ISEKI Food Association

ISSN: 2182-1054

International Journal *of* Food Studies

Volume 9 | April, 2020

International Journal of Food Studies

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INTERNATIONAL JOURNAL OF FOOD STUDIES

Volume 9, nº1 (2020)
(Published 18 April 2020)

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International Journal of Food Studies | IJFS April 2020 | Volume 9 | pages 135–145

Evaluation of Growth and Cereulide Production by *Bacillus cereus* Isolated from Cooked Rice

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Received: 25 March 2018; Published online: 18 April 2020

Abstract

Conditions influencing *Bacillus cereus* growth and cereulide production, such as temperature and pH, were evaluated at varying incubation periods. The growth and cereulide production at different temperatures and pH values ranging from 10 to 40 °C and 5.0 to 8.5, respectively showed that the temperature from 20 to 30 °C and at pH from 6.0 to 7.0 gave the optimum growth and cereulide production by *B. cereus* SA105. pH below 6.0 resulted in reduced growth and cereulide production. Cereulide production increased along with the incubation period, and the maximum cereulide titre (ng/mL) of 1219.1±8.90 was obtained after 6 days of incubation at 30 °C and pH 6.5 under static conditions. There was no quantifiable toxin at incubation temperatures of 10 and 40 °C by *B. cereus* SA105. This work further reveals that *B. cereus* growth and cereulide production was significantly affected by temperature and pH in relation to the incubation period. Furthermore, the findings of this study will serve as a means for reducing the diversity of the emetic toxin-producing *B. cereus* population in food and food products, thus preventing food poisoning.

Keywords: *Bacillus cereus*; Growth; Cereulide; pH; Temperature; Incubation period

1 Introduction

Bacillus cereus, a Gram-positive, rod-shaped, beta haemolytic, endemic soil-dwelling bacterium is a common cause of food poisoning around the world (Turnbull, 1996). Episodes of *B. cereus* food poisoning occur sporadically worldwide, resulting from ingestion of contaminated food containing the bacteria, which multiply in high levels (McKillip, 2000). Basically, *B. cereus* is the etiological agent of two distinct types of gastrointestinal disorders, the diarrhea and emetic syndromes. The two types of food-borne diseases are caused by toxins: the diarrhea type by protein toxins which are thermolabile and formed in the intestinal tract of the host by the growing organism (enterotoxin) and the emetic type by a cyclic peptide (non-protein) toxin that is thermostable and pre-formed in food (emetic toxin) (Agata et al., 1994; Agata, Ohta, Mori, & Isobe, 1995; Granum, 2007; Jay, Loessner, & Golden, 2005).

The emetic syndrome was first identified in the 1970s and was associated with the consumption of fried rice (Kramer & Gilbert, 1989). This syndrome is an intoxication caused by *B.*

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cereus emetic toxin, called cereulide which is secreted in foods before ingestion. The toxin is a ring shaped, dodecadepsipeptide consisting of four acids, repeating three times and oxy acids (Granum & Lund, 1997). Jay (1996) reported that the disease is more acute than the diarrhea syndrome with an incubation time of 1-5 hrs, manifesting nausea, vomiting (emesis) and sometimes diarrhea which lasts for 6-24 hrs (Ehling-Schulz, Fricker, & Scherer, 2004). However, for the transmission of this type of *B. cereus* food poisoning, the infective dose of *B. cereus* in implicated food is 10^5-10^8 cell/g of food (Wijnands, Dufrenne, Rombouts, In't Veld, & Van Leusden, 2006).

Furthermore, several studies have indicated that only a minority of *B. cereus* isolates may produce cereulide (Agata, Ohta, & Mori, 1996; Mikami et al., 1994; Pirttijärvi, Andersson, Scoging, & Salkinoja-Salonen, 1999). Yokoyama et al. (1999) suggested that more than 90% of food poisoning caused by *B. cereus* is of the emetic type in countries of the Far East. This may be partly due to the wide consumption of rice, which is a well-known food vehicle for the emetic toxin. Lund, De Buyser, and Granum (2000) reported the toxin produced by B. cereus, cereulide, as the most dangerous toxin to human health responsible for the deaths of young healthy individuals. The prolific growth of *B. cereus* in various food sources and its ability to produce heat stable, non-protein toxin calls for urgency to the microbiological quality and safety of food products. Several studies have been done on the effect of environmental factors on the growth and cereulide production of *B. cereus* (Agata et al., 1996; Agata, Ohta, & Yokoyama, 2002; Finlay, Logan, & Sutherland, 2002a, 2002b). However, it could be of interest to study the effect of environmental factors in relation to time for the growth and cereulide production of emetic toxin-producing *B. cereus*. In this article, we compare and assess the effects of temperature and pH on growth and cereulide production of *B. cereus* at different days of incubation.

2 Materials and Methods

2.1 Microorganism

The emetic toxin-producing strain of *Bacillus cereus* SA105 isolated from cooked rice in Ibadan, Oyo State, Nigeria was used for this study.

2.2 Effect of environmental factors on *B. cereus* growth

Effect of temperature on *B. cereus* growth

The effect of temperature on the growth of the toxigenic *B. cereus* strain was determined using the method of From, Pukall, Schumann, Hormazabal, and Granum (2005) and Chorin, Thuault, Cleret, and Bourgeois (1997). 10 mL of sterile Tryptone Soy Broth was inoculated with a suspension of vegetative cells of *B. cereus* to achieve a concentration of 10^3CFU/mL. The tubes were sealed with paraffin film to avoid evaporation and concentration of the liquid medium at higher temperatures. After inoculation of 3 replica tubes, for each condition tested, media was then incubated for 1, 2, 4, 6, 8 and 10 days at temperatures of 10, 20, 30 and 40 ^{o}C.

Effect of pH on *B. cereus* growth

The modified method of Chorin et al. (1997) was used to determine the effect of pH on the growth of the toxigenic isolate. Culture of the toxigenic *B. cereus* isolate was performed in Tryptone Soy Broth. 10 mL of sterile media was inoculated with a suspension of vegetative cells of *B. cereus* to achieve a concentration of 10^3CFU/mL. The media was buffered with M.E.S (2-(N-morpholino) ethane-sulfonic acid, for pH ranging from 5.0 to 6.7 and with M.O.P.S (3-(N-morpholino) propane-conse-sulfonic acid, from 6.5 to 7.9. The pH was adjusted using 0.1 M of HCl or NaOH. Precise volumes of sterile HCl or NaOH were added respectively for pH: 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5. The adjusted media from pH 5.0 to 8.5 was inoculated as previously described using 3 replicates for each condition

tested. The different media were later incubated at 30 ^{o}C for 24 hrs.

2.3 Effect of environmental factors on cereulide production

Effect of *B. cereus* growth and incubation temperature on cereulide production

Tryptone Soy Broth (100 mL) was inoculated with 10^3 CFU/mL of *B. cereus* SA105 overnight culture. Triplicate cultures were thereafter incubated at temperatures of 10, 20, 30, and 40 ^{o}C; static condition and agitation speed of 150 rpm. Following incubation, 1 mL was taken from each culture tube for serial dilution and the viable count was determined. Cereulide was extracted with pentane, and analysed using HPLC-MS after 1, 2, 4 and 6 days of incubation (Haggblom, Apetroaie, Andersson, & Salkinoja-Salonen, 2002).

Effect of pH on cereulide production

The pH (5.0-8.5) of Tryptone Soy Broth was adjusted and inoculated as previously described using 3 replicates for each condition tested. The different media were later incubated at 30 ^{o}C at 150 rpm. Cereulide was extracted with pentane, and analysed using HPLC-MS after 1, 2, 4 and 6 days of incubation.

2.4 Preparation of cell extracts

Incubated cultures were autoclaved (120 ^{o}C; 19 mins) to destroy heat-labile proteins and other substances. The liquid cultures were extracted twice, each time with an equal volume of pentane for 1 hr with mild agitation (25 rpm) in vertical motion and after shaking the tubes were frozen. Organic phase layer was then separated from the aqueous phase layer in a smaller test tube. The combined pentane phases were evaporated to dryness under a stream of nitrogen and the residue was dissolved in 1 mL of methanol (Andersson, Mikkola, Helin, Andersson, & Salkinoja-Salonen, 1998).

2.5 HPLC-MS analysis

High-performance liquid chromatography (HPLC)-MS analysis was performed on a Waters 2695 Separation Module HPLC equipped with a C_8 column (250 x 4.6 mm,5 μm Waters) and a solvent made up to 95 % Acetonitrile, 4.9 % H_2O, and 0.1 % Trifluoroacetic acid at a flow rate of 0.15 mL/min, with sample injection monitored with a Diode array detector. A full mass spectrum was recorded from 500 to 1,300 m/z in positive electron spray mode (ESI±). The total ion chromatogram was smoothed with Gaussian function. Valinomycin (Sigma) was used as the standard compound for quantification of cereulide. To quantify cereulide and valinomycin absorbance, Integrated Extracted Ion Current (EIC) chromatograms with ion ranges (m/z) of 1,170.5 to 1,193.5 for cereulide and 1,128.5 to 1,151.0 for valinomycin targeting the $NH4^{\pm}$ and $K^{\pm}$ adducts respectively, were used. Calibration curves extrapolated from integrated peak areas were plotted to calculate cereulide amounts via linear regression (Haggblom et al., 2002).

3 Results and discussion

3.1 Effect of temperature on *B. cereus* growth

he *B. cereus* SA105 growth profile at varying temperatures (10-40 ^{o}C) is shown in Figure 1, with maximum and minimum growth recorded at 30 and 10 ^{o}C after day 6 and 10 of incubation, respectively. The optimum temperature (30 ^{o}C) for *B. cereus* SA105 recorded is in accordance with the report of previous authors who observed the optimum temperature for the growth of *B. cereus* to be between 30-37 ^{o}C (European Food Safety Agency, 2005; Nguyen-The, Carlin, & Guinebretière, 2003). Similar to the present observation, Pielaat, Fricker, Nauta, and Van Leusden (2005), Carlin et al. (2006) and Wijnands et al. (2006) also reported that emetic toxin-producing *B. cereus* are found to be mesophilic in nature. The implication is that refrigeration will considerably increase the lag time and reduce the growth of emetic toxin-producing strains of *B. cereus.*

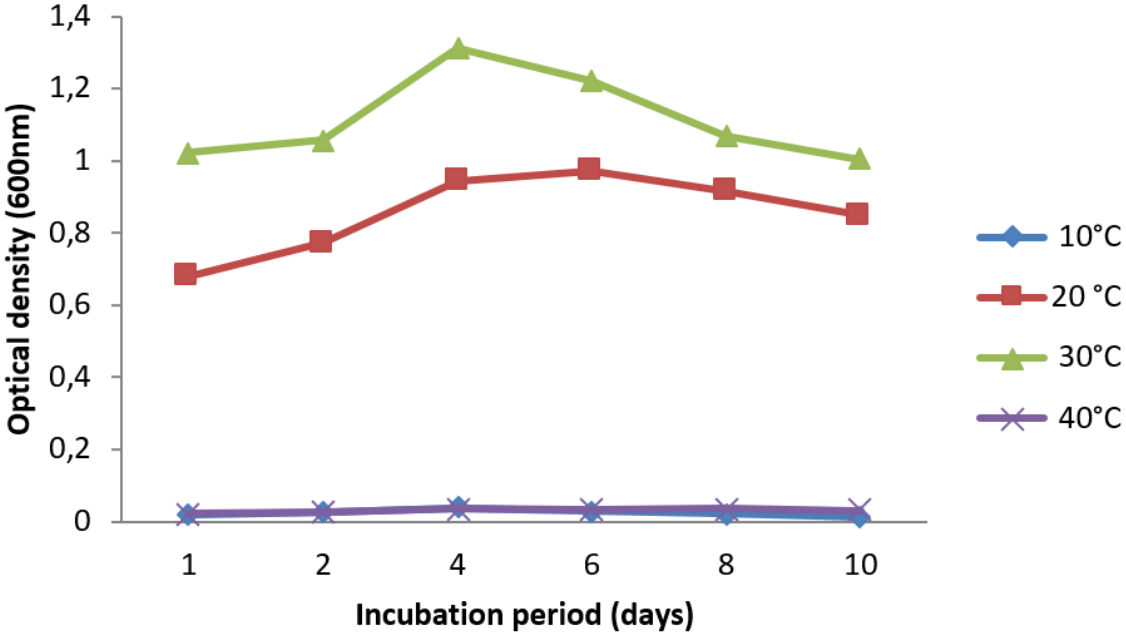

Figure 1: Effect of temperature on the growth of *B. cereus* SA105. Suspension of vegetative cells of the selected strain grown in Tryptone Soy Broth at varying incubation temperature and period (days) under static conditions. The values are presented as the mean ± SEM, n=3

3.2 Effect of pH on *B. cereus* growth

Results of the effect of pH on the growth of *B. cereus* SA105 are shown in Figure 2. The optimum pH for growth of *B. cereus* SA105 was pH 6.5. This was followed by pH 6.0 which gave the second highest absorbance while the least growth was observed at pH 5.0. The growth decreased with pH below 6.0 and above 8.5. Evaluation of the effect of pH on the growth rate of the emetic strain of *B. cereus* in this study revealed that it grew best from pH 6.0 to 7.0 and that it is not acid-tolerant. It is important to note that, at this optimum pH (6.0-7.0), the growth rate will be increased and lag time will be shorter (Benedict, Partridge, Wells, & Buchanan, 1993; Martinez, Borrajo, Franco, & Carballo, 2007). From the result obtained, it is indicated that pH below 5.5 was inhibitory to the strain thus, acidification is sufficient to prevent the growth of emetic strains of *B. cereus* during a longer storage period. This is useful in extending the shelf life and safety of processed foods but the significance to food protection is minimal since very few foods have such high acidity (Lindsay, Brozel, Mostert, & von Holy, 2000).

3.3 Effect of *B. cereus* growth and incubation temperature on cereulide production

The result (Figure 3) of viable counts and cereulide concentration at different incubation temperatures under static conditions revealed that *B. cereus* SA105 grew at all the incubation temperatures after 4 days of incubation. The highest cereulide concentration of 762.0±2.28 ng/mL and viable count of 6.3±0.23 $\log_{10}$CFU/mL was recorded by *B. cereus* SA105 at 30°C after 4 days. Increased temperature

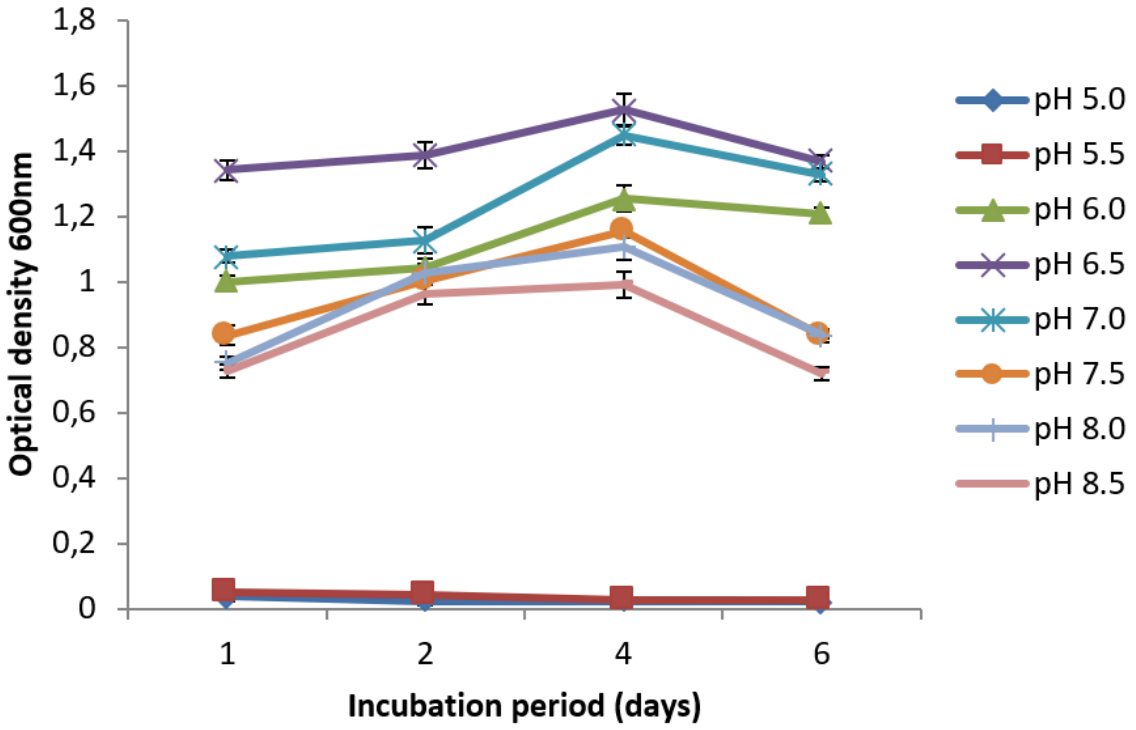

Figure 2: Effects of pH on the growth of *B. cereus* SA105. Suspension of vegetative cells of the selected strain grown in Tryptone Soy Broth, incubated at 30 ^{o}C and, at varying pH and incubation times (days) under static conditions. The values are presented as the mean± SEM, n=3

(above 10 ^{o}C) led to an increase in the growth of the test strain which influenced the production of cereulide. However, the mesophilic temperature has been previously observed to favor cereulide production in emetic strains of *B. cereus* (Dommel, Luecking, Scherer, & Ehling-Schulz, 2011). In our study, no quantifiable amount of cereulide was produced at 10 and 40 ^{o}C although the emetic strain was able to grow at these temperatures. In contrast, quantifiable amount of cereulide was produced in *B. weihenstephanensis* at 8-10 ^{o}C (Guerin et al., 2017). It has been previously reported that the production of cereulide below 10 ^{o}C does not seem possible and that temperatures above 37 ^{o}C also do not permit cereulide production (Finlay, Logan, & Sutherland, 2000, 2002a, 2002b; Jaaskelainen, Haggblom, Andersson, & Salkinoja-Salonen, 2004). The lack of quantifiable cereulide production by *B. cereus* at 10 and 40 ^{o}C implies that foods held for long periods at $\leq$10 ^{o}C and at raised temperatures are unlikely to be a risk for emetic food poisoning.

At 30 ^{o}C under static conditions, the growth curve of the tested strain (Fig. 4) attained its peak after day 4 with a mean viable count ($\log_{10}$CFU/mL) of 6.3±0.23. Cereulide production was first detectable after day 1 of incubation with the mean toxin titre of 117.5±4.45 ng/mL and gradually a maximum mean titre of 1131.7±0.90 ng/mL was attained after 6 days of incubation. In this study, the emetic strain of *B. cereus* analyzed started its exponential growth from day 2 to day 4 with a slight drop after day 4, probably from depletion of nutrients. Interestingly, cereulide was detectable after 24 hrs at 30 ^{o}C with an amount exceeding the minimum acute toxic level of cereulide (10 ng/g) as quanti-

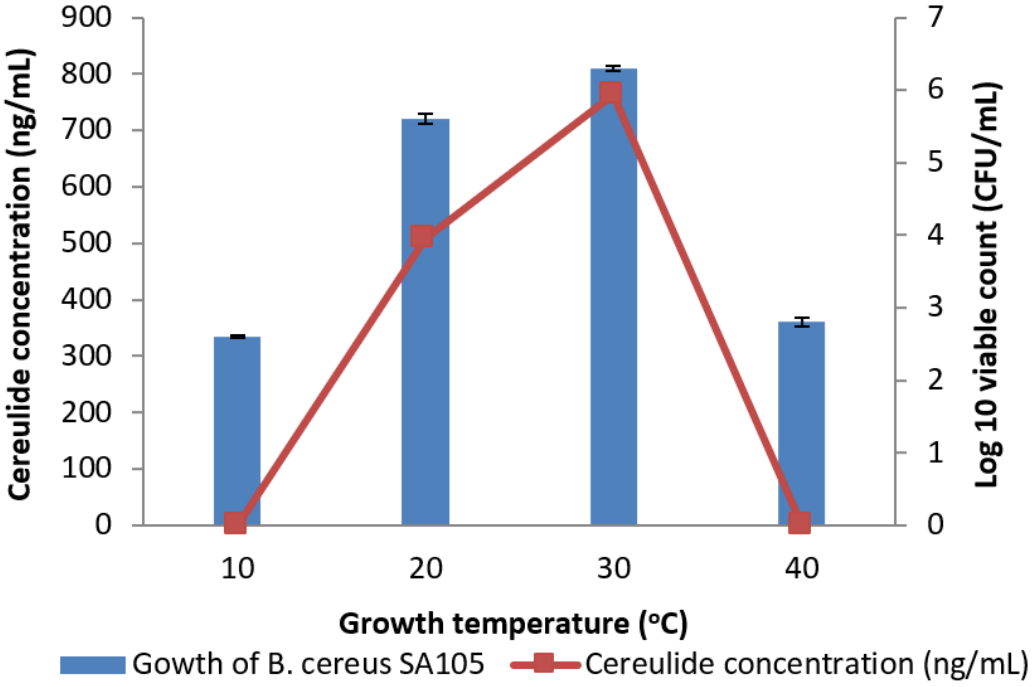

Figure 3: Effects of *B. cereus* growth and incubation temperatures on cereulide (ng/mL) production. The viable count was determined and cereulide was quantified using HPLC-MS after 4 days of incubation under static conditions. The values are presented as the mean± SEM, n=3

fied by Agata et al. (2002) in samples implicated in different outbreaks. Other studies have revealed that 20-30 ^{o}C is the optimal temperature for cereulide production and this supports the findings of the study (Thorsen, Budde, Henrichsen, Martinussen, & Jakobsen, 2009). The difference in the optimal temperature is influenced mainly by the bacterial growth rate and composition of the media or food (Agata et al., 2002; Rajkovic et al., 2006). Cereulide production in relation to temperature and time was analyzed in this study and it was discovered that the highest cereulide concentration was observed at the end of the growth period, which was between day 6-8. This agrees with the previous work of Haggblom et al. (2002) who reported that cereulide production is growth phase-dependent and that maximum cereulide was recorded at the end of the growth period. Hence, the incubation period (storage time) plays an important role in cereulide production since it was observed that cereulide production increased during the late stationary phase.

The effect of agitation on growth and cereulide production was established at 30 ^{o}C and 150 rpm for 10 days. The maximum mean viable count ($\log_{10}$CFU/mL) recorded was 8.1±0.31 (day 4) which was higher than the non-agitated broth at 30 ^{o}C. Cereulide was detectable after day 1 of incubation with a mean toxin titre of 317.5±2.99 ng/mL and the maximum mean toxin titre of 1670.7±7.43 ng/mL (day 8) was recorded (Fig. 5). The role of aeration in cereulide production cannot be underestimated, since the production of cereulide is greatly inhibited by a reduction in atmospheric oxygen. Oxygen acts as a terminal electron acceptor for oxidative reactions in providing energy for all cellular activities. Aeration

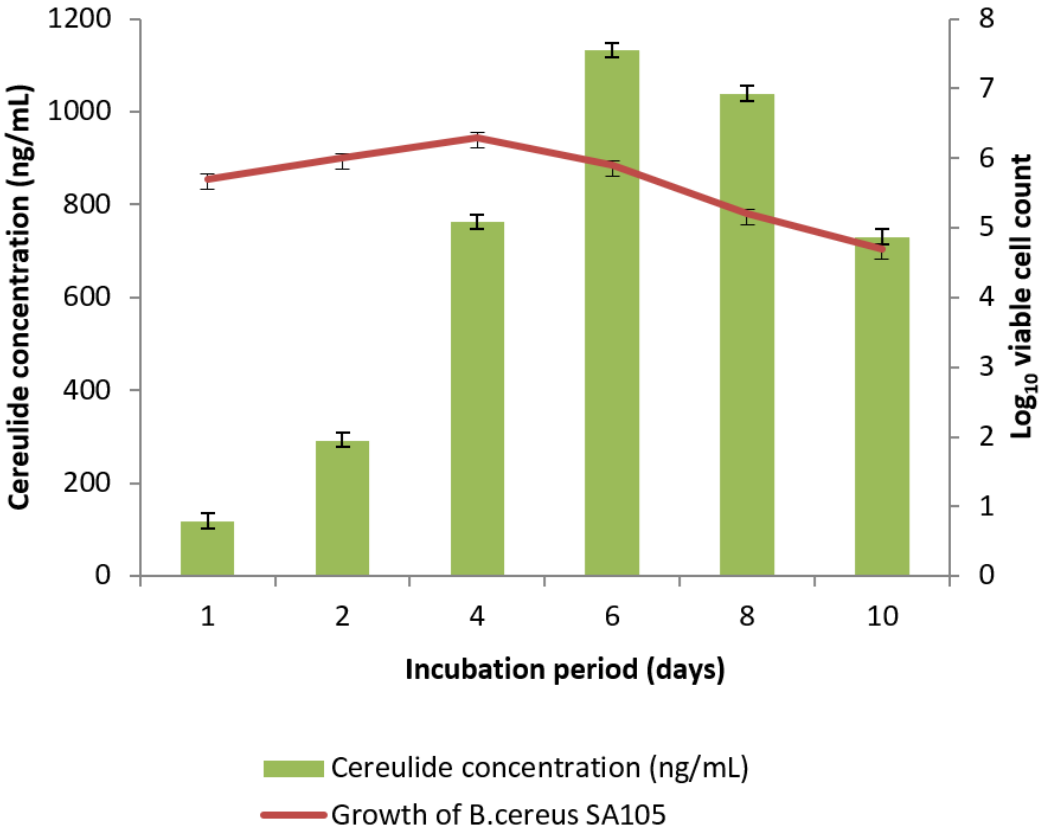

Figure 4: Production of cereulide (ng/mL) by *B. cereus* SA105 at 30 ^{o}C under static conditions. Vegetative cells of *B. cereus* SA105 were grown in broth and incubated at 30 ^{o}C under static conditions. The viable count was determined and cereulide was quantified using HPLC-MS after different incubation periods. The values are presented as the mean± SEM, n=3

influences the mixing and nutrient availability in shaking flasks (do Nascimento & Martins, 2004). Thus, oxygen is regarded as a stimulating factor in cereulide production (Agata et al., 2002; Finlay et al., 2002b). In contrast, Rajkovic et al. (2006) and Shaheen et al. (2006) reported an inhibitory effect of oxygen on cereulide production.

3.4 Effect of pH on cereulide production

The result (Table 1) of cereulide production in broth inoculated with *B. cereus* SA105 at different pH values incubated for 6 days. At pH 5.0 cereulide was not detected until day 4 while at pH 5.5 cereulide was detected at day 2. Quantifiable cereulide concentration was recorded after day 1 in pH ranging from 6.0-8.5. However, the highest mean toxin titre of 1219±8.9 ng/mL was produced at pH 6.5 followed by pH 6.0 reaching a mean toxin titre of 970.6±1.18 ng/mL after 6 days of incubation. Acidic pH inhibited cereulide production by the emetic strain of *B. cereus* in this study. In a previous study, Agata et al. (2002) reported that the addition of dressings like mayonnaise, vinegar or ketchup to rice for the purpose of acidification decreased *B. cereus* growth in these foods and cereulide produced was

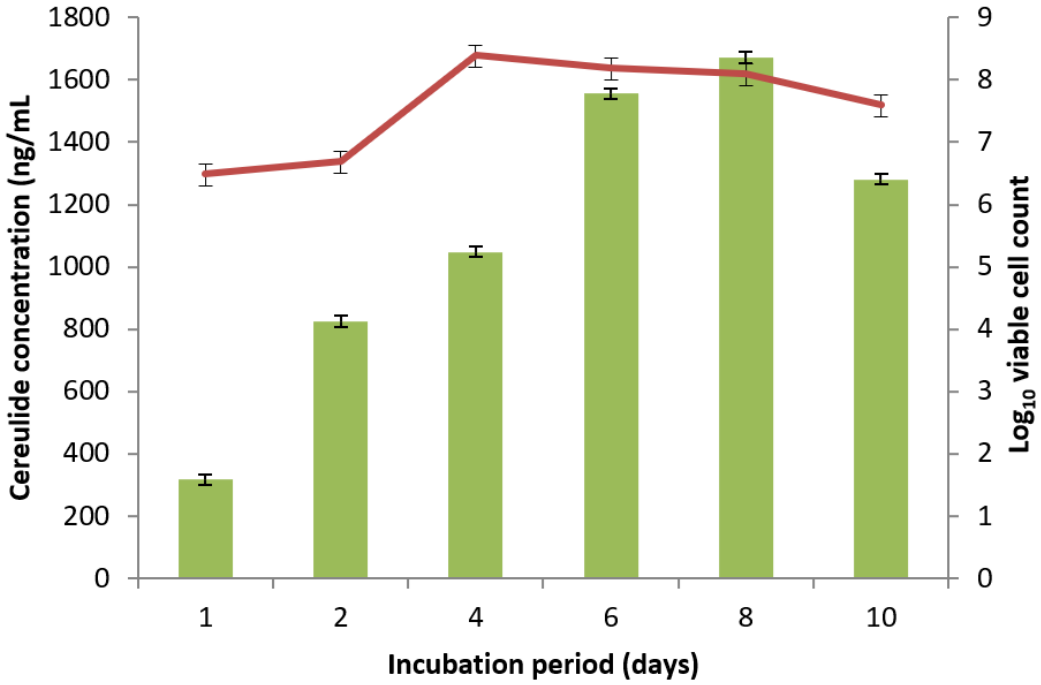

Figure 5: Production of cereulide (ng/mL) by *B. cereus* SA105 at 30 ^{o}C and 150 rpm. Vegetative cells of *B. cereus* SA105 were grown in broth and incubated at 30 ^{o}C and 150 rpm. The viable count was determined and cereulide was quantified using HPLC-MS after different incubation periods. The values are presented as the mean± SEM, n=3

Table 1: Effect of pH on cereulide production (ng/mL) by *B. cereus* SA105

	Incubation Period (days)/ Cereulide production (ng/mL)			
pH	1	2	4	6
5.0	ND	ND	15.4±2.28^{g}	24.4±0.90g$^{\pm}$
5.5	ND	31.7±0.30^{g}	39.4±4.90^{f}	44.8±3.50^{f}
6.0	115.6±3.55^{d}	476.2±1.15^{c}	732.2±4.48^{b}	1070.6±1.18^{b}
6.5	295.3±1.95^{b}	521.2±9.95^{a}	828.4±6.25^{a}	1219.1±8.90^{a}
7.0	108.5±4.75^{a}	431.5±2.75^{b}	673.3±4.75^{c}	1028.4±2.70^{c}
7.5	94.1±2.75^{c}	212.5±2.24^{d}	375.0±4.75^{d}	653.2±5.03^{d}
8.0	89.4±6.20^{d}	178.3±1.18^{e}	317±2.50^{c}	543.5±0.02^{e}
8.5	45.0±1.90^{e}	94.9±9.65^{f}	124.5±2.80^{e}	256.7± 0.85^{e}

The values are presented as the mean ± SEM, n=3. $^{\pm}$Values with different letters in the same column indicate a significant difference (P≤0.05) using Duncan's Multiple Range Test. ND: Not Detected

below 0.01 μg/g, which is similar to the effect of acidic pH in this study. This agrees with the work of Wong and Chen (1988) who reported the effect of pH on *B. cereus* growth and the experimental result of its growth and emetic toxin production on Brain Heart Infusion (BHI) bouillon and food products. Hence, a possible strategy to prevent growth and cereulide production by emetic strains of *B. cereus* is through the acidification of food.

4 Conclusions

This extensive study shows that the various environmental factors analyzed (temperature, pH, aeration, and incubation period) played an important role in influencing growth and cereulide production by the emetic strains of *B. cereus*. Hence, the findings of this study will serve as a means for reducing the diversity of emetic toxin-producing *B. cereus* populations able to multiply in food and food products thus preventing food poisoning.

Acknowledgements

This study was supported by the INSA-JRD TATA Fellowship administered by Centre for International Co-operation in Science (CICS), Chennai.
We are grateful to the Department of Microbiology and Fermentation Technology, CSIR-Central Food Technological Institute, Mysore, India for providing help with cereulide evaluation.

References

Agata, N., Mori, M., Ohta, M., Suwan, S., Ohtani, I., & Isobe, M. (1994). A novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in hep-2 cells. *Fems Microbiology Letters*, *121*(1), 31–34. doi:10.1016/0378-1097(94)90141-4

Agata, N., Ohta, M., & Mori, M. (1996). Production of an emetic toxin, cereulide, is associated with a specific class of *Bacillus cereus*. *Current microbiology*, *33*(1), 67–69.

Agata, N., Ohta, M., Mori, M., & Isobe, M. (1995). A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. *Fems Microbiology Letters*, *129*(1), 17–19.

Agata, N., Ohta, M., & Yokoyama, K. (2002). Production of *Bacillus cereus* emetic toxin (cereulide) in various foods. *International Journal of Food Microbiology*, *73*(1), 23–27. doi:10.1016/S0168-1605(01)00692-4

Andersson, M. A., Mikkola, R., Helin, J., Andersson, M. C., & Salkinoja-Salonen, M. (1998). A novel sensitive bioassay for detection of *Bacillus cereus* emetic toxin and related depsipeptide ionophores. *Applied and Environmental Microbiology*, *64*(4), 1338–1343.

Benedict, R. C., Partridge, T., Wells, D., & Buchanan, R. L. (1993). *Bacillus cereus* - aerobic growth-kinetics. *Journal of Food Protection*, *56*(3), 211–214. doi:10.4315/0362-028X-56.3.211

Carlin, F., Fricker, M., Pielaat, A., Heisterkamp, S., Shaheen, R., Salonen, M. S., ... Ehling-Schulz, M. (2006). Emetic toxin-producing strains of bacillus cereus show distinct characteristics within the *Bacillus cereus* group. *International Journal of Food Microbiology*, *109*(1-2), 132–138. doi:10.1016/j.ijfoodmicro.2006.01.022

Chorin, E., Thuault, D., Cleret, J. J., & Bourgeois, C. M. (1997). Modelling *Bacillus cereus* growth. *International Journal of Food Microbiology*, *38*(2-3), 229–234. doi:10.1016/S0168-1605(97)00110-4

do Nascimento, W. C. A., & Martins, M. L. L. (2004). Production and properties of an extracellular protease from thermophilic bacillus sp. *Brazilian Journal of Microbiology*, *35*(1-2), 91–96. doi:10.1590/S1517-83822004000100015

Dommel, M. K., Luecking, G., Scherer, S., & Ehling-Schulz, M. (2011). Transcriptional kinetic analyses of cereulide synthetase genes with respect to growth, sporulation and emetic toxin production in *Bacillus cereus*. *Food Microbiology*, *28*(2, SI), 284–290. doi:10.1016/j.fm.2010.07.001

Ehling-Schulz, M., Fricker, M., & Scherer, S. (2004). Bacillus cereus, the causative

agent of an emetic type of food-borne illness. *Molecular Nutrition & Food Research*, *48*(7), 479–487. doi:10.1002/mnfr.200100055

European Food Safety Agency. (2005). *Bacillus cereus* and other bacillus spp. in foodstuffs. EFSA Journal, 175, 1-48.

Finlay, W. J. J., Logan, N. A., & Sutherland, A. D. (2000). *Bacillus cereus* produces most emetic toxin at lower temperatures. *Letters in Applied Microbiology*, *31*(5), 385–389. doi:10.1046/j.1472-765x.2000.00835.x

Finlay, W. J. J., Logan, N. A., & Sutherland, A. D. (2002a). *Bacillus cereus* emetic toxin production in cooked rice. *Food Microbiology*, *19*(5), 431–439. doi:10.1006/yfmic.505

Finlay, W. J. J., Logan, N. A., & Sutherland, A. D. (2002b). *Bacillus cereus* emetic toxin production in relation to dissolved oxygen tension and sporulation. *Food Microbiology*, *19*(5), 423–430. doi:10.1006/yfmic.504

From, C., Pukall, R., Schumann, P., Hormazabal, V., & Granum, P. E. (2005). Toxin-producing ability among bacillus spp. outside the *Bacillus cereus* group. *Applied and Environmental Microbiology*, *71*(3), 1178–1183. doi:10.1128/AEM.71.3.1178-1183.2005

Granum, P. E. (2007). Food microbiology: Fundamentals and frontiers, third edition. In M. P. Doyle & L. R. Beuchat (Eds.), (Chap. *Bacillus cereus*, pp. 445–456). doi:10.1128/9781555815912

Granum, P. E., & Lund, T. (1997). *Bacillus cereus* and its food poisoning toxins. *Fems Microbiology Letters*, *157*(2), 223–228. doi:10.1111/j.1574-6968.1997.tb12776.x

Guerin, A., Ronning, H. T., Dargaignaratz, C., Clavel, T., Broussolle, V., Mahillon, J., … Nguyen-The, C. (2017). Cereulide production by bacillus weihenstephanensis strains during growth at different ph values and temperatures. *Food Microbiology*, *65*, 130–135. doi:10.1016/j.fm.2017.02.006

Haggblom, M. M., Apetroaie, C., Andersson, M. A., & Salkinoja-Salonen, M. S. (2002). Quantitative analysis of cereulide, the emetic toxin of *Bacillus cereus*, produced under various conditions. *Applied and Environmental Microbiology*, *68*(5), 2479–2483. doi:10.1128/AEM.68.5.2479-2483.2002

Jaaskelainen, E. L., Haggblom, M. M., Andersson, M. A., & Salkinoja-Salonen, M. S. (2004). Atmospheric oxygen and other conditions affecting the production of cereulide by *Bacillus cereus* in food. *International Journal of Food Microbiology*, *96*(1), 75–83. doi:10.1016/j.ijfoodmicro.2004.03.011

Jay, J. M. (1996). *Modern food microbiology* (5. ed. New York: Chapman & Hall, Eds.). doi:10.1007/978-1-4615-7476-7

Jay, J. M., Loessner, M. J., & Golden, D. A. (2005). Modern food microbiology. In I. New York USA: Springer Science ± Business media (Ed.), (Chap. *Bacillus cereus* gastroenteritis, pp. 583–590).

Kramer, J. M., & Gilbert, R. J. (1989). *Bacillus cereus* and other bacillus species. *Foodborne Bacterial Pathogens*, *19*, 21–70.

Lindsay, D., Brozel, V. S., Mostert, J. F., & von Holy, A. (2000). Physiology of dairy-associated bacillus spp. over a wide ph range. *International Journal of Food Microbiology*, *54*(1-2), 49–62. doi:10.1016/S0168-1605(99)00178-6

Lund, T., De Buyser, M. L., & Granum, P. E. (2000). A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Molecular Microbiology*, *38*(2), 254–261. doi:10.1046/j.1365-2958.2000.02147.x

Martinez, S., Borrajo, R., Franco, I., & Carballo, J. (2007). Effect of environmental parameters on growth kinetics of *Bacillus cereus* (atcc 7004) after mild heat treatment. *International Journal of Food Microbiology*, *117*(2), 223–227. doi:10.1016/j.pestbp.2006.08.002

McKillip, J. L. (2000). Prevalence and expression of enterotoxins in *Bacillus cereus* and other bacillus spp., a literature review. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, *77*(4), 393–399. doi:10.1023/A:1002706906154

Mikami, T., Horikawa, T., Murakami, T., Matsumoto, T., Yamakawa, A., Murayama, S., … Suzuki, M. (1994). An improved

method for detecting cytostatic toxin (emetic toxin) of *Bacillus cereus* and its application to food samples. *Fems Microbiology Letters*, *119*(1-2), 53–57.

Nguyen-The, C., Carlin, F., & Guinebretière, M. H. (2003). *Bacillus cereus* and food safety. *Bulletin of the Societe Francaise de Microbiologie*, *18*, 104–112. Retrieved from https://www.sfm-microbiologie.org/wp-content/uploads/2019/10/SFM_Bull2003_P104-112.pdf

Pielaat, A., Fricker, M., Nauta, M. J., & Van Leusden, F. M. (2005). Biodiversity in *Bacillus cereus*. *National Institute for Public Health and the Environment, The Netherlands. RIVM rapport 250912004*.

Pirttijärvi, T. S. M., Andersson, M. A., Scoging, A. C., & Salkinoja-Salonen, M. S. (1999). Evaluation of methods for recognising strains of the bacillus cereus group with food poisoning potential among industrial and environmental contaminants. *Systematic and Applied Microbiology*, *22*(1), 133–144. doi:10.1016/S0723-2020(99)80036-8

Rajkovic, A., Uyttendaele, M., Ombregt, S. A., Jaaskelainen, E., Salkinoja-Salonen, M., & Debevere, J. (2006). Influence of type of food on the kinetics and overall production of *Bacillus cereus* emetic toxin. *Journal of Food Protection*, *69*(4), 847–852. doi:10.4315/0362-028X-69.4.847

Shaheen, R., Andersson, M. A., Apetroaie, C., Schulz, A., Ehling-Schulz, M., Ollilainen, V. M., & Salkinoja-Salonen, M. S. (2006). Potential of selected infant food formulas for production of *Bacillus cereus* emetic toxin, cereulide. *International Journal of Food Microbiology*, *107*(3), 287–294. doi:10.1016/j.ijfoodmicro.2005.10.007

Thorsen, L., Budde, B. B., Henrichsen, L., Martinussen, T., & Jakobsen, M. (2009). Cereulide formation by bacillus weihenstephanensis and mesophilic emetic *Bacillus cereus* at temperature abuse depends on pre-incubation conditions. *International Journal of Food Microbiology*, *134*(1-2, SI), 133–139. Food Micro 2008 Conference, Aberdeen, SCOTLAND, SEP 01-04, 2008. doi:10.1016/j.ijfoodmicro.2009.03.023

Turnbull, P. C. B. (1996). *Bacillus* (B. S. et al., Ed.). Baron's Medical Microbiology. (4th ed.). University of Texas Medical Branch.

Wijnands, L. M., Dufrenne, J. B., Rombouts, F. M., In't Veld, P. H., & Van Leusden, F. M. (2006). Prevalence of potentially pathogenic *Bacillus cereus* in food commodities in the netherlands. *Journal of Food Protection*, *69*(11), 2587–2594. doi:10.4315/0362-028X-69.11.2587

Wong, H. C., & Chen, Y. L. (1988). Effects of lactic-acid bacteria and organic-acids on growth and germination of *Bacillus cereus*. *Applied and Environmental Microbiology*, *54*(9), 2179–2184.

Yokoyama, K., Ito, M., Agata, N., Isobe, M., Shibayama, K., Horii, T., & Ohta, M. (1999). Pathological effect of synthetic cereulide, an emetic toxin of bacillus cereus, is reversible in mice. *Fems Immunology and Medical Microbiology*, *24*(1), 115–120. doi:10.1016/S0928-8244(99)00017-6

International Journal of Food Studies | IJFS April 2020 | Volume 9 | pages 146–159

Chemical Constituents in Leaves and Aroma Products of *Nicotiana rustica* L. Tobacco

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Received: 4 May 2018; Published online: 18 April 2020

Abstract

Nicotiana rustica L. (Aztec tobacco) is the only *Nicotiana species*, except common tobacco (*N. tabacum* L.), which is cultivated for tobacco products. The leaves of *N. rustica*, however, accumulate various specialized metabolites of potential interest. Therefore, the objective of this study was to evaluate certain classes of metabolites (by HPLC and GC-MS) in the leaves, the essential oil (EO), concrete and resinoid of *N. rustica*. Three pentacyclic triterpenes were identified in the leaves (by HPLC): betulin (252.78 μg g^{-1}), betulinic (182.53 μg g^{-1}) and oleanolic (69.44 μg g^{-1}) acids. The dominant free phenolic acids in the leaves (by HPLC) were rosmarinic (4257.38 μg g^{-1}) and chlorogenic (1714.40 μg g^{-1}), and conjugated forms of vanillic (3445.71 μg g^{-1}), sinapic (1963.11 μg g^{-1}) and syringic (1784.96 μg g^{-1}). The major flavonoids in the leaves were luteolin (960.44 μg g^{-1}), apigenin (880.66 μg g^{-1}) and hyperoside (780.72 μg g^{-1}). The GC-MS profiling of the EO identified 19 components and the major ones were phytol (43.68 %), solanone (5.54 %), cis-5-butyl-4-methyldihydrofuran-2(3H)-one (5.23 %), dihydro-β-ionone (4.25 %), α-ionene (3.54 %) and β-damascenone (3.03 %). The major volatiles in the concrete were isoamyl alcohol (28.82 %), oxynicotine (9.02 %), phytol (7.80 %), 4-methyl-1-penthanol (6.33 %), cotinine (5.55 %) and 3-metyl-3-penthanol (4.09 %). Resinoid composition was dominated by nicotine (39.75 %), phytol (11.23 %), eicosane (4.88 %), diethyl phthalate (4.19 %), dibutyl phthalate (3.48 %) and solanone (3.27 %). Concrete and resinoid showed weak antibacterial activity. These results create grounds for considering *N. rustica* as a source to obtain aroma or other bioproducts.

Keywords: Essential oil; Extracts; *Nicotiana rustica* L.; Phytochemicals; Polyphenols; Triterpenes

1 Introduction

Nicotiana rustica L. (known also as "wild tobacco", "Aztec tobacco" or "makhorka") and *Nicotiana tabacum* L. (common tobacco) are the only two of the 76 *Nicotiana species* (Solanaceae) that are used for large-scale production of cured leaves intended for the manufacture of various tobacco products for human consumption (by smoking, chewing, snuffing). Reasonably, common tobacco (*N. tabacum* L.), the species being grown globally in a plethora of commercial types

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and varieties, has turned into one of the most studied plant materials in the world (Leffingwell, 1999; Rodgman & Perfetti, 2016). Considerably less is known about the chemical, technological and other characteristics of the other *Nicotiana species*.

N. rustica L. is an annual herbaceous plant, originating from Mexico and with natural habitats found in Peru and Bolivia. *N. rustica* L. was probably domesticated earlier than *N. tabacum*, and there is documented evidence of its historical use in religious rituals by the indigenous peoples of the Americas (Kishore, 2014; Winter, 2000). The plants are stout, 0.60 to 1.20 m high. The leaves are simple, broad-oval, petiolate and very dense. The flowers are pale-yellow to green. The vegetation period is comparatively short, 90 to 100 days, and the leaf yield may reach 1.5 to 2 tonnes per hectare (Knapp, Chase & Clarkson, 2004; Kostoff, 1941; Yadav, Rathi, Pednekar & Rewachandani, 2016).

