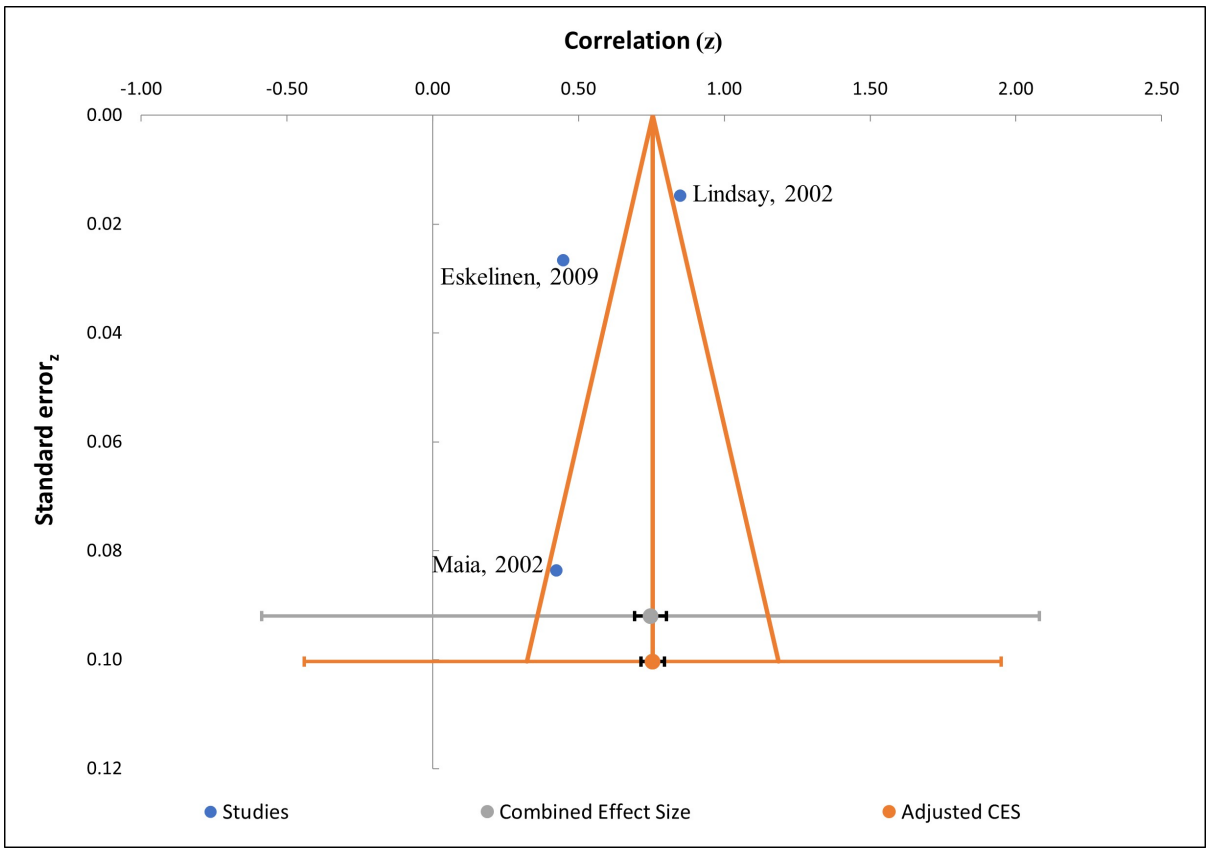

Figure 4: Funnel plot analysis of coffee consumption and Alzheimer's disease with 3 studies.

roprotective properties and regulates the Aβ-P metabolism (Carman et al., 2014). There are also genetic studies that discovered the E4 allele of the apolipoprotein E (APOE), a gene that is known to cause progress to late-onset AD. The locus of this E4 allele of the apolipoprotein E (APOE) gene was identified as rendering the carrier vulnerable to late-onset Alzheimer's disease (Barberger-Gateau et al., 2012). The 3C study is a genome-wide association study that discovered more hereditary genetic risk factors for Alzheimer's disease. Around 537,039 autosomal single nucleotide polymorphisms were genotyped on 2032 individuals who were diagnosed with Alzheimer's disease (Barberger-Gateau et al., 2012).