Although considered naturalized worldwide, nowadays *N. rustica* is grown and used in small amounts, in a limited number of countries, e.g. Turkey, India, Russia, South America, Vietnam and other Asian countries, where it is represented by a number of regionally defined varieties. Apart from being used in domestic smoking products (cigarette-like "papirossi", cigars or water-pipe tobacco), *N. rustica* L. is included in chewing tobacco, and moist and dry snuffs (e.g. in some regions of India or Pakistan) (Djordjevic & Doran, 2009; Kishore, 2014; Kurucu, Kartal & Erenmemişoğlu, 1998). One of the driving forces of *N. rustica* L. cultivation nowadays is its high alkaloid level and its suitability for an industrial production of nicotine and nicotine-related products (Jassbi, Zare, Asadollahi & Schuman, 2017; Kurucu et al., 1998; Saitoh, Noma & Kawashima, 1985; Sisson & Severson, 1990). The content of nicotine (3-[(2S)-1-methylpyrrolidin-2-yl] pyridine), the basic native alkaloid of *N. rustica* L. may reach as high as 8 to 10 % (in single reported cases even 15 to 18 %), depending greatly on variety, environmental and cultivation conditions. Tatarchenko, Mokhnachev and Kasyanov (2003) have summarized data about *N. rustica* L., emphasizing that different varieties grown under identical conditions accumulate considerably different amounts of nicotine in the leaves, from 3.4 to 8.32 %. Sisson and Severson (1990) in a study on the alkaloid levels and composition of 64 *Nicotiana species*, reported 5.4 μg g^{-1} total-alkaloid content in freeze-dried green leaves from greenhouse-grown plants (of which 98.2 % were nicotine, 0.5 % nornicotine, 0.8 % anabasine, 0.5 % anatabine), and considerably higher content in leaves from field-grown plants at 25.6 μg g^{-1} (97.1 % nicotine, 0.7 % nornicotine, 0.5 % anabasine, 1.7 % anatabine). Kurucu et al. (1998) found substantial differences in the alkaloid levels (nicotine, nornicotine and anabasine) of *N. rustica* leaves depending on plant cultivation conditions (for example, nicotine levels varying from 7.71 % to 2.12 % in the tobacco from two different regions of Turkey). In a comprehensive review on the secondary metabolites from the genus *Nicotiana* and their biological activities, Jassbi et al. (2017) pointed out that nicotine biosynthesis and aerial accumulation in *Nicotiana species* may increase substantially in response to environmental and other factors, such as herbivore attack and leaf damage. Stanfill et al. (2015) determined 5.09 % nicotine level in a Brazilian nasal snuff (rapé) product made entirely of *N. rustica* tobacco without flavorings, while according to Furbee (2009) *N. rustica* leaves contained up to 18 % nicotine. Studies of floral volatiles from *N. rustica* L. (Raguso, Levin, Foose, Holmberg & McDade, 2003; Schlotzhauer, Horvat, Chortyk, Nottingham & Jackson, 1995) observe that nicotine has been present in major amounts in flower oil as well (over 20 %), a coherent result of the well-documented intensive synthesis of this secondary metabolite by the species (Jassbi et al., 2017; Sisson & Severson, 1990).

Cured leaves of *N. rustica* L. are also a traditionally recognized source for obtaining citric acid, as its average content of 7 to 8 % may reach as high as 18 % if appropriate cultivation and post-harvest practices are applied (Tatarchenko et al., 2003).

In terms of the assessment of *N. rustica* L. leaf quality as a material for smoking and smokeless tobacco products, a number of basic indices of the chemical composition have been determined, e.g. ash (13.7 - 19.4 %), reducing substances (1.20 - 1.80 %), soluble carbohydrates (in trace amounts), total nitrogen (4.10 - 4.60 %), ammo-

nia (0.71 - 0.79 %) and organic acids (15.90 - 24.40 %) (Tatarchenko et al., 2003). Hristeva and Nikolov (2006) evaluated nine varieties of *N. rustica* L. as genetic donors in selection procedures aimed at enhancing the smoking quality of common tobacco, and provided the following data about their chemical characteristics: ash (13.08 - 15.87 %), reducing substances (6.77 - 15.00 %), soluble carbohydrates (6.13 - 14.06 %), total nitrogen (1.51 - 2.12 %), total alkaloids (0.78 - 2.87 %), nicotine (0.30 - 1.44 %), chlorogenic acid (0.43 - 0.83 %) and rutin (0.73 - 1.31 %).

In the last two decades the focus of phytochemical research on tobacco has been on the analysis of biologically active secondary metabolites such as plant volatiles, terpenes, carotenoids, sterols, saponins, phenolics and secondary alkaloids, and the results reveal the clear potential of *N. tabacum* L. and some other *Nicotiana species* in this aspect (Andersson, Wennstrom & Gry, 2003; Budzianowski, 2014; Chowański et al., 2016; Jassbi et al., 2017; Kodama, Fujimori & Kato, 1984; Rodgman & Perfetti, 2016; Zhou, Li, Feng & Li, 2013). The distinctive phytochemical profile of *Nicotiana species* justifies their processing by different extraction techniques (traditional, accelerated, microwave or ultrasound assisted solvent extraction, supercritical extraction with liquefied gasses and subcritical water extraction) (Huie, 2002). Extracts from *N. rustica* L. leaves and flowers revealed biological and pharmacological activities, such as antimicrobial, anti-proteolytic, antioxidant and insecticidal (Bakht, Azra & Shafi, 2013; Digrak, Alma & Ilçim, 2001; Ibrahim, Aliyu, Abusufiyanu, Bashir & Sallau, 2011; Jassbi et al., 2017).

Driven by these contemporary trends in tobacco science, in 2015 the Tobacco and Tobacco Products Institute in Bulgaria set up a research programme for the investigation of tobacco species that are not common to Bulgaria, including *N. rustica* L. and *N. alata* Link & Otto.

Therefore, the objective of the current study is to evaluate certain classes of biologically active secondary metabolites in the leaves of *N. rustica* L. tobacco, experimentally grown in Bulgaria, and to identify the chemical composition of different aroma products (essential oil, concrete and resinoid).

2 Materials and Methods

2.1 Materials

Plant material

Tobacco (*N. rustica* L. var. rustica) was grown on the experimental fields of the Tobacco and Tobacco Products Institute (a branch of the Bulgarian Agricultural Academy), in the region of Plovdiv, South Bulgaria ($42^o04'55.2"$N $24^o42'16.8"$E). Soil characteristics were: hummus-carbonate (rendzina) type, organic matter (by Turin) 2.31 %; total nitrogen (by Kjeldahl) 0.212 %; mobile forms of phosphorus P_2O_5 (by Egner - Reem) 14.85 mg 100 g^{-1} soil; available potassium K_2O 67.5 mg 100 g^{-1} soil and pH 8.2. The vegetation period (June - September 2016) was characterized by an average temperature of 22^oC and an average rainfall of 44.5 mm, during which two additional irrigations were carried out. Fresh leaves were picked by hand at maturity. The leaves were stringed and sun-cured in the open air for about two weeks, until the characteristic leaf coloration (green with bronze or brown hues) developed and the mid-rib became brittle. Until processing, leaf material was stored in cardboard boxes in an air-conditioned warehouse. For the preparation of analytical samples, the leaves were oven-dried (40^oC; 6 h) and ground in a laboratory mill. A portion of the ground sample, intended for polyphenol and triterpene analysis, was further powdered by a laboratory homogenizer. The moisture content (%) was determined by drying to constant weight at 103 ± 2^oC, and all results have been presented on a dry weight (DW) basis.

Chemicals

HPLC grade methanol and acetonitrile, as well as phenolic acid and flavonoid standards were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Germany).

2.2 Determination of leaf chemical composition

Nicotine, reducing carbohydrates, nitrogen, and ash content in the leaves

The chemical indices were determined by standardized analytical methods: nicotine ISO 15152:2003; reducing carbohydrates ISO 15154:2003; total nitrogen BDS 15836:1988 and mineral substances (ash) ISO 2817:1999. All analyses were done in triplicate and the respective mean values have been presented.

HPLC analysis of polyphenols in the leaves

Portions of 0.5 g homogenized leaf samples were taken and subjected to triple sonicated extraction with 70 % methanol at 70 ^{o}C for 3 hours. The combined extract was evaporated at 60 ^{o}C, the residue was dissolved in methanol and the solution (filtered through a 0.45 μm syringe filter) was transferred to the HPLC unit. For the extraction of conjugated phenolics, 2M HCl in methanol was used, and the other conditions were the same (Marchev, Georgiev, Ivanov, Badjakov & Pavlov, 2011).

The HPLC system used for the determination of phenolic acids and flavonoids consisted of a Waters 1525 Binary Pump (Waters, Milford, MA, USA), UV-VIS detector (Waters 2487 Dual λ Absorbance Detector (Waters, Milford, MA, USA) and SUPELCO Discovery HS C18 column (5 μm, 250 mm × 4.6 mm, operated at a temperature of 26 ^{o}C), and Breeze 3.30 software.

The determination of phenolic acids was done according to Marchev, Georgiev, Ivanov et al. (2011). The mobile phases were: Phase A 2 % acetic acid and Phase B 0.5 % acetic acid:acetonitrile = 1:1 (v/v). The gradient elution profile was (Phase B): 0 - 30 min increase from 5 % to 35 % at 0.8 mL min^{-1}; 30 - 45 min from 35 % to 70 % at 0.4 mL min^{-1}; 45 - 50 min from 70 % to 80 % at 1.2 mL min^{-1}; 50 - 60 min from 80 % to 100 % at 1.2 mL min^{-1}; 60 - 65 min decrease from 100 % to 5 % at 0.8 mL min^{-1} and 65 - 70 min maintained to equilibrate the column. The standards used for building the calibration curves were gallic, protocatechuic, salicylic, chlorogenic, vanillic, caffeic, syringic, ferulic, sinapic, p-coumaric and cinnamic acids. Detection was performed at wavelengths of 280 nm and 320 nm.

The mobile phases in the gradient elution of flavonoids were: Phase A 2 % acetic acid and Phase B methanol. The gradient elution profile was set up as follows (Phase B): 0 - 10 min increase from 30 % to 50 % at 1.0 mL min^{-1}; 10 - 15 min held at the same flow rate; 15 - 16 min increase to 52 % at a flow rate of 0.8 mL min^{-1}; 16 - 30 min increase to 80 % at the same flow rate; 30 - 35 min decrease to 30 % at 1.0 mL min^{-1} and then maintained to 40 min to equilibrate the column (Marchev, Georgiev, Ivanov et al., 2011). Myricetin, kaempferol, quercetin, hesperidine and apigenin were used as standards for plotting the calibration curves. Detection wavelengths were 308 nm and 380 nm.

The determination of the quercetin glycosides rutin and hyperoside was carried out under the following conditions: 2 % acetic acid (Phase A) and acetonitrile (Phase B) used as mobile phases, and elution gradient set up to the profile of 0-15 min 20 % Phase B; 15-17 min 50 % Phase B and 17-20 min 20% Phase B (Ivanov et al., 2014). Rutin and hyperoside were used as standards to build the calibration curves. The detection was carried out at 370 nm.

HPLC analysis of triterpenes in the leaves

Samples of 1.0 g finely ground tobacco were subjected to threefold extraction with acetone (each for 30 min), in an ultrasonic bath, at hydro module 1:20 (w/v) and temperature 45^{o}C. The solvent in the combined extract was evaporated at 60^{o}C, using a rotary vacuum evaporator, and the residue was transferred to 1 mL methanol, filtered through a 22 μm filter and analyzed by HPLC. The identification of triterpenes was carried out on the same HPLC system as described for polyphenols. The mobile phase was an aqueous solution of potassium dihydrogen phosphate (pH 2.8): methanol = 12:88 (v/v), the flow rate was: 0 - 18 min at 0.8 mL min^{-1}; 18 - 19 min at 0.6 mL min^{-1}; 19 - 30 min at 0.6 mL min^{-1}; 30 - 31 min at 0.8 mL min^{-1} and 31 - 40 min at 0.8 mL min^{-1}. The detection wavelength

was 210 nm. The determination of triterpenes was against a standard curve, with carnosic acid, betulin, betulinic, ursolic and oleanolic acid (97 %) (Extrasynthese, France) used as standards (Marchev, Georgiev, Badjakov et al., 2011).

2.3 GC-MS profiling of aroma products (essential oil, concrete and resinoid)

One and the same plant material can be processed by different methods, thus obtaining aroma products with specific chemical composition, olfactory, biological and other properties. Common aroma products obtained from essential oil-bearing and medicinal plants include essential oils, concretes, absolutes, resinoids, oleoresins and tinctures (Bauer, Garbe & Surburg, 2001). The flow chart (Fig. 1) illustrates the basic principles of obtaining the aroma products investigated in this study, essential oil, concrete and resinoid.

Essential oil was obtained by hydrodistillation of 100 g dry tobacco in a laboratory Clevenger-type glass apparatus of the British Pharmacopoeia, modified by Balinova and Diakov (Stoyanova, Georgiev & Atanasova, 2007). The distilled essential oil was dried over anhydrous sulfate and stored at 4^{o}C until analysis.

Resinoid was obtained by two-stage, static batch extraction of 100 g tobacco with 95 % ethanol. The extraction conditions were: hydro module (raw material:solvent) 1:10 (w/v); duration of the first and second stage of extraction, 2.5 h and 2 h, and temperature 70^{o}C. The solvent was evaporated using a rotary vacuum evaporator, at a temperature 55^{o}C (Stoyanova et al., 2007).

Concrete was obtained by two-stage, static batch extraction of 100 g tobacco with petroleum ether (FILLAB, Bulgaria) under the following conditions: hydro module (raw material:solvent) 1:10 (w/v); duration of the first and second extraction stage, 1 h and 0.5 h, and temperature 30^{o}C. The solvent was evaporated using a rotary vacuum evaporator at a temperature of 35 ^{o}C (Stoyanova et al., 2007).

The GC-MS was carried out with an Agilent 5975C MSD system coupled to an Agilent 7890A gas chromatograph (Agilent Technologies Inc., Santa Clara, CA). Agilent J&W HP-5MS column (0.25 μm, 30 m x 0.25 mm) was used with helium as a carrier gas (1.0 mL min^{-1}). The operational conditions were: oven temperature 35^{o}C for 3 min and 5^{o}C/min to 250^{o}C for 3 min, total run time 49 min; injector temperature 260^{o}C; ionization voltage 70 eV; ion source temperature 230^{o}C; transfer line temperature 280^{o}C; solvent delay 4.25 min and mass range 50-550 Da. The MS was operated in scan mode. One μL of the sample diluted with n-hexane (10 %, v/v) was injected into the GC-MS system at split ratio 30:1. The GC analysis was carried out using an Agilent 7890A GC system and FID temperature of 270^{o}C. In order to obtain the same elution order with GC-MS, simultaneous triplicate injections were done by using the same column and the same operational conditions.

The identification of compounds was made by comparing their mass spectra with those from mass spectra libraries (Adams, 2001) and by comparing the literature and estimated Kovat's (retention) indices that were determined using mixtures of homologous series of normal alkanes from C_8 to C_{40} in hexane, under the conditions described above. The percentage ratio of volatile components was computed using the normalization method of the GC/FID peak areas.

2.4 Antimicrobial activity of aroma products

The antimicrobial activity was determined against the Gram-positive bacteria *Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 6633, the Gram-negative bacteria *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella abony* NTCC 6017, the yeasts *Sacharomyces cerevisiae* ATCC 9763 and *Candida albicans* ATCC 10231, and the molds *Aspergillus niger* ATCC 16404. The test-microorganisms were obtained from the National Bank of Industrial Microorganisms and Cell Cultures (Sofia, Bulgaria) and were deposited in the microbial culture collection of the Department of Biotechnology and Food Technology, Razgrad Branch, Angel Kanchev University of Russe, Bulgaria. The antimicrobial activity was studied by the agar diffusion cup method using 8 mm cups

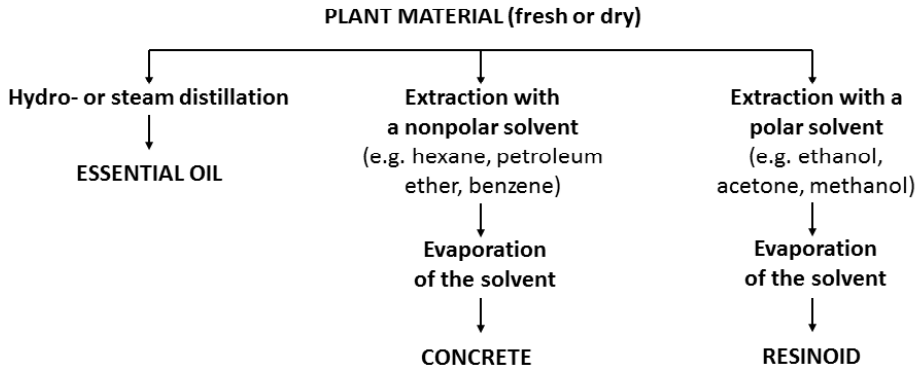

Figure 1: Principal scheme to obtain essential oil, concrete and resinoid from plant materials

and 50 μL of the samples. The respective media were soybean-casein digest agar medium (Biolife) for bacteria, and Sabouraud Dextrose Agar (Biolife) for yeasts and molds. The cultivation was carried out at 37^{o}C for 24 h (bacteria), at 27^{o}C for 24 h (yeasts) and for 72 h (molds), and the diameter of the inhibition zones was measured (Zaika, 1988). Blank dishes, with only solvent applied were also included as a negative control, in order to make the necessary corrections due to solvent activity. All tests were performed in triplicate.

2.5 Statistical analysis

All experiments were performed at least three times. All data were presented as mean ± standard deviation (SD). Statistical significance was assessed by Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison. Differences between means were considered statistically significant if $p < 0.05$.

3 Results and Discussion

3.1 Chemical composition of tobacco leaves

Basic chemical indices of tobacco

Leaf chemical composition is one of the decisive criteria in the evaluation of the quality of any tobacco type cultivated for commercial purposes (i.e. with the purpose to yield leaf material with certain, typical chemical, physical and sensory features). The assessment of the quality level and the typical nature of tobacco leaf material through its chemical composition are based on the complex consideration of some recognized, key indicators (Leffingwell, 1999; Stanfill et al., 2015). These include: the total alkaloid content (expressed as nicotine, free and/or ionized); the total content of reducing sugars (or alternatively of soluble carbohydrates); the total nitrogen content (or alternatively protein nitrogen); and ash (i.e. the total mineral matter, expressed as pure ash, corrected for sand and other stuck impurities), and are determined by standardized methods (by the ISO or national bodies). As it has been stated previously, *N. rustica* has been historically cultivated for tobacco products intended for human consumption, and is still an important cash crop in certain regions of the world. On the other hand, the species remains relatively

uncommon to Bulgaria and there is scarce data about its leaf chemical composition relevant to this particular geographical origin. Therefore, the first step in the current study was the determination of the basic leaf chemical indices of *N. rustica* tobacco experimentally grown in the country, in an attempt to assess its quality and compliance with the tobacco type profile (Table 1).

An analysis of the numerical data in Table 1 reveals that the chemical composition of the studied plant material can be considered as typical for *N. rustica*, with a chemical profile described by some authors (Tatarchenko et al., 2003) as resembling that of cigar tobacco which combines high alkaloid, nitrogen and ash levels with very low or trace amounts of soluble carbohydrates. Nevertheless, there were some characteristic leaf features, obviously influenced by environmental and other factors of the growing area or by the applied agricultural practices, which affected plant metabolism. The content of nicotine was reasonably high (3.09 %), but still closer to the lower limit of the characteristic 2 to 8 % nicotine range reported for commercial *N. rustica* tobacco (Tatarchenko et al., 2003). Similarly, the total nitrogen content was insufficiently high (2.68 %), being about a half of that found in the leaves of high quality *N. rustica* (Tatarchenko et al., 2003). On the other hand, leaves accumulated substantially higher amounts of reducing carbohydrates (3.82 %) than the typical range cited for the species by the same authors (0.5 - 1 %). These findings suggest that, in case of an interest in a commercially-oriented production of *N. rustica* in the country, the growing conditions (e.g. agricultural practices, soil, plant nutrition, curing and breeding) should be optimized so as to realise the full potential of the species in terms of leaf quality. It should be regarded, as well, that some of the analyzed leaf chemical indicators represent plant metabolites that are extractible and biologically active, and their levels would contribute to the composition and properties of plant-derived crude or processed extracts.

Table 1: Chemical indices of *N. rustica* L. leaves

Index	Content (% DW [a])
Nicotine	3.09 ± 0.03 [b]
Reducing sugars	3.82 ± 0.02
Total nitrogen	2.68 ± 0.01
Ash	17.52 ± 0.15

[a] DW: dry weight basis
[b] data expressed as mean (n=3) ± standard deviation

Triterpenes and polyphenols

The analysis of the two groups of biologically active metabolites, triterpenes and polyphenols, resulted in the qualitative and quantitative profile of *N. rustica* L. leaves as presented in Table 2 and Table 3.

Three pentacyclic triterpenes of the oleanane type were identified in the leaves, among which dominated the terpene alcohol betulin (50.08 % of the total triterpenoid content). The presence of ursolic and carnosic acids were not detected. All three triterpene secondary metabolites have well documented biological and pharmacological activities (Parikh et al., 2014; Patlolla & Rao, 2012).

In the group of free phenolic acids, cinnamic acid derivatives comprising mainly rosmarinic and chlorogenic acid were dominant. In the group of conjugated phenolic acids (determined after acid hydrolysis), hydroxybenzoic acid derivatives (vanillic acid, syringic acid) were dominant. The dominant free and conjugated flavonols were myrecitin and quercetin, respectively. Flavons were mainly in a conjugated form. In the group of quercetin glycosides, only the free form of hyperosid was identified, while the conjugated form of hesperetin was dominant in flavon glycosides. This profile of polyphenol metabolites in the leaves of *N. rustica* L. substantially differs from the profile established in *N. tabacum* L. leaves from Bulgaria, reflecting species distinctiveness.

3.2 Chemical composition of aroma products (essential oil, concrete and resinoid)

Beside the identification of phytochemicals in the cured leaves of *N. rustica* L. tobacco from

Table 2: Triterpenes in *N. rustica* L. leaves

Compound	Content (μg g^{-1} DW [a])
Carnosic acid	nd [b]
Betulin	252.78 ± 2.11 [c]
Betulinic acid	182.53 ± 1.51
Oleanolic acid	69.44 ± 0.55
Ursolic acid	nd

[a] DW: dry weight basis
[b] nd: not detected
[c] data expressed as mean (n=3)
± standard deviation

Bulgaria, our goal was also the characterization of the chemical composition of the essential oil (obtained by hydrodistillation) and that of two concentrated, ready-to-use aroma products obtained by solvent extraction, concrete and resinoid. We hypothesized that the comparative assessment of these three aroma and biologically active products would reveal distinct differences in their volatile profiles, due to the influence of temperature, pH of the medium, extractant nature and other factors relating to the transformations of plant metabolites during hydrodistillation and extraction.

The essential oil of *N. rustica* was a light brown liquid, and the final concentrated extraction products (concrete and resinoid) were dark brown waxy masses, all with specific tobacco odor. In these characteristics they did not differ from the respective aroma products obtained from common tobacco described elsewhere (Georgiev & Stoyanova, 2006; Popova et al., 2015). The yield of concrete was 1.50 ± 0.01 % (w/w) and that of resinoid was 15.62 ± 0.09 % (w/w). These results were sufficiently high, in a parallel with the yields of concrete (0.25 - 3.0 %) and resinoid (8 - 27 %) from common aromatic plants (Georgiev & Stoyanova, 2006). On a yield basis, the results suggest the potential of *N. rustica* leaves as a source to obtain aroma products by solvent extraction. The yield of essential oil, however, was considerably lower (0.09 ± 0.00 %, v/w), thus supporting some previous observations about only trace amounts of essential oil in *N. rustica* leaves (Tatarchenko et al., 2003). For comparison, leaves of common tobacco were found to contain between 0.2 % and 1.5 % essential oil (Alagić, "Selekcija", Palić, Stojanović & Nikolić, 2002; Tatarchenko et al., 2003; Zhang, Gao, Zhang, Liu & Ye, 2012), and in single cases of highly aromatic Bulgarian oriental varieties up to 2-2.5 % (Georgiev & Stoyanova, 2006).

Data from the identification of the volatile composition of the essential oil, concrete and resinoid are presented in Table 4.

GC-MS analysis identified 19 components in the essential oil of *N. rustica* L. leaves (constituting 75.49 % of the total oil content). Six of the identified volatiles were in quantities over 3 %: (E)-phytol (43.68 %), solanone (5.54 %), cis-5-butyl-4-methyldihydrofuran-2(3H)-one (5.23 %), dihydro-β-ionone (4.25 %), α-ionene (3.54 %) and β-damascenone (3.03 %). These results reveal an essential oil that is not significantly different from the one of Oriental type *N. tabacum* L. (Popova et al., 2015). *N. rustica* L., however, has not been referenced much as a source of essential oil or other aroma products, and it is hard to make parallels to previous results about leaf oil chemical composition. The established low oil accumulation also probably does not favor *N. rustica* as a promising aromatic plant. Overall, data in Table 4 showed that, in terms of aroma-active compounds, *N. rustica* essential oil could not be associated with distinct major compounds, responsible for its aroma profile. As previously reported, tobacco essential oil (*N. tabacum* L.) is different from other plant essential oils, which always contain a set of special profile-shaping components (Alagić et al., 2002; Zhang et al., 2012). One possible explanation of these results may be related to the particular distillation conditions of tobacco oil (i.e. distillation from a strongly acidified medium, pH 2.00), which stimulate the acidic hydrolysis of certain classes of plant metabolites (including carotenoids).

Nineteen compounds were identified in the concrete, representing 73.83 % of its composition. Six of them were in quantities over 3 % and could be considered as major components: isoamyl alcohol (28.82 %), oxynicotine (9.02 %), (*E*)-phytol (7.80 %), 4-methyl-1-penthanol (6.33 %), cotinine (5.55 %) and 3-metyl-3-penthanol (4.09 %). It is hard to draw comparisons, as to the

Table 3: Polyphenols in *N. rustica* L. leaves

No	Compounds	Free (μg g^{-1} DW [a])	Conjugated (μg g^{-1} DW)
	Phenolic acids		
1	Rosmarinic acid	4257 ± 100 [b]	394.6 ± 1.1
2	Chlorogenic acid	1714 ± 40	130.7 ± 1.0
3	Sinapic acid	725.5 ± 1.7	1963 ± 59
4	Ferulic acid	286.7 ± 1.2	388.5 ± 1.2
5	Caffeic acid	260.3 ± 1.2	nd [c]
6	*p*-Coumaric acid	99.64 ± 0.88	372.6 ± 1.2
7	Cinnamic acid	9.54 ± 0.08	110.2 ± 0.7
8	2-Hydroxybenzoic acid	1376 ± 30	343.3 ± 1.2
9	Syringic acid	287.7 ± 1.3	1785 ± 66
10	Vanillic acid	75.41 ± 0.51	3446 ± 100
11	3,4-Dihydroxybenzoic acid	34.41 ± 0.28	142.6 ± 1.0
12	Gallic acid	nd	67.72 ± 0.51
	Flavonoids		
1	Myricetin	107.5 ± 0.6	79.80 ± 0.50
2	Kaempferol	14.81 ± 0.09	94.75 ± 0.66
3	Quercetin	24.41 ± 0.11	389.4 ± 1.2
4	Apigenin	nd	880.7 ± 19.5
5	Luteolin	29.17 ± 0.12	960.4 ± 20.8
6	Hyperoside	780.7 ± 0.7	nd
7	Hesperetin	70.93 ± 0.49	567.8 ± 1.5

[a] DW: dry weight basis
[b] data expressed as mean (n=3) ± standard deviation
[c] nd: not detected

best of our knowledge no detailed research on the yield or composition of concrete from *N. rustica* L. from Bulgaria or other regions has been published.

In the resinoid, a total of 16 compounds were identified (85.44 % of its composition). The major constituents (over 3 %) were: nicotine (39.75 %), (E)-phytol (11.23 %), eicosane (4.88 %), diethyl phthalate (4.19 %), dibutyl phthalate (3.48 %) and solanone (3.27 %). The technology of obtaining, together with the high native alkaloid level in the leaves, make *N. rustica* L. resinoid a higher-nicotine product, with a more specific possible application. For example, a suitable one could be its inclusion in hair treatment products, as there is evidence for a favorable effect of tobacco alkaloids on hair growth (Murkute, Sahu, Mali & Rangari, 2010).

The distribution of the identified chemical constituents of essential oil, concrete and resinoid, by groups of chemicals (percentage of the identified) reveal the distinctive profile of each of the three aroma products. The essential oil was dominated by oxygenated diterpenes (57.86 %), followed by oxygenated monoterpenes (11.70 %) and nitrogenous compounds (7.51 %). The most abundant chemical constituents of the concrete belonged to the group of oxygenated aliphatics (64.72 %), followed by nitrogenous compounds (22.05 %) and oxygenated diterpenes (10.56 %). The major part of the resinoid was constituted by nitrogenous compounds (52.49 %, and mainly alkaloids), followed by diterpene (13.14 %), aromatic (8.98 %) and oxygenated aliphatic (8.84 %) compounds. The results show that some of the aroma-active classes of compounds (e.g. oxygenated terpenes and aromatic compounds) were found in the oil, but not in the concrete and res-

Table 4: Volatile composition of the aroma products from *N. rustica* L. leaves

No	Compound	RI [a]	Content (%)		
			Essential oil	Concrete	Resinoid
1	Acetic acid	673	nd [b]	nd	2.45 ± 0.02 [c]
2	3-Penthanone	701	nd	2.77 ± 0.02	nd
3	Ethylmethyl ketone	733	nd	0.68 ± 0.00	nd
4	Isoamyl alcohol	760	nd	28.82 ± 0.23	nd
5	2-Methyl-1-butanol	762	nd	1.70 ± 0.01	nd
6	2-Hexanol	812	nd	0.94 ± 0.00	nd
7	Furfural	838	nd	nd	2.21 ± 0.02
8	4-Methyl-1-penthanol	843	nd	6.33 ± 0.05	nd
9	3-Methyl-3-penthanol	846	nd	4.09 ± 0.03	nd
10	Furfuryl alcohol	865	nd	nd	2.89± 0.02
11	Isoamyl acetate	885	nd	0.59 ± 0.00	nd
12	2-Methylbutyric acid	898	nd	1.69 ± 0.01	nd
13	α-Pinene	939	0.10 ± 0.00 [d]	0.20 ± 0.00 [d]	nd
14	Benzaldehyde	965	nd	0.93 ± 0.00	nd
15	β-Pinene	979	0.14 ± 0.00	nd	nd
16	β-Myrcene	997	0.28 ± 0.00	nd	nd
17	6-Methyl-5-hepten-2-ol	1003	nd	0.17 ± 0.00	nd
18	Trimethylpyrazine	1008	nd	nd	1.40 ± 0.01
19	Limonene	1030	0.28 ± 0.00 [d]	0.47 ± 0.00 [e]	2.68 ± 0.02 [f]
20	Eucalyptol (1,8-cineole)	1032	0.11 ± 0.00 [d]	0.37 ± 0.00 [e]	0.65 ± 0.00 [f]
21	Benzyl alcohol	1041	0.23 ± 0.00	nd	nd
22	Linalool	1103	0.34 ± 0.00	nd	nd
23	α-Ionene	1256	3.54 ± 0.02	nd	nd
24	Linallyl acetate	1259	0.59 ± 0.00	nd	nd
25	2-Methylnaphthalene	1295	2.51 ± 0.02	nd	nd
26	1-Methylnaphthalene	1312	2.76 ± 0.02	nd	nd
27	cis-5-Butyl-4-methyldihydrofuran-2(3H)-one	1344	5.23 ± 0.04	nd	nd
28	Nicotine	1366	0.13 ± 0.00 [d]	0.32 ± 0.00 [e]	39.75 ± 0.12 [f]
29	Solanone	1374	5.54 ± 0.03 [d]	1.39 ± 0.01 [e]	3.27 ± 0.02 [f]
30	Oxynicotine	1396	nd	9.02 ± 0.08	nd
31	β-Caryophyllene	1419	0.19 ± 0.00	nd	nd
32	Dihydro-β-ionone	1443	4.25 ± 0.04	nd	nd
33	β-Farnesene	1448	nd	nd	2.46 ± 0.01
34	Dimethyl phthalate	1460	2.56 ± 0.02	nd	nd
35	β-Damascenone	1390	3.03 ± 0.02	nd	nd
36	Diethyl phthalate	1602	nd	nd	4.19 ± 0.03
37	Farnesylacetone	1922	nd	nd	1.20 ± 0.00
38	(E)-Phytol	1960	43.68 ± 0.18 [d]	7.80 ± 0.07 [e]	11.23 ± 0.07 [f]
39	Dibutyl phthalate	1972	nd	nd	3.48 ± 0.01
40	Cotinine	1981	nd	5.55 ± 0.05 [d]	0.43 ± 0.00 [e]
41	Eicosane	2000	nd	nd	4.88 ± 0.03
42	Isopropyl palmitate	2026	nd	nd	2.27 ± 0.01
	Total identified	-	75.47	73.83	85.44
By groups of compounds (% of the identified)					
	Aliphatic hydrocarbons	-	-	5.71	
	Oxygenated aliphatic compounds	-	64.72	8.84	
	Monoterpene hydrocarbons	1.06	0.91	3.14	
	Oxygenated monoterpenes	11.70	0.50	0.76	
	Sesquiterpene hydrocarbons	0.25	-	2.88	
	Oxygenated sesquiterpenes	4.01	-	1.40	
	Oxygenated diterpenes	57.86	10.56	13.14	
	Aromatic compounds	6.98	1.25	-	
	Oxygenated aromatic compounds	3.70	-	8.98	
	Nitrogenous compounds	7.51	22.06	52.49	
	Other compounds	6.93	-	2.66	
	Total	100	100	100	

[a] RI: retention (Kovat's) index
[b] nd: not detected
[c] data expressed as mean (n=3) ± standard deviation
[d–f] means with different superscripts in a row differ significantly ($p < 0.05$)

inoid (or present in much smaller amounts). Apparently this distribution is related to the different pathway of obtaining the respective aroma products and reflects the influence of factors such as temperature (being respectively 30^{o}C in concrete extraction, 70^{o}C in resinoid extraction and steam temperature in essential oil distillation), duration of treatment (1.5 h in the extraction of concrete and 3 h in the hydrodistillation of the oil), and solvent polarity (nonpolar solvent in the case of concrete and polar for resinoid). The identification of individual aroma-active compounds in the essential oil, such as α-ionene, dihydro-β-ionone and β-damascenone, seems to be related to the degradation of leaf carotenoids, as influenced by the high temperature and occurrence of acidic hydrolysis during hydrodistillation, previously reported for common tobacco and other plant materials (Leffingwell, 1999; Winterhalter & Rouseff, 2001). Such carotenoid-related compounds were not identified in *N. rustica* concrete and resinoid, whereas the latter contained more hydrocarbons and their oxygenated derivatives due to the selective extraction by the solvents, which gave them the characteristic waxy texture.

3.3 Antimicrobial activity

Two of the obtained aroma products, concrete and resinoid, were tested for antimicrobial activity against a set of test-microorganisms. They inhibited only the growth of the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*. Their activity, however, was weak to moderate, with diameters of the inhibition zones from 9.8±0.01 mm to 11.5±0.03 mm. The rest of the tested bacteria, yeasts and molds were not sensitive to the concrete and resinoid from *N. rustica* L. leaves. Similar findings of limited or no inhibition activity of ethanol and n-hexane extracts from *N. rustica* L. leaves on bacteria were reported by Bakht et al. (2013).

4 Conclusions

The study reveals the potential of *N. rustica* L. leaves as a plant material that is rich in biologically active compounds, expanding therefore its importance beyond the production of smoking or smokeless tobacco products. The findings justify its consideration as a valuable source for obtaining aroma products of the types traditionally used in cosmetics or other areas (essential oil, concrete, resinoid). Different classes of secondary metabolites with biological activity, alkaloids, triterpenes, phenolic acids and flavonoids, have been identified in the leaves of the tobacco species that is not traditional to Bulgaria. The analysis of the chemical composition of the distilled leaf essential oil and the two concentrated extraction products (concrete and resinoid) revealed their specific volatile profile. To the best of our knowledge, these are the first data about obtaining concrete and resinoid from *N. rustica* L. and their chemical identification, especially with regard to the one grown in Bulgaria. The results from the study create grounds for directing the respective aroma products to specific applications and for future investigations aimed at expanding the species' importance.

Acknowledgements

The authors are most grateful to the University of Food Technologies, Plovdiv and the Tobacco and Tobacco Products Institute, Markovo for providing the tobacco material, the laboratory facilities and all chemicals to carry out this study.

References

Adams, R. P. (2001). *Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy*. (R. P. Adams, Ed.). Carol Stream, USA: Allured Publishing Corporation.

Alagić, S., "Selekcija", I. S., Palić, R., Stojanović, G. & Nikolić, M. (2002). Chemical composition and antimicrobial activity of the essential oil of the oriental tobacco yaka. *Journal of Essential Oil Research*, *14*(3), 230–232. doi:10 . 1080 / 10412905 . 2002.9699832. eprint: https://doi.org/10.1080/10412905.2002.9699832

Andersson, C., Wennstrom, P. & Gry, J. (2003). Nicotine alkaloids in solanaceous food plants. *TemaNord*, *531*, 1–37.

Bakht, J., Azra & Shafi, M. (2013). Antimicrobial potential of different solvent extracts of tobacco (*nicotiana Rustica*) against gram negative and positive bacteria. *Pakistan Journal of Botany*, *45*(2), 643–648.

Bauer, K., Garbe, D. & Surburg, H. (2001). Common fragrance and flavor materials. *Preparation, Properties and Uses. Weinheim: Wiley–VCH*.

Budzianowski, J. (2014). Tobacco-a producer of recombinant interferons. *Przeglad lekarski*, *71*, 639–43.

Chowański, S., Adamski, Z., Marciniak, P., Rosiński, G., Büyükgüzel, E., Büyükgüzel, K., ... Bufo, S. A. (2016). A Review of Bioinsecticidal Activity of Solanaceae Alkaloids. *Toxins*, *8*(3). doi:10.3390/toxins8030060

Digrak, M., Alma, M. H. & Ilçim, A. (2001). Antibacterial and Antifungal Activities of Turkish Medicinal Plants. *Pharmaceutical Biology*, *39*(5), 346–350. doi:10.1076/phbi.39.5.346.5903. eprint: https://doi.org/10.1076/phbi.39.5.346.5903

Djordjevic, M. V. & Doran, K. A. (2009). Nicotine content and delivery across tobacco products. In J. E. Henningfield, E. D. London & S. Pogun (Eds.), *Nicotine psychopharmacology* (pp. 61–82). doi:10.1007/978-3-540-69248-5_3

Furbee, B. (2009). Clinical neurotoxicology e-book: Syndromes, substances, environments, expert consult-online and print. In M. R. Dobbs (Ed.), (Chap. Neurotoxic plants, pp. 523–542). Elsevier Health Sciences.

Georgiev, E. & Stoyanova, A. (2006). A guide for the specialist in aromatic industry. *Plovdiv, Bulgaria*.

Hristeva, T. & Nikolov, E. (2006). Interspecies distance with basic chemical and technological signs of tobacco - nicotiana rustica. papers presented at science in globalization in 21st century. stara zagora: Union of scientists. In *International science conference, held at the union of scientists, stara zagora* (Vol. 1, pp. 152–156). Retrieved from https://nrs.nacid.bg/register/search

Huie, C. W. (2002). A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. *Analytical and Bioanalytical Chemistry*, *373*(1-2), 23–30. doi:10.1007/s00216-002-1265-3

Ibrahim, M. A., Aliyu, A. B., Abusufiyanu, A., Bashir, M. & Sallau, A. B. (2011). Inhibition of *Naja nigricolis* (reinhardt) venom protease activity by *Luffa egyptiaca* (mill) and *Nicotiana rustica* (linn) extracts. *Indian Journal of Experimental Biology*, *49*(7), 552–554.

Ivanov, I., Vrancheva, R., Marchev, A., Petkova, N., Aneva, I., Denev, P., ... Pavlov, A. (2014). Antioxidant activities and phenolic compounds in Bulgarian *Fumaria* species. *Int. J. Curr. Microbiol. App. Sci*, *3*(2), 296–306. Retrieved from https://www.ijcmas.com/vol-3-2/Ivan%5C%20G.%5C%20Ivanov,%5C%20et%5C%20al.pdf

Jassbi, A. R., Zare, S., Asadollahi, M. & Schuman, M. C. (2017). Ecological roles and biological activities of specialized metabolites from the genus nicotiana. *Chemical Reviews*, *117*(19), 12227–12280. doi:10.1021/acs.chemrev.7b00001

Kishore, K. (2014). Monograph of tobacco *(Nicotiana tabacum). Indian Journal of Drugs*, *2*, 5–23.

Knapp, S., Chase, M. W.-. & Clarkson, J. J. (2004). Nomenclatural changes and a new sectional classification in *Nicotiana* (Solanaceae). *Taxon*, *53*(1), 73–82. doi:10.2307/4135490

Kodama, H., Fujimori, T. & Kato, K. (1984). Glucosides of ionone-related compounds in several *Nicotiana* species. *Phytochemistry*, *23*(3), 583–585. doi:10.1016/S0031-9422(00)80386-6

Kostoff, D. (1941). Cytogenetics of the genus nicotiana. karyosystematics, genetics, cytology, cytogenetics and phylogenesis of tobaccos. *Cytogenetics of the genus Nicotiana. Karyosystematics, genetics, cytology, cytogenetics and phylogenesis of tobaccos*.

Kurucu, S., Kartal, A. & Erenmemişoğlu, A. (1998). HPLC analysis of nicotiana rustica

and chewing tobacco (maraş powder) alkaloids. *FABAD Journal of Pharmaceutical Sciences*, *23*, 61–4. Retrieved from http://dergi.fabad.org.tr/pdf/volum23/Issue2/3.pdf

Leffingwell, J. (1999). Leaf chemistry-basic chemical constituents of tobacco leaf and differences among tobacco types. (pp. 265–284). doi:10.13140/2.1.5173.6645

Marchev, A., Georgiev, V., Badjakov, I., Kondakova, V., Nikolova, M. & Pavlov, A. (2011). Triterpenes production by rhizogenic callus of *Salvia Scabiosifolia Lam*. obtained via *Agrobacterium Rhizogenes* mediated genetic transformation. *Biotechnology & Biotechnological Equipment*, *25*(sup1), 30–33. doi:10.5504/BBEQ.2011.0109. eprint: https://doi.org/10.5504/BBEQ.2011.0109

Marchev, A., Georgiev, V., Ivanov, I., Badjakov, I. & Pavlov, A. (2011). Two-phase temporary immersion system for *Agrobacterium rhizogenes* genetic transformation of sage (*Salvia tomentosa Mill.) Biotechnology Letters*, *33*(9), 1873–1878. doi:10.1007/s10529-011-0625-5

Murkute, A., Sahu, M., Mali, P. & Rangari, V. (2010). Development and evaluation of formulations of microbial biotransformed extract of tobacco leaves for hair growth potential. *Pharmacognosy Research*, *2*(5), 300–303. doi:10.4103/0974-8490.72328. eprint: http://www.phcogres.com/article.asp?issn=0974-8490;year=2010;volume=2;issue=5;spage=300;epage=303;aulast=Murkute;t=6

Parikh, N. R., Mandal, A., Bhatia, D., Siveen, K. S., Sethi, G. & Bishayee, A. (2014). Oleanane triterpenoids in the prevention and therapy of breast cancer: Current evidence and future perspectives. *Phytochemistry Reviews*, *13*(4, SI), 793–810. doi:10.1007/s11101-014-9337-5

Patlolla, J. M. R. & Rao, C. V. (2012). Triterpenoids for cancer prevention and treatment: Current status and future prospects. *Current Pharmaceutical Biotechnology*, *13*(1), 147–155.

Popova, V., Gochev, V., Girova, T., Iliev, I., Ivanova, T. & Stoyanova, A. (2015). Extraction products from tobacco–aroma and bioactive compounds and activities. *Current Bioactive Compounds*, *11*(1), 31–37. doi:10.2174/1573407211011508041500 16

Raguso, R. A., Levin, R. A., Foose, S. E., Holmberg, M. W. & McDade, L. A. (2003). Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in *Nicotiana*. *Phytochemistry*, *63*(3), 265–284. doi:10.1016/S0031-9422(03)00113-4

Rodgman, A. & Perfetti, T. A. (2016). *The chemical components of tobacco and tobacco smoke*. CRC press.

Saitoh, F., Noma, M. & Kawashima, N. (1985). The alkaloid contents of sixty nicotiana species. *Phytochemistry*, *24*(3), 477–480. doi:10.1016/S0031-9422(00)80751-7

Schlotzhauer, W. S., Horvat, R. J., Chortyk, O. T., Nottingham, S. F. & Jackson, D. M. (1995). Comparison of the volatile flower oils of *Nicotiana rustica* and *N. forgetiana*. *Journal of Essential Oil Research*, *7*(3), 265–269. doi:10.1080/10412905.1995.9698517

Sisson, V. A. & Severson, R. F. (1990). Alkaloid composition of the *Nicotiana* species. *Beitrage zur Tabakforschung International/Contributions to Tobacco Research*, *14*(6), 327–339.

Stanfill, S. B., Oliveira da Silva, A. L., Lisko, J. G., Lawler, T. S., Kuklenyik, P., Tyx, R. E., ... Watson, C. H. (2015). Comprehensive chemical characterization of rape tobacco products: Nicotine, un-ionized nicotine, tobacco-specific n '-nitrosamines, polycyclic aromatic hydrocarbons, and flavor constituents. *Food and Chemical Toxicology*, *82*, 50–58. doi:10.1016/j.fct.2015.04.016

Stoyanova, A., Georgiev, E. & Atanasova, T. (2007). A handbook for laboratory practice in essential oils. *Acad. Publ. House of the University of Food Technologies, Plovdiv*.

Tatarchenko, I. I., Mokhnachev, I. G. & Kasyanov, G. I. (2003). Chemistry of subtropical and food products: A handbook for students. In *Academia, moscow* (pp. 5–79). Retrieved from https://www.elibrary.ru/item.asp?id=20796343

Winter, J. C. (2000). *Tobacco use by native north americans : Sacred smoke and silent killer*. Includes bibliographical references (p. 387-434) and index. Norman : University of Oklahoma Press. Retrieved from https://trove.nla.gov.au/work/6546660

Winterhalter, P. & Rouseff, R. (2001). Carotenoid-derived aroma compounds: An introduction. (pp. 1–17). doi:10.1021/bk-2002-0802.ch001

Yadav, R., Rathi, M., Pednekar, A. & Rewachandani, Y. (2016). A detailed review on Solanaceae family. *European Journal of Pharmaceutical and Medical Research*, *3*(1), 369–378. Retrieved from http://www.ejpmr.com/admin/assets/article_issue/1451902449.pdf

Zaika, L. L. (1988). Spices and herbs: Their antimicrobial activity and its determination. *Journal of Food Safety*, (2), 97–118. doi:10.1111/j.1745-4565.1988.tb00511.x

Zhang, X., Gao, H., Zhang, L., Liu, D. & Ye, X. (2012). Extraction of essential oil from discarded tobacco leaves by solvent extraction and steam distillation, and identification of its chemical composition. *Industrial crops and products*, *39*, 162–169. doi:10.1016/j.indcrop.2012.02.029

Zhou, R., Li, D.-L., Feng, G.-L. & Li, G.-Y. (2013). A new sesquiterpene glucoside from *Nicotiana rustica L. Natural Product Research*, *27*(14), 1261–1264. doi:10.1080/14786419.2012.725398

International Journal of Food Studies | IJFS April 2020 | Volume 9 | pages 160–177

Zizyphus lotus (L.) Extracts as Prebiotics in the Aggregation and Adhesion of Probiotic and Inhibition of Pathologic Bacteria from Patients with Colorectal Cancer

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Received: 22 July 2018; Published online: 18 April 2020

Abstract

The mucosal surfaces of the intestinal tract harbor a complex microbiota. Bacteria enter in contact with intestinal cells and bestow important nutritional, metabolic and protective functions which benefit the host. Various factors are able to alter the balances between different intestinal bacteria. Dysbiosis has been described in various pathologies and metabolic diseases in humans, such as cancer. Colorectal cancer pathology can benefit from gut microbial imbalance. Its overgrowth may lead to acute symptoms. An alternative strategy to restore this balance is the use of plant extracts which exhibit a prebiotic activity by stimulating probiotic bacteria and antimicrobial activity against pathogenic bacteria colonization. The present study aims to evaluate the *in vitro* antioxidant and antibacterial activity of phenolic extracts (Aqueous extract "AE" and Methanolic extract "ME") from *Zizyphus lotus*. It aims also to investigate the effect of this extract as a prebiotic on the aggregation of probiotic and inhibitory effects of pathogenic bacteria isolated from faeces of patients with colorectal cancer. Phytochemical analysis of different extracts from *Zizyphus lotus* by HPLC showed that all are rich in phenolic compounds (225.40 mgAGE/gDW for AE and 63.04 mgAGE/gDW for ME extracts). Moreover, all extracts showed an important antioxidant activity (227 μg/ml for AE and 195 μg/ml for ME). These extracts also exhibited a significant prebiotic effect and antibacterial activity. Characterization of the *in vitro* effect of the aqueous extract showed that the percentage of autoaggregation and adhesion of probiotic and inhibitory effects of pathogenic bacteria increased in the presence of aqueous extracts.

Keywords: *Zizyphus lotus(L.)*; Extracts; Colorectal cancer; Aggregation; Adhesion; Antimicrobial activity

1 Introduction

The human gut microbiota can be considered as a separate organ that has co-evolved with humans to achieve a symbiotic relationship leading to physiological homeostasis (Backhed, Ley, L Sonnenburg, Peterson & I Gordon, 2005). A number of gastrointestinal disease states or disorders have been proposed to be associated with changes in the composition or function of gut microbiota. However, opportunistic and pathogenic infections can benefit from gut microbial imbalance. Their overgrowth may lead to acute symptoms. This is implicated in many digestive diseases (van Nimwegen et al., 2011) like inflammatory bowel diseases and colorectal cancer.
Gut microbiota are an important factor associated with colorectal cancer (CRC), one of the

 10.7455/ijfs/9.1.2020.a3

most common malignant tumor types in the world (Azcarate-Peril, Sikes & Bruno-Barcena, 2011). Each year, nearly one million new CRC cases are diagnosed worldwide and more than 500,000 deaths are reported (Jemal et al., 2011). It is the third most-diagnosed malignancy in the world according to the GLOBOCAN study (Parkin, Bray, Ferlay & Pisani, 2001) and the second deadliest cancer after lung cancer in western countries. There is also emerging evidence that intestinal bacteria may play a role in the initiation of colon cancer through the production of carcinogenic chemicals (Rowland, 2009).
Recently, the use of probiotics, which are beneficial bacteria present in the intestine, is an alternative strategy to maintain the balance of the intestinal microbiota by the reduction of its pathogenic members and by increasing the potentially beneficial ones (Gomes, Pintado, Freitas & Silva, 2014). At the present time, some plant products have attracted the attention of researchers to exploit some phytochemicals as antimicrobials and anticancer drugs. Such plant products would be biodegradable and safe for human health (Kumar, Shukla, Singh, Shekhar Prasad & Kishore Dubey, 2008; Liu, 2004; Wang, Li, Cao & Jiang, 2010). Therefore, it is necessary to develop new alternative molecules from medicinal plants for their biological activity, such as the treatment of infectious diseases (Boominathan & Ramamurthy, 2009). At the present time, many new studies have confirmed the antimicrobial activity of polyphenols occurring in medicinal plants. They act as anti-tumour agents, antimicrobials and anti-inflammatory agents (Jayaprakasha, Singh & Sakariah, 2001; Muanda, Soulimani, Diop & Dicko, 2011). *Zizyphus lotus (L.)* was investigated in the present study. *Ziziphus lotus* (Jujube) is a medicinal plant found in the Mediterranean region, including Algeria (Pottier, 1981). The *Zizyphus lotus* is a fruit-bearing, thorny shrub belonging to the family Rhamnaceae (Rsaissi & Bouhache, 2002). In North Africa it is commonly called "Sedra" (Borgi, Ghedira & Chouchane, 2007). The different species of *Zizyphus* are widely used in the pharmacobiological field in the treatment of certain diseases such as inflammatory diseases, digestive disorders, weakness, liver diseases, obesity, urinary disorders, diabetes, skin infections, fever, diarrhea and insomnia (Abdel-Zaher, Salim, Assaf & Abdel Hady, 2005; Abu Zarga, Sabri, Al-Aboudi, Saleh Ajaz & Sultana, 2004; Suksamrarn et al., 2005).
The present study aims first to investigate the intestinal bacterial community in populations with colorectal cancer and compare it to the bacterial community of healthy adults. Secondly, the objective is to compare the phytochemical screening and evaluation of *in vitro* antioxidant and antibacterial activities of phenolic extracts from the leaves and stems of *Zizyphus lotus (L.)*. Finally, the effect of this extract as a prebiotic on the aggregation of probiotic and inhibitory effects of pathogenic bacteria isolated from adult patients with colorectal cancer will be investigated.

2 Materials and Methods

2.1 Subjects

A total of 20 adults (12 male and 8 female) were included and divided into 2 groups: group 1 of patients with colorectal cancer (n=10) and group 2 of healthy controls (n=10). Their age ranged between 21 and 34 years, with a mean of 27.6±2.73 years for cancerous patients and 26.2±4.53 years for healthy controls. Body Mass Index (BMI) had a mean and standard deviation of 22.10±0.65 kg/m^2 for cancerous patients and 22.05±1.10 kg/m^2 for healthy controls.

Clinical Characteristics

Clinical characteristics of groups included in this study are shown in Table 1.
All adults included in this analysis (male or female) were between 19 and 40 years old, having only colorectal cancer disease (Group 1); not having acute or chronic infectious diseases or problems that may affect the gut microbiota (diarrhea, constipation); and not having been treated with antibiotics or antifungals (three months), and chemotherapy.

Table 1: Clinical characteristics of groups (means ± SD)

Characteristics (n=10)	Cancerous patients (n=10)	Healthy controls
Male/Female	6/4	6/4
Age (years)	27.6±2.73	26.2±4.53
Weight (kg)	61.4±2.4	64.8±6.53
Height (m)	1.66±0.027	1.72±0.04
Body Mass Index (kg/m^2)	22.10±0.65	22.05±1.10

Ethics

This work was carried out with the patients' consent and the permission of the director of Yessad Khaled hospital of Mascara.

2.2 Research and isolation of probiotic and pathogenic Bacteria in patients with colorectal cancer

Sample preparation

A 10^{-1} dilution was prepared by adding 1g of fecal samples to 9 ml of sterile saline. The suspension was mixed for 2 minutes by a vortex. From this suspension serial decimal dilutions were performed up to 10^{-6}, and 0.1 ml of each dilution was spread on different selective agar media in duplicate.

Clinical isolation and enumeration of bacterial candidates

Microbiological analysis of the fecal samples was carried out, using various appropriate culture media, to characterize the gut microbiota of different populations. *Lactobacillus* on MRS (Man Rogosa and Sharpe agar), *Streptococcus* on M17agar, *Total Aerobic* and *Anaerobic Flora* was isolated on GN (Nutrient agar), *Enterobacteria* was isolated on EMB (Eosin Methylene Blue agar), *Staphylococcus* on CHAPMAN agar, *Enterococcus* on BEA (Bile Esculin agar), *Clostridium* on Liver Meat agar and *Bacteroides* Blood agar (Béraud, 2001). The plates were incubated at 37 ^{o}C for 24 to 72 hours under aerobic conditions for *Lactobacillus, Streptococcus, Total Aerobic Flora, Enterobacteria, Staphylococcus* and *Enterococcus*, and anaerobically for *Total Anaerobic Flora, Clostridium* and *bacteroide*. Identification of bacterial candidates found in each culture medium was confirmed by macroscopic examination, Gram staining and use of biochemical tests, API system. Probiotic strains were identified by API 50 CH and characterized for their probiotic potential by evaluation of growth at different temperatures and gastric pH, tolerance of bile salts, resistance to antibiotics, antibacterial activity, aggregation and adhesion capacity. The enumeration of viable bacteria in fecal specimens was performed on dishes with 30-300 colonies and expressed as log colony forming unit (CFU) per gram of fecal sample for statistical reasons and for better mathematical interpretation according to the following formula (Béraud, 2001):

$$\text{Log CFU/g} = \frac{\textit{Number of colony}}{\textit{Dilution} \times \textit{Volume seeded}} \quad (1)$$

2.3 Phytochemical analysis of *Zizyphus lotus*

The plant material consisted of leaves and stems of *Zizyphus lotus (L.)* from Sidi Benyekhlef in the region of Mascara (Algeria). The plants were collected in July – August 2015. Taxonomic identification was performed by Pr. Najat ELKHIATI (Biological and Health Laboratory, Faculty of Science Aïn Chock-University Hassan II, Casablanca).

Preparation of plant extracts

The collected plant samples were air dried for a few days, and then the leaves and stems were crushed into a powder and stored for use.

a **Preparation of aqueous extract**
A 5 g sample of plant material was suspended in 100 ml of distilled water and macerated for 24 h (Xiao, Han & Shi, 2008). The macerated material was filtered, and the filtrate was lyophilised and stored at ambient temperature until further use.

b **Preparation of methanolic extract**
The powder of plant material (10 g) was extracted with 100 ml of methanol (maceration) during 24 h. After filtration, the filtrate was concentrated under reduced pressure at 40 ^{o}C by rotary evaporator to eliminate the methanol, and the extract was then lyophilized and stored at ambient temperature until further use (Diallo, Sanogo, Yasambou, Traoré & Maiza, 2004).

Determination of total Phenolic Content

The amount of total polyphenols was determined according to the Folin-Ciocalteu method. Briefly, 200 μl of extract was mixed with 1 ml of 1/10th Folin–Ciocalteu reagent. After 4 min, 0.8 ml of Na_2CO_3 (7.5%) was added. The mixture was incubated at room temperature for 2 hours and the absorbance was then read using a spectrophotometer at 765 nm (SHIMADZU-1240 UV/ visible). All determinations were carried out in triplicate. A standard curve was prepared using Gallic acid. Total polyphenolic values are expressed in Gallic Acid Equivalents (GAE) per gram of dry weight (mg GAE/ g DW) (Li et al., 2007).

Estimation of total Flavonoid content

The determination of total flavonoid content in the various extracts was carried out by a method using aluminium trichloride ($AlCl_3$) (Barros, Carvalho & Ferreira, 2011). Briefly, a 1ml aliquot of the different extracts was mixed with 0.3ml of $NaNO_2$ solution (5%). After 5 min, the resulting mixture was added to 0.3 ml of $AlCl_3$ solution (10 %), and then after 6 min, this was mixed with 2 ml of NaOH (1M) and the total volume made up to 10 ml with distilled water. The absorbance was measured at 510 nm using a spectrophotometer (SHIMADZU-1240 UV/ visible). A standard curve was prepared using Catechin. Total flavanoid was expressed in milligram Catechin Equivalent (CE) per gram of dry weight (mg EC / g DW).

Estimation of total condensed Tannin content

The tannin content of plant extracts was determined by the method described by Heimler, Vignolini, Giulia Dini, Vincieri and Romani (2006), using Quercetin as a reference compound. Briefly, 400 μl of extract was added to 3 ml of methanolic solution, with 4% of vanillin, and 1.5 ml of concentrated hydrochloric acid. After 15 min of incubation, the absorbance was read at 550 nm using a spectrophotometer (SHIMADZU-1240 UV/ visible). The condensed tannin was expressed in milligram Quercetin Equivalent (QE) per gram of dry weight (mg EQ / g DW) (Heimler et al., 2006).

HPLC Analysis

Standardization of Phenolic extracts of *Zizyphus lotus (L.)* by High Performance Liquid Chromatography HPLC-UV was carried out using the optimized conditions. In this analysis, *Agilent Technologies 1200 series* chromatography equipment was used. For this system, 10 μl of each extract was injected onto a C18 reverse phase column. The mobile phase consisted of two eluents, water / methanol mixture (95/5), and the flow rate of the mobile phase was1 ml / min. The temperature was set at 25 ^{o}C. The detector used was a UV detector at a wavelength of 280-320 nm, for 70 min. The results are expressed by a comparison of the chromatograms of the standard with that of the sample.

Antioxidant activity evaluation: Scavenging ability towards DPPH radical

This technique depends on the reduction of the free radical DPPH (purple color) to a yellow coloured diphenyl picrylhydrazine in the presence of the antioxidant. Briefly, 25 μl of different concentrations of extract were mixed with 2.5 ml solutions of DPPH (0.004%). Reference antioxidant solutions (ascorbic acid) were also prepared under the same conditions to serve as a positive control. An equal amount of methanol and DPPH served as negative control. After 30 min of incubation at room temperature in the dark, the absorbance was recorded at 517 nm. The experiment was performed in duplicate (Es-Safi, Kollmann, Khlifi & Ducrot, 2007). The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{DPPH Scavenging activity}(\%) = \left[\frac{(A1–A2)}{A1}\right] \times 100 \quad (2)$$

where:
A1: Absorbance of negative control
A2: Absorbance of sample.

Determination of IC_{50}

The concentration of sample required to scavenge 50% of DPPH (IC_{50}) was determined graphically from the percent reduction versus concentration curve (Samartha et al., 2008). A decrease in the DPPH solution absorbance indicates an increase in the DPPH radical scavenging activity.

2.4 Antimicrobial activity evaluation of extract from *Zizyphus lotus*

Inoculum preparation

From a culture of 18 h, a bacterial suspension was prepared to obtain a density equivalent to the standard of 0.5 Mac Farland. This density corresponds to a concentration of 10^6 - 10^8 CFU / ml (Wade et al., 2001).

Antimicrobial Resistance Testing (Agar Diffusion Method)

The resistance of the strains to different antimicrobial agents was determined by the disc diffusion method and the antibiotic was chosen according to CASFM (2016). After 24 h of incubation at 37 ^{o}C, the diameter of inhibition zones was measured.

Agar Disc Diffusion Assay

The *in vitro* evaluation of antimicrobial activity was carried out using a disc diffusion method. The plant extracts were dissolved in DMSO at 200 mg / ml with binary dilution. The sterile paper discs (6 mm diameter), impregnated with 10 μl of extract of plant per disc, were sterile deposited on the agar surface. The discs were kept at 4 ^{o}C for 1 h and then incubated for 24 h at 37 ^{o}C (Warda et al., 2009). The disc impregnated with DMSO was used as a negative control. The diameter of the inhibition zone around each disc was measured for three replicates (Kumar et al., 2008).

Determination of MIC by microdilution

The minimal inhibitory concentration (MIC) of the plant extracts against the different strains was assessed using the microdilution method. Each well of microtiter plates was inoculated with 50 μl of Broth Muller Hinton (BMH), 50 μl of the extract (dissolved in DMSO) and 50 μl of bacterial suspension containing 10^8 CFU / ml adjusted to 0.5 McFarland so the final volume in each well was 150 μl. The positive control was prepared by 50 μl BMH and 50 μl of inoculum. The negative control was prepared with 50 μl of the extract in solution and 50 μl of BMH without inoculum. The microplates were then covered and incubated at 37 ^{o}C for 24 hours (Abdelrahman, Skaug & Francis, 2002). The turbidity was measured every 2 hours using a microplate reader at 620 nm. Each plant extract was run in duplicate.

Determination of minimal bactericidal concentration (MBC)

The minimum bactericidal concentration corresponds to the lowest concentration of extracts yielding negative cultures after incubation at 37 ^{o}C for 24 h. It was determined by re-plating 10 ul from culture negative wells on Muller Hinton Agar. After 24 h incubation at 37 ^{o}C, the number of colonies on the streaks was compared with those of the bacterial inoculum. All tests were done in triplicate. The MBC / MIC ratio was calculated for each extract. If the result was less than 4, the extract was considered bactericidal (Guinoiseau, 2010).

2.5 Effect of extracts from *Zizyphus lotuson* auto-aggregation and co-aggregation

Autoaggregation and coaggregation abilities of each strain were evaluated. The autoaggregation assay was performed according to the method of Collado, Meriluoto and Salminen (2007) and Tuo et al. (2013) with little modification. The *Lactobacillus* strains were grown for 18 h at 37 ^{o}C in MRS broth. Bacterial cells were recovered by centrifugation at 5000 g/15 min, washed twice and resuspended in phosphate buffered saline (PBS, pH 7.2) to obtain an optical density of 0.5 at 600 nm. Cell suspensions (4 ml) were enriched separately by three different volumes of aqueous extract (50, 100 and 200 μl). The absorbance at 600 nm of cell suspensions incubated at 37 ^{o}C was monitored with a spectrophotometer (SHIMADZU-1240 UV/ visible) for different times (2 h, 4 h and 24 h).
The results are expressed as a percentage by the following formula:

$$Autoaggregation(\%) = \left[1 - (\frac{A_t}{A_o})\right] \times 100 \quad (3)$$

where:
A_t represents the absorbance at time t = 2 h, 4 h, 24 h
A_o the absorbance at time t = 0 h.
The assay was carried out in triplicate (Collado et al., 2007; Tuo et al., 2013).

Coaggregation assays were also prepared as described for autoaggregation analysis. Briefly, 2 ml of each cell suspension of the different probiotic and pathogen strains were mixed and incubated at 37 ^{o}C. Samples were taken in the same way as in the autoaggregation assay. Absorbance was determined for the mixture and for the bacterial suspensions alone. The absorbance (600 nm) was monitored at different times (2 h, 4 h and 24 h). Percentages of coaggregation were determined as:

$$Coaggregation(\%) = \left\{ \frac{\left[\frac{(Ax+Ay)}{2-A(x+y)}\right]}{Ax} + \frac{Ay}{2} \right\} \times 100 \quad (4)$$

where x and y each represents one of the two strains in the control tubes, and (x + y) represents the absorbance of the mixed bacterial suspension.

2.6 Adhesive interaction assay

The adhesion ability of each strain was evaluated according to the method of Collado et al. (2007). The hydrophobicity of *Lactobacillus* strains was determined by Xylene and Toluene extraction. Overnight cultures were harvested by centrifugation, washed twice with PBS (pH 7.2), resuspended in the same buffer, and *A*600 of the cell suspension was measured to standardize the number of bacteria (approximately 10^8 CFU/ml). An equal volume of Xylene and Toluene was added, and the suspension was vortexed for 4 min. The phases were separated, and the absorbance at 600 nm of the aqueous phase was measured using a spectrophotometer (SHIMADZU-1240 UV/ visible). The affinity of the bacterial strains to hydrocarbons was reported as adhesion percentage according to the formula:

$$Hydrophobicity\% = \left[\frac{(A_0 - A)}{A_0}\right] \times 100 \quad (5)$$

where A_0 and A are absorbance values measured before and after Xylene / Toluene extraction.

3 Statistical analysis

All experiments were done in triplicate. All data are presented as means ±SD or as frequency in percentage (%). The means were compared using multivariate analysis of variance (ANOVA). P values <0.05 were considered significant.

4 Results and discussion

4.1 Microbiological analysis

The quantitative and qualitative distribution of the different microbial groups included in this study are shown in Figure 1.

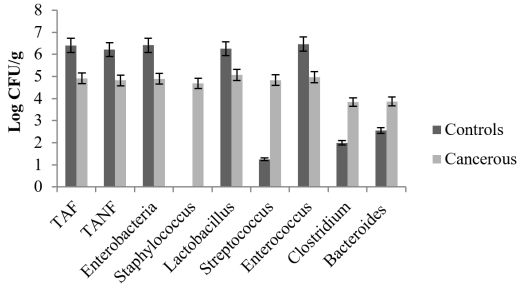

Figure 1: Intestinal microbiota composition in selected groups (cancerous and controls)

The results show that the gut microbiota of patients with colorectal cancer is significantly different than that of the healthy controls. The most difference is the presence of *Stapylococcus* for the group of cancer patients (4.69 log CFU/g) and its absence in the group of healthy controls. The also was a marked difference for *Streptococcus, Clostridium* and *Bacteroides* which were more abundant in patients with colorectal cancer (medians of 4.83 log CFU/g, 3.84 log CFU/g and 3.87 log CFU/g respectively) than in controls (medians of 1.24 log CFU/g, 1.99 log CFU/g and 2.54 log CFU/g respectively). However, the microbial groups belonging to the genus *Enterobacteria, Lactobacillus* and *Enterococcus* are significantly more abundant in healthy controls (medians of 6.41 log CFU/g, 6.25 log CFU/g and 6.46 log CFU/g respectively) than in patients with colorectal cancer (medians of 4.89 log CFU/g, 5.06 log CFU/g and 4.96 log CFU/g respectively).

4.2 Firmicutes/Bacteroidetes ratio

In this study for the Firmicutes/Bacteroidetes ratio, there was a significant difference between healthy controls and patients with colorectal cancer (0.6 and 0.7 respectively). The ratio is significantly higher in cancer patients that in healthy controls. Similar findings were reported in the study of Chen et al. (2012) which showed more abundant Firmicutes and less abundant Bacteroidetes in groups of patients with CRC. Gao, Guo, Gao, Zhu and Qin (2015) showed that cancerous tissue had a significantly higher abundance of Firmicutes and Fusobacteria than tissue found in healthy individuals. Another study reported that a reduction in *Bacteroidetes* diversity and an increase in Firmicutes and Fusobacteria diversity were observed in CRC samples, suggesting that specific bacteria could play a major role in CRC (Allali, 2017).

4.3 Microbial strains selection for aggregation and adhesion capacity

After identification, the following pathogenic microorganisms *Citrobacter braakii, Enterobacter cloacae, Escherichia coli, Staphylococcus aureus, Enterococcus faecalis* and *Clostridium leptum*, and the probiotic strains, *Lactobacillus salivarius* were selected for aggregation and adhesion tests.

4.4 Phytochemical analysis of *Zizyphus lotus* l

Extraction Yield

The results obtained show that the yield of extracts from *Ziziphus lotus* varies according to the solvent used. The greatest yield was observed

with the aqueous extract (32.92%), followed by the methanolic extract (30.84%). The yield of extracts depends on several factors, such as the origin of the plant (Ebrahimzadeh, Pourmorad & Hafezi, 2008), the variety, the harvest season, the climatic and environmental conditions, the geographical location, the different diseases that can affect the plant, the maturity of the plant (Park & Cha, 2003) and the method of extraction.

Phytochemical analysis

Aqueous and methanol extracts were prepared to examine the content of total phenolics, flavonoids and tannins. The results for extractive values are tabulated in Table 2.

The total phenolics content varied among the extracts as shown in Table 2. *Zizyphus lotus* extracts showed the higher polyphenol content (225.40 mg AGE / g DW) in the aqueous extract than in the methanolic extract (63.04 mg GAE/gDW). This could be due to different degrees of polarity of the solvents used for the extraction of the polyphenolic compounds. AE and ME extracts of leaves of *Zizyphus lotus* appear richer in polyphenols. In a study by Hossain, Uddin and Islam (2015), the extract of *Zizyphus lotus* contained a total phenolic content of 97.18± 12.81 mg of gallic acid equivalents /g DW. *Zizyphus lotus* extracts showed the higher flavonoids content (14.88 mg CE/gDW) in the methanolic extract than in the aqueous extract (11.30 mg CE/gDW). The concentration of flavonoids in plant extracts depends on the polarity of solvents used in the extract preparation (Gao & Liu, 2005). The presence of flavonoids in leaves of *Zizyphus lotus* extracts have been reported to be responsible for antioxidant activity (Braca et al., 2003).

For condensed tannin contents, higher values were observed in the aqueous extract (10.66 mg QE/gDW) than in the methanolic extract (8.66 mg QE/gDW). The presence of tannins in some plant extracts indicates that the plant may have the ability to be an antioxidant, antifungal and anti-inflammatory agent, and possess healing properties (Araujo, Alencar, Lucia Cavalcanti de Amorim & Albuquerque, 2008).

HPLC analysis

Preliminary analysis for the extracts of the aerial parts of this plant showed chromatographic profiles almost similar from a qualitative point of view but with a quantitative difference in the abundance of their majority compounds. The chromatographic profiles of the extracts EA and EM are shown in Table 3 and 4 respectively.

The chromatographic profile for quantitative and qualitative analysis (HPLC) of extracts is shown in Tables 3 and 4. During these analyses, 13 components were identified. The phytochemical analysis by HPLC showed that all extracts are rich in phenolic compounds. The aqueous extract is mainly composed of Quercetin glucosid (36.35%), Resveratrol (34.31%), Kaempferol glucosid (8.18%), Miricitine (3.19%), Kaempferol (2.9%), Gallic acid (1.06%) and Quercetin (1.03%). Other components such as Caffeic acid (0.70%), Epigalocatechin (0.34%), Epigalocatechin gallate (0.28%), Catechin (0.25%) and Procyanidin B2 (0.23%) are also present but with lower concentrations.

A comparison with the methanolic extract showed it to be composed of Quercetin glucosid (33.27%), Resveratrol (16.89%), Kaempferol glucosid (6.26%), Quercetin (3.33%), Kaempferol (3.13%), Miricitine (3.09%), Caffeic acid (2.87%), Catechin (2.83%), Procyanidin B2 (2.24%), Epigalocatechin gallate (1.1%) and Gallic acid (0.45%).

Other components such as Epigalocatechin (0.10%) and Catechin hydrate (0.08%) are also present but with lower concentrations. Some trace compounds can significantly increase the biological activity of medicinal plants (Vamanu & Nita, 2013). Plants with high levels of phenolic compounds have been shown to exhibit high antioxidant capacity (Razali, Razab, Junit & Abdul Aziz, 2008).

Antioxidant activity

The antioxidant activity of our extracts is expressed in IC_{50} (Table 5). This parameter has been used by several research groups to present their results. It defines the effective concentration of the substrate that causes the loss of 50% of the activity of DPPH. Lower IC_{50} values in-

Table 2: Total phenolic, flavonoid and tannin contents in the plant extracts

Sample	Polyphenol content (mgAGE/gDW)[a]*±SD	Flavonoid content (mgCE/gDW)[b]*±SD	Tannin content (mgQE/gDW)[c]*±SD
Aqueous extract (AE)	225.40 ± 0.28	11.30 ± 0.20	10.66 ± 0.33
Methanolic extract (ME)	63.04 ± 0.58	14.88 ± 0.37	8.66 ± 0.42

*Mean±SD of three determinations
[a] mg acid galic equivalent/g dry weight. [b] mg catechin equivalent/g dry weight.
[c] mg Quercetin equivalent / g dry weight

Table 3: Chromatographic profile of aqueous extracts

Components	Retention time (min)	Percentage (%)
Gallic Acid	7.30	1.0609
Catechin	17.59	0.3466
Epigalocatechin	17.92	0.3466
Procyanidin B2	19.94	0.2393
Caffeic acid	21	0.7062
Epigalocatechin gallate	22.54	0.2861
Resveratrol	36	34.3188
Quercetin glucoside	36.7	36.3544
Miricitine	38.5	3.1961
Kaempferol glucoside	39.7	8.1833
Quercetin	42	1.0331
Kaempferol	46	2.5935

Table 4: Chromatographic profile of methanolic extracts

Components	Retention time (min)	Percentage (%)
Gallic Acid	7.30	0.4573
Catechin hydrate	17	0.0807
Catechin	17.59	2.8301
Epigalocatechin	17.92	0.1047
Procyanidin B2	19.94	2.2410
Caffeic acid	21	2.8711
Epicatechin gallate	27.88	1.3186
Resveratrol	36	16.8941
Quercetin glucoside	36.7	33.2753
Miricitine	38.5	3.0955
Kaempferol glucoside	39.7	6.2677
Quercetin	42	3.3308
Kaempferol	46	3.1372

dicate higher antioxidant activity (Pokorny & Schmidt, 2001).
The IC_{50} value was determined from a graph of scavenging activity against the different concentrations of *Zizyphus lotus* extracts and ascorbic acid. The 50% inhibition concentrations (IC_{50}) are shown in Table 5. They are relatively low, reflecting the presence of compounds that can reduce the DPPH radical.
According to the results shown in Figure 2, our mostly methanolic extracts have more interesting DPPH free radical neutralization capabilities (195±0.02 μg/ml) than aqueous extracts (227±0.01 μg/ml). This finding is supported by the study of Sun, Powers and Tang (2007) which showed that methanol remains the best solvent to extract antioxidants from a plant, although still significantly lower than ascorbic acid (36±0.03 μg/ml). Much of the antioxidant potential of medicinal plants is due to the redox properties of phenolic compounds. They act as reducing agents, hydrogen donors and singlet oxygen scavengers (Hakkim, Gowri Shankar & Girija, 2007).

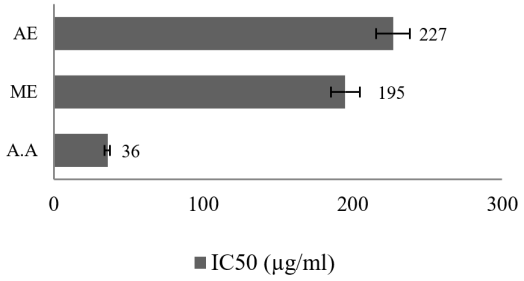

Figure 2: Inhibition concentrations (IC_{50}) of the plant extracts

4.5 Antimicrobial activity

Antimicrobial activity of selected antibiotics

The antibiotic resistance of strains was determined from the diameter of the zone of inhibition (mm) according to the Committee of the Antibiogram of the French Society of Microbiology (CASFM, 2016). The results of the antimicrobial activity of selected antibiotics are reported in Table 6.

Disc diffusion test

The results of the antimicrobial activity of extracts against the strains of bacteria tested by the method of aromatogram are shown in Table 7.
From the disc diffusion test results, it appears that all bacterial strains tested are inhibited by phenolic extracts, with various degrees of antimicrobial activity. This confirms the broad spectrum of antimicrobial activity of these extracts against Gram+ and Gram- bacteria. Large zones of inhibition appear with the methanolic extract against *Escherichia coli* and *Citrobacter braakii* (35-29 mm), respectively. The aqueous extracts are also more active against *Escherichia coli* (32mm). This plant extract exhibited a significant antibacterial activity against the different strains of antibiotics. However, these activities of extract are due to its content of active compounds.
The antimicrobial activity of these extracts against bacterial strains could be attributed to the presence of biologically active components such as flavonoids, phenolic acids and terpenoids as described in the chromatogram (Doudach et al., 2012).

Minimum inhibitory concentration (MIC)

Results of the antimicrobial activity of extracts against the strains of bacteria tested by microdilution are shown in Table 8.
According to Table 8, the plant extract had an important inhibitory activity against bacteria tested. The highest activity against strains tested with MIC was 6.25-25 mg/ml for aqueous extract and 12.5-25 mg/ml for methanolic extract. However, AE was more active against *E.coli, Enterobacter cloacae* and *Citrobacter braakii* (6.25 mg/ml) and ME were more active against *Enterococcus faecalis* and *Clostridium leptum* (12.5 mg/ml).
The antibacterial activity can be explained by

Table 5: The antioxidant activity of the plant extracts

Sample	DPPH (IC_{50}, μg/ml)
Aqueous extract (AE)	227±0.01
Methanolic extract (ME)	195±0.02
Ascorbic acid (AA)	36±0.03

Table 6: Antibiotic resistance profile of tested bacterial strains

	AMC	CN	OX	V	C	TE	SXT	L	ATM
Escherichia coli	R	I	/	/	S	R	R	/	R
Enterobacter cloacae	R	I	/	/	I	R	R	/	R
Citrobacter braakii	R	I	/	/	S	R	R	/	I
Staphylococcus aureus	/	I	R	R	S	I	R	R	/
Enterococcus faecalis	/	I	/	R	S	S	I	R	/
Clostridium leptum	R	I	/	R	I	/	/	R	R

R: Resistant, I: Intermediate, S: Susceptible, AMC: Amoxicillin+ Clavulanic acid, CN: Gentamycin, OX:Oxacillin, V: Vancomycin, C: Chloramphenicol, TE: Tetracyclin, SXT: Trimethoprin/Sulfamethoxazol, L: Lincomycin, ATM: Aztreonam

Table 7: The diameter of the zone of inhibition (mm) from plant extracts for pathogenic bacteria

	Inhibition diameter (ø mm)			
	Aqueous extract (AE)	Methanolic extract (ME)	OX 5 μg	CN 10 μg
Escherichia coli	32±0.8	35±0.12		14±0.1
Enterobacter cloacae	20±0.06	21±0.3		11±0.8
Citrobacter braakii	18±0.2	29±0.01		13±0.2
Staphylococcus aureus	16±0.17	22±0.15	-	
Enterococcus faecalis	23±0.35	20±0.08	-	11±0.09
Clostridium leptum	16±0.57	20±0.03		10±0

- :No inhibition zone

the mechanism of toxicity towards the half-organisms, which is done either by non-specific interactions such as the establishment of hydrogen bridges with the proteins of the cell walls or the enzymes, either by the chelation of metal ions (such as iron) and the imprisonment of the substances necessary for the growth of bacteria (Karou et al., 2005).

Minimum bactericidal concentrations (MBC)

Minimum bactericidal concentrations of extracts are reported in Table 9.
The highest activity against strains tested with MBC was 25-50 mg/ml for aqueous extracts and 25-100 mg/ml for methanolic extracts (Table 9). For the MBC / MIC ratio, the extract is validated as bactericidal when it is less than or equal to 4. Values of this ratio are variable for the various extracts according to the bacterial strains tested (Table 9), which makes it possible to establish a classification of these extracts according to their spectrum of action. Indeed, some work has shown that Flavonoids such as Quercetin and Apigenin are involved in the inhibition of D-alanine: D-alanine ligase, thus disrupting the synthesis of the bacterial wall (Wu et al., 2008). Other authors have speculated that certain Flavonoids belonging to the class of Flavonols (Cushnie & Lamb, 2005), the flavan-3-ols (Sirk, Brown, Sum & Friedman, 2008) cause an alteration of the cytoplasmic membrane leading to its lysis, or the inhibition of topoisomerase by Isoflavonoids, thus blocking the synthesis of deoxyribonucleic acid (DNA) (Gradisar, Pristovsek, Andreja & Jerala, 2007; Wang et al., 2010).

Effect of extract on autoaggregation and coaggregation

The *in vitro* effect of aqueous extract of *Zizyphus lotus* in auto and coaggregation of bacteria strains was evaluated. The viability of *Lactobacillus salivarius* was carried out on agar. The incorporation of 200 μl of aqueous extract had no effect on the development of probiotic strains tested.

As shown in Figure 3, autoaggregation of *Lactobacillus salivarius*, without aqueous extract, increased in time from 45.9% to 55.6% for 2 h and 4 h respectively to 79.6% and 91.2% for 2 h and 4 h respectively, in the presence of aqueous extract. After 24 h, the highest autoaggregation capacities were recorded of 81.20% and 93.4% respectively, without and with aqueous extract. The difference is statistically significant ($P<0.05$) compared to control (without AE).
The high autoaggregation capacity suggests that the probiotic strain is good at forming biofilms and/or gastrointestinal tract colonization, which are the ways of forming barriers against colonization by pathogenic microorganisms (Schachtsiek, P. Hammes & Hertel, 2005).
Coaggregation of *Lactobacillus salivarius* with potential gut pathogens could contribute to the positive properties of the probiotic. As shown in Figure 4, the strain *Lactobacillus salivarius* showed the highest coaggregating ability with *Clostridium leptum*, which without aqueous extract was 72.61% and in the presence of aqueous extract was 73.20%. Coaggregation is a process by which bacteria are attached to each other by means of specific molecules (Rickard, Gilbert, J. High, E. Kolenbrander & S. Handley, 2003). The percentage of coaggregation, without aqueous extract, of *Escherichia coli, Enterobacter cloacae, Citrobacter brakii, Staphylococcus aureus* and *Enterococcus fecalis* respectively was 8.66%, 57.75%, 72.40%, 56.65% and 48.98%. However, the respective percentage of coaggregation increased to 69.75%, 64.80%, 77.32%, 70.40% and 52.64% in the presence of aqueous extracts from *Zizyphus lotus (L)*. The difference is statistically significant ($P<0.05$) compared to control (without AE).
The coaggregation of probiotic-pathogen improved in the presence of the extract. A study by Alberto, Rinsdahl Canavosio and Manca de Nadra (2006) showed that flavonoids can prevent the expression of adhesion molecules, in particular human endothelial cells, and the inhibitory effect of the tumor factor NF-$\alpha\beta$ inducing the expression of adhesion molecules. This effect depends on the molecular structure, concentration and metabolic transformation of flavonoids. In another study, El Astal, Ashour and Kerrit (2005) suggested that plants have a variety

Table 8: The minimum inhibitory concentrations (MIC) of plant extracts and antibiotic

	Extracts of Zizyphus lotus (L.)(mg/ml)		Antibiotics (μg/ml)	
	Aqueous extract (AE)	Methanolic extract (ME)	OX	CN
Escherichia coli	6.25	25		62.5
Enterobacter cloacae	6.25	25		125
Citrobacter braakii	6.25	25		250
Staphylococcus aureus	25	25	62.5	
Enterococcus faecalis	12.5	12.5		125
Clostridium leptum	12.5	12.5		125

Table 9: The minimum bactericidal concentrations (MBC) and MBC/MIC ratio of plant extracts

	MBC (mg/ml)		MBC/MIC ratio	
	AE	ME	AE	ME
Escherichia coli	25	25	4	1
Enterobacter cloacae	50	25	8	1
Citrobacter braakii	25	25	4	1
Staphylococcus aureus	25	100	1	4
Enterococcus faecalis	25	25	2	2
Clostridium leptum	25	25	2	2

of potentially significant therapeutic compounds against human pathogenic bacteria.

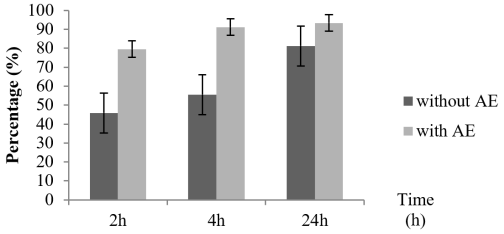

Figure 3: Effect of aqueous extract on autoaggregation of *Lactobacillus salivarius*

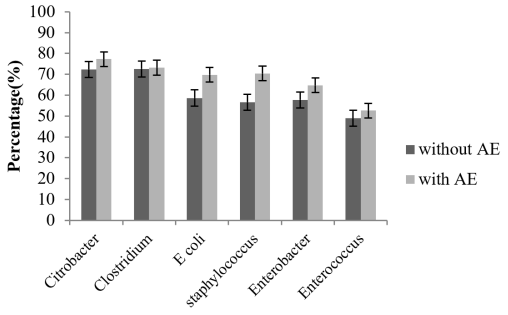

Figure 4: Effect of aqueous extract on coaggregation of bacteria strains tested

Capacity of Bacteria adhesion

As shown in Figure 5, *Lactobacillus salivarius* presents an important adhesion capacity of 67.52% and 65.31% for xylene and Toluene respectively. The hydrophobic nature of the outermost surface of microorganisms has been involved in the attachment of bacteria to host tissue. This property could confer an important advantage for bacterial maintenance in the human gastrointestinal tract (Schillinger, Guigas & Holzapfel, 2005). One of the main criteria in the selection of probiotic bacteria is the ability to adhere to the intestinal mucus because the adhesion extends their permanence in the intestine, which

allows them to exert a salutary effect (Apostolou et al., 2001) and to improve the antagonistic activity against enteropathogens (Leahy, Higgins, Fitzgerald & Van Sinderen, 2005).
The adhesion of probiotics to the intestinal mucosa and enterocytes is considered an important factor in the colonization of the intestinal tract and the modulation of the immune system of the host (Ouwehand, Isolauri & Salminen, 2002).

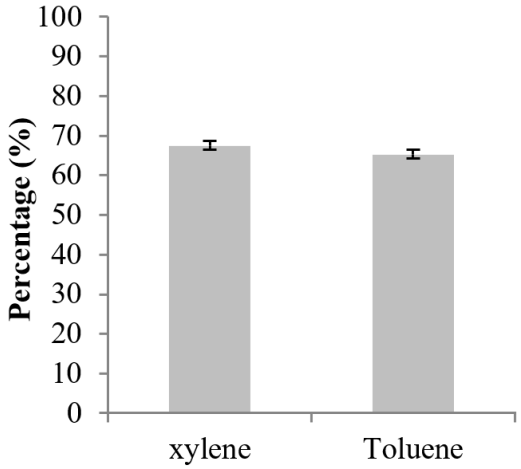

Figure 5: Percentage of adhesion of *Lactobacillus salivarius* to solvents

5 Conclusions

The presence of certain bacterial species has an impact on the development of CRC. Therefore, it is imperative to study the intestinal microbiota during CRC and the bacterial resistance to drugs, and to research a natural strategy that relieves the restoration and modulation of the gut microbiota.
This work confirms the prebiotic, antioxidant and antibacterial activity of extracts from *Zizyphus lotus*. It also confirms the stimulatory effect of the aqueous extract on probiotic aggregation and the inhibitory effect of *Lactobacillus salivarius* against the pathogenic bacteria tested (*Citrobacter braakii, Enterobacter cloacae, Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Clostridium leptum*) which were isolated from feces of patients with colorectal cancer. Autoaggregative and Coaggregative capacities can be used for preliminary selection of probiotic bacteria for potential applications in humans to modulate and keep the microflora balance by development of probiotics and the reduction of pathogenic bacteria responsible for many diseases.

Acknowledgements

The authors are grateful to MESRS and to DGRSDT from Algeria for their financial support to this project.

References

Abdel-Zaher, A. O., Salim, S. Y., Assaf, M. & Abdel Hady, R. (2005). Antidiabetic activity and toxicity of zizyphus spina-christi leaves. *Journal of Ethnopharmacology*, *101*, 129–38. doi:10.1016/j.jep.2005.04.007

Abdelrahman, H., Skaug, N. & Francis, G. (2002). In vitro antimicrobial effects of crude miswak extracts on oral pathogens. *Saudi Dent J*, *14*, 26–32.

Abu Zarga, M., Sabri, S., Al-Aboudi, A., Saleh Ajaz, M. & Sultana, N. (2004). New cyclopeptide alkaloids from *Zizyphus lotus*. *Journal of Natural Products*, *58*. doi:10.1021/np50118a004

Alberto, M. R., Rinsdahl Canavosio, M. A. & Manca de Nadra, M. C. (2006). Antimicrobial effect of polyphenols from apple skins on human bacterial pathogens. *Electronic Journal of Biotechnology*. doi:10.2225/vol9-issue3-fulltext-1

Allali, I. (2017). Etude d'association entre le microbiome intestinal et le cancer colorectal chez la population marocaine.

Apostolou, E., Kirjavainen, P., Saxelin, M., Rautelin, H., Valtonen, V., Salminen, S. & Ouwehand, A. (2001). Good adhesion properties of probiotics: A potential risk for bacteremia? *FEMS Immunology and Medical Microbiology*, *31*, 35–9. doi:10.1111/j.1574-695X.2001.tb01583.x

Araujo, T., Alencar, N., Lucia Cavalcanti de Amorim, E. & Albuquerque, U. (2008). A new approach to study medicinal plants with tannins and flavonoids contents from the local knowledge. journal of ethnopharmacology. *Journal of Ethnopharmacology*, *120*, 72–80. doi:10.1016/j.jep.2008.07.032

Azcarate-Peril, M., Sikes, M. & Bruno-Barcena, J. (2011). The intestinal microbiota, gastrointestinal environment and colorectal cancer: A putative role for probiotics in prevention of colorectal cancer? *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *301*, G401–424. doi:10.1152/ajpgi.00110.2011

Backhed, F., Ley, R., L Sonnenburg, J., Peterson, D. & I Gordon, J. (2005). Host-bacterial mutualism in the human intestine. *Science (New York, N.Y.) 307*, 1915–20. doi:10.1126/science.1104816

Barros, L., Carvalho, A. & Ferreira, I. (2011). Exotic fruits as a source of important phytochemicals: Improving the traditional use of rosa canina fruits in Portugal. *Food Research International*, *44*, 2233–2236. doi:10.1016/j.foodres.2010.10.005

Béraud, N. (2001). The biological analysis technician, theoretical and practical guide. *Tec Doc, Lavoisier*, 988–990.

Boominathan, M. & Ramamurthy, V. (2009). Antimicrobial activity of *Heliotropium indicum* and *Coldenia procumbens*. *Journal of Ecobiology*, *24*(1), 11–15.

Borgi, W., Ghedira, K. & Chouchane, N. (2007). Antiinflammatory and analgesic activities of *Zizyphus lotus* root barks. *Fitoterapia*, *78*, 16–9. doi:10.1016/j.fitote.2006.09.010

Braca, A., Politi, M., Sanogo, R., Sanou, H., Morelli, I., Pizza, C. & Tommasi, N. (2003). Chemical composition and antioxidant activity of phenolic compounds from wild and cultivated *Sclerocarya birrea* (anacardiaceae) leaves. *Journal of Agricultural and Food Chemistry*, *51*, 6689–95. doi:10.1021/jf030374m

CASFM. (2016). Committee of the antibiogram of the french society of microbiology. Retrieved from https://www.sfm-microbiologie.org/

Chen, Weiguang, Liu, Fanlong, Ling, Zongxin, ... Charlie. (2012). Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. *PLOS ONE*, *7*(6), 1–9. doi:10.1371/journal.pone.0039743

Collado, M. C., Meriluoto, J. & Salminen, S. (2007). Adhesion and aggregation properties of probiotic and pathogen strains. *European Food Research and Technology*, *226*, 1065–1073. doi:10.1007/s00217-007-0632-x

Cushnie, T. & Lamb, A. (2005). Detection of galangin-induced cytoplasmic membrane damage in *Staphylococcus aureus* by measuring potassium loss. *Journal of Ethnopharmacology*, *101*, 243–8. doi:10.1016/j.jep.2005.04.014

Diallo, D., Sanogo, R., Yasambou, H., Traoré, K., A. andCoulibaly & Maiza, A. (2004). Étude des constituants des feuilles de *Ziziphus mauritiana* Lam. (rhamnaceae) utilisées traditionnellement dans le traitement du diabète au mali. *Comptes Rendus Chimie - C R CHIM*, *7*, 1073–1080. doi:10.1016/j.crci.2003.12.035

Doudach, L., Meddah, B., Alnamer, R., Faouzi, M., Chibani, F., Abdelhakim, E. & Cherrah, Y. (2012). In vitro antibacterial potential activity of the methanolic and aqueous extracts of corrigiola telephiifolia pourr. and mesembryanthemum nodiflorum. *Journal of Biologically Active Products from Nature*, *2*(5), 284–291. Retrieved from 10.1080/22311866.2012.10719136

Ebrahimzadeh, M., Pourmorad, F. & Hafezi, S. (2008). Antioxidant activities of iranian corn silk. *Turkish Journal of Biology*, *32*, 43–49.