A case-control study involved 124 participants, and in the initial visit, the participants were neurologically assessed and blood samples were collected from them (Cao et al., 2012). After centrifugation of the blood, the blood plasma was measured for caffeine concentration using Enzyme-linked immunosorbent assay laboratory technique and the participants were monitored during the 2-4-year duration of follow-up. The caffeine level in the blood plasma was 51% lower in the participants who progressed from mild cognitive impairment to dementia than the participants who did not progress to dementia from mild cognitive impairment (Barberger-Gateau et al., 2012; Cao et al., 2012). The participants diagnosed with AD in these studies could have inherited the APOE gene which is a genetic risk factor to progression to AD (Lindsay et al., 2002). A study was conducted on 41,836 postmenopausal women with 15 years of follow up

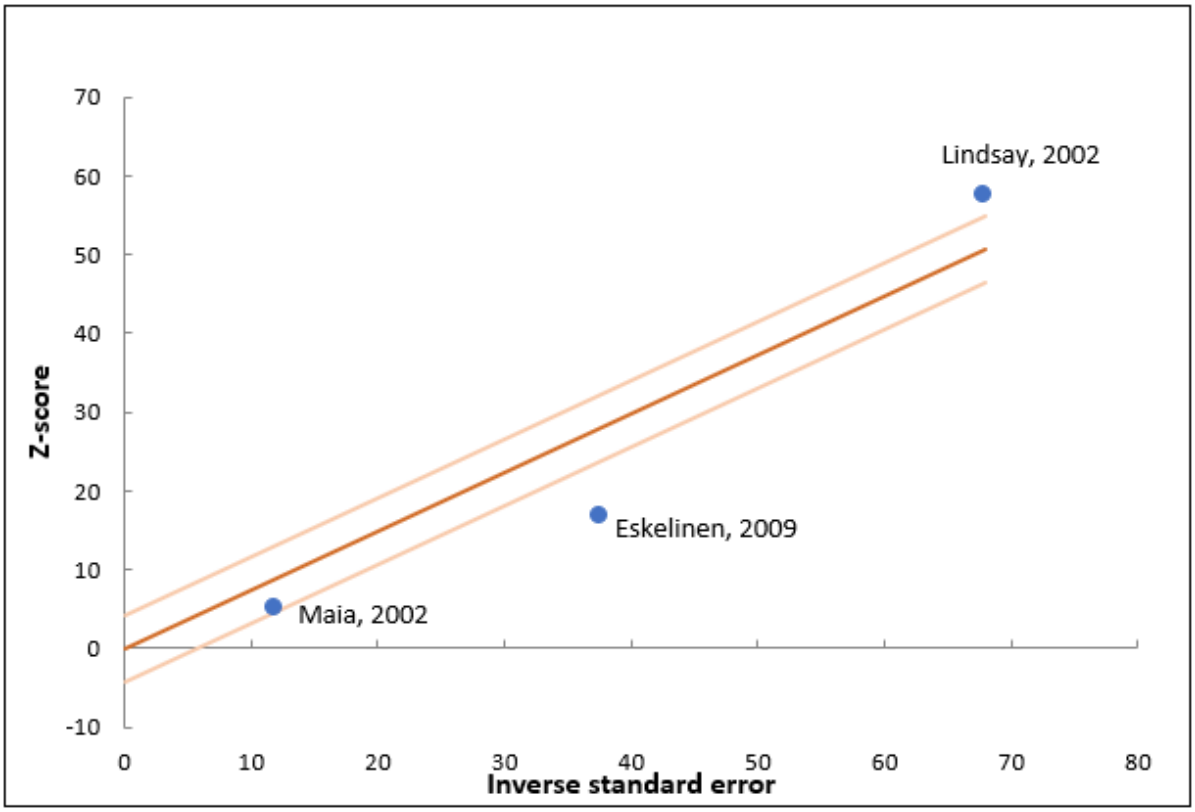

Figure 5: Galbraith plot analysis of coffee consumption and Alzheimer's disease with 3 studies.

(Andersen et al., 2006). This study showed that a moderate consumption of 3-5 cups of coffee in a day had a 30% reduced risk of developing an inflammatory disease or Alzheimer's disease with a summary risk estimate of 0.67 (0.50, 0.90) (Andersen et al., 2006). A review showed that consuming 3-5 cups of coffee in a day reduces Alzheimer's disease by 64% and 3 cups of coffee in a day prevents vascular dementia, meta-analysis on case control and cohort studies had an inverse association, and a literature review pointed out that coffee is a medical boon against Alzheimer's disease (Wierzejska, 2017).

The 90+ longitudinal study with 587 participants was conducted in California and had a 3 years duration of follow-up (Paganini-Hill et al., 2016). This study showed that elderly people who consumed 200+ mg/day caffeine per day reduced the risk of developing dementia to 34%, and the summary risk estimate was 0.66 (0.43, 0.99) (Paganini-Hill et al., 2016). A longitudinal study was conducted in Italy with a total sample size of 1445 (Solfrizzi et al., 2015), the method of assessment of coffee intake was aninterviewer-administered questionnaire and food free questionnaire. This study suggested that when consuming 1-2 cups of coffee per day, the relative risk of developing cognitive impairment was the lowest, risk estimate being 0.11 (0.02, 0.84) (Solfrizzi et al., 2015). A cohort study was conducted in Japan on 13,137 Japanese participants and the duration of follow up was 5.7 years (Sugiyama et al., 2015). The method of assessment of coffee intake was a self-administered and food free questionnaire. The study showed that when 1-2 cups of coffee per day are consumed, there was a minimum risk of getting incident dementia, and the risk estimate was 0.58 (0.43, 0.78) (Sugiyama

et al., 2015). The three-city study was a cohort study conducted in France with a 3.47 years follow-up and a sample size of 7017 (Ritchie et al., 2007). The method of assessment coffee intake was a standardized interview. The study showed that women who consumed more than 3 cups of coffee in a day had improvements in verbal retrieval, with a summary risk estimate of 0.67 (0.53, 0.85) and visuospatial memory, risk estimate 0.73 (0.53, 1.02) (Ritchie et al., 2007). A population-based study was conducted in the Netherlands and the method of assessment of coffee intake was a home interview and a partially food free questionnaire. The sample size was 5408 and the number of years of follow-up was 13.2 years (Mirza et al., 2014). This study showed that consuming coffee cannot progress to incident dementia in the long run (Mirza et al., 2014). The Rancho Bernardo was a cross-sectional study and the sample size was 1528 participants (Rosso et al., 2008). The female participants who were over 80 years of age who drink coffee regularly performed well on 11 cognitive tests (Rosso et al., 2008). A study that was conducted on 716 Finnish men reported that low coffee consumption was related to a reduction in cognitive decline after the participants were assessed by the Mental Status Questionnaire (MSQ) during the 25-year follow-up (Panza et al., 2015).

There are also epidemiological animal studies and clinical studies which have reported that caffeine helps to reduce cognitive decline in elderly patients diagnosed with Alzheimer's disease (van Gelder et al., 2007). In an animal study, when rats or transgenic mice were treated with caffeine, it was noted that they had bigger dendritic length and bigger spine density in distal dendritic branches in the basal dendrites of CA1 pyramidal neurons (Arab et al., 2013). Caffeine can inhibit GABA receptor signaling, increase intracellular calcium release, inhibit multiple phosphodiesterases, has pleiotropic effects on the central nervous system and can decrease Aβ production in rodents (Carman et al., 2014).