El Astal, Z. Y., Ashour, A. E. R. A. & Kerrit, A. A. M. (2005). Antimicrobial activity of some medicinal plant extracts in palestine. *Pak. J. Med. Sci*, *21*(2), 187–193. Retrieved from www.pjms.com.pk

Gao, M. & Liu, C.-Z. (2005). Comparison of techniques for the extraction of flavonoids from cultured cells of *Saussurea medusa* Maxim. *World Journal of Microbiology and Bio-*

technology, *21*, 1461–1463. doi:10.1007/s11274-005-6809-1

Gao, Z., Guo, B., Gao, R., Zhu, Q. & Qin, H. (2015). Microbiota disbiosis is associated with colorectal cancer. *Frontiers in Microbiology*, *6*, 20. doi:10.3389/fmicb.2015.00020

Gomes, A., Pintado, M., Freitas, A. & Silva, J. P. (2014). Gastrointestinal tract: Microflora and transit aspects. (pp. 7–45). doi:10.4032/9789814411639

Gradisar, H., Pristovsek, P., Andreja, P. & Jerala, R. (2007). Green Tea Catechins Inhibit Bacterial DNA Gyrase by Interaction with Its ATP Binding Site. *Journal of Medicinal Chemistry*, *50*, 264–271. doi:10.1021/jm060817o

Guinoiseau, E. (2010). *Molécules antibactériennes issues d'huiles essentielles: Séparation, identification et mode d'action* (Doctoral dissertation).

Hakkim, L., Gowri Shankar, C. & Girija, S. (2007). Chemical composition and antioxidant property of holy basil (*Ocimum sanctum* l.) leaves, stems, and inflorescence and their in vitro callus cultures. *Journal of Agricultural and Food Chemistry*, *55*, 9109–17. doi:10.1021/jf071509h

Heimler, D., Vignolini, P., Giulia Dini, M., Vincieri, F. & Romani, A. (2006). Antiradical activity and polyphenol composition of local brassicaceae edible varieties. *Food Chemistry*, *99*, 464–469. doi:10.1016/j.foodchem.2005.07.057

Hossain, M. S., Uddin, N. & Islam, A. (2015). Evaluation of in vitro antioxidant and brine shrimp lethality activities of different stem extracts of *Zizyphus rugosa* Lam. *Food Measure*, *9*, 454–462. doi:10.1007/s11694-015-9253-4

Jayaprakasha, G., Singh, R. & Sakariah, K. (2001). Antioxidant activity of grape seed (vitis vinifera) extracts on peroxidation models in vitro. *Food Chemistry*, *73*, 285–290. doi:10.1016/S0308-8146(00)00298-3

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E. & Forman, D. (2011). Global cancer statistics. *CA: A Cancer Journal for Clinicians*, *61*(2), 69–90. doi:10.3322/caac.20107

Karou, D., Dicko, M. H., Simporé, J., Yameogo, S., Sanon, S. & Traoré, A. S. (2005). Activités antioxydantes et antibactériennes des polyphénols extraits de plantes médicinales de la pharmacopee traditionnelle du burkina faso. *Maitrise des procédés en vue d'améliorer la qualité et la sécurité des aliments, utilisation des OGM, analyse des risques en agroalimentaire. ouagadougou*, 8–11.

Kumar, A., Shukla, R., Singh, P., Shekhar Prasad, C. & Kishore Dubey, N. (2008). Assessment of *Thymus vulgaris* L. essential oil as a safe botanical preservative against post harvest fungal infestation of food commodities. *Innovative Food Science & Emerging Technologies*, *9*, 575–580. doi:10.1016/j.ifset.2007.12.005

Leahy, S., Higgins, D., Fitzgerald, G. & Van Sinderen, D. (2005). Getting better with bifidobacteria. *Journal of Applied Microbiology*, *98*, 1303–15. doi:10.1111/j.1365-2672.2005.02600.x

Li, H.-B., Cheng, K.-W., Wong, C.-C., Fan, K.-W., Chen, F. & Jiang, Y. (2007). Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. *Food Chemistry*, *102*, 771–776. doi:10.1016/j.foodchem.2006.06.022

Liu, R. H. (2004). Potential synergy of phytochemicals in cancer prevention: Mechanism of action. *The Journal of Nutrition*, *134*(12), 3479S–3485S. doi:10.1093/jn/134.12.3479S

Muanda, F., Soulimani, R., Diop, B. & Dicko, A. (2011). Study on chemical composition and biological activities of essential oil and extracts from *Stevia rebaudiana Bertoni* leaves. *LWT - Food Science and Technology*, *44*, 1865–1872. doi:10.1016/j.lwt.2010.12.002

Ouwehand, A., Isolauri, E. & Salminen, S. (2002). The role of the intestinal microflora for the development of the immune system in early childhood. *European Journal of Nutrition*, *41 Suppl 1*, I32–7. doi:10.1007/s00394-002-1105-4

Park, H.-J. & Cha, H.-C. (2003). Flavonoids from leaves and exocarps of the grape ky-

oho. *Korean Journal of Biological Sciences*, *7*, 327–330. doi:10.1080/12265071.2003.9647723

Parkin, D. M., Bray, F., Ferlay, J. & Pisani, P. (2001). Estimating the world cancer burden: Globocan. *Int J Cancer*, *94*, 153–156.

Pokorny, J. & Schmidt, S. (2001). Antioxidants in food: Practical applications. *Antioxidants in Foods*, 331–354. doi:10.1533/9781855736160.4.331

Pottier, A. (1981). Flore de la tunisie. *Programme Flore et Végétation Tunisiennes, Publications Scientifiques Tunisiennes, Tunis*, *1*, 402–405.

Razali, N., Razab, R., Junit, S. & Abdul Aziz, A. (2008). Radical scavenging and reducing properties of extracts of cashew shoots (anacardium occidentale). *Food Chemistry*, *111*, 38–44. doi:10.1016/j.foodchem.2008.03.024

Rickard, A., Gilbert, P., J. High, N., E. Kolenbrander, P. & S. Handley, P. (2003). Bacterial coaggregation: An integral process in the development of multi-species biofilms. *Trends in Microbiology*, *11*, 94–100. doi:10.1016/S0966-842X(02)00034-3

Rowland, I. R. (2009). The role of the gastrointestinal microbiota in colorectal cancer. *Current Pharmaceutical Design*, *15*, 1524–7. doi:10.2174/138161209788168191

Rsaissi, N. & Bouhache, M. (2002). La lutte chimique contre le jujubier. *Transfert de technologie en agriculture*, *94*, 1–4.

Es-Safi, N. E., Kollmann, A., Khlifi, S. & Ducrot, P.-H. (2007). Antioxidative effect of compounds isolated from *Globularia alypum* L. structure-activity relationship. *LWT - Food Science and Technology*, *40*, 1246–1252. doi:10.1016/j.lwt.2006.08.019

Samartha, D. R., Samartha, M., Kumar, M., Soni, A., Kumar, M. & Kumar, A. (2008). Evaluation of antioxidant and radical-scavenging activity of certain radioprotective plant extracts. *Food Chemistry*, *106*, 868–873. doi:10.1016/j.foodchem.2007.05.005

Schachtsiek, M., P. Hammes, W. & Hertel, C. (2005). Characterization of lactobacillus coryniformis dsm 20001t surface protein cpf mediating coaggregation with and aggregation among pathogens. *Applied and Environmental Microbiology*, *70*, 7078–85. doi:10.1128/AEM.70.12.7078-7085.2004

Schillinger, U., Guigas, C. & Holzapfel, W. (2005). In vitro adherence and other properties of lactobacilli used in probiotic yoghurt-like products. *International Dairy Journal*, *15*, 1289–1297. doi:10.1016/j.idairyj.2004.12.008

Sirk, T., Brown, E. F., Sum, A. & Friedman, M. (2008). Molecular dynamics study on the biophysical interactions of seven green tea catechins with lipid bilayers of cell membranes. *Journal of Agricultural and Food Chemistry*, *56*, 7750–8. doi:10.1021/jf8013298

Suksamrarn, S., Suwannapoch, N., Aunchai, N., Kuno, M., Ratananukul, P., Haritakun, R., ... Ruchirawat, S. (2005). Ziziphine n, o, p and q, new antiplasmodial cyclopeptide alkaloids from *Ziziphus oenoplia var. brunoniana*. *Tetrahedron*, *61*, 1175–1180. doi:10.1016/j.tet.2004.11.053

Sun, T., Powers, J. R. & Tang, J. (2007). Evaluation of the antioxidant activity of asparagus, broccoli and their juices. *Food Chemistry*, *105*(1), 101–106. doi:10.1016/j.foodchem.2007.03.048

Tuo, Y., Yu, H., Ai, L., Wu, Z., Guo, B. & Chen, W. (2013). Aggregation and adhesion properties of 22 lactobacillus strains. *Journal of Dairy Science*, *96*. doi:10.3168/jds.2013-6547

Vamanu, E. & Nita, S. (2013). Antioxidant capacity and the correlation with major phenolic compounds, anthocyanin, and tocopherol content in various extracts from the wild edible boletus edulis mushroom. *BioMed Research International*, *2013*, 313905. doi:10.1155/2013/313905

van Nimwegen, F., Penders, J., E Stobberingh, E., S Postma, D., Koppelman, G., Kerkhof, M., ... Thijs, C. (2011). Mode and place of delivery, gastrointestinal microbiota, and their influence on asthma and atopy. *The Journal of Allergy and Clinical Immunology*, *128*, 948–55.e1. doi:10.1016/j.jaci.2011.07.027

Wade, D., Silveira, A., Rollins-Smith, L., Bergman, T., Silberring, J. & Lankinen, H. (2001). Hematological and antifungal properties of temporin a and a cecropin a-temporin a hybrid. *Acta Biochimica Polonica*, *48*, 1185–9.

Wang, J., Li, J., Cao, J. & Jiang, W. (2010). Antifungal activities of neem (*Azadirachta indica*) seed kernel extracts on postharvest diseases in fruits. *African Journal of Microbiology Research*, *4*.

Warda, K., Markouk, M., Bekkouche, K., Abbad, M. L. A., Romane, A. & Bouskraoui, M. (2009). Antibacterial evaluation of selected moroccan medicinal plants against *Streptococcus pneumoniae*. *African Journal of Pharmacy and Pharmacology*, *3*(3), 101–104. Retrieved from https://academicjournals.org/journal/AJPP/article-abstract/35C476B33535

Wu, D., Kong, Y., Han, C., Chen, J., Hu, L., Jiang, H. & Shen, X. (2008). D-alanine: D-alanine ligase as a new target for the flavonoids quercetin and apigenin. *International Journal of Antimicrobial Agents*, *32*, 421–6. doi:10.1016/j.ijantimicag.2008.06.010

Xiao, W., Han, L. & Shi, B. (2008). Microwave-assisted extraction of flavonoids from radix astragali. *Separation and Purification Technology*, *62*, 614–618. doi:10.1016/j.seppur.2008.03.025

International Journal of Food Studies | IJFS April 2020 | Volume 9 | pages 178–192

Effect of Olive Pulp Enrichment on Physicochemical and Antioxidant Properties of Wheat Bread

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Received: 8 August 2018; Published online: 18 April 2020

Abstract

Black and green olive pulp was added to wheat bread formulation at different levels (5, 10, 15%) with the aim to improve its nutritional value by enhancing the phenolic content and antioxidant capacity. Additionally, the effects of the fortification with olive pulp on the physical characteristics, staling rate and overall consumer acceptability of the formulated breads were explored. Both olive pulps exhibited significantly higher antioxidant activity than refined wheat flour. Baking imparted an impressive increase in TPC, TFC and antioxidant activity of breads as revealed by comparison of experimental with theoretical values but returned significant differences only in the case of TPC when a two-tailed t-test for paired data was applied. Texture measurements showed a substantial increase in hardness with storage along with decreasing loaf volume and increased density. Hydroxytyrosol was the major phenolic compound of fortified breads followed by tyrosol. Olive pulp could be incorporated in a bread formulation without interfering with the general sensory acceptability.

Keywords: Olive pulp; Bread; HPLC; Antioxidants; Staling

1 Introduction

Traditional Mediterranean eating culture relies on ancient recipes composed of ingredients mainly derived from agricultural material from the rural area. The last few years have seen a tremendous back-to-nature demand in the market, probably because of an increased production of food with little nutritional value or even unhealthy food infected with some form of toxicity. The later has led to an increase in the use of natural antioxidants, especially those of vegetable origin like the case of table olives.

For several years now, the term "functional foods" has been used to define natural and naturally produced food with enrich characteristics that enhance consumer well-being. At this point, the demand for wheat-based products with value-added is growing rapidly (Bhattacharya, Langstaff, & Berzonsky, 2003). In the last decade, there is an increased tendency to produce functional breads made from whole grain

 10.7455/ijfs/9.1.2020.a4

flour or other functional ingredients (Dewettinck et al., 2008). In the light of this, many ingredients or extracts have been included in bread formulations to increase their diversity, nutrition or product appeal. Among functional ingredients, antioxidants occupy a place of prominence since they may inhibit lipid peroxidation and improve bread quality and safety (Duan, Zhang, Li, & Wang, 2006). However, in the literature there are no studies concerning the addition of table olives in bread formulations. Table olives are a traditional component of the Mediterranean diet and their antioxidant properties and other biological activities are well studied. Part of the beneficial nutritional value of this diet has been attributed to olive oil owing to its high content of monounsaturated fatty acids and its minor constituents such as tocopherols and various phenolic components (Galli & Visioli, 1999). Although, olive products and by-products are known for their biological properties, there is a lack of research concerning the incorporation of pulp from processed green or black table olives in a bread formulation. The aim of this study was to improve the nutritional value of wheat bread with the incorporation of green or black olive pulps thus enhancing the phenolic content and antioxidant capacity. Additionally, the effect of the supplementation of green or black olive pulp on the physical characteristics and staling rate of the formulated breads was explored.

2 Materials and Methods

2.1 Materials

Wheat flour (70% of milling yield) was purchased from a local milling company. The samples of green (Spanish type, cv. Conservolea) and black (Greek type, cv. Kalamon) table olives were purchased from the local market and used throughout in the present study. Both types of olives were pulped in a laboratory homogenizer after removal of the cores.

Reagents and chemicals

Hydroxytyrosol (HTR), tyrosol (TYR), oleuropein (OLE), protocatechuic acid (PRCA), 4-hydroxybenzoic acid (4HBA), vanillic acid (VA), caffeic acid (CA), syringic acid (SRA), *p*-coumaric acid (pCA) and ferulic acid (FA) were supplied by Sigma-Aldrich (Steinheim, Germany). Galic acid (GA) and catechin (CAT) were obtained from Extrasynthese (Genay Cedex, France). Analytical grade supplies of Folin-Ciocalteu, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxyl-2,5,7,8-tetramethychromane-2-carboxylic acid (Trolox) and 2, 4, 6-tripyridyl-s-triazine (TPTZ) were from Sigma-Aldrich (Steinheim, Germany). All other solvents/chemicals obtained from Chem-Lab (Zedelgem, Belgium) were of analytical grade or high-performance liquid chromatography (HPLC) grade.

2.2 Methods

Proximate chemical analysis

Moisture, protein and ash content in flour and formulated breads, were determined according to the official methods AACC 44-15A, ICC 105/2 and 104/1, respectively (AACC, 2000; ICC, 1994). Moisture content of olive pulps was determined by drying at 70^{o}C until constant weight, whereas their ash content was determined by incineration at 550±15^{o}C (AOAC 955.04 method) (AOAC, 1990). Total fat content was determined in a Soxhlet apparatus according to AOAC 948.22 method (1990), using petroleum ether as solvent with a minimum extraction time of 24h. Protein content was determined by the Kjeldahl method after multiplying by a factor of 5.7 for wheat flour and 6.25 for olive pulps. The carbohydrate content was calculated by difference as the percent remaining after all the other components have been measured.

Farinograph and bread making procedure

The mixing properties of the doughs obtained from the different flour-olive pulp blends were examined with a Brabender farinograph (OHgG, Duisburg, Germany), equipped with a 300 g mixing bowl according to ICC standard method No 115/1 (ICC, 1994).
All bread formulations contained wheat flour

(300 g, 14% moisture basis), olive pulp (black or green), salt (2% flour basis), dried yeast (1.5% f.b.) and water as determined by the farinograph water absorption in order to obtain 500 BU consistency. Green and black olive pulps were added fresh (not dried) at 0, 5, 10 and 15% supplementation levels (on dry matter basis). The salt content of the green and black olives was 8.5 and 7.0%, respectively, according to the labeling of the product by the manufacturer. Thus, no salt was added to the formulations where the salt of the pulp exceeded 2% of salt on flour basis, required by the recipe. Each fortification level experiment was carried out in duplicate.
Bread doughs were prepared using a two-step bulk fermentation and proofing, up to optimum volume increase of 100 g dough. Loaf volume was determined by rapeseed displacement. Subsequently, breads were sealed in polyethylene bags to monitor changes in bread characteristics upon storage (5^oC). Freeze-dried samples of bread crumbs were ground and homogenized, and finally used for measuring phenolic compounds and antioxidant activity.

Texture profile analysis (TPA)

A two bite TPA test was performed to measure the hardness, cohesiveness and chewiness of bread crumbs, from slices of the center of each bread loaf using a texture analyzer TA-XTplus (Stable Micro Systems, Gudaiming, Surrey, UK), equipped with P/50 probe. TPA analysis was carried out according to the AACC Method 74-09 (2000). For each test, 40% strain at a distance of 1.0 cm was applied to 25 mm thick samples. The test speed was 2.0 mm/s. TPA and bread moisture measurements were performed after 1, 3 and 5 days of storage at 5^oC in order to monitor staling and moisture loss. Mean values of at least three bread loaves were utilized for statistical purposes.

Color measurement

The color of bread crumbs was measured one-day after baking with a Hunterlab colorimeter, model MiniScan XE plus. Color readings were expressed by CIElab (L*, a*, b* and ΔE^*). Total color difference ΔE was calculated as $\Delta E^*=[(\Delta L^*)2+(\Delta a^*)2+(\Delta b^*)2]1/2$. Reported values are the means of independent triplicate measurements; two values obtained from the same loaf were averaged into one replicate.

Image acquisition and analysis

Images of bread slices were scanned using a scanner (HP ScanJet 3400C, Hewlett Packard,California, USA) in order to perform image analysis as described by Skendi, Biliaderis, Papageorgiou, and Izydorczyk (2010) with an UTHSCSA ImageTool programme (Version 3.0).

Total phenolic (TPC), total flavonoid content (TFC) and antioxidant activity assays

Estimation of free phenolic and flavonoid contents as well as antioxidant activity were performed in 80% methanol extracts. A double extraction (ratio 1:10) in an ultrasonic bath for 10min at 60^oC was applied on each freeze-dried sample (bread crumb or olive pulp). The mixture was then centrifuged at 4000 rpm, at 4^oC for 10min and the prepared extracts were stored in the freezer until analysis. Extraction was carried out at least in triplicate.
The amount of TPC in extracts was determined according to the Folin-Ciocalteu method (Irakli, Samanidou, Katsantonis, Biliaderis, & Papadoyannis, 2016; Singleton, Orthofer, & Lamuela-Raventos, 1999). The results were expressed as mg of GA equivalents per 100 g of the dried sample (mg GAE/100 g dw). The concentration of TFC was determined using the aluminum chloride colorimetric method (Bao, Cai, Sun, Wang, & Corke, 2005; Irakli et al., 2016). The TFC was estimated using a standard calibration curve, with catechin as a standard, and expressed as milligrams catechin equivalent (CATE) per 100 g of dry weight (mg CATE/100 g dw). The DPPH free radical scavenging activity (RSA) was based on the protocol described by Yen and Chen (1995). Results were expressed as mg Trolox equivalents per 100 g of dry sample (mg TE/100 g dw). The ferric reducing antioxidant power assay (FRAP) was performed according Benzie and Strain (1996) and Irakli et al. (2016). The results were expressed as mg Trolox equivalents per 100

g of dry sample (mg TE/100 g dw).
The ABTS radical scavenging activity was evaluated by employing the ABTS assay (Re et al., 1999). The ABTS•+ scavenging capacity was expressed as previously described for the DPPH assay.

HPLC profile of phenolic compounds

The analyses were performed on an HPLC Agilent 1200 system (Agilent Technology, Urdorf, Switzerland) equipped with a 250 x 4.6 mm i.d., 5 μm Nucleosil 100 C_{18} column (MZ, Mainz, Germany) maintained at 30^{o}C, a 20μL loop and diode-array detector (DAD). The mobile phase consisted of three solvents: (A) 1% acetic acid in water, (B) acetonitrile and (C) methanol and the following gradient program was performed: 0min, 90% A-0% B; 10min, 80% A-4% B; 25min, 75% A-5% B; 30min, 65% A-5% B; 31min, 40% A-0% B; 37min, 35% A-20% B; 50min, 20% A-80% B. The flow rate of mobile phase was 1.3mL/min. The DAD recorded the spectra at 260, 280 and 320 nm and the chromatograms were analyzed using the Agilent Chemstation software (version B.04.01, Agilent Technologies). Identification of phenolics was obtained by comparison of retention times and UV/VIS spectra with those of authentic standards (Cabrera-Banegil et al., 2017).

Sensory evaluation

Sensory evaluation was carried out on the breads on the same day of baking. Seven breads were evaluated (control, fortified with 5, 10 & 15% green or black olive pulp) by a 20-member trained panel using a nine-point hedonic scaling method (1-9 scoring): 1=extremely dislike (or lowest quality), 5=either like or dislike (or medium quality) and 9=extremely like (or highest quality), respectively. The breads were sliced into equally sized pieces (1cm thick) and served coded, randomized. Crust and crumb color, flavor/aroma and taste, as well as overall acceptability, were evaluated. Breads were considered acceptable if their mean score for overall acceptability was above 5 (neither like nor dislike).

Statistical analysis

All experiments, unless otherwise mentioned, were performed in triplicate. Data were analyzed by analysis of variance (ANOVA) using the Duncan's multiple range test to detect significant differences ($p<0.05$). Differences between the mean values of nutritionally important constituents measured experimentally in the breads and the theoretical values expected from the contribution of each individual raw material (flour and olive pulp) in the bread recipe were evaluated by the two-tailed t-test for paired data, in order to appreciate the impact of the baking process on these components. The statistical analyses were performed using SPSS statistical software (IBM SPSS Statistics version 19, 2010).

3 Results and Discussions

3.1 Proximate chemical composition of olive pulps and wheat flour

The proximate chemical composition, TPC, TFC and total antioxidant activity (ABTS, DPPH and FRAP assays) of black or green olive pulps and of wheat flour are presented in Table 1. The results showed that there were significant differences in the examined parameters among the samples (wheat flour, black olive, green olive). Both moisture and crude fat content were significantly different among the black and green olive pulp. Similar results were observed for the total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity assays (ABTS, FRAP and DPPH). The differences observed are probably related to factors such as cultivation practices, ripeness, harvesting time, climatic conditions and storage time of table olives (Pereira et al., 2006). In particular, due to the different ripening stage, the fat content of the green olives was 45% less than of the black, while crude protein, ash and polysaccharide contents were similar in both pulps. Black olive pulp had a richer TPC when compared to the green and both accounted for 5 and 3 times the TPC of wheat flour, respectively. The TFC of the black olive pulp was more than 1.4 times higher than that of the green

Table 1: Proximate chemical composition, total phenolic content, total flavonoid content and antioxidant activity (ABTS, FRAP, DPPH) of wheat flour, green and black olive pulp

Parameters (%)	*Wheat flour*	Black olive pulp	Green olive pulp
Moisture content	13.29 ($\pm$0.12)^1	60.24 ($\pm$0.55)^2	75.08 ($\pm$0.07)^3
Crude protein	13.11 ($\pm$0.23)^2	2.89 ($\pm$0.13)^1	2.85 ($\pm$0.10)^1
Crude fat	1.30 ($\pm$0.03)^1	32.11 ($\pm$0.06)^3	17.65 ($\pm$0.38)^2
Ash content	0.58 ($\pm$0.01)^1	1.61 ($\pm$0.01)^2	1.50 ($\pm$0.21)^2
Polysaccharide content (by difference)	71.72	3.45	2.92
TPC (mg GAE/100g)	70.23 ($\pm$0.32)^1	344.75 ($\pm$28.64)^3	246.10 ($\pm$17.11)^2
TFC (mg CAT/100g)	52.92 ($\pm$0.02)^1	320.60 ($\pm$30.69)^3	219.90 ($\pm$24.89)^2
ABTS (mg TE/100g)	25.61 ($\pm$2.62)^1	434.90 ($\pm$28.14)^3	288.20 ($\pm$18.95)^2
FRAP (mg TE/100g)	24.35 ($\pm$0.22)^1	476.75 ($\pm$28.78)^3	302.40 ($\pm$16.40)^2
DPPH (mg TE/100g)	20.60 ($\pm$0.85)^1	442.45 ($\pm$16.67)^3	272.10 ($\pm$15.41)^2

*Reported values are the mean of at least 3 replicates.
**Different numbers in the same line used as superscripts indicate differences ($p<0.05$) amongst the means, as determined by the Duncan's multiple range test.

olive pulp but they are both richer in flavonoids when compared to refined wheat flour. Antioxidant activity measurements from ABTS, DPPH and FRAP assays show that again the black olive pulp exhibited higher antioxidant activity than its green counterpart. Both olive pulps exhibit significantly higher antioxidant activity than refined wheat flour and can serve their role as nutritional enhancers in a bread recipe.

3.2 Mixing characteristics of dough

Fortification with olive pulp resulted in lower water absorption and longer development time, and produced more stable dough with a lower degree of softening than the control (data not shown). The decreasing water absorption on the farinograph follows the pattern of increased intrinsic moisture content of the olive pastes (Table 1) and the increase in supplementation level. More than 5% inclusion of olive pulp in wheat dough increased the dough development time (DDT) by 0.6-1.5min, in the farinograph, as well as the mixing stability from 4.3 to 7.0min. There is an obvious delay in the development of the gluten network in the presence of olive pulp. However, the dough remained consistent for a longer time when supplemented with olive pulp as evidenced by longer stability times and by lower values for degree of softening.

3.3 Physicochemical characteristics of bread

The characteristics of breads prepared after fortification with green or black olive pulp are summarized in Table 2. As it can be seen there is a significant progressive decrease in moisture content of all loaves on storage. However, the formulated breads with black olive pulp at higher levels of pulp addition managed to retain their moisture for longer. The higher moisture content of breads containing olive pulp may be related to the presence of fat which prevents water evaporation during the baking process (Pareyt, Finnie, Putseys, & Delcour, 2011). The weight loss of all breads was ~10%, while there was a pronounced progressive decrease of loaf volume with increasing addition of olive pulp. This may be attributed to the dilution of the gluten network and the resulting decrease of gas retention capacity. Thus, olive pulp although a high in fat ingredient does not act as a typical shortening which is expected to entrap air during mixing thus enhancing the loaf volume. In the case of

Table 2: Moisture content on storage, weight loss, loaf volume, crumb color and gas characteristics of control bread and of formulated breads with the addition of olive pulp at different supplementation levels (5, 10, 15%)*

	Control	Black olive pulp			Green olive pulp		
		5 %	10%	15%	5 %	10%	15%
Moisture content (%)**							
Day 1	40.06 ($\pm$0.3)^{1,2,a}	42.15 ($\pm$0.4)^{3,a}	42.60 ($\pm$0.4)^{3,4,a}	43.70 ($\pm$0.2)^{4,a}	40.25 ($\pm$0.1)^{1,2,a}	41.47($\pm$0.6)^{2,3,a}	39.69($\pm$0.1)^{1,a}
Day 3	38.05 ($\pm$0.1)^{1,b}	39.80 ($\pm$0.1)^{2,b}	40.23 ($\pm$0.3)^{2,a,b}	41.85 ($\pm$0.3)^{3,a,b}	37.56 ($\pm$0.2)^{1,b}	40.48($\pm$0.2)^{2,a,b}	37.60($\pm$0.1)^{1,b}
Day 5	34.34 ($\pm$0.1)^{1,c}	38.11 ($\pm$0.4)^{2,c}	38.16 ($\pm$0.6)^{2,b}	39.93 ($\pm$0.5)^{2,b}	34.56 ($\pm$0.2)^{1,c}	38.19($\pm$0.4)^{2,b}	33.18($\pm$0.1)^{1,c}
Weight	10.0	9.3	10.0	10.1	9.9	10.1	10.6
Loss (%)^a							
Loaf volume**							
Volume (cm^3)	235 ($\pm$10.0)^1	225 ($\pm$2.8)^{1,2}	195 ($\pm$10.6)^{13}	160 ($\pm$15.2)^{4,5}	215 ($\pm$21.2)^2	170 ($\pm$3.5)^4	150 ($\pm$5.0)^5
Decrease (%)^b	-	4.30	17.02	31.91	8.50	27.66	36.17
Crumb color**							
L*	67.22 ($\pm$0.62)^1	62.95 ($\pm$0.30)^2	62.48 ($\pm$0.13)^2	59.45 ($\pm$0.96)^3	48.02 ($\pm$0.53)^4	44.23 ($\pm$0.57)^5	38.12 ($\pm$0.34)^6
a*	1.70 ($\pm$0.28)^5	2.51 ($\pm$0.23)^{3,4}	3.62 ($\pm$0.02)^2	4.75 ($\pm$0.06)^1	2.27 ($\pm$0.12)^4	2.50 ($\pm$0.23)^{3,4}	2.61 ($\pm$0.31)^3
b*	15.85 ($\pm$0.23)^3	9.56 ($\pm$0.17)^4	9.34 ($\pm$0.09)^{4,5}	9.13 ($\pm$0.16)^5	17.16 ($\pm$0.04)^2	17.37 ($\pm$0.28)^2	20.46 ($\pm$0.18)^1
ΔE*	-	20.22	23.97	30.02	4.50	5.04	9.08
Gas characteristics							
Total cells	632 ($\pm$58)^1	366 ($\pm$25)^3	365 ($\pm$19)^3	397 ($\pm$21)^4	328 ($\pm$70)^4	338 ($\pm$60)^{3,4}	348 ($\pm$38)^{3,4}
Nr of cells $\leq$**4mm^2**	371($\pm$23)^1	240($\pm$38)^3	214($\pm$29)^{3,4}	222($\pm$33)^{3,4}	189($\pm$41)^4	190($\pm$35)^4	224($\pm$31)^{3,4}
Total cell area (mm^2)	101.1($\pm$14)^1	49.5($\pm$23)^{2,3}	68.2($\pm$16)^2	37.3($\pm$28)^3	57.4($\pm$24)^{2,3}	51.7($\pm$17)^{2,3}	35.6($\pm$32)^3
Mean cell area (mm^2)	0.16($\pm$0.09)^{1,2}	0.14($\pm$0.10)^{1,2}	0.19($\pm$0.07)^1	0.09($\pm$0.06)^2	0.17($\pm$0.08)^{1,2}	0.10($\pm$0.09)^{1,2}	0.10($\pm$0.09)^{1,2}
Nr cells/cm^2	70.2($\pm$3.28)^1	40.7($\pm$1.12)^3	40.5($\pm$1.03)^3	44.2($\pm$1.02)^2	36.4($\pm$2.42)^4	37.5($\pm$2.12)^{3,4}	38.6($\pm$1.01)^4

*Reported values are the mean of at least 3 replicates.

**Different numbers in the same line used as superscripts indicate differences ($p<0.05$) amongst the means, as determined by the Duncan's multiple range test.

^{a} Weight loss calculation based on 100 g dough used for each loaf of bread

^{b} Compared with the control

olive oil ~75% percent of its fat content is oleic acid (Bartoli et al., 2000). The highest value of L^* corresponding to lightness was obtained for the control bread. Addition of olive pulp significantly decreased L^* proportionally to the level of supplementation and the type of olive pulp. In the case of a^*, the addition of olive pulp signified predominance of red over green in the bread crumb. In all cases, the value of b^* was positive, which corresponds to a more intense yellow hue over blue both in control and fortified breads. Introduction of black or green olive pulp even at the lowest supplementation level resulted in a dramatic decrease of ≈42 and ≈48% of total cells, respectively. It seems that the air bubbles entrapped in oil cannot withstand baking or even proofing as opposed to those incorporated in the gluten matrix. The observed decrease in total cell area was confirmed by the decrease in volume yield of breads containing olive pulp as well as by the visual detection of higher density breads. A significant decrease was also observed in the number of cells $\leq 4mm^2$, and number of cells per cm^2 with introduction of olive pulp. During mixing, the incorporated air forms gas cells as nucleation sites for the CO_2 gas generated by yeast activity during proofing. The three-dimensional protein network traps gasses and the embedded gasses expand the dough, leading to a porous structure after baking (Campbell, 2003). In the case of breads with olive paste the significant fat content of both black and green olives also incorporate air on mixing. As was previously reported (Jacob & Leelavathi, 2007), the air incorporated into oil cannot be retained thus leading to an increase in hardness of bread. The later is in agreement with our findings in the following section.

3.4 Texture profile analysis of wheat flour bread and of breads containing olive pulp

Fig. 1 shows the texture parameters of the control bread and of breads containing black or green olive pulp. Hardness of breads containing black olive pulp (Fig. 1a_1) did not differ from the control as a function of increased supplementation level up to 10% olive pulp during the whole storage period. In the case of breads with green olive pulp only the bread with 5% green olive pulp had similar hardness values with the control during the whole storage period. Generally, there was a substantial increase in hardness with storage time for breads at 10 and 15% supplementation (Fig. 1a_2). The increase in bread hardness may result from a decrease in the total area of the gas cell; greatest crumb hardness is usually observed in breads with the lowest loaf volume. Besides, hardness values of breads with the addition of 5, 10 or 15% green olive pulp (Fig. 1a_2) were higher than the corresponding breads with black olive pulp (Fig. 1a_1). The higher fat content of the black pulp could act as a moisture barrier or can be engaged in retardation of starch retrogradation (Provost, Colabroy, S. Kelly, & Wallert, 2016). Results of cohesiveness (Fig. 1b_1, b_2) indicated that for all breads, cohesiveness followed a decreasing trend on storage and increased supplementation with olive pulp. This is due to the crumb offering less resistance to the compression force. The observed decrease of cohesiveness on storage could be attributed to moisture loss of the breads (Table 2). Besides, the decreased cohesiveness may be related to the loss of intramolecular attraction among ingredients as postulated by Gomez, Ronda, Caballero, Blanco, and Rosell (2007) thus resulting in increased susceptibility of the bread to crumbliness (Boz & Karaoglu, 2013). As far as the chewiness of breads is concerned (Fig. 1c_1, c_2), a similar pattern to hardness was observed.

3.5 Antioxidant properties of breads

The TPC of the control and fortified breads with black or green olive pulp is shown in Fig. 2. The highest TPC values were observed in breads fortified with 15% olive pulp. The TFC of the breads containing black olive pulp was higher than those with green olive pulp. Besides, the results indicated that as the supplementation level increases the ABTS values of fortified breads increased significantly ($p<0.05$). As regards the values of the DPPH assay for the fortified breads with black and green olive pulp they ranged between 20.7 to 65.1 mg TE/100 g and 11.9 to 40.5 mg TE/100

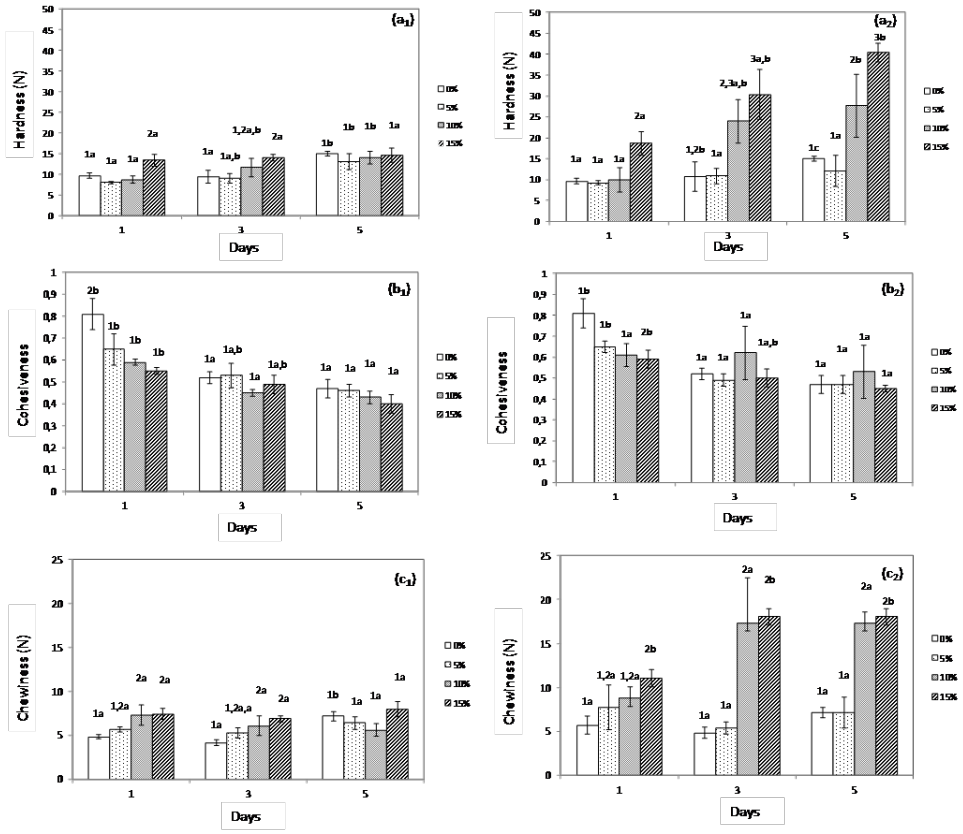


Figure 1: Texture profile parameters: Hardness (a_1, a_2); Cohesiveness (b_1, b_2); Chewiness (c_1, c_2) of control bread (0%) and of breads containing black (a_1, b_1, c_1) or green (a_2, b_2, c_2) olive pulp. Different numbers within the same day group or letters for the same recipe (on different days) used as superscripts indicate differences ($p<0.05$) amongst the means, as determined by the Duncan's multiple range test.

g, respectively, whereas the control bread showed 6.46 mg TE/100 g DPPH radical scavenging activity. This was expected since Maillard reaction products also possess certain free radical scavenging activity (Jing & Kitts, 2000). The same trend was observed for the FRAP assay. As for the effect of storage time, according to Fig. 2, there is no significant difference of antioxidants, irrespective of the assay employed during the whole period of storage.

Many researchers, observed an increase of the TPC and the antioxidant activity of the baked product (bread) compared to raw flour (Gelinas & McKinnon, 2006; Holtekjolen, Baevre, Rodbotten, Berg, & Knutsen, 2008; Yu, Nanguet, & Beta, 2013). In an attempt to investigate the role of the baking process, Table 3 shows the experimental and the theoretical values (by calculation) of TPC and antioxidant activity (ABTS, FRAP, DPPH) of the control bread and of fortified breads, on the first day of storage. Instead of comparing the antioxidants of raw materials (wheat flour and olive pulps) to those of the final product (bread) we proceeded to the calculation of theoretically expected values, based on the actual contribution of dry ingredients per 100 g of dry bread while taking into account weight loss from dough to bread (Table 1). Furthermore, we employed the paired sample t test to reveal if mean differences between the experimental and theoretical values were significant ($p<0.05$). This test was employed in two groups; group 1

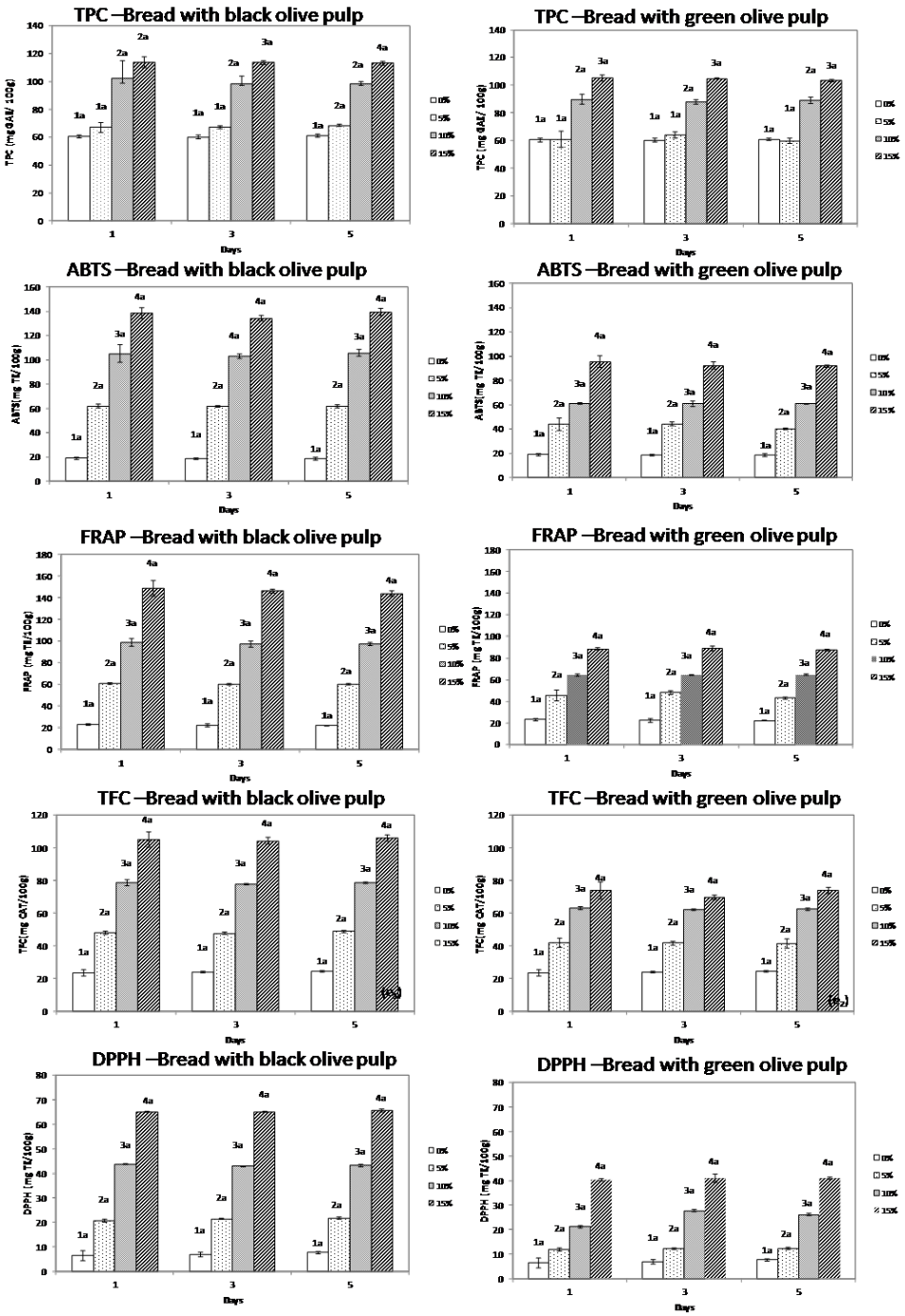
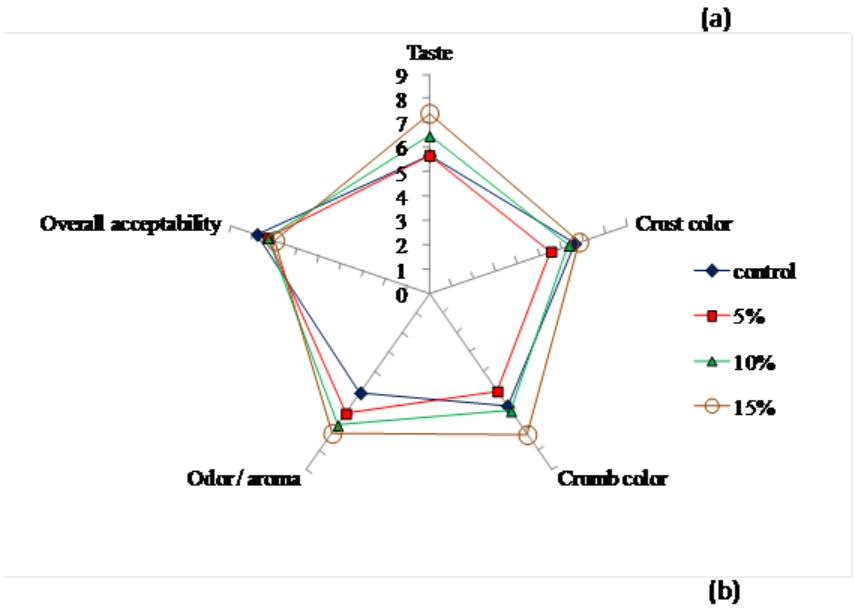


Figure 2: Total phenolic content, total flavonoid content and antioxidant activity (ABTS, FRAP, DPPH) of control bread and of breads containing black or green olive pulp at 5, 10 or 15% supplementation levels. Different numbers in the same day group or letters among the same recipe (on different days) used as superscripts indicate differences ($p<0.05$) amongst the means, as determined by the Duncan's multiple range test.



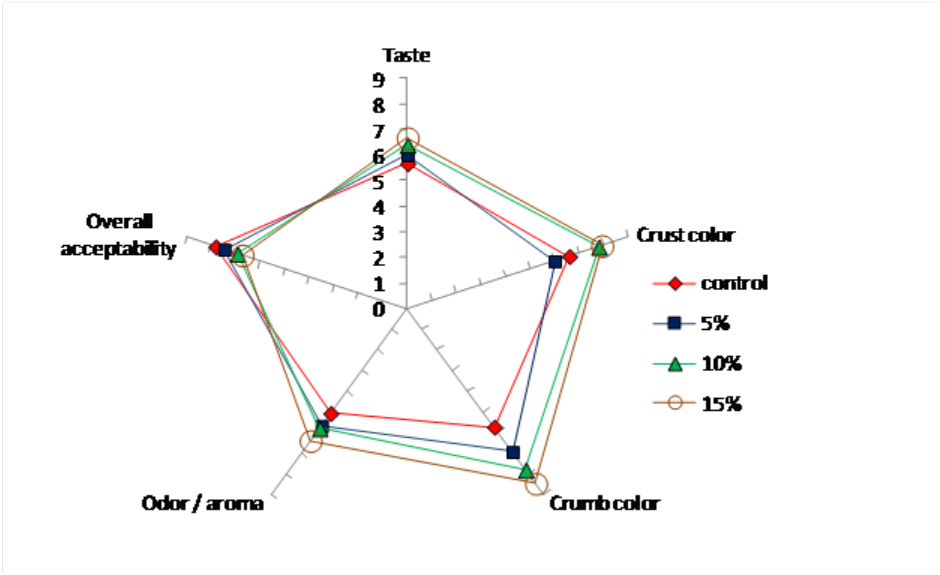


Figure 3: Sensory evaluation of black (a) and green (b) olive pulp supplemented breads

Table 3: Experimental mean value and theoretical values (by calculation) of TPC, TFC and antioxidant activity (ABTS, FRAP, DPPH) of control bread and of breads containing black or green olive pulp at supplementation levels 5, 10, 15% on storage day 1. Values in brackets show standard deviation

		Formulated breads with black olive pulp			**Formulated breads with green olive pulp**		
	Control	5%	10%	15%	5%	10%	15%
TPC (mg GAE/100 g)			Group 1*			Group 2*	
Experimental	60.5 (±1.3)	67.0 (±3.5)	102.2 (±13.0)	114.1 (±3.9)	60.9 (±5.8)	89.7 (±3.6)	105.2 (±2.2)
Theoretical	24.2	30.1	35.3	40.3	29.1	32.5	38.5
TFC (mg CAT/100 g)			Group 1			Group 2	
Experimental	23.6 (±2.0)	48.1 (±1.0)	78.6 (±1.7)	105.1 (±4.6)	41.9 (±2.0)	62.9 (±2.9)	73.8 (±1.0)
Theoretical	18.3	23.4	28.0	32.3	18.3	24.7	29.7
FRAP (mg TE/100 g)			Group 1			Group 2*	
Experimental	22.9 (±0.8)	60.9 (±0.8)	98.8 (±3.7)	148.7 (±7.0)	45.5 (±4.9)	64.4 (±0.9)	88.4 (±0.9)
Theoretical	8.4	17.3	25.5	33.1	14.5	19.6	26.3
ABTS (mg TE/100 g)			Group 1			Group 2	
Experimental	18.8 (±1.2)	61.9 (±1.5)	105.2 (±7.3)	138.5 (±14.4)	43.7 (±5.0)	61.1 (±0.9)	95.5 (±4.7)
Theoretical	8.8	16	24.3	31.3	14.6	19.4	25.7
DPPH (mg TE/100 g)			Group 1			Group 2	
Experimental	6.5 (±2.0)	20.7 (±0.5)	43.7 (±0.2)	65.1 (±0.1)	11.9 (±0.6)	21.2 (±0.4)	40.5 (±0.5)
Theoretical	16.4	23.8	30.4	36.7	21.8	26.0	32.3

* Significant mean differences between the experimental and theoretical values if $p<0.05$.

comprised the control and the formulated breads with black olive pulp and group 2 comprised the control and the formulated breads with green olive pulp.