Caffeine acts as a neuro-stimulatory substance

Methylxanthine caffeine has a psychostimulant property by acting on neurotransmission in different parts of the brain like an antagonist of the adenosine receptors A1 and A2A subtypes (Flaten et al., 2014; Haller et al., 2014). Caffeine can also act as an excitatory neurostimulator, controls the cerebral perfusion and is a vasoconstrictor that causes a low pressure of cerebral blood flow in the participants (Haller et al., 2014). When the 3C cohort study was conducted, women who consumed caffeine showed an inverse dose-response association towards cognitive decline (Barberger-Gateau et al., 2012). The side effects of caffeine include cardiovascular side effects, inhibition of phosphodiesterase, and rise in intracellular calcium but these side effects disappear after 3-4 days and consuming a moderate number of cups of coffee per day (Marques et al., 2011). This also indicates the corroboration with other studies with the results of this systematic literature review and meta-analysis, consuming a moderate number of cups of coffee a day prevents side effects in the participants of the included studies. Caffeine can also protect against a diagnosis of AD by increasing the activity of the Na^+-K^+-ATPase pump and also there is a rise in cerebrospinal fluid production (Yenisetti & Muralidhara, 2016).

The significant hallmarks of Alzheimer's disease are that the amyloid plaques and neurofibrillary tangles are deposited, the neurons disappear and the synapse does not function properly (Marques et al., 2011). The amyloid-β plaques are heterogeneous peptides produced from the amyloid-β protein precursor and these plaques can lead to excess accumulation of Aβ42 (Marques et al., 2011; Tabaton, 2009). The neurofibrillary tangles arise from the hyperphosphorylation of the tau protein which damages the neurons and the function of the synapse also ceases. In tau pathology, Aβ deposits occur which is eventually followed by the late tangle pathology stages of AD (Marques et al., 2011). Factors that can trigger or that can occur in Alzheimer's disease include inflammation, tau protein phosphorylation or amyloid precursor protein expression and processing; together all these can lead to changes

Table 3: The study characteristics of the 3 included articles

First author (year)	Coffee Intake		Incident cognitive Disorders				
	Method of Assessment	Coffee Consumption	Type	Method of Ascertainment	No of cases	Coffee consumption → Risk estimate (95% CI)	Adjustment factor
(Eskelinen et al., 2009)	Self-administered questionnaire	0-2;				0-2 cups/day → 1.0 (Reference)	dementia and Alzheimer's disease, age, sex, education, follow up time, community of residence, midlife smoking, systolic blood pressure, serum total cholesterol, BMI, physical activity, ApoEe4, late-life myocardial infarction, stroke, diabetes mellitus, beck depressive scale
			Dementia; Alzheimer's	DSM-IV; NINCDS-ADRDA	61; 48	3-5 cups/day → 0.42(0.12-1.46)	
		3-5;				>5 cups/day → 1.01(0.33-3.09)	
		>5 cups/day					
(Lindsay et al., 2002)	Self-administered questionnaire	0;	Alzheimer's	NINCDS-ADRDA	194	Regular vs. not regular coffee consumption → 0.69 (0.50-0.96)	age, sex, education
(Maia & de Mendonca, 2002)	Self-administered questionnaire	>1 cup/day Average daily intake (mg) in 20 yrs before AD; cases, 73.9 [97.9] controls, 198.7 [35.7]. Average daily intake (mg) from 25 yrs old to 20 yrs before AD; cases, 69.6 [94.1] controls, 184.5[137.2]. Average daily intake after AD; cases, 36.3[64.1] controls, 177.1[123.7]	Alzheimer's	MMSE, NINCDS-ADRA	54	Coffee in mg → 0.40(0.24-0.65)	sex, age, hypertension, diabetes, stroke, head trauma, smoking habits, alcohol consumption, non-steroid anti-inflammatory drug use, vitamin E, gastric disorder, heart disease, education and family history of Alzheimer's disease