The TPC of fortified breads with black olive pulp at 5% supplementation level was more than 2 times higher than that obtained by calculation (theoretical), while those at 10 or 15% supplementation level were 3 times higher than the theoretical one. In the formulated breads with green olive pulp at 5, 10 or 15% supplementation levels the experimental TPC was ∼2.5 times higher than those of the theoretical one. Similarly, the TFC of the breads with the addition of black olive pulp at 5, 10 or 15% level were 2.0, 2.8 or 3.2 times higher compared to the theoretical ones, while those with green olive pulp were 1.7, 2.1 or 2.0 times higher, respectively. The formulated breads with black green olive pulp at 15% supplementation level had an antioxidant capacity (ABTS, FRAP) ∼4.4 times more than the theoretical and with green olive pulp ∼3.7 times. To the contrary DPPH experimental values of formulated breads with black or green olive pulp were only 1.7 or 1.2 times higher than that of the theoretical. As for the control bread, the experimental values of TFC, TPC, ABTS & FRAP were also higher in comparison with the theoretical. Despite the impressive increase that baking imparted in all the examined parameters for all breads, the paired sample t test showed that the mean differences between the experimental and theoretical values (i.e. effect of baking) were only significant for the TPC of Groups 1 and 2 breads and for FRAP values of Group 2 breads. The observed differences between the experimental and the theoretical values of the control bread and of the formulated breads are attributed to the bread making process. In the literature, studies on the effect of baking on the TPC, TFC and antioxidants activity of bread are contradictory (Gelinas & McKinnon, 2006; Holtekjolen et al., 2008; Yu et al., 2013). The antioxidant composition or capacity of bakery products derives from the intrinsic phenolic compounds of flour, added phenolic ingredients, other ingredients physically containing phenolics, intermediate phenolic products newly generated during baking for example, via Maillard reactions (Michalska, Amigo-Benavent, Zielinski, & del Castillo, 2008), thermal-induced degradative products (Rupasinghe, Wang, Huber, & Pitts, 2008), and/or polyphenol polysaccharides complexes (Shahidi & Naczk, 1995). In accordance with our results, Chandrasekara and Shahidi (2011) reported that products of Maillard reactions could increase the TPC of baked breads when compared to raw flour. However, it cannot be specified whether the observed dif-

Table 4: Content (μg/g) of phenolic compounds on dry-weight basis of flour, black or green olive pulps, and of control and formulated breads with black or green olive pulp at supplementation levels of 5, 10, 15%

Analyte	Flour	Black olive pulp	Green olive pulp	Control (Bread)	Breads with Black olive pulp			Breads with Green olive pulp		
Phenolic acids & their derivatives				0%	5%	10%	15%	5%	10%	15%
HTR	ND	788.6(±17.1)	321.9(±17.1)	ND	135.2(±12.1)	278.6(±10.3)	481.4(±20.5)	57.0(±6.3)	77.0(±4.1)	98.4(±8.8)
PRCA	0.2(±0.0)	2.7(±0.1)	1.6(±0.2)	0.2(±0.0)	0.7(±0.1)	1.0(±0,2)	1.4(±0.3)	0.8(±0,1)	1.0(±0,1)	0.7(±0,1)
TR	ND	280.6(±34.6)	49.1(±5.7)	ND	25.2(±2.5)	74.1(±6.3)	124.2(±10.1)	23.6(±1.8)	28.2(±6.5)	32.8(±5.6)
4HBA	0.5(±0.1)	2.7(±0.2)	1.7(±0.5)	0.5(±0.1)	0.8(±0.1)	2.0(±0.2)	2.1(±0.3)	1.0(±0.2)	0.8(±0.1)	0.7(±0.2)
VA	0.9(±0.2)	12.8(±2.8)	2.8(±0.6)	0.9(±0.2)	2.5(±0.4)	6.2(±0.8)	8.5(±0.5)	2.1(±0.3)	2.3(±0.2)	2.4(±0.1)
CA	ND	2.7(±0.1)	2.7(±0.4)	ND	0.8(±0.1)	1.0(±0.2)	1.0(±0.3)	0.5(±0.1)	0.4(±0.0)	0.3(±0.0)
SRA	0.1(±0.1)	2.0(±0.1)	0.5(±0.1)	1.0(±0.3)	0.6(±0.1)	0.6(±0.1)	0.7(±0.2)	0.2(±0.0)	0.6(±0.2)	0.2(±0.0)
PCA	0.3(±0.0)	ND	5.7(±0.1)	0.3(±0.0)	1.1(±0.3)	1.4(±0.4)	1.3(±0.2)	0.8(±0.1)	0.6(±0.1)	0.4(±0.1)
FA	0.5(±0.1)	ND	ND	0.5(±0.1)	0.5(±0.1)	0.6(±0.1)	0.6(±0.1)	0.6(±0.1)	0.6(±0.2)	0.4(±0.1)
OLE	ND	105.6(±0.4)	ND	ND	ND	ND	ND	ND	ND	ND
Total μg/g	2.0(±0.4)	1197.7(±60.7)	385.8(±55.6)	3.4(±0.1)	167.2(±15.5)	364.8(±18.5)	620.7(±33.3)	86.0(±9.1)	110.7(±11.6)	135.8(4.8)

ND-Not detected.
Values are means of duplicate analysis.

ferences in the TPC could be attributed to the fermentation, kneading or the baking process.

3.6 HPLC profile of phenolic compounds

Table 4 shows the content of phenolic acids and their derivatives for wheat flour, black or green olive pulp and formulated breads. The black olive pulp contained higher quantities of phenolic compounds compared to its green counterpart. Hydroxytyrosol was the major phenolic compound existing in both black and green olives, with the black olive pulp exhibiting the highest concentration. This was expected since hydroxytyrosol is considered the major phenolic compound existing in processed olives followed by tyrosol (Boskou, 2017). OLE was detected only in black olives with a mean value of 105.6 μg/g. The detectable content of the rest phenolic compounds (sum of PRCA, 4HBA, VA, CA, SRA and PCA) ranged between 15.0 and 22.9 μg/g for green and black olive, respectively, whereas FA was not detected in both types of olives. The formulated breads with 5 to 15% black or green olive pulp presented approximately 50 to 200 times or 25 to 40 times greater amount of phenolic compounds compared to the control. In particular, the HPLC analysis showed that HTR was the major phenolic compound in formulated breads with 5 to 15% black or green olive pulp ranging from 135.2 to 481.4 μg/g and 57 to 98.3 μg/g, respectively. A similar trend was observed for the TR, the second primary phenolic compound in formulated breads with black and green olive pulp, ranging from 25.2 to 124.2 μg/g and 23.6 to 32.8 μg/g, respectively as the fortification level increased from 5 to 15%.

3.7 Sensory evaluation of wheat flour bread and of breads containing olive pulp

The sensory evaluation scores of breads with different supplementation levels of green or black olive pulp is shown in Fig. 3. The highest taste scores were obtained in the formulation with 15% black olive pulp. Crust and crumb color as well as the taste of breads containing olive pulp scored higher than the control. The remarkable color difference measured instrumentally did not seem to affect the scoring of the breads for crust or crumb color. In general, the rating in taste, crust and crumb color, and odor/aroma increased with the supplementation level. Breads at 10 and 15% supplementation level of black olive pulp scored significantly higher. All formulations with olive pulp were acceptable, since they received scores for overall acceptability, much higher than 5, ranging from 6.73 to 7.47. The results suggest that supplementation of olive pulp in a wheat bread formulation would not interfere with general bread acceptability.

4 Conclusion

Fortification of wheat bread with preferably black rather than green olive pulp resulted in tasty breads with satisfactory overall acceptability despite the denser structure and smaller loaf volumes observed. The formulated breads with black olive pulp at higher levels of pulp addition managed to retain their moisture for longer, possibly due to the higher fat content to its green olive counterpart, and also exhibited a slower staling rate. As shown by TFC, TPC, FRAP, ABTS and DPPH assays, it can be concluded that the formulated breads with olive pulp, had significantly higher antioxidant activity as compared to the control whereas the formulated breads with black olive pulp had higher antioxidant activity than the green-olive counterparts. The observed antioxidant activity remained unchanged during the 5 days of storage, for all bread formulations with olive pulp. HPLC results showed that HTR was the major phenolic compound in formulated breads, followed by TR.

Acknowledgements

The authors would like to greatly thank the technical staff of the Institute of Plant Breeding and Genetic Resources, Thermi-Thessaloniki, Greece for their assistance.

References

AACC. (2000). Approved Methods of the AACC. American. Association of Cereal Chemists. St. Paul MN USA: AACC.

AOAC. (1990). Official Methods of Analysis. Association of Official Analytical Chemists. Washington DC.

Bao, J. S., Cai, Y. Z., Sun, M., Wang, G. Y., & Corke, H. (2005). Anthocyanins, flavonols, and free radical scavenging activity of chinese bayberry (myrica rubra) extracts and their color properties and stability. *Journal of Agricultural and Food Chemistry*, *53*(6), 2327–2332. doi:10.1021/jf048312z

Bartoli, R., Fernandez-Banares, F., Navarro, E., Castella, E., Mane, J., Alvarez, M., … Gassull, M. A. (2000). Effect of olive oil on early and late events of colon carcinogenesis in rats: Modulation of arachidonic acid metabolism and local prostaglandin e-2 synthesis. *GUT*, *46*(2), 191–199. doi:10.1136/gut.46.2.191

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (frap) as a measure of ''antioxidant power'': The frap assay. *Analytical Biochemistry*, *239*(1), 70–76. doi:10.1006/abio.1996.0292

Bhattacharya, M., Langstaff, T. M., & Berzonsky, W. A. (2003). Effect of frozen storage and freeze-thaw cycles on the rheological and baking properties of frozen doughs. *Food Research International*, *36*(4), 365–372. doi:10.1016/S0963-9969(02)00228-4

Boskou, D. (2017). Table olives: A vehicle for the delivery of bioactive compounds. *Journal Exp Food Chemistry*, *3*(123), 2472–0542.

Boz, H., & Karaoglu, M. M. (2013). Improving the quality of whole wheat bread by using various plant origin materials. *Czech Journal of Food Sciences*, *31*(5), 457–466. doi:10.17221/410/2012-CJFS

Cabrera-Banegil, M., Schaide, T., Manzano, R., Delgado-Adamez, J., Duran-Meras, I., & Martin-Vertedor, D. (2017). Optimization and validation of a rapid liquid chromatography method for determination of the main polyphenolic compounds in table olives and in olive paste. *Food Chemistry*, *233*, 164–173. doi:10.1016/j.foodchem.2017.04.052

Campbell, G. M. (2003). Bread making: Improving quality. In S. P. Cauvain (Ed.), (Chap. Bread aeration, pp. 352–374).

Chandrasekara, N., & Shahidi, F. (2011). Effect of roasting on phenolic content and antioxidant activities of whole cashew nuts, kernels, and testa. *Journal of Agricultural and Food Chemistry*, *59*(9), 5006–5014. PMID: 21438525. doi:10.1021/jf2000772. eprint: https://doi.org/10.1021/jf2000772

Dewettinck, K., Van Bockstaele, F., Kuhne, B., de Walle, D. V., Courtens, T. M., & Gellynck, X. (2008). Nutritional value of bread: Influence of processing, food interaction and consumer perception. *Journal of Cereal Science*, *48*(2), 243–257. doi:10.1016/j.jcs.2008.01.003

Duan, X.-J., Zhang, W.-W., Li, X.-M., & Wang, B.-G. (2006). Evaluation of antioxidant property of extract and fractions obtained from a red alga, polysiphonia urceolata. *Food Chemistry*, *95*(1), 37–43. doi:10.1016/j.foodchem.2004.12.015

Galli, C., & Visioli, F. (1999). Antioxidant and other activities of phenolics in olives olive oil, typical components of the mediterranean diet. *Lipids*, *34*(S), S23–S26. 3rd Congress of the International-Society-for-the-Study-of-Fatty-Acids-and-Lipids, LYON, FRANCE, JUN 01-05, 1998. doi:10.1007/BF02562224

Gelinas, P., & McKinnon, C. M. (2006). Effect of wheat variety, farming site, and bread-baking on total phenolics. *International Journal of Food Science and Technology*, *41*(3), 329–332. doi:10.1111/j.1365-2621.2005.01057.x

Gomez, M., Ronda, F., Caballero, P. A., Blanco, C. A., & Rosell, C. M. (2007). Functionality of different hydrocolloids on the quality and shelf-life of yellow layer cakes. *Food Hydrocolloids*, *21*(2), 167–173. doi:10.1016/j.foodhyd.2006.03.012

Holtekjolen, A. K., Baevre, A. B., Rodbotten, M., Berg, H., & Knutsen, S. H. (2008). Antioxidant properties and sensory profiles of breads containing barley flour. *Food*

Chemistry, *110*(2), 414–421. doi:10.1016/j.foodchem.2008.02.054

ICC. (1994). Standard methods of the International Association for Cereal Chemistry. International Cereal Chemistry, (4th supplement). Vienna, Austria: ICC.

Irakli, M. N., Samanidou, V. F., Katsantonis, D. N., Biliaderis, C. G., & Papadoyannis, I. N. (2016). Phytochemical profiles and antioxidant capacity of pigmented and non-pigmented genotypes of rice (oryza sativa l.) *Cereal Research Communications*, *44*(1), 98–110. doi:10.1556/0806.43.2015.033

Jacob, J., & Leelavathi, K. (2007). Effect of fat-type on cookie dough and cookie quality. *Journal of Food Engineering*, *79*(1), 299–305. doi:10.1016/j.jfoodeng.2006.01.058

Jing, H., & Kitts, D. (2000). Comparison of the antioxidative and cytotoxic properties of glucose-lysine and fructose-lysine maillard reaction. *Food Research International*, *33*, 509–516. doi:10.1016/S0963-9969(00)00076-4

Michalska, A., Amigo-Benavent, M., Zielinski, H., & del Castillo, M. D. (2008). Effect of bread making on formation of maillard reaction products contributing to the overall antioxidant activity of rye bread. *Journal of Cereal Science*, *48*(1), 123–132. doi:10.1016/j.jcs.2007.08.012

Pareyt, B., Finnie, S. M., Putseys, J. A., & Delcour, J. A. (2011). Lipids in bread making: Sources, interactions, and impact on bread quality. *Journal of Cereal Science*, *54*(3), 266–279. doi:10.1016/j.jcs.2011.08.011

Pereira, J. A., Pereira, A. P. G., Ferreira, I. C. F. R., Valentao, P., Andrade, P. B., Seabra, R., ... Bento, A. (2006). Table olives from portugal: Phenolic compounds, antioxidant potential, and antimicrobial activity. *Journal of Agricultural and Food Chemistry*, *54*(22), 8425–8431. doi:10.1021/jf061769j

Provost, J., Colabroy, K., S. Kelly, B., & Wallert, M. (2016). The science of food and cooking. (pp. 1–54). doi:10.1002/9781118910771.ch1

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved abts radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9-10), 1231–1237. doi:10.1016/S0891-5849(98)00315-3

Rupasinghe, H. P. V., Wang, L., Huber, G. M., & Pitts, N. L. (2008). Effect of baking on dietary fibre and phenolics of muffins incorporated with apple skin powder. *Food Chemistry*, *107*(3), 1217–1224. doi:10.1016/j.foodchem.2007.09.057

Shahidi, F., & Naczk, M. (1995). Nutritional and pharmacological effects of food phenolics. *Food phenolics: Sources, chemistry, effects and applications*, 171–191.

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In L. Packer (Ed.), *Oxidants and antioxidants, pt a* (Vol. 299, pp. 152–178). Methods in Enzymology. 525 B STREET, SUITE 1900, SAN DIEGO, CA 92101-4495 USA: Elsevier Academic Press Inc.

Skendi, A., Biliaderis, C., Papageorgiou, M., & Izydorczyk, M. (2010). Effects of two barley beta-glucan isolates on wheat flour dough and bread properties. *Food Chemistry*, *119*, 1159–1167. doi:10.1016/j.foodchem.2009.08.030

Yen, G. C., & Chen, H. Y. (1995). Antioxidant activity of various tea extracts in relation to their antimutagenicity. *Journal of Agricultural and Food Chemistry*, *43*(1), 27–32. doi:10.1021/jf00049a007

Yu, L., Nanguet, A. L., & Beta, T. (2013). Comparison of antioxidant properties of refined and whole wheat flour and bread. *Antioxidants*, *2*, 370–383. doi:10.3390/antiox2040370

International Journal of Food Studies | IJFS April 2020 | Volume 9 | pages 193–202

Mango Peel Ingredient as Salt and Phosphate Replacement in Chicken Breast Marinade

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Received: 14 August 2018; Published online: 18 April 2020

Abstract

Whole mango peel powder and fibre extracted from mango peel were investigated as potential ingredients to replace salt and phosphate in chicken marinade. Whole mango peel powder possessed higher water absorption capacity than mango peel fibre, 4.57% and 1.47%, respectively ($P < 0.05$). Similar oil absorption capacity was observed in both mango peel ingredients ($P > 0.05$). Purified pectin and sorbitol syrup were also used to compare the water holding capacity in marinated chicken breasts. Mango peel ingredients exhibited less cooking loss than pectin and sorbitol syrup, however, higher cooking loss than sodium tripolyphosphate (STPP) and NaCl ($P > 0.05$). Marinating with mango peel ingredients significantly increased a* value of chicken breasts after cooking, while L* and b* values were similar in marinating with NaCl ($P > 0.05$). Hardness of frozen fully cooked chicken breast was measured after thawing. The lowest shear force of 7.13 N was observed in chicken breasts marinated with mango peel fibre ($P < 0.05$), while the hardness of chicken breasts marinated with whole mango peel powder was not significant different from STPP and NaCl treatments. Results from this study revealed a potential approach of utilizing fruit waste as an ingredient to substitute for phosphate and salt in chicken marinade.

Keywords: Low sodium; Low phosphate; Mango peel; Marinade; Chicken breast

1 Introduction

Frozen, fully cooked chicken products are typically consumed worldwide, both in households and in restaurant chains. In order to boost up flavour, marination is becoming a crucial step in preparing chicken products. Marinades not only develop the flavour and texture of the chicken products, but reduce weight loss in further processes such as roasting, baking, frying, and thawing. In addition to taste, the marinade recipe that could be absorbed and well retained in the chicken meat is very desirable. Most marinade solutions regularly add salt and phosphates. Salt or sodium chloride (NaCl) provides a negative charge (Cl^- ion) to interact with positive charge on the myosin protein in chicken meat. This interaction induces the dissolving of insoluble myosin, hence the meat swells and is able to hold water. Moreover, salt and phosphates extract myofibrilla proteins to form a cohesive network on the meat surface, which could trap water upon cooking (Acton & Jensen, 1994; Tarté, 2009). Although salt and phosphates have been generally used as food additives, consumers have currently a raised awareness of the sodium and

 10.7455/ijfs/9.1.2020.a5

phosphate content in their foods. Sodium levels in food have become a serious concern due to its potential to cause high blood pressure and cardiovascular diseases. Additionally, excessive phosphate consumption could lead to hyperphosphataemia with kidney disease. Several research efforts have been made to test potential approaches to replacing or reducing salt and phosphate levels in the marinade, whilst maintaining percent yield and organoleptic properties. Uchupaj, Malila, Petracci, Benjakul and Visessanguan (2017) reported that using a proprietary recipe of non-phosphate and low-sodium salt (NPLS) marinade significantly reduced cooking loss, and it was found to be more effective on breast meat than thighs and drumsticks. The NPLS marinade contained potassium bicarbonate, potassium chloride and sorbitol. Jarvis et al. (2012) proposed dried plum ingredients to replace sodium tripolyphosphate (STPP) in marinating chicken breast fillet, providing similar sensory results and yield to the STPP marinade. With more health awareness, plant-based ingredients are gaining more attention. Mango is one of the tropical fruits available for consumption throughout the year, particularly in Thailand. In Thailand, mango is usually consumed when it is still green and just starts ripening. Mango peel is always discarded as waste. Ajila, Naidu, Bhat and Rao (2007), and Kanatt and Chawla (2018) revealed antioxidant, and antibacterial properties in mango peel extract. The incorporation of mango peel powder was found to increase total dietary fibre, and firmness in a macaroni preparation (Ajila, Aalami, Leelavathi & Rao, 2010). In addition, mango peel has been reported to be a potential source of good pectin (Geerkens et al., 2015; Kermani, Shpigelman, Huong, Van Loey & Hendrickx, 2015; Nagel et al., 2015; Wang et al., 2016). These investigations have revealed the health benefit component in mango peel, and its application. However, publications regarding the application of mango peel as a substitute for salt and phosphate are still limited. Therefore, this research was aimed to determine a potential approach of utilizing the natural ingredient for reducing salt and phosphate in chicken breast marinade. Physicochemical properties of frozen fully cooked chicken breast marinated with mango peel ingredients were evaluated compared to marination with salt, phosphate, pectin, and sorbitol.

2 Materials and Methods

2.1 Whole mango peel powder and fibre extracted from mango peel

Mango peels of Fah Lan mango (*Mangifera indica* L.) were collected from a fruit trolley in Maha Sarakham province, Thailand. Mango peel was from mature stage of mango with fully developed green skin, and white colour of firm flesh. The mango peel was washed with tap water, drained, and dried in hot air oven (Memmert, Schwabach, Germany) at 60 ^{o}C for 4 hours. The dried peel was finely ground and kept in polyethylene bags. Fibre from mango peel was extracted using 250 grams of whole mango peel powder in 1.5 L of acidified water (pH 2.5 - 3, pH adjusted using citric acid), and stirred until the mango powder was well dispersed. The solution was heated at 65 ^{o}C for 1 hour in a water bath (Memmert W200, Schwabach, Germany) with continuously stirring. The hot mixture was filtered through cheese cloth (fine grade, 100% cotton). Pectin was coagulated from the filtrate using equal volume of 95% ethanol (Analytical grade, VWR chemicals, Leighton Buzzard, England). The solution was left for 1 hour to let pectin float to the surface. Extracted pectin was skimmed off and washed 2 - 3 times with ethanol to remove remaining impurities. Finally, the pectin was dried at 40 ^{o}C for 4 hours in a hot air oven. The dried pectin was ground and kept in polyethylene bags for further use and analysis.

2.2 Frozen fully cooked chicken breast

Packaged raw chicken breasts (Tesco brand) were purchased from Tesco Lotus supermarket in Maha Sarakham province. The meat was cut, and each individual piece weighed to 50±3 g. The temperature of the raw chicken breast was controlled by keeping it in a refrigerator (4 ^{o}C) for 3 hours before marination. There

Table 1: Six treatments of marinade

Ingredient lists	Ingredient amount (%)					
	Trt #1	Trt #2	Trt #3	Trt #4	Trt #5	Trt #6
Water	90	90	90	90	90	90
Corn starch	8	8	8	8	8	8
NaCl	2	0	0	0	0	0
STPP	0	2	0	0	0	0
Mango peel powder	0	0	2	0	0	0
Mango peel fibre	0	0	0	2	0	0
Pectin powder	0	0	0	0	2	0
70% Sorbitol syrup	0	0	0	0	0	2

All treatments were prepared in triplicate.

were 6 marinade treatments as shown in Table 1. All 6 treatments contained 90% distilled water with 8% food graded corn starch (Krauwangthip brand, Smutsakorn Thailand), the remaining 2% of each treatment was sodium chloride (NaCl, Univar®, Ajax Finechem Laboratory Chemicals, Australia), or sodium tripolyphosphate (STPP, Chemipan Corp, Bangkok, Thailand), or mango peel powder, or mango peel fibre, or purified pectin powder (Chemipan Corp, Bangkok, Thailand), or sorbitol syrup (70% sorbitol syrup, Krungthepchemi Co., Ltd, Thailand). The 200 g chicken breasts (4 pieces of each 50 g meat cut) were mixed with 15% marinade solution. The mixing was gently done by hand in a stainless steel bowl for 2 minutes, the bowl wrapped with plastic and was then put in a refrigerator for 45 minutes to let the meat absorb the marinade. Marinated chicken breasts were baked in a conventional oven (MEX oven, Pen K Life Center, Bangkok, Thailand) at 220 ^{o}C for 20 minutes, and internal temperature of the meat reached 75 ^{o}C. The cooked breasts were frozen using an air blast freezer (iRiNOX, Aerosia interpac Co., Ltd, Bangkok, Thailand) for 15 minutes. The frozen fully cooked products were packed in freezer bags and stored at -18 ^{o}C. All six marinating treatments were done in triplicate.

2.3 Physicochemical analysis

Moisture content

Moisture content of whole mango peel powder or extracted fibre from mango peel was analyzed based on the AOAC (2005) method. Briefly, the sample (5 g) was weighed in a pre-weighed moisture can, and dried at 105 ^{o}C overnight in a hot air oven. The moisture content was expressed as a percentage.

Water and oil absorption capacities

The water absorption capacity and oil absorption capacity of mango peel ingredients were determined by the methods described by Cheng and Bhat (2016), and Jan, Saxena and Singh (2016) with some modifications. Mango peel samples (2 g) were mixed in 20 mL distilled water or refined oil in a pre-weighed centrifuge tube. The mixture was stirred well and allowed to stand at room temperature (28±2 ^{o}C) for an hour. Then the samples were centrifuged at 2000 x g for 20 minutes. The supernatant was drained off and the sediment was weighed. The water absorption capacity or oil absorption capacity was expressed as weight of absorbed water or oil by one gram of mango peel sample.

pH and Colour measurement

The pH value of raw chicken breasts was recorded before marination using a pH meter (FiveEasy Plus, Mettler-Toledo, Kowloon, Hong Kong).

Meanwhile, colour (L*, a*, b*) of chicken breast was measured before marination, after cooking, and after thawing, using a handheld colorimeter. L*, a*, b* values of mango peel ingredients were also measured using a handheld colorimeter (CR-400 Chroma meter, Konica Minolta). All 4 chicken breast samples of each treatment were measured for colour L* a* b*. The experiment was conducted in triplicate.

2.4 Water holding capacity

Water holding capacity of each treatment was determined by comparing % marinade pick up, % cooking loss, and % thawing loss. The calculations were done using following equations;

$$\%\text{marinade pick up} = \frac{m_{marinated} - m_{raw}}{m_{raw}} \times 100 \quad (1)$$

$$\%\text{cooking loss} = \frac{m_{marinated} - m_{cooked}}{m_{marinated}} \times 100 \quad (2)$$

$$\%\text{thawing loss} = \frac{m_{cooked} - m_{thawed}}{m_{cooked}} \times 100 \quad (3)$$

$$\%\text{total loss} = \%\text{cooking loss} + \%\text{thawing loss} - \%\text{marinade pick up} \quad (4)$$

Where:
m_{raw} = weight of raw chicken breast before marination
$m_{marinated}$ = weight after 45-minute marination
m_{cooked} = weight after cooking in an oven
m_{thawed} = weight after thawing overnight in a refrigerator
The overall mass (4 chicken breast samples) of each treatment was determined, with three replicates.

Texture (hardness) analysis

Hardness of thawed chicken breast was measured by Texture Profile Analysis (TPA) following the method of Li (2006). The thawed chicken breast was cut into 1.5-cm cubes. Hardness of the sample was measured using a texture analyzer model TA-XT plus (Stable Micro Systems, United Kingdom), fitted with a 5-mm-diameter P/6 stainless steel cylindrical probe. The sample was placed on the plate so that its muscle fibres were aligned horizontally. The compression settings were: 2 mm s-$^{-1}$ pre-test speed, 1 mm s^{-1} test speed, 10 mm s^{-1} post-test speed, and distance was 70% strain. In each treatment, all 4 chicken breast samples were tested. The treatments were prepared in triplicate.

2.5 Statistical analysis

All data were reported as mean with standard deviation, and subjected to analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was used to identify significant differences, with defined statistical level as $\alpha = 0.05$. The statistical analysis was performed in SPSS Statistica 17.0 software.

3 Results and discussion

3.1 Physicochemical properties of whole mango peel powder and mango peel fibre

Water absorption capacity (WAC) of whole mango peel powder (4.57 g water/ g dry peel powder) (Table 2) was found to be higher than WAC of extracted mango fibre (1.47 g water/g fibre) ($P < 0.05$). However, the oil absorption capacities (OAC) of the two samples were not significantly different (0.7 - 0.9 g oil/ g dry sample). Essmat, Marwa and Ferial (2017) reported WAC and OAC of citrus (orange and lemon) peel powder from four different cultivars, which varied from 1.53 - 3.83 g water/ g dry sample, and 0.89 - 1.37 g oil/ g dry sample, respectively. In comparison to those results, whole mango peel powder exhibited outstanding WAC over peel powder from some citrus fruits. The ingredient with high WAC would be preferable to use in marinating, since it could help to increase water holding in the marinated meat. Moisture content of whole mango peel powder was significantly higher than moisture content of fibre extracted from mango peel. This may have been due to the double drying in fibre extraction process. This excess heat could have also given a darker colour in the extracted fibre sample ($P < 0.05$).

Table 2: Physicochemical properties of mango peel ingredients

Analysis	Mango peel powder	Mango peel fibre
WAC (g water/ g dry powder)	4.57 ± 0.08^{a}	1.47 ± 0.01^{b}
OAC (g oil/ g dry powder)	0.91 ± 0.19^{a}	0.70 ± 0.12^{a}
Moisture content (%)	7.23 ± 0.20^{a}	5.13 ± 0.21^{b}
L* value	62.96 ± 0.54^{a}	52.82 ± 0.49^{b}
a* value	4.19 ± 0.24^{a}	0.23 ± 0.09^{b}
b* value	26.13 ± 1.46^{a}	20.50 ± 0.21^{b}

Values are mean ± standard deviation (n=3). Mean values in the same row sharing different superscripts were significantly different ($P < 0.05$).

Table 3: Water holding capacity of chicken breast marinated in different treatments

Treatment	% Marinade pick-up (1)	% Cooking loss (2)	% Thawing loss (3)	% Total loss (3)+(2)-(1)
1. STPP	3.90 ± 0.56^{ab}	30.37 ± 3.37^{a}	1.01 ± 0.45^{a}	27.47 ± 3.75^{bc}
2. NaCl	4.74 ± 1.32^{a}	28.93 ± 6.04^{a}	2.10 ± 1.24^{a}	26.29±6.39 c
3. Mango peel fibre	3.14 ± 0.23^{ab}	33.61 ± 2.46^{a}	2.34 ± 0.54^{a}	32.81 ± 2.55^{abc}
4. Mango peel powder	3.16 ± 0.73^{ab}	35.01 ± 1.26^{a}	2.08 ± 0.78^{a}	33.93 ± 1.94^{ab}
5. Pectin	3.76 ± 0.29^{ab}	36.65 ± 2.26^{a}	2.16 ± 0.42^{a}	35.05 ± 2.51^{a}
6. Sorbitol Syrup	2.14 ± 1.15^{b}	36.30 ± 2.19^{a}	1.95 ± 0.31^{a}	36.11 ± 3.53^{a}

Values are mean ± standard deviation (n=3). Mean values in the same row sharing different superscripts (a, b, c) were significantly different ($P < 0.05$).

3.2 Water holding capacity

Water holding capacity of samples in different marinades were determined in terms of %marinade pick up, %cooking loss, %thawing loss, and %total loss (Table 3). After 45-minute marinating in a refrigerator, chicken breast marinated with 2% NaCl showed the highest marinade pick up. Meanwhile, 2% sorbitol syrup gave the lowest pick up ($P < 0.05$), in contrast to its well-recognized absorbent functionality. The marinade pick up of all treatments reported in this study ranged from 2.14 to 4.74%, which corresponded well to the result with chicken carcass of 4.09% (U-chupaj et al., 2017). However, vacuum marinating could increase marinade uptake to be 5.6 - 11.8% as reported by Jarvis et al. (2012). Water holding capacity in samples marinated with whole mango peel powder and extracted mango fibre were similar ($P > 0.05$). Chicken breast in 2% pectin marinade presented the highest cooking loss (36.65%), while the highest thawing loss was found in the sample with 2% mango peel fibre (2.34%). The highest cooking loss was found in 2% pectin marinade solution, which contrasted to the hypothesis in which pectin could help holding water through interaction between negative charge of carboxylic group and calcium ions to form an "egg box" structure, according to Rolin (1993). Zheng, Toledo and Wicker (1999) reported the limitation of using pectin in a chicken breast marinade due to the difficulty of pectin distribution. However, mango peel ingredients might provide pectin with better distribution, since lower cooking loss was found. According to %total loss shown in Table 3, as expected, NaCl and STPP were the best two ingredients which gave the least loss. However, whole mango peel powder and mango peel fibre tended to provide better yields than pectin and sorbitol syrup.

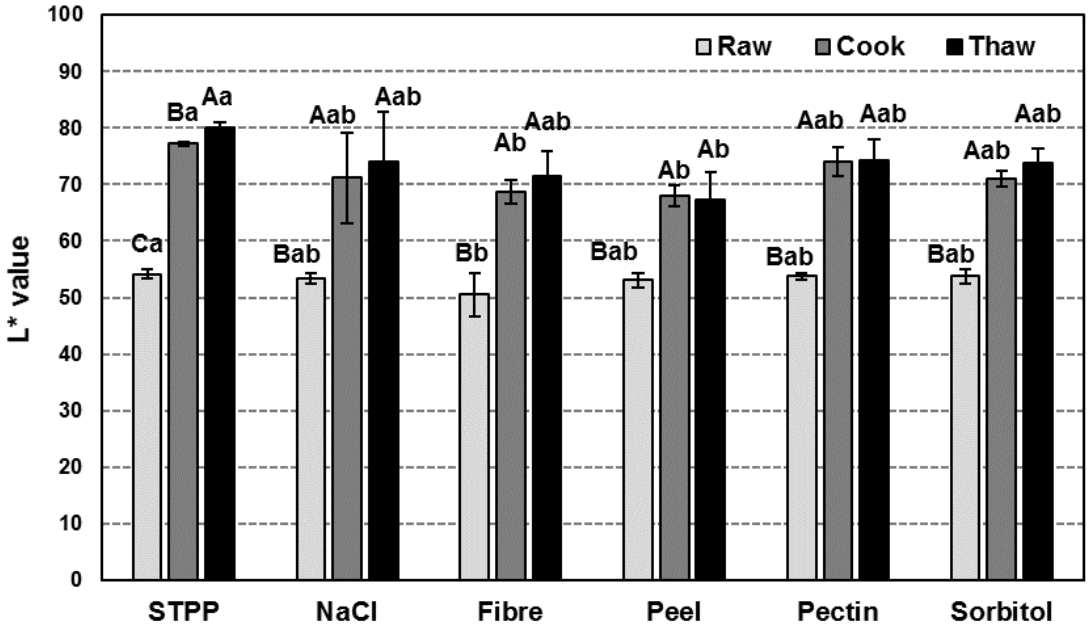

Figure 1: Effect of marinade treatments on lightness (L* value) of chicken breasts. Treatments sharing different letters (A, B, C) and (a, b) were significantly different from each other within the same marinade and across different marinades, respectively ($P < 0.05$).

3.3 pH and colour

The pH values of raw chicken breasts used in all treatments varied in a narrow range of 6.02 - 6.07 (data not shown), and the L*, a*, b* values of raw chicken breast used in the six treatments were not significantly different, which indicated the colour uniformity of raw material. Colour measurements are shown in Figures 1 - 3. Cooking significantly increased lightness (L* value), and thawing did not affect the lightness ($P > 0.05$) except STPP treatment where the lightness significantly increased after thawing (Figure 1). The lowest L* value was found in the treatments with both mango ingredients ($P < 0.05$), which corresponded to the darker colour of dried mango peel and extracted mango fibre. A similar darker colour in chicken breast was made with addition of plum extract puree (Lee & Ahn, 2005). However, organoleptic testing would be necessary to identify whether this darker colour affected consumer acceptance. The a* values (redness) of chicken breast decreased after cooking, except for the two treatments with mango peel ingredients ($P < 0.05$) (Figure 2). Moreover, in 5 out of 6 treatments, the redness slightly increased after thawing ($P > 0.05$). The change in b* values from raw meat to cooking and thawing in all six treatments followed the same trend (Figure 3). As shown, yellow colour developed on chicken breast after cooking ($P < 0.05$), and the colour after thawing remained more intense than the raw. Among six treatments, marinating with STPP seemed to give a whiter colour, considering the highest lightness, and lowest red and yellow intensity. According to Figure 3, the levels of colour intensity in the yellow region were higher than the levels of redness (Figure 2), hence the yellow colour seemed to be dominant over the redness.

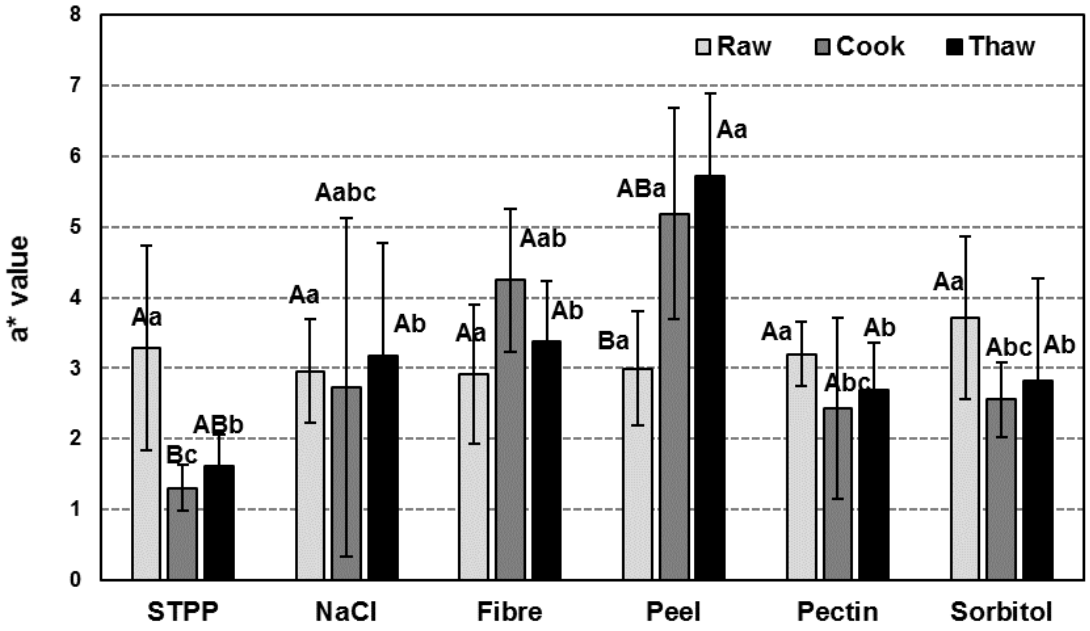

Figure 2: Effect of marinade treatments on red colour intensity (a* value) of chicken breasts. Treatments sharing different letters (A, B) and (a, b, c) were significantly different from each other within the same marinade and across different marinades, respectively ($P < 0.05$).

3.4 Texture profile analysis

With respect to ready-to-eat aspects, the hardness of the samples was measured after thawing (Table 4). Highest shear force was found in samples with sorbitol syrup (10.45 N), and STPP (10.38 N) marination, while the lowest force (7.13 N) was in mango peel fibre ($P < 0.05$). The hardness recorded in this study was similar to that reported by Jarvis et al. (2012), in which the shear force varied from 7.2 - 9.2 N. Nunez de Gonzalez, Boleman, Miller, Keeton and Rhee (2008) concluded that increasing plum ingredient in ham marinade contributed to higher cooking loss and higher shear force. However, their conclusion did not seem to apply to the results in this study, especially when marinating with mango ingredients. To clarify, hardness of chicken breasts marinated with mango fibre was less than the samples in STPP marinade ($P < 0.05$), even the STPP marinade exhibited higher water holding capacity. Although water-holding capacity in chicken breast samples marinated with whole mango peel powder could not fully match STPP and NaCl, hardness of the samples was similar ($P > 0.05$).

4 Conclusions

Mango peel ingredients were investigated for potential ingredients that could replace salt and phosphate in chicken marinade. Purified pectin and sorbitol syrup were used in this study to compare the water holding capacity in marinated chicken breasts. It was found that mango peel ingredients provided better water holding capacity than purified pectin and sorbitol syrup. After thawing, L*, a*, b* values of chicken breasts marinated with mango peel ingredients were similar to the colour values of chicken breasts marinated with STPP and salt ($P > 0.05$). Although both mango peel powder and mango fibre were better than STPP and salt in chicken marination, when

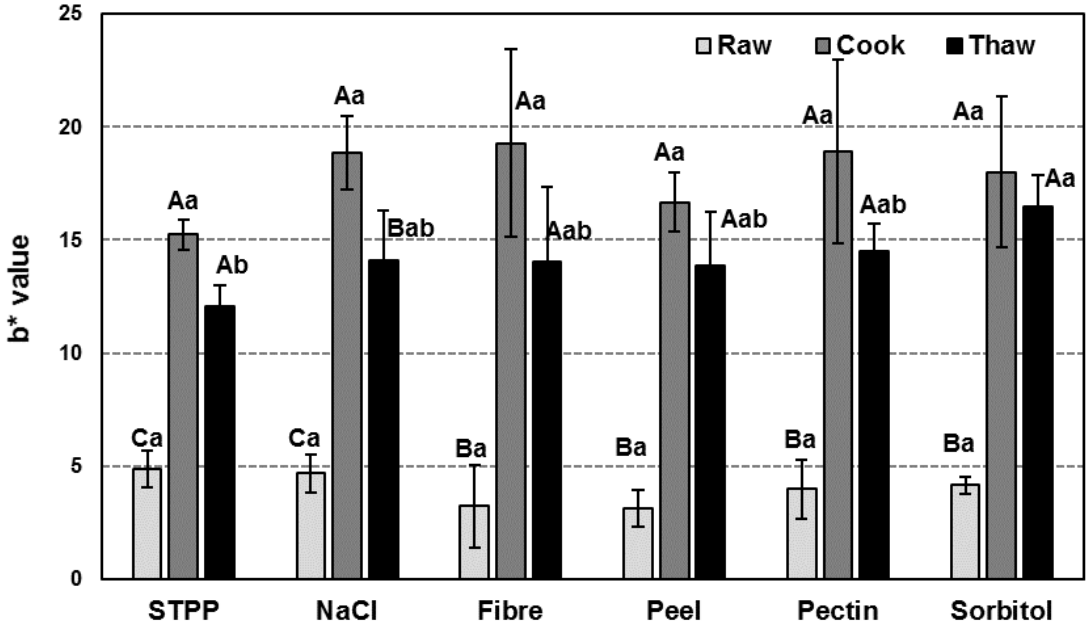

Figure 3: Effect of marinade treatments on yellow colour intensity (b* value) of chicken breasts. Treatments sharing different letters (A, B, C) and (a, b) were significantly different from each other within the same marinade and across different marinades, respectively ($P < 0.05$).

Table 4: Shear force of chicken breast marinated in different treatments

Treatment	Hardness (N)
1. STPP	10.38 ± 0.54^{a}
2. NaCl	8.39 ± 0.45^{ab}
3. Mango peel fibre	7.13 ± 1.12^{b}
4. Mango peel powder	9.94 ± 0.73^{a}
5. Pectin	8.94 ± 0.12^{ab}
6. Sorbitol Syrup	10.45 ± 1.17^{a}

Values are mean ± standard deviation (n=3). Mean values in the same row sharing different superscripts (a, b, c) were significant different ($P < 0.05$).

considering the feasibility in use, whole mango peel powder would be a better choice over mango peel fibre, due to the higher yield and lower cost of ingredient preparation. The findings from this study should encourage an interest in utilizing mango peel in the food industry. This would not only add value to the waste, but also incorporate a low-sodium, low-phosphate, and natural plant-based ingredient to address current consumers' requirements.

Acknowledgements

Thanks are due to technicians from Department of Food Technology and Nutrition, Maha Sarakham University, for providing chemicals and analytical training.

References

Acton, J. C. & Jensen, J. M. (1994). Understanding marinade technology. *Poult. Proc. 9*, 18–21.

Ajila, C. M., Aalami, M., Leelavathi, K. & Rao, U. J. S. P. (2010). Mango peel powder: A potential source of antioxidant and dietary fiber in macaroni preparations. *Innovative Food Science & Emerging Technologies*, *11*(1), 219–224. doi:10.1016/j.ifset.2009.10.004

Ajila, C. M., Naidu, K. A., Bhat, S. G. & Rao, U. P. (2007). Bioactive compounds and antioxidant potential of mango peel extract. *Food Chemistry*, *105*(3), 982–988. doi:10.1016/j.foodchem.2007.04.052

AOAC. (2005). Official methods of analysis (18th ed.). Association of Official Analytical Chemist. Arlington, VA, USA.

Cheng, Y. F. & Bhat, R. (2016). Functional, physicochemical and sensory properties of novel cookies produced by utilizing underutilized jering (pithecellobium jiringa jack.) legume flour. *Food Bioscience*, *14*, 54–61. doi:10.1016/j.fbio.2016.03.002

Essmat, A. A.-A., Marwa, H. M. & Ferial, M. A.-S. (2017). Functional properties of citrus peel as affected by drying methods. *American Journal of Food Technolog*, *12*, 193–200. doi:10.3923/ajft.2017.193.200

Geerkens, C. H., Nagel, A., Just, K. M., Miller-Rostek, P., Kammerer, D. R., Schweiggert, R. M. & Carle, R. (2015). Mango pectin quality as influenced by cultivar, ripeness, peel particle size, blanching, drying, and irradiation. *Food Hydrocolloids*, *51*, 241–251. doi:10.1016/j.foodhyd.2015.05.022

Jan, R., Saxena, D. C. & Singh, S. (2016). Physico-chemical, textural, sensory and antioxidant characteristics of gluten - free cookies made from raw and germinated chenopodium (chenopodium album) flour. *LWT - Food Science and Technology*, *71*, 281–287. doi:10.1016/j.lwt.2016.04.001

Jarvis, N., Clement, A. R., O'Bryan, C. A., Babu, D., Crandall, P. G., Owens, C. M., ... Ricke, S. C. (2012). Dried plum products as a substitute for phosphate in chicken marinade. *Journal of Food Science*, *77*(6), S253–S257. doi:10.1111/j.1750-3841.2012.02737.x

Kanatt, S. R. & Chawla, S. P. (2018). Shelf life extension of chicken packed in active film developed with mango peel extract. *Journal of Food Safety*, *38*(1). doi:10.1111/jfs.12385

Kermani, Z. J., Shpigelman, A., Huong, T. T. P., Van Loey, A. M. & Hendrickx, M. E. (2015). Functional properties of citric acid extracted mango peel pectin as related to its chemical structure. *Food Hydrocolloids*, *44*, 424–434. doi:10.1016/j.foodhyd.2014.10.018

Lee, E. J. & Ahn, D. U. (2005). Quality characteristics of irradiated turkey breast rolls formulated with plum extract. *Meat Science*, *71*(2), 300–305. doi:10.1016/j.meatsci.2005.03.017

Li, C. T. (2006). Myofibrillar protein extracts from spent hen meat to improve whole muscle processed meats. *Meat Science*, *72*(3), 581–583. doi:10.1016/j.meatsci.2005.08.008

Nagel, A., Mix, K., Kuebler (Nee Wulfkuehler), S., Bogner, H., Kienzle, S., Elstner, P., ... Neidhart, S. (2015). The arabinogalactan of dried mango exudate and its co-extraction during pectin recovery from mango peel. *Food Hydrocolloids*, *46*, 134–143. doi:10.1016/j.foodhyd.2014.11.029

Nunez de Gonzalez, M. T., Boleman, R. M., Miller, R. K., Keeton, J. T. & Rhee, K. S. (2008). Antioxidant properties of dried plum ingredients in raw and precooked pork sausage. *Journal of Food Science*, *73*(5), H63–H71. doi:10.1111/j.1750-3841.2008.00744.x

Rolin, C. (1993). Pectin, industrial gums: Polysaccharides and their derivatives. Academic Press, New York.

Tarté, R. (2009). Meat-derived protein ingredients. (pp. 145–171). doi:10.1007/978-0-387-71327-4_7

U-chupaj, J., Malila, Y., Petracci, M., Benjakul, S. & Visessanguan, W. (2017). Effect of tumbling marination on marinade uptake of chicken carcass and parts quality. *Brazilian Journal of Poultry Science*, *19*(1), 61–67. doi:10.1590/1806-9061-2016-0380

Wang, M., Huang, B., Fan, C., Zhao, K., Hu, H., Xu, X., ... Liu, F. (2016). Characterization and functional properties of mango peel pectin extracted by ultrasound assisted citric acid. *International Journal of Biological Macromolecules*, *91*, 794–803. doi:10.1016/j.ijbiomac.2016.06.011

Zheng, M., Toledo, R. & Wicker, L. (1999). Effect of phosphate and pectin on quality and shelf-life of marinated chicken breast. *Journal of Food Quality*, *22*(5), 553–564. doi:10.1111/j.1745-4557.1999.tb00186.x

International Journal of Food Studies | IJFS April 2020 | Volume 9 | pages 203–212

The Effect of Gamma Irradiation on the Essential Oils and Antioxidants in Dried Thyme

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Received: 27 September 2018; Published online: 18 April 2020

Abstract

This research was undertaken to investigate the effect of gamma rays at dose rates of 5.0, 10.0 and 15.0 kGy on the chemical composition of essential oils, total antioxidants, total flavonoids and total phenols, as well as the antioxidant activity and the thiobarbituric acid (TBA) and the free radical-scavenging activity (DPPH) of thyme. Radiation processing increased the total phenols, total flavonoids and total antioxidants of thyme, and moderate changes were detected at doses of 5 and 10 kGy for the essential oils. Thymol was sensitive to irradiation, especially at 15.0 kGy doses. In addition, the evaluation of antioxidant activity using DPPH radical-scavenging activity indicated some decreases of antioxidant activity in irradiated samples, while thyme exposed to doses of 10 and 15 kGy exhibited a significant increase in TBA values. The irradiation process can facilitate the utilisation of thyme as a preservative ingredient in the food and pharmaceutical industry.

Keywords: Irradiation; Thyme; Essential oil; Antioxidant; Flavonoid; Phenol

1 Introduction

The long history of herbs and spices has demonstrated their safe usage and excellent source for antioxidants. Thyme (*Thymus vulgaris* L.) is an herb descended from the *Lamiaceae* family. Thyme can be consumed as a whole spice/herb, or it can be ground, extracted, encapsulated or used as an emulsion. Thyme is characterized by its phytochemicals, efficacy as an antioxidant, and possession of phenolic compounds and flavonoids. Therefore, it has been determined to be a prominent herb from a medicinal and aromatic perspective (Embuscado, 2015).

Antioxidants are essential substances that inhibit other compounds from being oxidized (Aqil, Ahmad & Mehmood, 2006). Furthermore, the antioxidants produced by spices and herbs usually act with free radicals created in the initiation phase of autoxidation (Lee, Umano, Shibamoto & Lee, 2005). The antioxidants from thyme methanolic extracts can significantly prevent peroxidation in lipids (Fejes et al., 2000).

DPPH radical-scavenging activity is employed worldwide as an antioxidant activity assay; using this assay, a correlation between phenolic content and free radical-scavenging in nine plant extracts was observed. The chemical analysis of the extracts indicated the presence of phenolics, tannins, flavonoids, glycosides and alkaloids. The phenolic concentrations in dry plant extracts varied from 28.66 to 169.67 mg g^{-1} (Aqil et al., 2006).

Thyme's essential oils are distinguished by having a high content of important compounds, such as *thymol, carvacrol, y-terpinene* and *p-cymene.* These compounds range from 57.3% to 62.5% of the total oil content (Senatore, 1996). How-

 10.7455/ijfs/9.1.2020.a6

Nomenclature

TBA	Thiobarbituric acid number	FID	flame ionization detectors
DPPH	2, 2-diphenyl-1-picrylhydrazyl	GAE	Gallic acid equivalent
GLC	gas-liquid chromatography	IC 50	The half maximal inhibitory concentration

ever, it has been reported that thyme contains thymol and carvacrol in the 44-60% and 2.2-4.2% range, respectively, which can therefore be used to control lipid oxidation in foods (Alcicek, 2011). In addition, the prominent compounds quantitatively constitute approximately three-quarters of the total volatile compounds: *thymol* (72%) and *carvacrol (isothymol)* (5.7%) (Baranauskiene, Venskutonis, Viskelis & Dambrauskiene, 2003).

Although synthetic antioxidants can be used to prevent oxidation, they may not contribute additional nutritional benefits. On the contrary, the body can easily assimilate natural antioxidants, which are produced by spices and herbs, such as thyme (Embuscado, 2015).

Furthermore, thyme has long been used to preserve food and beverages via its phytochemicals. It has also been used as a natural food ingredient due to its colour and aroma (Alcicek, 2011).

Numerous aromatic substances have been generated from thyme extract. The extract consists of three acids, three aldehydes, four ketones and esters, seven alcohols, 14 aromatic compounds, 16 sesquiterpenes and 43 monoterpenes. In fact, the primary aroma in thyme is derived from phenolic compounds (Baranauskiene et al., 2003).

Food irradiation is a process involving the use of ionizing radiation, such as gamma rays, to generate various beneficial effects. The process can minimise post-harvest and storage losses, ensure hygienic quality, extend shelf life, simplify the trade of food products and enhance the parasitological and microbiological safety of foods by diminishing the effect of spoilage from microorganisms. Moreover, dried foods, herbs and spices can be exposed to ionizing radiation as an accredited preservation process (Nagy, Solar, Sontag & Koenig, 2011). Any of these effects depends on the radiation dose absorbed. Among all irradiated commercial products, irradiated spices and vegetable seasonings are the most commonly used. However, for commercial use, the typical dose is 10 kGy (Shurong, Meixu & Chuanyao, 2006).

Due to the importance of antioxidants in herbs and spices, Nagy et al. (2011) confirmed that radiation has no significant effect on their antioxidant properties nor on the division in the bonds of the glycoside. In fact, many types of herbs and spices exhibit a level of radioprotection even when consumed before radiation exposure (Farag, 2013).

The present study aimed to investigate the effect of gamma rays at dose rates of 5, 10 and 15 kGy on the chemical composition of essential oils, total antioxidants, total flavonoids and total phenols, as well as on the antioxidant activity of dried thyme (*Thymus vulgaris* L.).

2 Materials and Methods

2.1 Plant material

Samples of fresh aerial parts of thyme (*Thymus vulgaris* L.) were collected from a grocery market in Riyadh city, Saudi Arabia. The samples were prepared by washing and drying them in the shade. Then, 250 grams of plant material were placed in a series of polyethylene bags. For the chemical analysis, the bags were divided into groups; each sample had five replicates.

2.2 Irradiation process

A Gamma Cell was used for irradiation, delivering a dose rate of 14.2514 kGy h^{-1} at the time of the experiment. (Model Gamma Cell-220 from MDS, Nordion Initial, Canada: Activity source (Co-60) was 24,000 Ci of the production in December 1993) The Gamma Cell at King Abdul Aziz City for Science and Technology in Riyadh was used to expose the thyme samples, except the control sample, to 5, 10 or 15 kGy of gamma radiation.

2.3 Chemical analysis

After extracting the essential oils from the treated samples of thyme, the samples were analysed via gas-liquid chromatography (GLC).

2.4 Essential oil extraction

Each sample of dried thyme was placed in a flask filled with double-distilled water. Steam distillation was continuously applied for three hours until the oil was isolated; then, it was dried over anhydrous sodium sulphate (Pellegrini et al., 2003).

2.5 GLC analysis

Authentic essential oils were obtained from Dragoc (Holzminden, Germany) and were analysed using GC Pye-Unicam gas chromatography dual-flame ionization detectors (FID) with a chromate graph fitted with a coiled glass column (1.5 m x 4 mm) and packed with a 100-120 diatomitec mesh coated with 10% PEGA. The oven was programmed to gradually increase in temperature from 60 ^{o}C to 180 ^{o}C by 4 ^{o}C min^{-1}, and the isothermal process was continued for 15 minutes at 180 ^{o}C. The temperature for the detector was 220 ^{o}C, while that for the injector was 30 ^{o}C. The gas flow rate was 33 mL min^{-1} for hydrogen and 30 mL min^{-1} for nitrogen and air. After mixing the extracted essential oils with their main components, they were injected into the GLC to verify the resultant peaks (Jayaprakasha, Rao & Sakariah, 2002). For accuracy, the analysis was repeated five times.

2.6 Preparing samples for chemical analysis

Thirty grams of dried thyme was exposed to radiation in various doses and then weighed; they were then extracted by mixing them with distilled water, then stirring and turning them for 15 minutes, after which they were separated in centrifugal concentrators for 10 minutes (1000 × g). Afterwards, they were re-extracted several times and kept as an aqueous extract for subsequent tests. After filtering and extraction, 110 mL were obtained, and five replicates were made after each test analysis (Pellegrini et al., 2003).

2.7 Total antioxidant activity

The antioxidant content was estimated as equivalent to quercetin, which was used by Meda, Lamien, Romito, Millogo and Nacoulma (2005), by adding 0.75 mL of aqueous extract to 1.5 mL of a 2,2,-diphenyl-2-picryl-hydrazil (DPPH) solution in methanol at a 0.02-mg mL^{-1} concentration. Then, the mixture was left at room temperature for 15 minutes, after which the absorbance was read via a spectrophotometer with a wavelength of 517 nm and with 0.75 mL of water + 1.5 mL of methanol as a blank. The results were compared with similar cases when using the quercetin 6 mg mL^{-1} concentration.

2.8 Total phenolic assay

Determination the total phenolic content of thyme samples was accomplished using the Folin-Ciocalteu assay (Singleton & Rossi, 1965). One mL of the extract or a standard solution of gallic acid was added to 9 mL of distilled water in a 25 mL volumetric flask. A reagent blank was prepared using distilled water. The mixture was shaken after adding 1 mL of Folin-Ciocalteu phenol reagent. Then, 10 mL of 7% Na_2CO_3 solution was added to the mixture after 5 minutes. After incubating the mixture at room temperature for 90 minutes, the prepared reagent blank had a specified absorbance at 750 nm. One mg of gallic acid equivalent to GAE.100 g^{-1} of dried weight of thyme was used to express the

total phenolic content. For each determination, five samples were used (Meda et al., 2005).

2.9 Total flavonoid assay

An aluminum chloride solution (5 mL) was added to methanol at 2%, and the mixture was left for 10 minutes. Absorbance was read at a wavelength of 415 nm. A mixture of 5 mL of each methanol and the extraction was used as a blank. The results were then compared to quercetin at a concentration of 6.25 μg mL^{-1}. For each determination, five samples were used (Meda et al., 2005).

2.10 Radical scavenging activity

Antioxidant activity was estimated using the method of Meda et al. (2005), with some modification, i.e., the use of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The formula used to calculate the activity was:

$$\%Inhibition = [(AC - AS).AC^{-1}] \times 100 \quad (1)$$

AC: the absorbance value of the control.
AS: the absorbance value of the test solution.
Subsequently, the half maximal inhibitory concentration IC_{50} was calculated by evaluating the DPPH radical scavenging activity at different concentrations of the water extracts.

2.11 Thiobarbituric acid value

The analysis was accomplished by the oxidative reaction products, which were determined as the thiobarbituric acid number (TBA). Two mL of trichloroacetic acid (20% aq.) and 2 mL of thiobarbituric acid solution (0.67% aq.) were added to 1 mL of thyme extract. The mixture was submerged in a boiling water bath for 10 minutes. Then, the mixture was cooled to ambient temperature before centrifugation of the 500 g mixture at 3000 rpm for 20 minutes. For each determination, five samples were used (Zin, Abdul-Hamid & Osman, 2002).

2.12 Statistical analysis

The data from the experiment were analysed using SAS system version 9.1.3. (Cary, NC), to calculate means, standard deviations and least significant differences. Results were expressed as mean ± (standard deviation) (SD) considering a P value of ≤ 0.05 as significant (Ott, 1984).

3 Results and Discussion

3.1 Essential oils

At low doses, the irradiation process is considered to be a cold, physical treatment for food because no significant heating occurs as a result of treating the samples. Therefore, irradiation has no direct effect on flavour compounds (Al-Bachir, 2016).
The important compounds were thymol (39.52 ± 0.15 mg .100 g^{-1}), *p-cymene* (21.60 ± 0.28 mg .100 g^{1}) and y-terpinene (18.41 ± 0.07 mg .100 g^{-1}), which constituted almost three-quarters of the total quantified volatiles, followed by monoterpenes, P-caryophyllene (2.71 ± 0.08 mg .100 g^{-1}), carvacrol (2.45 ± 0.07 mg .100 g^{-1}) and trans-sabinene hydrate (2.30 ± 0.14 mg .100 g^{-1}). Major volatile constituents, such as thymol, y-terpinene and carvacrol, were distinctive for *T. vulgaris* species and were also found as major compounds in other published results (Baranauskiene et al., 2003).
The results showed that radiation had significant effects on the concentrations of some compounds in the content of the dried thyme before and after different radiation doses. Table 1 shows the chemical composition of the essential oils of thyme after radiation at 5, 10 and 15 kGy compared with non-irradiated samples.
Thymol was stable when exposed to doses of 5 and 10 kGy (39.0 ± 0.165, 39.11 ± 0.087 mg .100 g^{-1}, respectively), yet its concentration decreased significantly when exposed to higher doses as compared with non-irradiated samples (38.50 ± 0.068, 39.52 ± 0.150 mg .100 g^{-1}, respectively). This result is consistent with the determination of 10 kGy as the commercial dose of dried spices, set by the World Health Organization World Health Organization (1988), needed

to reduce the loss of thymol, which is considered one of the most important compounds for inhibiting oxidation (Lee et al., 2005).
The concentrations of trans-sabinene hydrate were stable when exposed to doses of 5, 10 and 15 kGy (2.29 ± 0.224, 2.28 ± 0.035, 2.18 ± 0.012 mg .100 g^{-1}, respectively) compared with non-irradiated samples (2.30 ± 0.141 mg .100 g^{-1}).
The concentrations of *p-cymene* increased after exposure to a dose of 10 kGy (22.24 ± 0.230 mg .100 g^{-1}); a tendency towards reduction after exposure to a dose of 15 kGy (22.20 ± 0.135 mg .100 g^{-1}) was also shown. The lack, or small effect, of irradiation on the thyme aroma compounds is in agreement with Pereira et al. (2016).
The concentrations of y-terpinene decreased in samples exposed to a dose of 5 kGy compared with the control sample, while there was no obvious difference when exposed to doses of 10 and 15 kGy. There was also a reduction of carvacrol for all samples compared to the control, with the least-affected samples receiving a dose of 10 kGy. Generally, irradiation adversely affected the total content of the essential oils of dried thyme.

3.2 The total phenols, total flavonoids and total antioxidants

The results show the effects of Y-irradiation treatments on total phenols, flavonoids and antioxidants (Table 2).
The results illustrated that irradiated dried thyme at 5, 10 and 15 kGy had higher levels of phenolic compounds than the non-irradiated control sample in the methanolic extract. The significant increase in the phenolic content was 4954.67 ± 0.072, 5010.71 ± 0.015 and 4986.22 ± 0.075 mg .100 g^{-1} for the samples irradiated at 5, 10 and 15 kGy, respectively, compared to their content in the non-irradiated control (4925.73 ± 0.048 mg .100 g^{-1}). The highest total phenolic content occurred at a dose of 10 kGy.
The increase in the total phenolic content could be attributed to the inducement of a chemical reaction that decomposed the large molecules into small molecules, which are easily soluble in methanol and thus produce more solutes. This explanation is correlated with Huang and Mau (2006) findings. Moreover, Kim, Yook and Byun (2000) found that the total methanolic extract in 15 kinds of Korean medicinal herbs using various solvents increased by 5-25% at a dose of 10 kGy of γ-irradiation.
Gamma radiation causes an increase of soluble phenols in some spice extracts (Variyar, Limaye & Sharma, 2004). On the contrary, in some studies, no significant change was observed when comparing the phenolic content in the non-irradiated samples with that of irradiated samples at a dose of 20 kGy in *Agaricus blazei* (Huang & Mau, 2007), 30 kGy in *Rosmarinus officinalis* L. powder (Perez, Calderon & Croci, 2007), and 5 to 30 kGy in *Carum carvi* L. and *Laurus nobilis* L. (Polovka & Suhaj, 2010).
The total flavonoid content for thyme irradiated with a dose of 5, 10 and 15 kGy increased significantly by 43.66 ± 0.035, 47.42 ± 0.043 and 40.81 ± 0.039 mg .100 g^{-1}, respectively. Whereas in the non-irradiated sample, it was 36.41 ± 0.026 mg .100 g^{-1}. The maximum increase was obtained at the 10 kGy dose: 47.42 ± 0.043 mg .100 g^{-1}. However, these findings are in contrast to those of Zhu, Cai, Bao and Corke (2010), who reported a decrease in flavonoid content at a dose of 2 kGy and a minimum content at a dose of 10 kGy.
Total antioxidant content for thyme irradiated with 5, 10 and 15 kGy doses significantly increased, reaching levels of 2402 ± 0.880, 2419.66 ± 0.152 and 2408 ± 0.234 mg .100 g^{-1}, respectively. Whereas in the non-irradiated sample, it was 2392.25±0.027 mg .100 g^{-1}, with a maximum increase obtained at a 10 kGy dose, 2419.66 ± 0.152 mg .100 g^{-1}, as shown in Table 2. However, Taipina, Lamardo, Rodas and del Mastro (2009) reported no antioxidant content loss when irradiating pecan nuts with doses between 1-3 kGy.
The DPPH radical-scavenging activity and IC_{50} for irradiated thyme are shown compared with non-irradiated samples in Table 3.
The results indicate that the DPPH radical-scavenging activity of methanolic thyme extracts for an irradiated sample at doses of 5, 10 and 15 kGy were 0.58 ± 0.001, 0.56 ± 0.001 and 0.60 ± 0.003 % less than that for the non-irradiated control: 0.62 ± 0.001 %. A similar trend was observed in Abolhasani, Barzegar and Sahari

Table 1: The chemical composition of the essential oils of dried thyme irradiated with various doses of γ-irradiation

Compound	Quantity (mg.100 g^{-1} dry weight) Radiation Dose (kGy)				
	Control	5 KGy	10 KGy	15 KGy	*P*- Value
a-Thujene	1.61 ± 0.175	1.75 ± 0.156	1.99 ± 0.069	2.10 ± 0.025	0.00
a-Pinene	1.32 ± 0.109	1.90 ± 0.069	2.16 ± 0.089	2.01 ± 0.022	0.00
Myrcene	2.11 ± 0.077	2.15 ± 0.036	2.62 ± 0.168	2.60 ± 0.115	0.00
a-Teroinene	1.74 ± 0.139	1.99 ± 0.010	2.23 ± 0.154	2.33 ± 0.064	0.00
p-cymene	21.60 ± 0.282	21.90 ± 0.07	22.24 ± 0.230	22.20 ± 0.135	0.00
y-Terpinene	18.41 ± 0.076	18.21 ± 0.044	18.39 ± 0.048	18.36 ± 0.038	0.00
trans-Sabinene hydrate	2.30 ± 0.141	2.29 ± 0.224	2.28 ± 0.035	2.18 ± 0.012	0.482*
Linalol	1.42 ± 0.025	1.30 ± 0.052	1.21 ± 0.06	1.15 ± 0.018	0.00
Borneol	1.18 ± 0.01	1.22 ± 0.014	1.01 ± 0.01	0.98 ± 0.034	0.00
Thymol	39.52 ± 0.150	39.0 ± 0.165	39.11 ± 0.087	38.50 ± 0.068	0.001
Carvacrol	2.45 ± 0.079	1.14 ± 0.046	1.76 ± 0.063	1.55 ± 0.044	0.00
P-Caryophyllene	2.71 ± 0.08	3.55 ± 0.015	2.30 ± 0.041	2.13 ± 0.078	0.00
Total	96.37 ± 1.288	96.436 ± 1.20	97.33 ± 0.571	96.09 ± 0.104	0.212

(*) There was no significant difference between *trans-Sabinene hydrate* compound groups.
Values expressed as means ± SD (standard deviation). Repetition number = 5.
Values are significant differences (P $\leq$ 0.001).

Table 2: Total phenols, flavonoids and antioxidants of methanolic thyme extracts irradiated with doses of gamma radiation

Radiation Dose (kGy)	Contents (mg.100 g^{-1} dry weight)		
	Total Phenols	Total Flavonoids	Total Antioxidants
Control	4925.73 ± 0.048	36.41 ± 0.026	2392.25 ± 0.027
5 kGy	4954.67 ± 0.072	43.66 ± 0.035	2402 ± 0.880
10 kGy	5010.71 ± 0.015	47.42 ± 0.043	2419.66 ± 0.152
15 kGy	4986.22 ± 0.075	40.81 ± 0.039	2408 ± 0.234
P - Value	0.00	0.00	0.00

Values expressed as means ± SD. Repetition number = 5.
Values are significant differences (P $\leq$ 0.05).

Table 3: Scavenging activity and IC_{50} values of methanolic extract of thyme irradiated (10 mg mL^{-1}) against DPPH radicals

Radiation Dose (kGy)	% of DPPH scavenging activity	IC_{50} (mg mL^{-1})
Control	0.62 ± 0.001	0.27 ± 0.002
5	0.58 ± 0.001	0.46 ± 0.001
10	0.56 ± 0.001	0.51 ± 0.001
15	0.60 ± 0.003	0.37 ± 0.001
P - Value	0.00	0.00

IC_{50} value: the effective concentration at which the antioxidant activity was 50%; the (DPPH) radical was scavenged by 50%.
Values expressed as means ± SD. Repetition number = 5.
Values are significant differences ($P \leq 0.05$).

Table 4: TBA values of methanolic extract of thyme as a function of irradiation dose.

Radiation Dose (kGy)	TBA number (A_{532}) mean±SD
Control	0.77 ± 0.002
5	0.51 ± 0.002
10	0.98 ± 0.003
15	0.90 ± 0.003
P - Value	0.00

Values expressed as means ± SD. Repetition number = 5.
Values are significant differences ($P \leq 0.05$).

(2018), who found that DPPH activity decreased for irradiated pistachio green hull extracts at a dose of 10 kGy, then increased at a dose of 20 kGy. In contrast, there was an increase in the IC_{50} values of methanolic thyme extracts for the same doses, with the highest value recorded at a dose of 10 kGy.

A study conducted by Huang and Mau (2006) revealed that DPPH radical-scavenging activity exhibited no significant change as a result of irradiating freeze-dried mushrooms at doses from 2.5 to 20 kGy. Conversely, some studies reported an increase in DPPH radical-scavenging activity as a result of irradiating soybeans, green tea leaf extracts and rosemary leaf powder extracts at doses of 0.5-5 kGy, 10-20 kGy and 30 kGy, respectively (Jo, Son, Lee & Byun, 2003; Perez et al., 2007; Variyar et al., 2004).

Table 4 shows that exposing thyme to doses of 10 and 15 kGy gave a significant increase in the TBA numbers, which reached 0.98 ± 0.003 and 0.90 ± 0.003, respectively. On the other hand, at 5 kGy, the TBA number decreased to 0.51 ± 0.00 compared to the control sample, which was 0.77 ± 0.002. This result agreed with Suhaj, Rácová, Polovka and Brezová (2006), who studied black pepper methanolic extract irradiated at doses ranging from 5-30 kGy. Moreover, the current results agreed with Polovka and Suhaj (2010), who reported that the highest value for the TBA of irradiated caraway samples was achieved at a dose of 10 kGy.

An analysis of some irradiated edible and medicinal herb extracts at doses of 10, 20 and 30 kGy showed a change in the active components (Koseki et al., 2002). Under their experimental conditions, the only dose that had an inducement to the chemical substances of the extracts was at 10 kGy.

4 Conclusions

Radiation processing increased the total phenols, total flavonoids and total antioxidants of thyme, with moderate changes detected at doses of 5 and 10 kGy for the essential oils. Thymol was sensitive to irradiation, especially at a 15.0 kGy dose. In addition, the evaluation of antioxidant activity using DPPH radical-scavenging activity indicated some loss of antioxidant activity in irradiated samples, while exposing thyme to doses of 10 and 15 kGy significantly increased the TBA numbers.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

References

Abolhasani, A., Barzegar, M. & Sahari, M. A. (2018). Effect of gamma irradiation on the extraction yield, antioxidant, and antityrosinase activities of pistachio green hull extract. *Radiation Physics and Chemistry*, *144*, 373–378. doi:10.1016/j.radphyschem.2017.09.025

Alcicek, Z. (2011). The effects of thyme (thymus vulgaris l.) oil concentration on liquid-smoked vacuum-packed rainbow trout (oncorhynchus mykiss walbaum, 1792) fillets during chilled storage. *Food Chemistry*, *128*(3), 683–688. doi:10.1016/j.foodchem.2011.03.087

Aqil, F., Ahmad, I. & Mehmood, Z. (2006). Antioxidant and free radical scavenging properties of twelve traditionally used indian medicinal plants. *Turkish Journal of Biology*, *30*, 177–183.

Al-Bachir, M. (2016). Some microbial, chemical and sensorial properties of gamma irradiated sesame (sesamum indicum l.) seeds. *Food Chemistry*, *197*(A), 191–197. doi:10.1016/j.foodchem.2015.10.094

Baranauskiene, R., Venskutonis, P. R., Viskelis, P. & Dambrauskiene, E. (2003). Influence of nitrogen fertilizers on the yield and composition of thyme (thymus vulgaris). *Journal of Agricultural and Food Chemistry*, *51*(26), 7751–7758. doi:10.1021/jf0303316

Embuscado, M. E. (2015). Spices and herbs: Natural sources of antioxidants - a mini review. *Journal of Functional Foods*, *18*(B), 811–819. doi:10.1016/j.jff.2015.03.005

Farag, M. F. S. (2013). Utilization of basil extract as a radioprotector in male rats. *Arab J Sci Appl*, *46*, 274–281.

Fejes, S., Blazovics, A., Lemberkovics, E., Petri, G., Szoke, E. & Kery, A. (2000). Free radical scavenging and membrane protective effects of methanol extracts from anthriscus cerefolium l. (hoffm.) and petroselinum crispum (mill.) nym. ex a. w. hill. *Phytotherapy Research*, *14*(5), 362–365. doi:10.1002/1099-1573(200008)14:5⟨362::AID-PTR554⟩3.0.CO;2-G

Huang, S.-J. & Mau, J.-L. (2006). Antioxidant properties of methanolic extracts from agaricus blazei with various doses of gamma-irradiation. *LWT - Food Science and Technology*, *39*, 707–716. doi:10.1016/j.lwt.2005.06.001

Huang, S.-J. & Mau, J.-L. (2007). Antioxidant properties of methanolic extract from antrodia camphorate with various doses of ??-radiation. *Food Chemistry*, *105*, 1702–1710. doi:10.1016/j.foodchem.2007.04.046

Jayaprakasha, G. K., Rao, L. J. & Sakariah, K. K. (2002). Chemical composition of volatile oil from cinnamomum zeylanicum buds. *Zeitschrift Fur Naturforschung C-a Journal of Biosciences*, *57*(11-12), 990–993.

Jo, C., Son, J. H., Lee, H. J. & Byun, M. W. (2003). Irradiation application for color removal and purification of green tea leaves extract. *Radiation Physics and Chemistry*, *66*(2), 179–184. doi:10.1016/S0969-806X(02)00273-6

Kim, M. J., Yook, H. S. & Byun, M. W. (2000). Effects of gamma irradiation on microbial contamination and extraction yields of korean medicinal herbs. *Radiation Physics*

and Chemistry, *57*(1), 55–58. doi:10.1016/S0969-806X(99)00298-4

Koseki, P. M., Villavicencio, A. L. C. H., Brito, M. S., Nahme, L. C., Sebastiao, K. I., Rela, P. R., ... Freitas, P. C. D. (2002). Effects of irradiation in medicinal and eatable herbs. *Radiation Physics and Chemistry*, *63*(3-6), 681–684. doi:10.1016/S0969-806X(01)00658-2

Lee, S. J., Umano, K., Shibamoto, T. & Lee, K. G. (2005). Identification of volatile components in basil (ocimum basilicum l.) and thyme leaves (thymus vulgaris l.) and their antioxidant properties. *Food Chemistry*, *91*(1), 131–137. doi:10.1016/j.foodchem.2004.05.056

Meda, A., Lamien, C. E., Romito, M., Millogo, J. & Nacoulma, O. G. (2005). Determination of the total phenolic, flavonoid and proline contents in burkina fasan honey, as well as their radical scavenging activity. *Food Chemistry*, *91*(3), 571–577. doi:10.1016/j.foochem.2004.10.006

Nagy, T. O., Solar, S., Sontag, G. & Koenig, J. (2011). Identification of phenolic components in dried spices and influence of irradiation. *Food Chemistry*, *128*(2), 530–534. doi:10.1016/j.foodchem.2011.03.037

Ott, R. L. (1984). An introduction to statistical methods and data analysis, 807–837.

Pellegrini, N., Serafini, M., Colombi, B., Del Rio, D., Salvatore, S., Bianchi, M. & Brighenti, F. (2003). Total antioxidant capacity of plant foods, beverages and oils consumed in italy assessed by three different in vitro assays. *Journal of Nutrition*, *133*(9), 2812–2819.

Pereira, E., Pimenta, A. I., Calhelha, R. C., Antonio, A. L., Verde, S. C., Barros, L., ... Ferreira, I. C. F. R. (2016). Effects of gamma irradiation on cytotoxicity and phenolic compounds of thymus vulgaris l. and mentha x piperita l. *LWT - Food Science and Technology*, *71*, 370–377. doi:10.1016/j.lwt.2016.04.004

Perez, M. B., Calderon, N. L. & Croci, C. A. (2007). Radiation-induced enhancement of antioxidant activity in extracts of rosemary (rosmarinus officinalis l.) *Food Chemistry*, *104*(2), 585–592. doi:10.1016/j.foodchem.2006.12.009

Polovka, M. & Suhaj, M. (2010). Detection of caraway and bay leaves irradiation based on their extracts' antioxidant properties evaluation. *Food Chemistry*, *119*(1), 391–401. doi:10.1016/j.foodchem.2009.07.005

Senatore, F. (1996). Influence of harvesting time on yield and composition of the essential oil of a thyme (thymus pulegioides l) growing wild in campania (southern italy). *Journal of Agricultural and Food Chemistry*, *44*(5), 1327–1332. doi:10.1021/jf950508z

Shurong, L., Meixu, G. & Chuanyao, W. (2006). Use of irradiation to ensure hygienic quality of fresh pre-cut and blanched vegetables and tofu. *Use of Irradiation to Ensure the Hygienic Quality of Fresh, Pre-Cut Fruits and Vegetables and Other Minimally Processed Food of Plant Origin*, 87.

Singleton, Y. I. & Rossi, J. A. J. (1965). Colorimetry of total phenolics with phophomolybdic-phosphotunstic acid reagents. *Amer. J. Enol. Vitic*, *16*, 144–158.

Suhaj, M., Rácová, J., Polovka, M. & Brezová, V. (2006). Effect of ??-irradiation on antioxidant activity of black pepper (piper nigrum l.) *Food Chemistry*, *97*, 696–704. doi:10.1016/j.foodchem.2005.05.048

Taipina, M. S., Lamardo, L. C. A., Rodas, M. A. B. & del Mastro, N. L. (2009). The effects of gamma irradiation on the vitamin e content and sensory qualities of pecan nuts (carya illinoensis). *Radiation Physics and Chemistry*, *78*(7-8), 611–613. doi:10.1016/j.radphyschem.2009.03.019

Variyar, P. S., Limaye, A. & Sharma, A. (2004). Radiation-induced enhancement of antioxidant contents of soybean (glycine max merrill). *Journal of Agricultural and Food Chemistry*, *52*(11), 3385–3388. doi:10.1021/jf030793j

World Health Organization. (1988). Food irradiation: A technique for preserving and improving the safety of food.

Zhu, F., Cai, Y.-Z., Bao, J. & Corke, H. (2010). Effect of gamma-irradiation on phenolic compounds in rice grain. *Food Chemistry*,

120, 74–77. doi:10.1016/j.foodchem.2009.09.072

Zin, Z. M., Abdul-Hamid, A. & Osman, A. (2002). Antioxidative activity of extracts from mengkudu (morinda citrifolia l.) root, fruit and leaf. *Food Chemistry*, *78*(2), 227–231. doi:10.1016/S0308-8146(01)00402-2

International Journal of Food Studies | IJFS April 2020 | Volume 9 | pages 213–224

Flax Seeds and Finger Millet Enriched Functional Rusk

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Received: 22 November 2018; Published online: 18 April 2020

Abstract

Bakery products are generally made up of refined wheat flour that may be deficient in both fibre and essential fatty acids. In this study α-linolenic acid and fibre enriched rusk using flax seeds and finger millet were developed. The proportions of finger millet, flax seed and wheat flours were optimized using Response Surface Methodology (RSM). On the basis of fibre and ALA content and baking quality characteristics of rusk 13.13%, 6.0% and 80.6% of finger millet, flax seed and wheat flours, respectively was finalized. The developed rusk contained 4.81% fibre and 1.36% α-linolenic acid. Wet and dry gluten content, SDS sedimentation, falling number, dough raising capacity of yeast and baking time of mixed flour of these components were statistically ($p>0.05$) similar to that of wheat flour (control). However, higher loaf weight and lower loaf height was observed in mixed flour rusk in comparison to control rusk. α-linolenic acid and fibres enriched rusk were developed with similar quality parameters and improved functional properties.

Keywords: Fortification; Flax seeds; Finger millet; Rusk; α-linolenic acid

1 Introduction

The baking Industry is the largest organized sector within the Indian Food Industry (Nasirullah, Marry & Shariff, 2013). With wheat reported as the most popular cereal grain for baked goods. The elasticity of wheat dough and its rheological properties make wheat superior to other cereal grains (Kaushik et al., 2013). The major factor sought for the suitability of wheat varieties for making different types of bakery products is the ability of the flour used to form a gluten network (Kumar, Khatkar & Kaushik, 2013).which is the protein component of flour that gives the dough elasticity and strength (Sharma, Khatkar, Kaushik, Sharma & Sharma, 2017). In wheat products such as bread, the gluten network formation is desirable for gas retention that in turn enhances the volume of product, while in products such as biscuits, extensibility is required, so gluten formation and its gas retention network is undesirable. Getting the desired quality of wheat flour for making specific kinds of bakery products is a challenging task for the bakery industry (Kaushik, Chawla, Kumar, Janghu & Lohan, 2018; Kaushik, Chawla, Kumar & Kumar, 2017).

The rusk is a bakery product that has low residual moisture and is rapidly soluble in warm liquids (Liu, Bates, Yin, Wang & Lu, 1993). Williams (1976) defined the rusk as any cooked product of a grain based dough, whether it has been raised with yeast or not. Rusks are formed

 10.7455/ijfs/9.1.2020.a7

when bread is baked twicee and these are generally made by baking leavened bread in small portions and then reducing the baked bread to crumbs. The most commonly used grains is wheat, however maize and barley grains may be used. Rusk is widely consumed in all countries, the production procedure includes mixing, fermentation, baking and roasting (Yaseen, 2000). Mallik and Kulkarni (2010) prepared rusk by replacing water with concentrated whey. The rusk produced showed good appearance, flavour, body and texture and overall acceptability during storage (37 ^{o}C) for 9 days. Nasirullah et al. (2013) determined the physicochemical characteristics of fat present in rusk and reported 5.8 % fat, 53.5 butyrometer reading (BR), 31.5 ^{o}C slip melting point and 73.5 Iodine value (IV). Liu et al. (1993) checked/determined /characterized the nutritional efficacy of a fortified weaning rusk thrhuman trails on 2026 full term infants (6-13 months). Rusks were fortified with zinc, iron, calcium, vitamin A, D, B12, thiamin, riboflavin, niacin and folic acid. Infants who received fortified rusks exhibited no decline in haemoglobin concentrations during 3 months study.

Fortification of diets with food materials rich in bioactive compounds were shown to impart several health benefits that can be exploited in development of health foods (Devi, Vijayabharathi, Sathyabama, Malleshi & Priyadarisini, 2014). F seed consumption increased due to its potential functional properties on health (Carraro, de Souza Dantas, Espeschit, Martino & Ribeiro, 2012). Flax seed oil is rich in omega-3 (α-linolenic acid), digestible proteins and soluble fibre (lignin). Pohjanheimo, Hakala, Tahvonen, Salminen and Kallio (2006) reported that with addition of flaxseed, the bread improves moisture retention and softness of bread without any off order (rancidity and mustiness) up to 6 days storage. They further reported a positive impact of flaxseed on textural properties of bread during storage along with improvement in nutritional composition (fibre and α-linolenic acid).

Finger millet is one of the minor cereals known for several health benefits such as anti-diabetic, antitumerogenic, anti-diarrheal, antiulcer, anti-inflammatory, atherosclerogenic effects, antioxidant and antimicrobial properties (Chandra, Chandra, Pallavi & Sharma, 2016). Finger millet contains several phenolic acids and their derivatives, flavonoids and tannins, and showed multifunctional activity and free radical terminator, metal chelator, and singlet oxygen quencher properties. Hegde, Anitha and Chandra (2005) reported that whole finger millet meal flour protected wistar rats against hyperglycaemic and alloxan- induced oxidative stress.

Therefore, the present study was designed for development and evaluation of finger millet and flaxseed enriched rusk to develop new healthy bakery products. The optimization was carried out using Response Surface Methodology. (RSM)

2 Materials and Methods

2.1 Materials

Wheat cultivar C-306, and finger millet were procured form a Wheat Breeding Farm, Haryana Agriculture University, Hisar, India. Flax seeds, sugar, salt, shortening and baker's yeast (*Saccharomyces cerevisae*) were procured from a local market, Hisar, India. Airtight plastic containers were used for grain storage and flour storage. Parad tablets (Himalya, India) were put into grains container enclosed in cloth for protection of wheat grains during storage.

2.2 Milling of wheat, finger millet and flex seeds

Clean wheat and finger millet grains were conditioned by steeping the grains in hot water (60 ^{o}C) for 4 h. The water was drained off and grains were dried in sunlight. The moisture content was adjusted by tempering the grains to 16%. The above conditioned wheat and finger millet were milled using a roller-mill (Chopin Laboratory CD-1 mill, France). Flax seeds were milled without any conditioning treatment and the flour obtained was stored in airtight plastic containers under ambient conditions for further analysis. To ensure the purity of the roller-milled flour samples from each lot, mechanical and manual cleaning of the roller-mill, including air was performed between milling of each sample.

2.3 Proximate Analysis

Wheat, finger millet and flex seed flour obtained were then analysed for moisture, ash, protein and fat contents using Official Methods of Analysis of the Association of Official Analytical Chemists (2012). The α-linolenic acid was determined using method no. 2012.13 (Official Methods of Analysis of the Association of Official Analytical Chemists, 2012) and total dietary fibre content was determined using method no. 991.43 (Official Methods of Analysis of the Association of Official Analytical Chemists, 2012).

2.4 Quality characteristics of wheat flour

Quality characteristics of wheat flour was determined using wet and dry gluten yield, gluten index, SDS sedimentation value and falling number. Gluten extraction was carried out by adopting the procedure as described by Kumar et al. (2013). Wet gluten yield, gluten index and dry gluten yield was determined as per method described by Kaushik et al. (2013). The falling number and dough rising capacity were determined by the approved method as described by AACC (2012) and the results were expressed as time in seconds. Sodium dodecyl sulphate (SDS Solution) sedimentation volume of flour samples was estimated according to the method as described by Axford, McDermott and Redman (1978).

2.5 Optimization of ingredients and rusk preparation

The Straight dough method was used for preparation of dough. The ingredients composition (formula) used was according to the experimental runs given by the Box-Behnken design of Response Surface Methodology (RSM) within the range as wheat (90 to 100 g), finger millet (0 to 10g) and flax seeds flour (2 to 6g). Salt, water, yeast, sugar and fat were added at the rate of 1.0, 48.0, 7.5, 28.0 and 2.0 % of flour, respectively. The concentration of different ingredients taken for optimization was selected on the basis of pre-analysis and literature reviewed for the preparation of rusk.

A second order polynomial equation was used to express the responses as a function of the independent variables given below:

$$Y_k = \beta_{k0} + \sum_{i=1}^{n} \beta_{ki} X_i + \sum_{i=1}^{n} \beta_{kii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{kij} X_i X_j \quad (1)$$

where Y_k = response variable; Y_1 = Fat content (%); Y_2 = Yeast (%); Y_3 = Sugar content (%) Y_4 = water content (%); x_i represent the coded independent variables (x_1 = wheat flour content (g), x_2 = finger millet flour content (g), x_3 = flax seed flour (g); where β_{ko} was the value of the fitted response at the centre point of the design, i.e., point (0,0,0), β_{ki}, β_{kii} and β_{kij} were the linear, quadratic and cross-product regression coefficients, respectively. The test of statistical significance was performed on the total error criteria, with a confidence level of 95%.

The significant terms in the model were found by analysis of variance (ANOVA) for each response. The adequacy of the model was checked by calculating the R^2 and adjusted-R^2. The numerical optimization techniques of the Design-Expert software were used for the simultaneous optimization of the multiple responses. The desired goals for each variable and response were chosen. All of the independent variables were kept within range, while the responses were either maximized or minimized. Optimization of ingredients data using RSM is presented in table 1.

2.6 Rusk preparation

Rusks were prepared using method as described by Yaseen (2000) with some modifications. Yeast and water were mixed in a bowl and kept it for 30 min then sugar was added for activation of yeast and. Flour and other ingredients except fat were added to the yeast water solution. Fat was added in last. Mixing was carried out for 10-12 min. Two stage proofing was carried out. First proofing for 105 minutes, knock back and a further 45 minutes second proofing. Baking was done at 200 ^{o}C for 15 minutes and the loaf was left to cool overnight. The next day, the loaf was sliced and then baked a second time at 160 ^{o}C for 20 minutes and finally the prepared rusks were

Table 1: Box-Behnken Design arrangement and percentage of ingredient

Std	Run	X1	X2	X3	Salt 1%	Fat 2%	Yeast 7.5 %	Sugar 28%	Water 48%
15	1	95	5	4	1.04	2.08	7.80	29.12	49.92
8	2	100	5	6	1.11	2.22	8.33	31.08	53.28
13	3	95	5	4	1.04	2.08	7.80	29.12	49.92
12	4	95	10	6	1.11	2.22	8.33	31.08	53.28
14	5	95	5	4	1.04	2.08	7.80	29.12	49.92
4	6	100	10	4	1.14	2.28	8.55	31.92	54.72
2	7	100	0	4	1.04	2.08	7.80	29.12	49.92
6	8	100	5	2	1.07	2.14	8.03	29.96	51.36
3	9	90	10	4	1.04	2.08	7.80	29.12	49.92
5	10	90	5	2	0.97	1.94	7.28	27.16	46.56
9	11	95	0	2	0.97	1.94	7.28	27.16	46.56
10	12	95	10	2	1.07	2.14	8.03	29.96	51.36
16	13	95	5	4	1.04	2.08	7.80	29.12	49.92
17	14	95	5	4	1.04	2.08	7.80	29.12	49.92
7	15	90	5	6	1.01	2.02	7.58	28.28	48.48
1	16	90	0	4	0.94	1.88	7.05	26.32	45.12
11	17	95	0	6	1.01	2.02	7.58	28.28	48.48

Where: X1- Factor 1A: wheat flour (g), X2-Factor 2 B: finger millet (g), X3-Factor 3 C: flax seed (g)

packed in polyethylene packs and stored at room temperature for further processing.

2.7 Sensory evaluation

Sensory evaluation of rusk was carried out using a nine (9) point Hedonic scale with 1 indicating an extremely disliked and 9 indicating an extremely liked sample. Sample scoring a mean value of 6 and above were taken as acceptable (Ranganna, 1986). Test panel composition and procedures were approved for sensory analysis by the ethics committee, Shoolini University, Solan. The samples were evaluated by the panels for colour, taste, appearance and overall acceptability. The panel scored quality characteristics of each sample using preference test on a nine point hedonic scale as previously described. The sample with highest mean score for all characteristics was chosen to be the most preferred one. The sensory evaluation of the rusk was performed with an evaluation panel of 10 trained members.

2.8 Textural Properties of rusk

Rusk hardness/crispiness was determined with a Texture Analyser TAXT2i (Stable Micro Systems Ltd., Surrey, UK) equipped with a 25 kg load cell in compression mode with a Knife Edge (HDP/BS) attached to the load cell carrier and lowered into the slotted insert. The Heavy Duty Platform (HDP/90) was repositioned so that there was no contact between the blade and slot surfaces and a 'blank' test run as a check. The blade was then raised to allow placement of the sample. Pre- and post-test speeds were 1.5 and 10 mm/s, respectively while test speed was 2.0 mm/s. The maximum force reading (i.e. highest peak) was observed within the first seconds of the test. At this point the rusk fractured into two large pieces. Hardness of rusk (n=8) was determined after 30 minutes of second baking.

2.9 Physical measurements

The time required for first and second baking was recorded in minutes. Volumes of cooled loaves

were measured by the rape seed displacement method. Specific volumes were calculated from loaf volume and loaf weight taken after 1 h of baking (Yaseen, 2000).