Abbreviations: AD; Alzheimer's Disease; DSM; Diagnostic and Statistical Manual of Mental Disorders; MMSE; Mini-Mental State Examination; NINCDS-ADRDA; National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association; mg; milligrams; Ave; Average; yrs; years

in the neurotransmission, synaptic loss, neurodegeneration including clinical and neuropathological changes (Profenno et al., 2010). Caffeine can also increase the use of cerebral glucose rate which helps to improve cognitive functioning (Rosso et al., 2008). Caffeine possesses the ability to bind to many types of receptors that are found in the synaptic membranes for cytoplasmic phosphodiesterases (Santos et al., 2010). Caffeine is a nonselective antagonist of adenosine receptors (van Gelder et al., 2007). Caffeine can block A2A receptors that reduce the synaptotoxic effect of Aβ (van Gelder et al., 2007). The mechanism of caffeine as a protective factor against Alzheimer's disease is that it initially enters the circulatory system, then acts like an antagonist on the A2A adenosine receptors that are located in the brain and this action immediately stimulates the cholinergic neurons which have the ability and play the role of defending against β-amyloid induced neurotoxicity, the precursor of cognitive decline (Rosso et al., 2008).

Systematic Literature Review and Meta-Analysis of Population-Based Cohort Studies and Case-Control Study

The significant result that was obtained from the dose-response curve was a non-linear inverse correlation between coffee consumption or coffee intake and the risk of developing AD. The case-control study that was selected had the sources of caffeine specified such as coffee (5 oz), instantaneous coffee and decaffeinated (Maia & de Mendonca, 2002). All the included studies had relative risk and 95% confidence intervals stated. The number of follow-ups in years was long for Eskelinen et al. (2009) study and Maia and de Mendonca (2002) study. The sample size was the largest for Lindsay et al. (2002) study. All the 3 included studies used the self-administered

questionnaire method for assessing coffee intake for both male and female genders. The study were prospective studies (Eskelinen et al., 2009; Lindsay et al., 2002).
The epidemiologic studies conducted on humans and animals show that coffee is a neurostimulant, coffee consumption plays a significant role in neuroprotection and has a reduced risk of developing AD. The results of the 3 included studies graphically plotted on the forest plot helped to detect any heterogeneity between the studies and the results between the studies was homogenous and more reliable. The results of the 3 included studies graphically plotted on the funnel plot and the Galbraith plot helped to detect publication bias between the studies. In conclusion, most of the studies are based on study data from 2 population-based cohort studies and 1 case-control study, an exact relationship between consuming coffee and the risk of developing AD cannot be judged with the current systematic literature review and meta-analysis which has been conducted.

4 Conclusions

The major strengths of the current systematic literature review and meta-analysis are the inclusion of 2 population-based cohort studies and 1 case-control study. The large sample size with a total number of 6170 participants and a total number of 296 cases diagnosed with AD helped plot the dose-response curve. The large sample size from a population makes the study data more reliable, less uncertainty and errors are avoided. In conclusion, the results and the current systematic literature review and meta-analysis of the 3 included studies which were conducted gives evidence that consuming a moderate number of 3-5 cups of coffee per day has a reduced risk of developing Alzheimer's disease. The Forest plot graphically summarized the results of the 3 included studies and the 3 studies had low heterogeneity. A U-shaped curve was obtained after plotting the dose-response curve. Publication bias between the studies exists after plotting the funnel plot and the Galbraith plot. Epidemiological studies such as human and animal studies prove that coffee can be a therapeutic agent for Alzheimer's disease. More prospective and long-term studies have to be conducted in other regions of the world to determine the exact relationship between coffee and Alzheimer's disease. The mechanism of how coffee carries out its neuroprotective effects in the human body might guide to invent new treatments for Alzheimer's disease in the future.

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