2.10 Statistical analysis

The data was analysed using the method described by Kaushik, Sachdeva and Arora (2014). Means, standard error of the mean (SEM), linear regression analysis and 95% confidence intervals were calculated using Microsoft Excel 2007 (Microsoft Corp., Redmond, WA). Data was subjected to a single way analysis of variance (ANOVA) to calculate critical difference.

3 Results and discussion

Finger millet and flax seed flour along with refined wheat flour were used for the preparation of rusks containing high fibre content and α-linolenic acid, respectively. Three types of flours in different proportions were used during trials for preparation of the rusk and finally a range was fixed for each type of flour on the basis of characteristic properties required for dough preparation. Response Surface Methodology was used for optimization of the best combination containing maximum fibre and α-linolenic acid content. The physicochemical properties of all three types of flours used for rusk preparation were determined and represented as below:

3.1 Proximate analysis of flour samples

The moisture content was significantly higher in finger millet, and lowest in flax seed flour. Moisture content is an important factor as it affects the water activity which in turn affects the microbial proliferation and oxidative degradationJanghu, Ray, Bansal and Kaushik (2014). Fat content of flax seed was highest (37.10%), and lowest in refined wheat flour (0.40%). Flax seed showed higher protein content, while finger millet showed lowest protein content. Moreover, ash content was highest in finger millet, and lowest in refined wheat flour. Flax seed showed highest amount of fibre content, while refined wheat flour showed least amount of fibre content (Table 2). We could not use higher amount of flax seed flour due to its fat content which restricted the development of gluten networks in the dough. The main purpose of including the flaxseed flour was its higher content of α-linolenic acid content which was not detected in wheat and finger millet flour, respectively.

Effect of different flours on fibre content

The fibre content in different rusk samples ranged between 4.1 to 5.36 g with an average value of 4.69g, respectively (Table 3). High fibre content exhibit important role in lowering serum cholesterol and glucose level, and essential for intestinal health (Miremadi, Sherkat & Stojanouska, 2016). The interactive effect of flours on fibre content have been shown in 3D graphs of figure 1(a) which indicates that variation in proportions of millet flour and flaxseed flour showed great effect on fibre content as compare with refined wheat flour. The fibre content was found to be increased with increasing proportion of finger millet and flaxseed flour in the mixture of flour, however increasing the proportion of refined wheat flour did not affect the fibre content. The reason behind this was low fibre content of refined wheat flour as compare with finger millet and flaxseed flour.

Effect of flours amount on α-linolenic acid content:

α- linolenic acid in different rusk samples ranged in between 0.531 to 1.355 g with an average value of 0.889 g (Table 3). The variation of α-linolenic acid was mainly due to level of addition of flaxseed flour, so varied proportion of flaxseed flour showed significant effect on the α-linolenic acid content of rusk. Increased proportion of wheat and finger millet flour keeping the flaxseed flour constant lowered the α-linolenic acid content and vice versa (figure 1 b).

Table 2: Chemical Composition of Raw Materials

Sample	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	Fibre (%)	ALA (mg/g)
Wheat Flour	12.8±0.92^{b}	0.40±0.02^{a}	11.20±0.81^{b}	0.50±0.04^{a}	2.7±0.54^{a}	ND
Finger millet	13.20±0.79^{b}	1.30±0.57^{b}	7.30±0.45^{a}	4.00±0.13^{c}	4.5±0.67^{b}	ND
Flax Seeds	6.50±0.65^{a}	37.10±1.21^{c}	20.30±0.78^{c}	3.50±0.20^{b}	8.12±0.58^{c}	230.63+4.7

ALA= Alpha Linolenic Acid; ND= Not Detected
Data are presented as mean ± SEM (n=3).
$^{a-b}$Means with same superscript in column do not vary significantly ($p<0.05$) from each other.

Table 3: Box Behnken design experiments for optimization of flour amount

Independent Variables			Dependent Variables	
Wheat flour (g)	Finger millet flour (g)	Flaxseed flour (g)	Fibre content (g)	ALA (g)
100	10	4	4.77	0.9
90	15	4	4.66	0.86
90	20	6	4.95	1.241
90	10	2	4.37	0.555
80	15	6	4.38	1.355
80	10	4	4.10	0.94
90	10	6	4.37	1.31
90	20	2	4.95	0.552
100	15	2	5.06	0.535
100	15	6	5.06	1.211
80	15	2	4.38	0.531
90	15	4	4.68	0.85
90	15	4	4.72	0.852
80	20	4	4.68	0.88
100	20	4	5.36	0.838
90	15	4	4.64	0.855
90	15	4	4.65	0.858

3.2 Optimization of flour amounts

Optimum amount of flour samples were tested to obtain the criteria of maximum fibre and α-linolenic acid content (Table 3). Second order polynomial models were used for each response to determine the optimum flour amount of samples. The optimization was applied on selected range of wheat, finger millet and flax seed flour samples as 80-100, 10-20, and 2-6g, respectively. The desirability function method was used to obtain the optimum amount of flour in the mixture. This method gave the desirability value of 0.891g with amount of flours as 80.06g for wheat, 13.13g for finger millet, and 6.0 g for flaxseed. By using these proportions of flour, 4.81g of fibre and 1.358g of α-linolenic acid were predicted. Small deviations were recorded between the experimental and predicted values, therefore, the model obtained in this study could be utilized to optimize the flour amounts.

Table 4: Functional and baking properties of refined wheat flour and optimized mixed flour

Sample	Functional properties					Baking properties			
	Wet Gluten (%)	Dry Gluten (%)	SDS-Sedimentation Volume (ml)	Falling Number (sec.)	Dough Raising Capacity of Yeast (%)	Loaf Weight (gm)	Loaf Weight (cm)	First Baking Time (min)	Second Baking Time (min)
Refined Wheat flour	31.96±2.19^a	10.51±0.99^a	35.85±1.42a	425.66±5.09^a	249.07±7.28^a	134.58±3.5^a	8.5±0.9^a	15.1±0.7^a	20.2±1.2^a
Optimized mixed flour	31.50±2.31^a	10.43±1.04^a	35.02±1.36^a	428.33±5.3^a	249.07±7.28^a	140.56±2.9^b	8.2±1.1^a	15.1±0.5^a	20.2±1.1^a

Data are presented as mean ± SEM (n=3).
$^{a-b}$Means with same superscript in column do not vary significantly ($p<0.05$) from each other.

Table 5: Compositional analysis of rusk prepared from optimized mixed flour and control (Refined wheat flour)

Sample	Carbohydrate (%)	Fat (%)	Protein (%)	Ash (%)	Energy (KJ/100g)	Moisture (%)	ALA content	Fibre content
Refined Wheat flour	73.5±2.1^a	5.8±0.9^a	9.3±1.4^a	1.5±0.4^a	1594±36.2^a	7.8±0.7^a	0.0±0.00^a	2.78±0.41^a
Optimized mixed flour	74.6±1.6^a	6.3±1.2^b	9.2±1.3^a	1.6±0.3^a	1631±41.3^a	8.2±0.9^b	1.363±0.17^b	4.86±0.0.37^b

Data are presented as mean ± SEM (n=3).

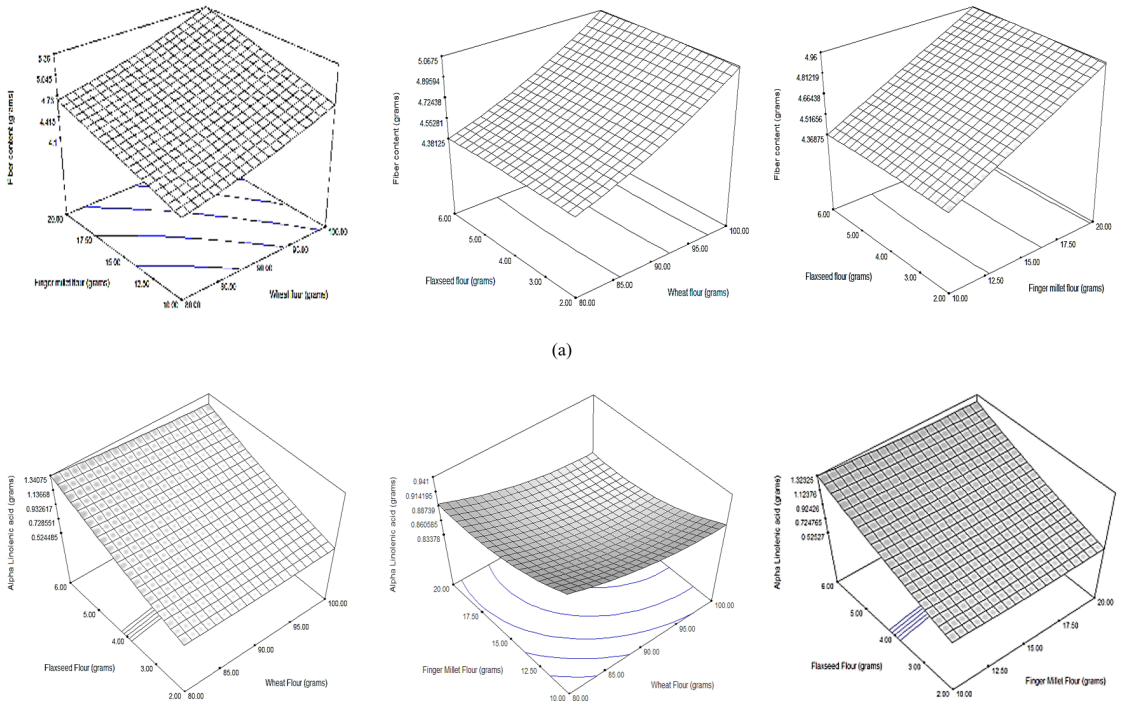

Figure 1: (a) Effect of different amount of flour on fiber content (b) Effect of different amount of flour on Alpha Linolenic acid content.

Table 6: Sensory properties and cost of rusk prepared from optimized mixed flour and control (Refined wheat flour)

Sample	Sensory analysis of rusk (9 point hedonic scale)					Cost of production (in Rs)
	Colour	Texture	Crispiness	Taste	Overall acceptability	
Refined wheat flour (Control)	8.21 ± 0.08^a	7.91 ± 0.13^b	7.79 ± 0.08^b	8.09 ± 0.08^a	8.04 ± 0.10^a	47.29
Optimized Mixed flour	7.90 ± 0.05^b	8.10 ± 0.14^a	8.30 ± 0.12^a	8.10 ± 0.09^a	8.10 ± 0.06^a	50.46

Data are presented as mean ± SEM (n=3).

3.3 Comparison of optimized flour mixture with control flour for functional parameters

The optimized flour mixture was compared with control (only refined wheat flour) for preparation of rusks and its quality characteristics. The results obtained are presented in Table 4 and discussed as follows:
The functional testing values of the raw component material i.e. wheat flour and yeast were studied. Wet and dry gluten yield of refined wheat flour was 31.96 and 10.51% while of optimized mixed flour was 31.50 and 10.43%, respectively. There was non-significant difference in between these values as the lesser protein content of finger millet flour was compensated by higher protein content of flaxseed flour. Our observations were in accordance with Singh and Singh (2006) and Kaushik et al. (2013). The wet and dry gluten yield indicates the quality of protein and baking quality of flour.Autran, Hamer, Plijter and Pogna (1997) observed that pentosans and hemicelluloses in flours have a strong effect on gluten yield and that flour processing properties are strongly determined by the way flour milling fractions are blended. SDS values and Falling number for refined wheat and optimized mixed flour were 35.85 ml, 425.66 s and 35.02 ml, 428.33 s, respectively. Similar to protein content, no significant difference was obtained between these values of SDS and falling number value. Present SDS values were in agreement with the values obtained by Supekar, Patil and Munjal (2005) and Kaushik et al. (2014). The SDS sedimentation value of wheat flours is based on the fact that the gluten protein absorbs water and swells considerably when treated with lactic acid. Falling number indicates flour quality. More the falling number low will be the amylase activity and vice-versa. According to AACC (2012) high falling number (>300 s) indicates minimal enzyme activity and sound quality of wheat flour. A low falling number (<250 s) indicates substantial enzyme activity and sprout-damaged wheat or flour. Dough raising capacity of yeast was 249.07 %.

3.4 Baking quality characteristics of rusk

Rusks prepared with incorporation of finger millet flour and flax seed flour were analysed for their baking quality characteristics and compared with the control sample (refined wheat flour) which is presented in Table 4. Weight and height of the prepared loaves were measured just before second baking of rusk. In the case of the refined wheat flour dough before baking, the initial dough weight was 148g which reduced to 134.58g, while in case of mixed flour it reduced from 156g to 140.56g, respectively. During the experiment where the concentration of fibre material was increased i.e. flax seed, the weight of loaf samples also increased and the reason behind this was the high fibre content in the flax seeds. The fibre content has strong water binding ability which results in more water absorption. So high water retention causes increase in bread weight (Chen, Ratnayake & Cunnane, 1994). Loaves height was observed to reduce from 8.5 to 8.2cm in refined wheat flour and mixed flour dough, respectively. The variation in height was due to the quantity of the wheat flour used. It was observed that due to the gluten network the height of loaf increased when the wheat flour concentration was increased. It was found that the samples with high concentration of wheat flour produced a better gluten network, and hence resulted in more puffing and good height. The baking time was kept constant for both samples as first baking was lasted for 15 min and second for 20 min, respectively.

3.5 Compositional characteristics of rusk

Non-significant difference was observed in the composition of rusk prepared from both type of flours i.e. refined wheat and optimized mixed flour. The results of nutritional composition i.e. moisture, carbohydrates, protein, fat, ash and energy were presented in Table 5. Table 5 showed that that rusks prepared from mixed flour contain similar carbohydrate, protein, ash and energy in comparison to control wheat rusks, however, significantly higher fat, moisture, ALA and

fibre contents compared to that of refined wheat flour were observed. The reason of high fat was the incorporation of flaxseed flour which is a rich source of fat and this high fat content was also responsible for slightly higher energy value of these rusks as fat provides 9 Kcal/g energy after metabolism in the body. The higher moisture content in the mixed flour rusks was due to the high fibre content that has the ability to absorb moisture. This is because of the lack of expansion of gas bubble to their full potential due to rupture of cell walls by fibre particles (Lue, Hsieh & Huff, 1991). No ALA was detected in the control Rusk. The ALA content of the optimized mixed flour rusk was significantly ($p<0.05$) higher than control. Similarly, significantly ($p<0.05$) higher fibre content was observed in optimized mixed flour rusk in comparison to control. It was also observed that both ALA and fibres were stable in rusks after baking, however, slightly higher content was observed due to decrease in moisture content. Ratnayake et al. (1992) determined the thermal stability of flaxseed oil upon 350 ^{o}C and also oxidative stability up to 10 months and reported that the oil was heat and oxidative stable. Similar results were also reported by Chen et al. (1994) and Cunnane et al. (1995). Fibres are highly stable components and its stability during baking was reported by several researchers. Rupasinghe, Wang, Huber and Pitts (2008) reported that fibres were stable after baking of muffins.

3.6 Sensory analysis of rusk

Control rusks and optimized mixed flour rusks were compared for sensory acceptability and results are presented in Table 6. It was observed that Optimized mixed flour rusks obtained significantly ($p<0.05$) lower scores for colour in comparison to control rusks. It may be due to dark colour of flaxseed and finger millet, however in texture, crispiness, taste and overall acceptability mixed flour rusks showed higher sensory scores than that of control. The sensory scores revealed that rusks prepared from the mixed flour sample was superior the control rusks. Rusks are a highly consumable bakery product among the poor and middle class people. They are comparatively simple and inexpensive to prepare when compared to other bakery items. The cost of mixed flour rusks were higher than control sample as finger millet and flaxseed flour cost was higher than that of wheat flour. The cost of control rusk and mixed flour rusk were 47.29 and 50.46 rupees per unit or kilogram, respectively. The cost of mixed flour rusk was slightly higher, but it supplies ALA and higher fibres in comparison to control rusk. Therefore, consumers can spent even more to buy such health beneficial rusk.

4 Conclusions

It has been observed that the developed rusks were a good source of fibre and α-linolenic acid with excellent processing and storage stability. With time the market demand changes which, leads to new and innovative products. Traditional food could, be replaced by reformulated traditional functional food products which, in the present study was achieved with finger millet and flax seeds. The enriched Rusks have similar quality characteristics and sensory properties as the control sample and potentially provide more variety and health attributes to the human population. Also, there is need of such products in the market as the burden of disease is increasing day by day. Mineral fortification and essential amino acids addition to other rusks will also be carried on in future research.

Acknowledgements

The authors acknowledge the financial support provided by Department and University for providing all chemicals, glassware and instruments.

References

AACC. (2012). Approved Methods of Analysis, 11th Ed. Method 56-81.04. Determination of Falling number. St. Paul, MN, U.S.A. Retrieved from http://methods.aaccnet.org/summaries/56-81-04.aspx

Autran, J. C., Hamer, R. J., Plijter, J. J. & Pogna, N. E. (1997). Exploring and improving the industrial use of wheats. *Cereal Foods World*, *42*(4), 216–227. Retrieved from https://www.researchgate.net/publication/287559740%20_Exploring_and_improving_the_industrial_use_of_wheats

Axford, D. W. E., McDermott, E. E. & Redman, D. G. (1978). Small scale tests of bread making quality. *Milling Feed Fert.*

Carraro, J. C. C., de Souza Dantas, M. I., Espeschit, A. C. R., Martino, H. S. D. & Ribeiro, S. M. R. (2012). Flaxseed and human health: Reviewing benefits and adverse effects. *Food Reviews International*, *28*(2), 203–230. doi:10.1080/87559129.2011.595025. eprint: https://doi.org/10.1080/87559129.2011.595025

Chandra, D., Chandra, S., Pallavi & Sharma, A. K. (2016). Review of finger millet (eleusine coracana (l.) gaertn): A power house of health benefiting nutrients. *Food Science and Human Wellness*, *5*(3), 149–155. doi:10.1016/j.fshw.2016.05.004

Chen, Z. Y., Ratnayake, W. M. N. & Cunnane, S. C. (1994). Oxidative stability of flaxseed lipids during baking. *Journal of the American Oil Chemists Society*, *71*(6), 629–632. doi:10.1007/BF02540591

Cunnane, S. C., Hamadeh, M. J., Liede, A. C., Thompson, L. U., Wolever, T. M. S. & Jenkins, D. J. A. (1995). Nutritional attributes of traditional flaxseed in healthy-young adults. *American Journal of Clinical Nutrition*, *61*(1), 62–68.

Devi, P. B., Vijayabharathi, R., Sathyabama, S., Malleshi, N. G. & Priyadarisini, V. B. (2014). Health benefits of finger millet (eleusine coracana l.) polyphenols and dietary fiber: A review. *Journal of Food Science and Technology-mysore*, *51*(6), 1021–1040. doi:10.1007/s13197-011-0584-9

Hegde, P. S., Anitha, B. & Chandra, T. S. (2005). In vivo effect of whole grain flour of finger millet (eleusine coracana) and kodo millet (paspalum scrobiculatum) on rat dermal wound healing. *Indian Journal of Experimental Biology*, *43*(3), 254–258.

Janghu, S., Ray, A., Bansal, V. & Kaushik, R. (2014). Dehydration process optimization for maximum lycopene retention in tomato slices using response surface methodology. *Asian Journal of Dairy and Food Research*, *33*(3), 204–208.

Kaushik, R., Chawla, P., Kumar, N., Janghu, S. & Lohan, A. (2018). Effect of premilling treatments on wheat gluten extraction and noodle quality. *Food Science and Technology International*, *24*(7), 627–636. PMID: 29911411. doi:10.1177/1082013218782368

Kaushik, R., Chawla, P., Kumar, N. & Kumar, M. (2017). Effect of pre-milling treatments on wheat flour quality. *Annals of the University Dunarea De Jos of Galati, Fascicle Vi-food Technology*, *41*(2), 141–152.

Kaushik, R., Sachdeva, B. & Arora, S. (2014). Heat stability and thermal properties of calcium fortified milk. *Cyta - Journal of Food*, *13*(2), 305–311. doi:10.1080/19476337.2014.971346

Kaushik, R., Sharma, N., Swami, N., Sihag, M., Goyal, A., Chawla, P., ... Pawar, A. (2013). Physico-chemical properties, extraction and characterization of gluten from different indian wheat cultivars. *Research & Reviews: A Journal of Crop Science and Technology*, *2*, 37–42.

Kumar, N., Khatkar, B. S. & Kaushik, R. (2013). Effect of reducing agents on wheat gluten and quality characteristics of flour and cookies. *Annals of the University Dunarea de Jos of Galati Fascicle VI - Food Technology*, *37*(2), 68–81. Retrieved from http://search.ebscohost.com/login.aspx?direct=true&AuthType=ip,uid&db=bth&AN=95805839&site=eds-live&scope=site&authtype=ip,cookie,uid

Liu, D. S., Bates, C. J., Yin, T. A., Wang, X. B. & Lu, C. Q. (1993). Nutritional efficacy of a fortified weaning rusk in a rural area near beijing. *American Journal of Clinical Nutrition*, *57*(4), 506–511.

Lue, S., Hsieh, F. & Huff, H. E. (1991). Extrusion cooking of corn meal and sugar-beet fiber - effects on expansion properties, starch gelatinization, and dietary fiber content. *Cereal Chemistry*, *68*(3), 227–234.

Mallik, J. & Kulkarni, S. (2010). Quality of rusks prepared by incorporation of concentrated whey. *Journal of Food Science and Technology-mysore*, *47*(3), 339–342. doi:10.1007/s13197-010-0055-8

Miremadi, F., Sherkat, F. & Stojanouska, L. (2016). Hypocholesterolaemic effect and anti-hypertensive properties of probiotics and prebiotics: A review. *Journal of Functional Foods*, *25*, 497–510. doi:10.1016/j.jff.2016.06.016

Nasirullah, Marry, S. R. J. & Shariff, R. (2013). Studies on the trans-fatty acids and the stability of the fats present in indian bakery products. *Grasas Y Aceites*, *64*(1), 36–40. doi:10.3989/gya.058812

Official Methods of Analysis of the Association of Official Analytical Chemists. (2012). North fredrick avenue gaithersburg, maryland, usa. Retrieved from https://www.aoac.org/official-methods-of-analysis-19th-edition-2012/#

Pohjanheimo, T. A., Hakala, M. A., Tahvonen, R. L., Salminen, S. J. & Kallio, H. P. (2006). Flaxseed in breadmaking: Effects on sensory quality, aging, and composition of bakery products. *Journal of Food Science*, *71*(4), S343–S348. doi:10.1111/j.1750-3841.2006.00005.x

Ranganna, S. (1986). *Handbook of analysis and quality control for fruit and vegetable products*. Tata McGraw-Hill. Retrieved from https://books.google.pt/books?id=jQN8Kpj0UOMC

Ratnayake, W. M. N., Behrens, W. A., Fischer, P. W. F., Labbe, M. R., Mongeau, R. & Bearerogers, J. L. (1992). Chemical and nutritional studies of flaxseed (variety linott) in rats. *Journal of Nutritional Biochemistry*, *3*(5), 232–240. doi:10.1016/0955-2863(92)90045-K

Rupasinghe, H. P. V., Wang, L., Huber, G. M. & Pitts, N. L. (2008). Effect of baking on dietary fibre and phenolics of muffins incorporated with apple skin powder. *Food Chemistry*, *107*(3), 1217–1224. doi:10.1016/j.foodchem.2007.09.057

Sharma, N., Khatkar, B. S., Kaushik, R., Sharma, P. & Sharma, R. (2017). Isolation and development of wheat based gluten edible film and its physicochemical properties. *International Food Research Journal*, *24*(1), 94–101.

Singh, B. & Singh, N. (2006). Physico-chemical, water and oil absorption and thermal properties of gluten isolated from different indian wheat cultivars. *Journal of Food Science and Technology Mysore*, *43*(3), 251–255. Retrieved from https://eurekamag.com/research/004/460/004460147.php

Supekar, D. T., Patil, S. R. & Munjal, S. V. (2005). Comparative study off some important aestivum, durum and dicoccum wheat cultivars for grain, flour quality and suitability for chapati making characteristics. *Journal of Food Science and Technology - Mysore*, *42*(6), 488–492.

Williams, D. A. (1976). Rusk based food snack. US Patent 3,989,858. Google Patents.

Yaseen, A. A. E. (2000). Formulating a new high fiber rusk for production on commercial scale. *Nahrung-food*, *44*(2), 110–113. doi:10.1002/(SICI)1521-3803(20000301)44:2⟨110::AID-FOOD110⟩3.0.CO;2-2

International Journal of Food Studies | IJFS April 2020 | Volume 9 | pages 225–237

The Fortification of Biscuits with Coriander Leaf Powder and its Effect on Physico-Chemical, Antioxidant, Nutritional and Organoleptic Characteristics

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Received: 12 December 2018; Published online: 18 April 2020

Abstract

The aim of this study was to examine the effects of coriander powder substitutions at various proportions in biscuits. The coriander leaf powder (CLP) was rich in essential minerals like calcium (2805.46 mg/100 g), iron (42.1 mg/100 g) and phosphorous (44.36 mg/100 g). The scavenging activity of CLP was 93.34% and the total phenolic content was found to be 40.43 μg/ ml GAE. The biscuits were prepared using 10, 20 and 30% CLP and were baked at 180 °C for 17 minutes. Biscuits were evaluated for their nutritional composition, antioxidant activities, phenolic content, texture, sensory quality and shelf life over 21 days. The macronutrients, antioxidant activities, phenolic content and hardness increased with increasing concentrations of coriander powder. According to the overall acceptability scores from sensory evaluation, 10% and 20% CLP supplemented biscuits were more acceptable than 30% CLP supplemented biscuits.

Keywords: Biscuits; Coriander powder; Antioxidant activity; Shelf life; Fortification

1 Introduction

Coriander (*Coriandrum sativum*) is a member of the carrot family (*Umbelliferae*), and is considered an annual herb and a spice, since both its leaves and seeds are used as a seasoning. The root, stem, leaves and fruits all have a pleasant aromatic odour, and are widely used in flavorings. Leaves are particularly rich in vitamin A, vitamin C and vitamin K, with moderate content of dietary minerals (Dhanapakiam, Joseph, Ramaswamy, Moorthi & Kumar, 2008). Coriander is a valuable herb in promoting digestion and treating gastro-intestinal disorders such as dyspepsia, flatulence, loss of appetite, griping pain and vomiting (Jabeen, Bashir, Lyoussi & Gilani, 2009). Dry coriander treats diarrhoea and chronic dysentery, as well as being useful in preventing acidity (Kaium, 2013). The seeds in particular are recommended as a cure for anxiety and insomnia (Emamghoreishi & Heidari-Hamedani, 2005). Various parts of this plant such as leaves, flower seed, and fruit possess antioxidant activity, diuretic, anti-diabetic, sedative, anti-microbial activity, anti-convulsant activity, hypnotic activity, anti-helminthic and anti-mutagenic activity (Aissaoui, Zizi, Israili & Lyoussi, 2011; Rajeshwari & Andallu, 2011). Polyphenolic compounds, caffeic acid, protocatechinic acid and glycitin are present in high concentration in coriander aerial parts (Melo, Mancini & Guerra, 2005). They are known

 10.7455/ijfs/9.1.2020.a8

to be excellent antioxidants. It has been demonstrated that coriander extract was able to decrease hyperglycemia and increase glucose uptake and metabolism, and insulin secretion (Jelodar, Mohsen & Shahram, 2007). It was observed that incorporation of coriander in the diet lowered the LDL (Low density lipoprotein) and VLDL (Very low density lipoprotein), and significantly increased the HDL (High density lipoprotein) (Dhanapakiam et al., 2008).

The bakery industry in India is growing very rapidly and its products are very popular. Biscuits are the products which have attractive features, with wider consumption, relatively longer shelf life and good eating quality (Hooda & Jood, 2005; Krystyjan, Gumul, Ziobro & Korus, 2015). Biscuits are basically thin, flat and baked products made from flour, salt, sweetening agents, fat and food additives. As far as palatability is concerned, serious consideration is given to shelf life. Development of fortified biscuits or other composite flour bakery products is the latest trend in the bakery industry. Most of the bakery products are used as a source for incorporation of different nutritionally rich ingredients for their diversification (Hooda & Jood, 2005). In recent years, there is a considerable body of research on biscuit ingredients and their proportions which could be modified to make biscuits more nutritious and healthy (Galla, Pamidighantam, Karakala, Gurusiddaiah & Akula, 2017; Giarnetti, Paradiso, Caponio, Summo & Pasqualone, 2015; Pasqualone, Bianco & Paradiso, 2013; Yamsaengsung, Berghofer & Schoenlechner, 2012).

In India, green leafy vegetables are common and are considered most economical because they are available throughout the year. The present dietary scenario necessitates the possible exploration of incorporating novel ingredients in commonly consumed foods rather than developing new food products. As the demands for nutritious food is increasing, efforts are needed to enhance the nutritional value of biscuits (Zaker, Aleem & T. R., 2012).

The present work aims to estimate the nutritional composition and antioxidant activity of coriander leaf powder. The incorporation of CLP to formulate cost effective nutritionally enriched biscuits was followed by the analysis of the biscuit products in terms of nutritive value, microbial safety, sensory characteristics and shelf life over 21 days.

2 Materials and Methods

2.1 Raw materials

Whole coriander leaves were purchased from a local vegetable market in Navi Mumbai. Whole wheat flour, butter, sugar, vanilla essence, sodium bicarbonate, baking powder and salt were purchased from a supermarket store in Navi Mumbai. All chemicals and solvents used were of analytical grade, obtained from Sd Fine-Chem Ltd. (Mumbai, India).

2.2 Packaging

Food grade metallized polyester polyethylene laminate zip-lock pouches, with dimensions of 15 cm length x 12 cm width (12 μm metallized polyester, 7.5 μm polyethylene), were used for packaging of biscuits and coriander leaf powder. The biscuits were placed and zip locked in the metallized polyester polyethylene laminate zip-lock pouches to give air tight packs. The oxygen permeability and water vapour permeability of the metalized polyester were 1.2 ml/m^2 day and $<$1 g/m^2 day respectively.

2.3 Preparation of Coriander Leaf Powder (CLP)

Whole coriander leaves were sorted to reject over matured and insect affected portions, and then washed with water, soaked in 6% sodium hypochlorite solution (1ml/L) for 20 minutes and dried in a tray dryer at 60$\pm$ 2 ^{o}C for 8 hours. The dried material was ground to a fine powder, using a grinder (Philips HL7555-00), to obtain coriander leaf powder (CLP) which passed through a BS 72 (220 μm) mesh. The powder was then packed in metallized polyester polyethylene laminate pouches and stored at room temperature for further chemical analysis and application in biscuits.

2.4 Preparation of coriander supplemented biscuits

The product formulations for the control and coriander supplemented biscuits are presented in Table 1. Control biscuits and coriander powder supplemented biscuits were prepared according to the method mentioned in the literature, with minor modifications (Galla et al., 2017). Coriander powder was supplemented at 10%, 20%, 30% levels in wheat flour and dough was prepared using sugar, butter, ammonium carbonate, sodium bicarbonate, baking powder, salt and water. A creaming process or premixing of butter, sugar and vanilla essence was undertaken for 2-3 minutes until a pale yellow color was obtained. Then dry flour was added to this premixed butter and sugar, with final addition of water to prepare the dough. The dough was manually kneaded for 5-7 minutes, and rested or conditioned at room temperature for 20-30 minutes. The biscuit dough was sheeted to a thickness of 3 mm and cut using a circular cutter of 5 cm diameter. The biscuits, for all four different concentrations, were arranged on a butter paper coated aluminum tray to minimize any effect of tray location and baked at 180 ^{o}C for 17-18 minutes. Then the baked biscuits were cooled to room temperature, and packed and stored in metallized polyester polyethylene laminate ziplock pouches for further analysis and shelf life investigation. The samples were tagged as CB (control biscuits); (CPB-10) 10% supplemented coriander biscuits; (CPB-20) 20% supplemented coriander biscuits and(CPB-30) 30% supplemented coriander biscuits. All the samples were prepared in triplicates.

2.5 Proximate analysis of biscuits

Moisture content and ash content of the samples were estimated by employing the standard methods of analysis (AOAC, 1995). The crude fat content of samples was determined by using Soxhlet apparatus. The method is recognized by the (AOAC, 1995). Crude protein was performed and analyzed by a certified analytical lab (Varni Analytical Laboratory Mira Road, Thane), and was estimated by the standard method of IS 7219:1973(RA-2010) using a KEL PLUS Automatic Nitrogen Estimation System. Crude fibre was performed and analyzed by a certified analytical lab (Varni Analytical Laboratory Mira Road, Thane), and was estimated by the standard method of FSSAI Manual-3. The percentage of carbohydrate content and energy value were calculated using the standard method of analysis (AOAC, 1995). The values for the proximate analysis are the average of triplicates, with standard deviation.

2.6 Antioxidant Assays

Sample Extraction for Antioxidant Assay

The biscuits' samples were extracted according to the method reported in the literature, with minor modifications (Chakraborty & Bhattacharyya, 2015). 1g ground biscuit sample was extracted in 50 ml of 60% aqueous methanol, at 40-50 ^{o}C, in a beaker. The mixture was shaken vigorously for 5 minutes on a magnetic stirrer and was allowed to stand for 20 minutes at room temperature. The mixture was centrifuged at 8000rpm for 10 minutes. The supernatant was collected for further analysis. Fresh samples were prepared for each extraction.

DPPH assay

DPPH (2, 2-Diphenyl-1-picrylhydrzyl) radical activity of coriander leaf powder (CLP), control biscuits & coriander powder supplemented biscuits i.e. CPB: 10%, CPB: 20%, CPB: 30% were performed by following a method mentioned in the literature, with minor modifications (Chakraborty & Bhattacharyya, 2015). 1 ml of DPPH solution was mixed with 0.5 ml of extracted sample solution. The samples were vortexed and incubated at room temperature in the dark for 30minutes. The decrease in color intensity, during incubation of the samples, was monitored at 517nm in a spectrophotometer. Ascorbic acid was used as a standard. The assay was performed in triplicate. The results of scavenging activity were expressed as μM AAE (Ascorbic Acid Equivalent).

Table 1: Product Formulation for control biscuits (CB); 10% supplemented coriander biscuits (CPB-10); 20% supplemented coriander biscuits (CPB-20) and 30% supplemented coriander biscuits (CPB-30)

Parameters(g)	Control Biscuits (CB)	Coriander leaf powder supplemented biscuits		
		CPB:10%	CPB:20%	CPB:30%
Wheat Flour	100	90	80	70
Coriander Leaf Powder	-	10	20	30
Butter	50	50	50	50
Sugar	36	36	36	36
Ammonium Carbonate	4	4	4	4
Sodium Bicarbonate	1	1	1	1
Baking Powder	2	2	2	2
Salt (NaCl)	1	1	1	1
Vanilla Essence (ml)	2	2	2	2
Water	60	60	60	60

Total phenolic content (TPC) assay

Total phenolic content of coriander leaf powder (CLP), control biscuits and coriander powder supplemented biscuits i.e. CPB:10%, CPB:20%, CPB:30% were performed by following a method mentioned in the literature, with minor modifications (Chakraborty & Bhattacharyya, 2015). 0.5 ml of extracted sample was added to 2.5 ml of 10% Folin Ciocalteu reagent and kept at room temperature for 8 minutes. 2 ml of 7.5% Na2CO3 was then added to the sample, vortexed and incubated at room temperature in the dark for 1 hour. The absorbance of the reaction mixture was then measured at 765nm in a spectrophotometer. Gallic acid was used as the standard. Each sample was analyzed in triplicate and results expressed as μg/ ml GAE.

2.7 Estimation of mineral content

The mineral solution was prepared by dissolving the ash, obtained after ashing the samples in a muffle furnace, in diluted hydrochloric acid and then making the volume to 100 ml with distilled water in a standard volumetric flask. Calcium in the samples was determined by the Versenate Titration (EDTA) method according to (AOAC, 1995). Phosphorus content in the samples was determined by Fiske-Subbarao method or ANSA method according to (AOAC, 1995). The absorbance of solution was taken at 510 nm in a spectrophotometer (Shimadzu, UV-1700 model). The assay was performed in triplicate and results expressed as the average, with standard deviation.

2.8 Texture analysis

Texture analysis of control biscuits and sample biscuits were carried out using a TA-XT2i texture analyzer (Stable Micro System, Ltd. in Godalming, Surrey, UK), with a 50 kg load cell, equipped with a 5 mm diameter cylindrical probe. The test speed was 0.5 mm/s, pretest speed 10 mm/s, probe reversing speed 10 mm/s and trigger force was 15g and the distance travelled by the probe inside the sample was 1 mm. The average of three values, with standard deviation, was taken for each set of samples.

2.9 Sensory analysis

The control biscuits and coriander powder supplemented biscuits were subjected to sensory evaluation by 15 panelists (7 males and 8 females), aged between 20 to 35 years, who had earlier experience in quality evaluation of bakery products. Also the panelists were trained via six pre-test sessions according to Pasqualone et al. (2011). The parameters evaluated were appearance, aroma, texture, taste and overall acceptability. The coded samples were served in

clean white plates. The panelists scored for different parameters using a 9-point Hedonic scale:9 for like extremely, 8-like very good, 7-like good, 6-like moderately, 5-neither like nor dislike, 4-dislike moderately, 3- dislike fairly, 2-dislike very much and 1 for dislike extremely (Amerine, Pangborn & Roessler, 2013).

2.10 Shelf life analysis

The analysis of control and coriander leaf supplemented biscuits was carried for 21 days, at an interval of 7 days, under room temperature(30-35 ^{o}C) storage, at a relative humidity of 60-65%. The samples were stored in the metallized polyester polyethylene laminate pouches for different storage intervals to check the efficiency of packaging. The samples were checked every 7 days for the moisture content, mineral content, antioxidant activity, total phenolic content, texture, color analysis, organoleptic evaluation and microbiological analysis throughout the 21 days of storage.

Microbiological Analysis

The total microbial loads of the control biscuits and the coriander leaf supplemented biscuit samples were determined for 21 days, at an interval of 7 days, by the method as described by APHA (2005). The microbiological quality of control biscuit and coriander leaf supplemented biscuit samples was determined by enumerating total viable organisms, which includes the total aerobic count of bacteria and molds. Nutrient agar was used for total viable count and potato dextrose agar was used for the presence of yeasts and molds.1g of biscuit samples were mixed with 9 ml of sterile saline (0.85%) to obtain 10^{-1} dilution. Further serial dilution was made up-to 10^{-4} using a fresh sterile pipette every time. The spread plate technique was used to assess the microbial population. 0.1 ml of each dilution was poured plated onto prepared, sterile and dried nutrient agar plates for total viable count and potato dextrose agar plates for yeasts and molds. The sample was spread by using a sterile glass spreader in circular movements in different directions for 10 sec. After inoculation, the plates were incubated in an incubator at 37 ^{o}C for 48hours for total viable count and for 3-5 days for yeasts and molds.

2.11 Statistical analysis

All data were expressed as means ± standard errors of triplicate measurements and analysed by SPSS for Windows (ver. 16.0). One-way analysis of variance (ANOVA): Post Hoc multiple comparison were carried out to test significant differences. Statistical comparisons between variables were performed with the Least Square Differential method (LSD). Differences were considered significant at $p < 0.05$.

3 Results and discussion

3.1 Proximate composition

The mineral content and antioxidant activity of coriander leaf powder are presented in Table 2. The Calcium, Iron and Phosphorus content in coriander leaf powder was found to be 2805.46 mg/100g, 42.1 mg/100g and 44.36 mg/100g respectively. The determined scavenging activity and Total phenolic content of CLP were 93.34 μM/mlAAE and 40.43 μg/mlGAE. The nutritional composition of CB and CPB-10-30% is presented in Table 2. CPB-30% possessed high quantities of protein (17.84%) and fiber (15.9%). Increasing addition of coriander (10-30%) enhanced the protein and fiber in biscuits as compared to the control. A decrease in fat content was observed as the concentration of coriander in the biscuits increased and hence the least amount of fat was seen in CPB-30% (22.71%). The carbohydrate content in the given samples ranged from 40.18 %-54.68 % and was highest in the control biscuit (54.68%). It was also observed that the energy value of samples ranged from 428.67 kcal to 470.42 kcal. The highest energy value was recorded in the control biscuit (470.42kcal). The difference in proximate composition of the value added biscuits developed from dehydrated coriander leaf powder was basically due to the difference in the proximate composition of coriander powder.

3.2 Shelf Life Study of coriander supplemented biscuits

Effect of storage period on moisture content

Moisture content data for CB, CPB-10%, 20% and 30% are presented in Figure 1. The slight increase in moisture content in optimized biscuits, supplemented with coriander powder, as compared to the control biscuit might be due to the higher water binding capacity of dehydrated coriander powder. A similar increase in moisture content of biscuits by addition of spinach powder was reported by Galla et al. (2017). The moisture content in a food product influences the water activity and hence the shelf life of the product. The lower the moisture content in the product, the longer the shelf life and quality of the product. The moisture content over the storage period increased for CB, CPB-10%, CPB-20%, CPB-30%. The maximum moisture content for CB, CPB-10%, CPB-20%, CPB-30% was seen on day 21 (1.3±0.02, 1.43±0.06, 1.59±0.015, 1.7±0.05) with the lowest on day 0 (1.09±0.03, 1.22±0.07, 1.38±0.02, 1.46±0.025) respectively. The increase in moisture content might be due to the hygroscopic nature of biscuits, storage environment or the ineffective moisture impermeability of the packaging material. A similar increase inmoisture content in wheat defatted coconut flour was seen throughout the storage period (Nadarajah & Mahendran, 2015).

Effect of storage period on scavenging activity

The scavenging activity of biscuits during storage is shown in Figure 3. The figure shows that the antioxidant activity increased with the supplementation of CLP in the biscuits. Initially, the highest scavenging activity was seen in CPB-30% samples (84.26%) which decreased to 78% on day 21. Throughout the storage period, all samples decreased slightly in their antioxidant activity. The scavenging activity of fresh product on day 0 was 21.16±0.15, 78.33±0.3, 80.36±0.35 and 84.26±0.25 for CB, CPB-10%, CPB-20% and CPB-30% biscuits respectively. Whereas, the scavenging activity on day 21 was found to be 17±0.15, 70±0.15, 75.36±0.15 and 78.26±0.3 for CB, CPB-10%, CPB-20% and CPB-30% biscuits respectively. Storage period significantly affected the antioxidant activity of coriander biscuits. Due to ineffective oxygen impermeability of the packaging material, the effect in antioxidant activity was observed. A similar decreasing trend in antioxidant activity during storage was observed in defatted coconut cookies due to the oxidation of unsaturated fatty acids with atmospheric oxygen and moisture uptake (Nadarajah & Mahendran, 2015). Hence, the results of this assay indicated a decrease in the scavenging activity, with an increase in days of storage.

Effect of storage period on Total Phenolic content

The changes in total phenolic content for all the samples are shown in Figure 4. It was observed that the phenolic content increased in the biscuits supplemented with CLP. The highest phenolic content was estimated in CPB-30% samples on day 0 (5.09 μg/ ml GAE) and this decreased to 4.6 μg/ ml GAE on day 21. Throughout the storage period, all samples slightly decreased in their antioxidant activity. The results of this assay indicated the slight decrease in the phenolic content in the CB, CPB-10%, CPB-20%, CPB-30% biscuits on day21 (1.03±0.05, 3.5±0.1, 3.9±0.1, 4.6±0.1) as compared to the fresh products (1.43±0.20, 4.15±0.03, 4.60±0.01, 5.09±0.01) respectively. Storage period significantly affected the phenolic content of coriander biscuits in agreement with the results of scavenging activity.

Texture measurements

The data for the textural measurements are presented in the Figure 2. A slight difference was observed in the hardness of the biscuits throughout the storage period as compared to the fresh product. The hardness went on increasing as the concentration of CLP increased. A similar increase in texture values of biscuits by addition of spinach powder was reported by Galla et al. (2017). The values for hardness of the biscuits decreased throughout the storage

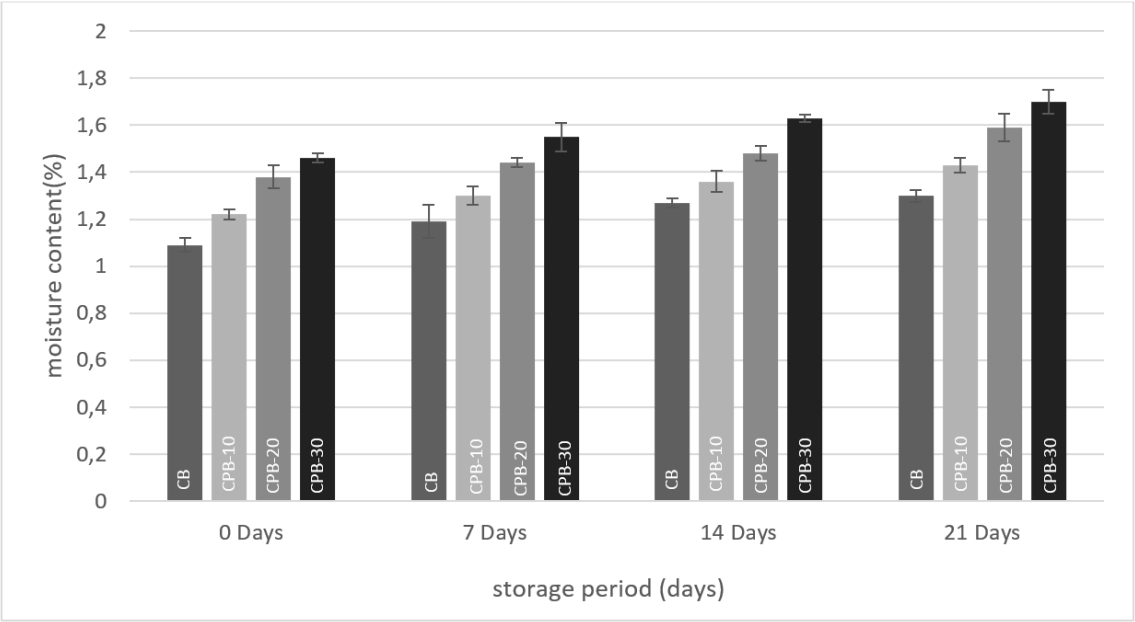

Figure 1: Effect of coriander powder on moisture content (means of three replicates ± standard deviation) control biscuits (CB); 10% coriander supplemented biscuits (CPB-10); 20% coriander supplemented biscuits (CPB-20); 30% coriander supplemented biscuits (CPB-30) during storage at room temperature.

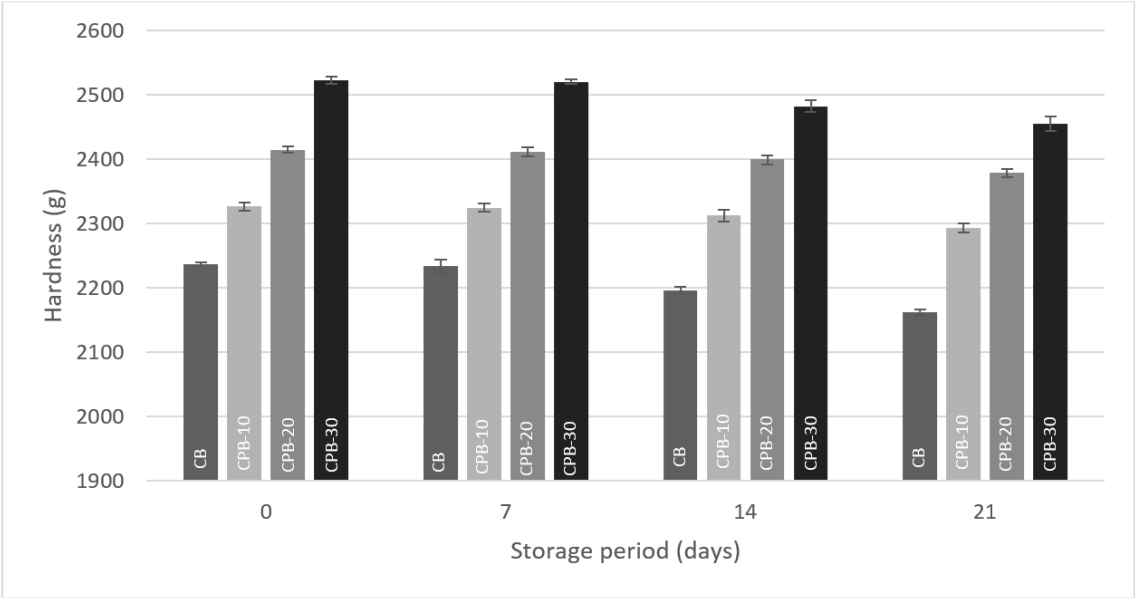

Figure 2: Effect of coriander powder on texture (means of three replicates ± standard deviation) control biscuits (CB); 10% coriander supplemented biscuits (CPB-10); 20% coriander supplemented biscuits (CPB-20); 30% coriander supplemented biscuits (CPB-30) during storage at room temperature.

Table 2: Nutritional composition of control biscuits (CB); 10% supplemented coriander biscuits (CPB-10); 20% supplemented coriander biscuits (CPB-20) and30% supplemented coriander biscuits (CPB-30), mineral composition and antioxidant activity of coriander leaf powder (CLP)

Nutritional composition (%)	Control (CB)	coriander leaf powder supplemented biscuits		
		CPB:10%	CPB:20%	CPB:30%
Moisture	1.09±0.3a	1.22±0.1a	1.38±0.3a	1.46±0.2a
Ash	2.61±0.1a	3.16±0.2b	3.68±0.1c	4.21±0.3d
Fat	23.72±0.3a	23.84±0.7a	23.16±0.6a	22.71±0.5a
Crude Protein	9.52±0.1a	13.49±0.8b	15.40±0.5c	17.84±0.4d
Crude Fibre	8.31±0.4a	11.41±0.3b	13.91±0.06c	15.90±0.7d
Carbohydrates	54.68±0.7a	46.76±0.3b	42.86±0.4c	40.18±0.8d
Energy (kcal/100g)	470.42±1.3a	456.2±2.1b	439.85±0.9c	428.67±1.6d

	Mineral content			Antioxidant activity	
	Calcium (mg/100g)	Iron (mg/100g)	Phosphorus (mg/100g)	DPPH(AAE, μM/ml)	TPC(GAE, μg/ml)
Coriander Leaf Powder	2805.46±0.4a	42.1±0.1b	44.36±0.5c	93.34±0.1d	40.43±0.3e

Values are average of triplicate analysis with ±SD. DPPH: 2, 2-Diphenyl-1-picrylhydrzyl; AAE: Ascorbic Acid Equivalent. TPC: Total Phenolic Content; GAE: Gallic Acid Equivalent. Different small letters in a row indicate differences for control and supplemented coriander biscuits (P<0.05).

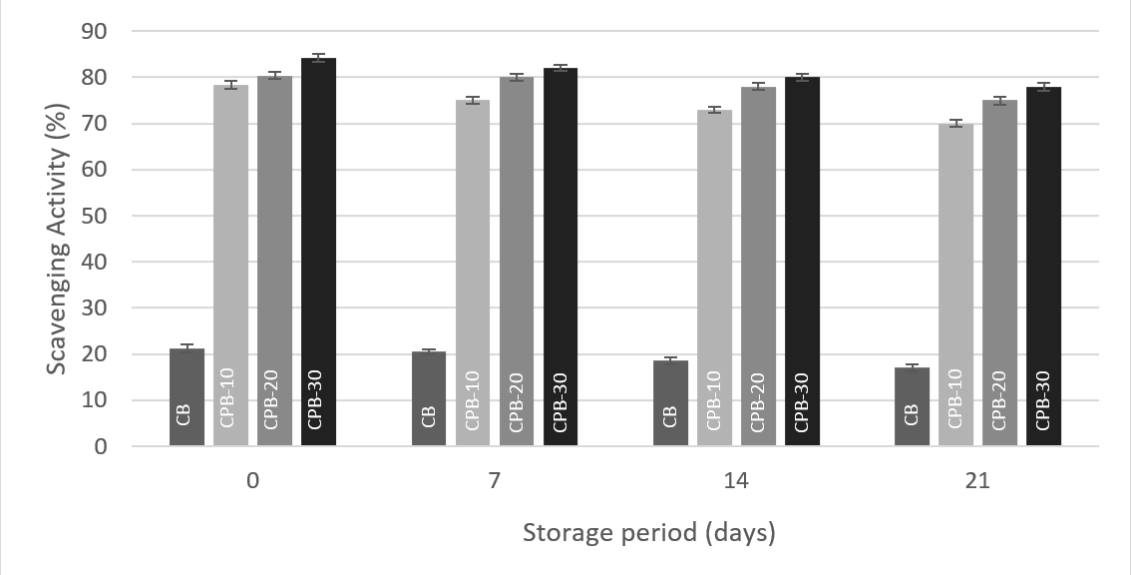

Figure 3: Effect of coriander powder on scavenging activity (means of three replicates ± standard deviation) control biscuits (CB); 10% coriander supplemented biscuits (CPB-10); 20% coriander supplemented biscuits (CPB-20); 30% coriander supplemented biscuits (CPB-30) during storage at room temperature.

Table 3: Effect of coriander powder on sensory attributes (means of three replicates ± standard deviation) of control biscuits (CB); 10% coriander supplemented biscuits (CPB-10); 20% coriander supplemented biscuits (CPB-20); 30% coriander supplemented biscuits (CPB-30) during storage at room temperature.

Sensory attributes	Storage days	Control Biscuits (CB)	Coriander supplemented biscuits		
			CPB-10%	CPB-20%	CPB-30%
Appearance	0	8.6±0.3^a	8.3±0.2^a	8.1±0.3^a	7.3±0.3^b
	7	8.3±0.1^a	8.0±0.3^a	7.6±0.4^a	7.1±0.2^a
	14	7.8±0.2^a	7.6±0.2^a	7.5±0.2^a	6.6±0.6^b
	21	7.5±0.4^a	7.1±0.4^a	7.1±0.1^a	6.2±0.4^b
Aroma	0	8.4±0.2^a	8.1±0.3^a	8.1±0.6^a	7.7±0.2^a
	7	8.1±0.2^a	7.7±0.5^a	7.5±0.4^a	7.4±0.4^a
	14	7.6±0.4^a	7.2±0.3^a	7.0±0.2^a	6.9±0.5^a
	21	7.2±0.5^a	6.8±0.2^a	6.6±0.4^a	6.2±0.6^a
Texture	0	8.6±0.4^a	8.4±0.4^a	8.0±0.5^a	7.4±0.4^a
	7	8.2±0.3^a	8.1±0.3^a	7.7±0.6^a	7.1±0.5^a
	14	7.5±0.2^a	7.4±0.5^a	7.3±0.5^a	6.8±0.2^a
	21	7.1±0.3^a	7.2±0.4^a	7.0±0.3^a	6.5±0.6^a
Taste	0	8.5±0.4^a	8.4±0.4^a	8.1±0.5^a	7.1±0.2^b
	7	8.1±0.3^a	8.2±0.2^a	7.7±0.2^b	6.6±0.4^c
	14	7.8±0.2^a	7.6±0.3^a	7.3±0.5^a	6.3±0.3^b
	21	7.6±0.3^a	7.5±0.4^a	7.0±0.3^a	6.1±0.4^b
Overall Acceptability	0	8.4±0.4^a	8.1±0.5^a	8.0±0.5^a	7.2±0.2^b
	7	8.1±0.3^a	7.6±0.4^a	7.6±0.4^a	6.8±0.4^b
	14	7.7±0.3^a	7.2±0.4^a	7.1±0.3^a	6.4±0.3^b
	21	7.3±0.4^a	6.8±0.5^a	6.5±0.6^a	6.0±0.1^a

*Values are average of triplicate analysis with ±SD; CB: Coriander Biscuits
Different small letters in a row indicate differences for control and coriander supplemented biscuits ($P<0.05$)

period. The hardness of CB, CPB-10%, CPB-20%, CPB-30% biscuits on day 0 were 2236.3±4, 2326.6±6.2, 2414.6±4.09 and 2522.667±5.04 respectively, compared to 2162.3±4.07, 2293±7.08, 2379±6.5, and 2455±10.98 respectively on day 21. This may be due to variation in the moisture content of the control and coriander supplemented biscuits throughout the shelf life of the biscuits.

Sensory Analysis

Sensory scores for all attributes were quite affected, as the days of storage increased, as shown in Table 3. Darkness in the samples increased with an increase in CLP content of biscuits. There was a negligible difference in texture and taste of all the samples when compared to fresh products. Overall acceptability was slightly higher for fresh products as compared to products stored until 21 days. This may be due to the non- enzymatic browning reaction and fat oxidation. It was reported that moisture uptake and gas exchange were causes of off odour development in biscuits (Berger & Fisek, 1970). The sensory score was slightly affected by appearance when products were stored for 21 days. All the samples showed slight change in color up to 21 days as per the results presented in Table 3. Aroma refers to the intensity associated with coriander powder and ingredients sufficiently heated to caramelize some starches and sugars. Sensory score for aroma ranged from 7.7

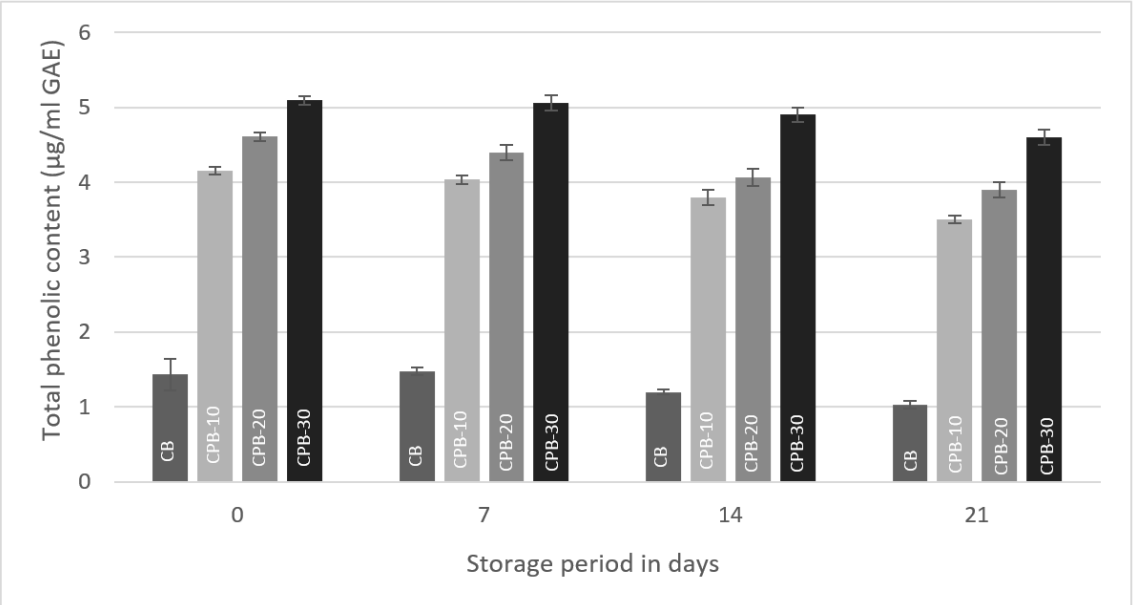

Figure 4: Effect of coriander powder on total phenolics content (means of three replicates ± standard deviation) control biscuits (CB); 10% coriander supplemented biscuits (CPB-10); 20% coriander supplemented biscuits (CPB-20); 30% coriander supplemented biscuits (CPB-30) during storage at room temperature.

Table 4: Effect of coriander powder on mesophilic aerobics and yeasts and molds (means of three replicates ± standard deviation) of control biscuits (CB); 10% coriander supplemented biscuits (CPB-10); 20% coriander supplemented biscuits (CPB-20); 30% coriander supplemented biscuits (CPB-30) during storage at room temperature

Storage in days	Microbial load ($x10^3$cfu/ml)	Control (CB)	CPB-10%	CPB-20%	CPB-30%
0	TPC	x	x	x	x
	Yeasts & molds	x	x	x	x
7	TPC	0.2	0.25	0.2	0.13
	Yeasts & molds	x	x	x	x
14	TPC	0.37	0.32	0.33	0.28
	Yeasts & molds	x	x	x	x
21	TPC	0.44	0.2	0.4	0.4
	Yeasts & molds	x	x	x	x

*TPC- Total Plate Count; x- no visible growth; cfu- colony forming unit

to 8.4 for all the freshly prepared samples. The minimum score was obtained for sample CPB-30%, and the maximum was obtained for CB. The score for texture of the biscuits was slightly decreased after 14 and 21 days when compared to the texture of the fresh products. There was a decrease in the taste score during the storage period of the CB, CPB-10%, 20% and 30% biscuits. The overall acceptability depends on color, flavor and taste score of the products. The overall acceptability score ranged from 8.4 to7.3, 8.1 to 6.8, 8.0 to 6.5 and 7.2 to 6.0 for the control, CPB-10%, CPB-20% and CPB-30% samples. The least score was observed for the CPB - 30% sample. The values presented in the table clearly indicate that the highest overall acceptability score was for the control sample, followed by CPB-10%, CPB-20% and CPB-30%.

Microbiological Analysis

The results for microbiological analysis of the samples are shown in Table 4. There was no fungal growth observed on potato dextrose agar media in the coriander powder supplemented biscuits which were packed in the metallized polyethylene zip lock batches during the entire storage period of 21 days. Lack of fungal growth might be due to effective packaging. The bacterial growth was observed in the control, CPB-10 %, CPB-20 % and CPB-30% biscuits on storage for 7, 14 and 21 days. Total viable count results showed negligible growth on all the storage days. This is an indication that the biscuits were prepared under good hygienic condition and the integrity of the packaging material used was not compromised. Therefore, it can be concluded that the products remained safe for consumption up to 21 days of the storage period.

4 Conclusions

It has been observed that dehydrated coriander powder could be safely employed as a protein, mineral and fibre source in biscuits at 20% level. The scavenging activity of CPB-20% biscuits up to 81% and calcium content almost 1202 mg calcium/100g was achieved by the addition of 20% of dehydrated coriander. A reduction in fat content in the biscuits can be highlighted with increased addition of coriander. It is found that the sensory score and the overall acceptability of the coriander biscuits were acceptable up to 20% level. The coriander leaves can be readily selected and utilized as a remarkable food ingredient to formulate a wide range of products considering its valuable nutrient composition, with noticeable amounts of both macronutrients (carbohydrate and protein) and micronutrients (minerals and antioxidants). Presence of heat stable antioxidants increase the potential of the bioactive compounds based coriander leaf to become a valuable food ingredient. Hence, coriander leaves may be used as a potential source of food flavoring and antioxidants.

Acknowledgements

The authors greatly appreciate Dr. Debjani Dasgupta, Director of School of Biotechnology and Bioinformatics, Navi Mumbai for her constant encouragement throughout the work.

References

Aissaoui, A., Zizi, S., Israili, Z. H. & Lyoussi, B. (2011). Hypoglycemic and hypolipidemic effects of *Coriandrum sativum* L. in meriones shawi rats. *Journal of Ethnopharmacology*, *137*(1), 652–661. doi:10.1016/j.jep.2011.06.019

Amerine, M. A., Pangborn, R. M. & Roessler, E. B. (2013). *Principles of sensory evaluation of food*. Elsevier.

AOAC. (1995). Official Methods of Analysis 16th edn. Association of official AnalyticalChemists, Washington, DC.

APHA. (2005). Standard methods for the Examination of Water and Wastewater, 21st Edition.American Public Health Association, Washington, D.C.

Berger, J. & Fisek, M. H. (1970). Consistent and inconsistent status characteristics and the determination of power and prestige orders. *Sociometry*, 287–304.

Chakraborty, K. & Bhattacharyya, S. (2015). Evaluation of in vitro antioxidant potential of green tea (*Camelia sinensis*) infusions with leaves collected from different heights of darjeeling hill, west bengal. *Euro J. Biomed Pharm Sci*, *2*, 736–749.

Dhanapakiam, P., Joseph, J. M., Ramaswamy, V. K., Moorthi, M. & Kumar, A. S. (2008). The cholesterol lowering property of coriander seeds (*Coriandrum sativum*): Mechanism of action. *Journal of Environmental Biology*, *29*(1), 53–56.

Emamghoreishi, M. & Heidari-Hamedani, G. (2005). Sedativehypnotic activity of extracts and essential oil of coriander seeds. *Iran. J. Med. Sci*, *31*.

Galla, N. R., Pamidighantam, P. R., Karakala, B., Gurusiddaiah, M. R. & Akula, S. (2017). Nutritional, textural and sensory quality of biscuits supplemented with spinach (*Spinacia oleracea* L.) *International Journal of Gastronomy and Food Science*, *7*, 20–26. doi:10.1016/j.ijgfs.2016.12.003

Giarnetti, M., Paradiso, V. M., Caponio, F., Summo, C. & Pasqualone, A. (2015). Fat replacement in shortbread cookies using an emulsion filled gel based on inulin and extra virgin olive oil. *LWT - Food Science and Technology*, *63*(1), 339–345. doi:10.1016/j.lwt.2015.03.063

Hooda, S. & Jood, S. (2005). Organoleptic and nutritional evaluation of wheat biscuits supplemented with untreated and treated fenugreek flour. *Food Chemistry*, *90*(3), 427–435. doi:10.1016/j.foodchem.2004.05.006

Jabeen, Q., Bashir, S., Lyoussi, B. & Gilani, A. H. (2009). Coriander fruit exhibits gut modulatory, blood pressure lowering and diuretic activities. *Journal of Ethnopharmacology*, *122*(1), 123–130. doi:10.1016/j.jep.2008.12.016

Jelodar, G., Mohsen, M. & Shahram, S. (2007). Effect of walnut leaf, coriander and pomegranate on blood glucose and histopathology of pancreas of alloxan induced diabetic rats. *African Journal of Traditional Complementary and Alternative Medicines*, *4*(3), 299–305.

Kaium, A. (2013). *Yield and yield contributes of coriander (Coriandrum Sativum L.) as influenced by spacing and variety* (Doctoral dissertation, Department of Agricultural Botany, Sher-e-Bangla Agricultural University).

Krystyjan, M., Gumul, D., Ziobro, R. & Korus, A. (2015). The fortification of biscuits with bee pollen and its effect on physicochemical and antioxidant properties in biscuits. *LWT - Food Science and Technology*, *63*(1), 640–646. doi:10.1016/j.lwt.2015.03.075

Melo, E. D., Mancini, J. & Guerra, N. B. (2005). Characterization of antioxidant compounds in aqueous coriander extract (*Coriandrum sativum* L.) *LWT - Food Science and Technology*, *38*(1), 15–19. doi:10.1016/j.lwt.2004.03.011

Nadarajah, S. & Mahendran, T. (2015). Influence of storage conditions on the quality characteristics of wheat-defatted coconut flour biscuits packed in metalized polypropylene. *International Journal of Engineering Research and Technology (IJERT)*. *4*.

Pasqualone, A., Piergiovanni, A. R., Caponio, F., Paradiso, V. M., Summo, C. & Simeone, R. (2011). Evaluation of the technological characteristics and bread-making quality of alternative wheat cereals in comparison with common and durum wheat. *Food Science and Technology International*, *17*(2), 135–142. doi:10.1177/1082013210381547

Pasqualone, A., Bianco, A. M. & Paradiso, V. M. (2013). Production trials to improve the nutritional quality of biscuits and to enrich them with natural anthocyanins. *Cyta-Journal of Food*, *11*(4), 301–308. doi:10.1080/19476337.2012.753113

Rajeshwari, C. U. & Andallu, B. (2011). Oxidative stress in niddm patients: Influence of coriander (*Coriandrum sativum*) seeds. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, *2*, 31–41.

Yamsaengsung, R., Berghofer, E. & Schoenlechner, R. (2012). Physical properties and sensory acceptability of cookies made from chickpea addition to white wheat or whole wheat flour compared to gluten-free amaranth or buckwheat flour. *International*

Journal of Food Science and Technology, *47*(10), 2221–2227. doi:10.1111/j.1365-2621.2012.03092.x

Zaker, M. D., Aleem & T. R., G. (2012). Effects of defatted soy flour incorporation on physical, sensorial and nutritional properties of biscuits. *Journal of Food Processing & Technology*, *03*. doi:10.4172/2157-7110.1000149

International Journal of Food Studies | IJFS April 2020 | Volume 9 | pages 238–250

Bacterial Diversity, Biogenic Amines and Lipids Oxidation in Traditional Dried Anchovy (*Encrasicholina punctifer*) during Ambient Storage

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Received: 18 February 2019; Published online: 18 April 2020

Abstract

This study aimed to elucidate the effect of ambient storage (23±2°C, 68% RH) on the bacterial load and diversity, biogenic amines and lipids oxidation in traditional dried anchovy (*E. punctifer*) in order to evaluate its safety, quality and stability during 12 weeks of storage. Total aerobic bacteria (TAB), *Staphylococcus aureus*, *Enterobacteriaceae* (ENT), histidine decarboxylating bacteria (HDB), lysine decarboxylating bacteria (LDB) and ornithine decarboxylating bacteria (ODB) were enumerated and identified by conventional, VITEK 2 compact and sequencing of 16S rRNA gene methods. Histamine, cadaverine and putrescine contents were determined by high performance liquid chromatography. Lipid oxidation was evaluated by peroxide value (PV). Total aerobic bacteria, *S. aureus*, ENT, HDB, LDB and ODB initial counts of $\log_{10}$ 4.9 ± 0.85, 3.7 ± 0.57, 4.2 ± 0.05, 3.7 ± 0.72, 3.9 ± 0.40 and 4.1 ± 0.24 CFU/g respectively did not significantly change ($p > 0.05$) during 12 weeks of storage. A high bacterial diversity of 27 species belonging to 20 genera was found, with the dominance of *S. aureus*, *Acinetobacter lwoffii* and *S. warneri* and the first incidence of *Psychrobacter celer*, *Desemzia incerta*, *Granulicatella elegans* and *Bhargavaea indica* in dried fish. Initial histamine, cadaverine and putrescine contents and PV of 5.2 ± 4.3, 8.5 ± 1.9 and 5.8 ± 0.6 mg/100g and 0.19 ± 0.02 meq/kg respectively did not significantly change ($p > 0.05$) during 12 weeks of storage. This study found that ambient storage at 23±2°C, 68% RH for 12 weeks did not affect the bacterial load, biogenic amines and lipids, and that the dried anchovy remained microbiologically safe and of good quality.

Keywords: Traditional anchovy; Diversity; Ambient; Biogenic amines

1 Introduction

Traditionally, fresh anchovy is caught by trawler fishing, handled under unhygienic conditions and kept at ambient temperature for several hours, spread on sandy/clay sites and sun-dried for 3-5 days in open coastal areas. Pre-drying treatments such as washing and salting are not con-

 10.7455/ijfs/9.1.2020.a9

Nomenclature

TAB	total aerobic bacteria	LDB	lysine decarboxylating bacteria
ENT	*Enterobacteriaceae*	ODB	ornithine decarboxylating bacteria
HDB	histidine decarboxylating bacteria	PV	peroxide value

ducted in some developing countries such as Oman.

Salting, fermenting, drying and smoking are the common fish preservation methods used in the developing countries, particularly Africa and Asia, where these methods represented 12 percent of all fish destined for human consumption in the developing countries in 2016 (Food and Agriculture Organization, 2018).

These practices are expected to increase bacterial load and diversity and to heavily expose fish products to contamination with pathogenic, spoilage and biogenic amine producing bacteria from different sources during catching and prior to and post processing. However, this diversity is not well understood in dried fish products such as dried anchovy. In a single study, *S. aureus* was found in some dried fish products (Moon, Min, Park, Park & Yoon, 2017).

Moreover, since fish is exposed to different contamination sources in traditional drying and dried fish products are stored at ambient temperature in many developing countries, high bacterial diversity in dried fish is expected as a result of these conditions. Nevertheless, this diversity has not been explored and elucidated in these products in order to assess their microbial safety and quality.

Histamine levels higher than 5 mg/100g were found in some dried fish such as dried flying fish (Kung et al., 2015). In addition, histamine-producing bacteria such as *Raoultell ornithinolytica*, *Pantoea agglomerans*, *Proteus vulgaris* and *Enterobacter amnigenus* isolated from dried mahi-mahi were found to be capable of producing 1.25-56.2 mg/100 g of histamine (Lin et al., 2014). Besides their association in scombroid food poisoning, biogenic amines can be used to evaluate the hygienic handling and quality of fish. In this regard, histamine can be used to evaluate the quality of dark muscled fish, whereas putrescine and cadaverine are more subjective parameters to evaluate the quality of white muscled fish and other seafood products (Prester, 2011). Moreover, cadaverine and putrescine have been shown to be involved in the formation of nitrosamines, nitrosopiperidine (NPIP), and nitrosopyrrolidine (NPYR), respectively in in-vitro studies and factors such as impure salts and high temperature have been found to enhance nitrosamine formation (Al Bulushi, Poole, Deeth & Dykes, 2009). Since dried fish are stored at ambient temperature for months before consumption, the stability of biogenic amines during storage needs to be well understood in order to ensure the safety of these products.

Fresh anchovy contains 12.79±0.53% lipid (Gencbay & Turhan, 2016). Fish with this lipids' content is subject to lipid oxidation during processing and storage. Peroxide value is used as a main parameter to assess lipid oxidation (Milijašević, Babić Milijašević, Đinović-Stojanović, Vesković Moračanin & Slobodan, 2017). Information on the lipid oxidation in dried fish processed and stored traditionally which is required to evaluate the quality and storage stability of the products is also limited.

Therefore, this study aimed to elucidate the effect of ambient storage (23±2°C, 68% RH) on bacterial load and diversity, content of biogenic amines and lipid oxidation in order to evaluate the safety, quality and storage stability of tradi-

tional dried anchovy.

2 Materials and Methods

2.1 Dried anchovies and storage conditions

Fresh anchovies were caught from the Sea of Oman and the whole ungutted anchovies were dried by spreading on sandy/clay open sites under the sun for 3-5 days at a temperature of approximately 25^{o}C - 35^{o}C. In Arabian Gulf countries such as Oman and UAE, fish are not salted prior to drying. Moreover in traditional practices, the end point of drying, the quality and safety of fresh anchovies are not evaluated. Two storage studies were conducted. For each storage study about 15 kg of dried anchovy (*Encrasicholina punctifer*) was purchased from a local processing site in UAE immediately after drying. Dried anchovies were dispensed into 500 g samples in closed polyethylene bags and stored at ambient temperature (23±2^{o}C, 68% RH) for 12 weeks of storage. This temperature was selected because 23±2^{o}C is a common storage temperature for this product in many developing counties. Samples were analyzed at three-week intervals over 12 weeks. At each sampling occasion, three samples of dried anchovies, each weighing 500 g, were used for analyses. Prior to analyses, dried anchovies were aseptically chopped manually and mixed. The same chopped sample was used for all analyses.

2.2 Bacterial enumeration and identification

Total aerobic bacteria were enumerated on tryptone soya agar (TSA) (Oxoid, UK), supplemented with 2% NaCl (Oxoid, UK) and incubated aerobically at 32 ^{o}C for three days (Al Bulushi, Poole, Deeth & Dykes, 2008). To recover the injured bacteria of specific groups, a thin agar layer method (TAL) was used in the selective media (Wu, 2008). In this method, Baird-Parker agar and ISO violet red bile glucose agar were overlaid with 6 mL of TSA before inoculation. *Staphylococcu S. aureus* was enumerated on Baird-Parker agar (Oxoid, UK), supplemented with rabbit plasma fibrinogen (Oxoid, UK) and incubated at 37^{o}C for 48 ± 2 h (Reginald & Gayle, 2016). *Staphylococcus aureus* production of coagulase as a means of identification was confirmed by the Staphytect plus system (Oxoid, UK). *Enterobacteriaceae* were enumerated on ISO violet red bile glucose agar (VRBG, Oxoid, UK) and incubated at 37^{o}C for 24 ± 2 h (ISO, 2004). Histidine decarboxylating bacteria, LDB and ODB were enumerated on HD-medium and plates were incubated aerobically at 30 ^{o}C for two and four days (Yamani & Untermann, 1985). This medium consisted of 0.5% tryptone (Oxoid, UK), 0.5% yeast extract (Oxoid, UK), 2.7% L-histidine.2HCl (Sigma, Germany), 0.5% NaCl (Oxoid, UK), 0.1% $CaCO_3$ (Sigma, Germany), 2% agar (Oxoid, UK) and 0.006% bromocresol purple (Sigma, Germany), and the pH was adjusted to pH 5.3. For enumeration of LDB and ODB, L-histidine.2HCl was replaced by L-lysine monohydrochloride (Sigma, Germany) and L-ornithine monohydrochloride (Sigma, Germany) respectively. To facilitate the enumeration of LDB and ODB, 0.01% pyridoxine hydrochloride (Sigma, USA) was added as a coenzyme (Frank, Baranowski, Chongsiriwatana, Brust & Premaratne, 1985).

Eighteen isolates were randomly selected using Harrison's disc for randomized colony selection (Harrison 1938, cited by Harrigan, 1998) at each sampling occasion. The isolates were purified twice in TSA, supplemented with 2% agar and stored on beads (Abtek, UK) at -80 ^{o}C until identified. Isolates were identified to the genus and species levels by VITEK 2 compact (bioMérieux, France), using GP and GN cards and software version of 05.02 according to the manufacturer's instructions, and by sequencing of the 16S rRNA gene. The 16S rRNA gene of selected strains was amplified by the PCR procedure described by Ayyash et al. (2018). PCR primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were employed during amplification. PCR mixtures were prepared following the manufacturer's protocol (Qiagen, Cat No./ID: 201443) and subjected to initial denaturation at 94^{o}C for 2 min followed by 35 cycles of

heating at 94^{o}C (20 sec), primer annealing at 53^{o}C (20 sec) and extension at 70^{o}C for 1.5 min. The final extension was carried out at 70^{o}C for 5 min for 1 cycle. Presence of specific PCR products was confirmed by agarose electrophoresis. The DNA sequence of PCR products was carried out by Macrogen Sequencing Facilities (http://dna.macrogen.com, Seoul, Korea). Sequence results were aligned with the NCBI database using the BLAST algorithm. BLAST and probabilities were carried out by Macrogendna.com. A probability level of $\geq$ 93% was considered for bacterial identification by VITEK 2 compact.

2.3 Determination of water content, water activity, peroxide value and color

Water content was determined gravimetrically by drying at 105^{o}C in an air convection drier (Gallenkamp, UK) to a consistent weight (Association of Official Analytical Chemists, 1990). Water activity was measured by a water activity meter (Rotronic, USA). Peroxide value was measured using a method provided by Egan, Kirk and Sawyer (1981). Briefly, 0.5 g of dried anchovies was mixed with 25 mL of a mixed solution (acetic acid (Sigma, Germany): chloroform (Sigma, Germany), 3:2). Then, 1 mL of saturated potassium iodide (Sigma, Germany) was added and the sample was kept in a dark place for 10 min. A total of 30 mL of water was added and the liberated iodine was titrated with 0.01 N sodium thiosulfate (Sigma, Germany) in the presence of 1 mL of freshly prepared 1% starch (Sigma, Germany) until the disappearance of the blue color. Peroxide value was calculated as meq/kg lipid according to the following formula:

$$PV = (A - B)/S \times 10 \quad (1)$$

Where, A is the titration value for the sample, B is the titration value for the blank, and S is the weight of the sample.

The color of dried anchovy was measured using a color meter, Minolta Chroma meter (Model CR-310, Japan) and a method followed by Rahman, Al-Amri and Al-Bulushi (2002). Briefly, the equipment was calibrated with a white standard calibration plate provided by the manufacturer. Six dried anchovies were placed on a flat surface, the tip of the measuring head was pointed on the sample and the color measurement was taken. Five readings for each value from each sample were recorded. The results were expressed in Hunter as L, a and b values, where L is lightness or darkness (black L = 0; white L = 100), a is intensity of red color and b is intensity of yellow color.

2.4 Determination of amino acids decarboxylation activity

The abilities of the isolates to decarboxylate histidine, lysine and ornithine were assessed using HD-medium developed by Yamani and Untermann (1985). This medium was composed of 2 g peptone, 1 g Lab-lemco powder, 5 g NaCl, 10 g L-histidine monohydrochloride monohydrate (Sigma, Germany), 10 mL bromo-cresol green solution 0.1%, 10 mL chlorophenol red solution 0.2% and 1000 mL deionized water. Pyridoxine hydrochloride was added to the medium to facilitate the decarboxylation of lysine and ornithine. Briefly, 100 μL aliquot of 24 h old isolates was inoculated in HD-medium; the medium was immediately sealed with mineral oil and incubated at 32 ^{o}C for 4 days. The presence of amino acid decarboxylase was assessed by changing the color of the medium from green to violet.

2.5 Determination of biogenic amines

Whole dried anchovy was ground using a commercial blender (Black and Decker, USA). Ground dried anchovies (5 g) were homogenized for 2 min at high speed in a homogenizer (Black and Decker, USA) with 20 mL chilled 6% trichloroacetic (TCA) (Sigma, USA) in a 50-mL centrifuge tube for 3 min. The homogenates were centrifuged at 10,000 g for 10 min at 4 ^{o}C and filtered through Whatman No. 2 filter paper (Sigma, USA). The filtrates were transferred in a 50-mL volumetric flask and brought to a final volume of 50 mL with TCA. Aliquots (20 mL) were placed into storage vials and stored at -50

^{o}C until use. Histamine, cadaverine and putrescine contents were determined by HPLC using a Lichrospher100 RP-18 reversed-phase column (5 lm, 125·4.6 mm, E. Merck, Damstadt, Germany). The mobile phase consisted of water and methanol (Sigma, Germany). The gradient elution program was started with 50/50 (methanol : water) at a low rate of 0.8 mL/min for 5 min. Then the gradient elution was followed by a linear increase to 85/15 (methanol : water) at the same flow rate for 6.5 min. The latter protocol was held for another 5 min and then decreased to 50/50 (0.8 mL/min) for the last 2 min.
The standards consisted of putrescine dihydrochloride (Put), cadaverine dihydrochloride (Cad) and histamine dihydrochloride (Him) (Sigma, Germany). Put (91.5 mg), Cad (85.7 mg) and Him (82.8 mg) were prepared in 50 mL of 0.1 M HCl and used as the standard stock solution (each at 1.0 mg/mL). Before injection to HPLC, the sample and standards were derivatized and the biogenic amines contents were determined as described by Tsai et al. (2005).

2.6 Statistical analysis

Bacterial numbers are reported as $\log_{10}$ CFU/g. A one-way ANOVA test was used to evaluate the effect of ambient storage on the parameters, whereas Tukey Simultaneous Test was used to evaluate the differences between the initial and final values of each parameter during storage. These tests were conducted in Minitab release 14 software (Minitab Inc., USA), and a level $p < 0.05$ was considered statistically significant. Each sample was run in 2-6 replicates.

3 Results and Discussion

3.1 Bacterial counts

Changes in bacterial counts during ambient storage can be seen in Table 1. The total aerobic bacterial count of log 4.9 ± 0.85 CFU/g indicates the good microbial quality of dried anchovies as compared with log 5 CFU/g set for good quality foods (International Commission on Microbiological Specifications for Foods, 1986). This count may also indicate the dominance of mesophilic bacteria in dried anchovies during ambient storage as anchovies are heavily contaminated during handling at ambient temperature. In fact, the dominancy of mesophilic bacteria in dried anchovies prior and during ambient storage should be expected since dried anchovies are handled at ambient temperature and this group grow at ambient temperature with an optimum temperature of 35^{o}C (Ray & Bhunia, 2014). It is expected that the main sources of mesophilic bacteria in dried anchovies are drying surfaces, air born bacteria, human contact and packaging. The practices of traditional catching such as exposure to poor hygienic conditions during catching, handling and drying were expected to heavily increase TAB in dried anchovies, however, the TAB were found to be within the limit of good quality food (International Commission on Microbiological Specifications for Foods, 1986).
It is quite possible that traditional drying times e.g. 5 days and direct sunlight either killed some bacteria or caused cell injury to others. Moreover, the water activity of about 0.5 (Table 2) was another inhibitory factor to limit bacterial growth to log 4.9 ± 0.85 CFU/g. Total aerobic bacterial count in our study was lower than the log 8 CFU/g found in some dried fish (Jakhar, Kumar & Vardia, 2015). This discrepancy may reflect the effect of fish environment and hygienic handling status on the bacterial load of dried fish. Anchovies which were used in this study were harvested from the sea whereas those used in that study (Jakhar et al., 2015) were harvested from fresh water. These environments have different microbial flora which can be a source of microbial flora in fish besides handling (Al Bulushi et al., 2009). Total aerobic bacteria counts did not significantly change ($p > 0.05$) during ambient storage for 12 weeks. This trend can be expected as water activity (Table 2) did not exceed 0.5 during storage which is an inhibitory value for bacterial growth.
Dried anchovies were initially loaded with log 3.7 ± 0.57 CFU/g and 4.2 ± 0.05 CFU/g of *S. aureus* and *Enterobacteriaceae* respectively. The *S. aureus* count in the current study was higher than that found in some dried fish (Kakati, Sharma & Goswami, 2017). However, this count is not expected to create any safety risk factor as a typical count of 10^5 -

Table 1: Bacterial counts in dried anchovies during storage at 23±2^{o}C, 68% RH

	Bacterial count, $\log_{10}$ CFU/g					
Time, week	TAB	*S. aureus*	ENT	HDB	LDB	ODB
0	4.9 ± 0.85^a	3.7 ± 0.57^a	4.2 ± 0.05^a	3.7 ± 0.72^a	3.9 ± 0.40^a	4.1 ± 0.24^a
3	4.5 ± 0.80^a	3.5 ± 0.34^a	3.1 ± 1.1^a	2.7 ± 0.73^a	3.7 ± 0.31^a	3.5 ± 0.02^a
6	4.6 ± 0.92^a	3.9 ± 0.27^a	3.2 ± 0.49^a	3.0 ± 0.22^a	4.2 ± 0.40^a	4.0 ± 0.85^a
9	4.1 ± 1.5^a	3.7 ± 0.23^a	3.3 ± 0.49^a	3.8 ± 0.65^a	4.7 ± 0.04^a	4.7 ± 0.15^a
12	4.8 ± 0.51^a	4.0 ± 0.04^a	3.2 ± 0.16^a	3.8 ± 0.38^a	3.9 ± 0.69^a	4.0 ± 0.40^a

Each mean was compared with that at week 0. Means with different alphabetical superscripts in the same column are significantly different ($p< 0.05$), n = 3
TAB: total aerobic bacteria, EN: *Enterobacteriaceae*, HDB: histidine decarboxylating
LDB: lysine decarboxylating bacteria, ODB: ornithine decarboxylating bacteria

Table 2: Water content, water activity and biogenic amines contents in dried anchovies during storage at 23±2^{o}C, 68% RH

Time, week	% water	a_w	PV, meq/kg	His, mg/kg	Cad, mg/kg	Put, mg/kg
0	8.3 ± 0.03^a	0.48 ± 0.00^a	0.19 ± 0.02^a	5.252 ± 4.3^a	8.585 ± 1.9^a	5.858 ± 0.6^a
3	8.3 ± 0.04^a	0.48 ± 0.00^a	0.34 ± 0.15^a	8.282 ± 5.5^a	5.656 ± 0.75^a	5.252 ± 0.5^a
6	8.5 ± 0.43^a	0.49 ± 0.00^b	0.28 ± 0.12^a	3.838 ± 2.6^a	7.676 ± 4.0^a	9.090 ± 3.5^a
9	7.9 ± 0.34^a	0.50 ± 0.00^b	0.20 ± 0.01^a	2.424 ± 0.84^a	6.666 ± 1.3^a	5.656 ± 1.3^a
12	9.1 ± 0.14^b	0.51 ± 0.00^b	0.30 ± 0.11^a	5.656 ± 3.8^a	3.838 ± 1.0^a	3.838 ± 00^a

Each mean was compared that at week 0. Means with different alphabetical superscripts in the same column are significantly different ($p< 0.05$), n = 2-6
PV: peroxide value, His: histamine, Cad: cadaverine, Put: putrescine

10^8 CFU/g of *S. aureus* is required for enterotoxin production and secondly, the enterotoxins production requires a water activity level of 0.85-1.0 which was not provided by dried anchovies in the current study (Montville & Matthews, 2008; Stewart, 2003). The *Enterobacteriaceae* load indicated that the product had been exposed to cross-contamination of sanitary sources, especially from feces of animals. In fact, as per traditional processing, anchovies are processed in open sites where different animals have access to the products during processing. Although most *Enterobacteriaceae* are heat-sensitive (Baylis, Uyttendaele, Joosten & Davies, 2011), the presence of these microorganisms in anchovies indicates that traditional drying temperature and time did not destroy *Enterobacteriaceae* totally and dried anchovies served as a vehicle for this group of microorganisms. The common *Enterobacteriaceae* sanitary sourced pathogens such as *Salmonella* sp. and *E. coli* which are expected to be among the flora due to contamination from humans during handling were not found among the *Enteriobacteriacea* in the current study. This absence may be attributable to the effect of the drying temperature. In fact, the counts of some Enteriobacteriacea, such as *Salmonella* sp. and *Salmonella* typhimurium, were found to decrease during drying (Ingham, Searls & Buege, 2006).

During ambient storage for 12 weeks, neither *S. aureus* nor *Enterobacteriaceae* showed statistic-

Table 3: Bacterial diversity in dried anchovies during storage at 23±2^{o}C, 68% RH

Bacteria	AC/Bio #	Storage, week					#
		0	3	6	9	12	
Macrococcus sp.	KP181835.1	1					1
Psychrobacter sp.	FJ984919.1	2	1	1	2		6
Staphylococcus sciuri	KT955004.1	3				4	7
*Staphylococcus aureus**		13	10	15	11	5	54
Pseudomonas fluorescens	5000001100101240	3					3
Alloiococcus otitis	000002000000000	5	6		3	10	24
Aeromonas salmonicida	0000000000000200	2				1	3
Kocurica kristinae	04000203220031	10	1	1		2	14
Acinetobacter lwoffii	0000000100000000	5	15	11	3	10	44
Serratia fonticola	616363563561101	1					1
Staphylococcus xylosus	430046057773231	6	4				10
Micrococcus luteus	041032301000000	1					1
Staphylococcus warneri	050002003220231	2	5	1	10	19	37
Streptococcus alactolyticus	000030310270021	1					1
Staphylococcus saprophyticus	050002012670231	1				1	2
Sphingomonas paucimobilis	0001200150300210	1	4			3	8
Staphylococcus hominis	000000000320231		2		1		3
Staphylococcus gallinarum	430446056373331		1				1
Comamonas testosteroni	0000000100500001		1				1
Aerococcus viridans	020103000042031		1				1
Staphylococcus epidermidis	KP236244.1		1	1			2
Arthrobacter sp.	JX047437.1		1			1	2
Psychrobacter celer	KR051247.1			5		1	6
Pseudomonas oryzihabitans	4 001600140100210			1			1
Kocuria rosea	010010300000000				1	2	3
Staphylococcus caprae	010002002461221				1		1
Sporosarcina aquimarina	KT922020.1				1		1
Desemzia incerta	LN867201.1					1	1
Methylobacterium sp.	0000000200000000					1	1
Granulicatella elegans	010030300000000					1	1
Pantoea sp.	AY659872.1					1	1
Bhargavaea indica	KT008289.1					1	1
Total							243

AC : Accession number for sequencing of 16S rRNA gene
BIO : Bionumber in VITEK 2 compact
*: Identified by Staphytect plus system

ally significant change ($p > 0.05$). This trend was attributed to the low water activity of the product of around 0.5 whereas the minimum water activities required for the growth of *S. aureus* and *Enterobacteriaceae* are 0.8 and 0.94 respectively (Baylis et al., 2011). *S. aureus* viability in the current study agreed with Moon et al. (2017) who found a significant reduction in *S. aureus* only after 5 months of storage at 24^{o}C.
The counts of HDB, LDB and ODB ranged from log 3.7 ± 0.72 CFU/g, log 3.9 ± 0.40 CFU/g and log 4.1 ± 0.24 CFU/g at the beginning of storage to log 3.8 ± 0.38 CFU/g, log 3.9 ± 0.69 CFU/g and log 4.0 ± 0.40 CFU/g at the end of storage respectively; these changes were not statistically significant ($p > 0.05$). The counts of HDB, LDB and ODB may indicate the potential of these flora to produce histamine, cadaverine and putrescine in fresh anchovies prior to drying.

3.2 Bacterial diversity

In total, 243 isolates were identified (Table 3). Twenty-seven species belonging to 20 genera were found, where the *Staphylococcus* genus was dominant with 49% of total bacteria, followed by the *Acinetobacter* genus with 18% of total bacteria. Throughout the storage, *S. aureus*, *Acinetobacter lwoffii* and *S. warneri* dominated the bacterial flora with 22%, 18% and 15% of total bacteria respectively. *S. aureus* was the dominant species within the *Staphylococcus* genus with 45% followed by *S. warneri* with 31%. Most of bacteria maintained their viability during ambient storage, indicating high diversity of bacteria in dried anchovy. In general, the incidences of Gram-positive bacteria in dried anchovy were higher than those of Gram-negative bacteria. To our knowledge, this study is the first to report the incidences of some bacteria such as *Psychrobacter celer*, *Desemzia incerta*, *Granulicatella elegans* and *Bhargavaea indica* in dried fish. High bacterial diversity and incidences in dried anchovies could be attributed to high exposure to cross-contamination, mainly from sand and humans during handling and processing. In fact, high *S. aureus* and *Alloiococcus otitis* incidence indicates high cross-contamination from humans (El-Jakee et al., 2008). Incidence and viability of *S. aureus* in dried anchovy in the current study coincided with that found in some dried fish (Moon et al., 2017).
Maintaining viability in dried anchovy confirmed the earlier finding that *S. aureus* resists drying (Beardpegler, Stubbs & Vickery, 1988). *S. aureus* enterotoxins have not been assessed in the current study, however, their presence in dried fish has not been reported in other studies to our knowledge. *S. warneri*, found at high levels in the current study has been reported in various marine fish (Musharrafieh, Tacchi, Trujeque, LaPatra & Salinas, 2014). Its viability during the storage of dried product indicates its resistance to drying conditions. Among the Gram-negative bacteria, *A. lwoffii* dominated the flora. This infectious bacterium, which originates from humans, was found to resist the drying conditions; this could explain its viability and dominance in the current study (Jawad, Heritage, Snelling, GascoyneBinzi & Hawkey, 1996).
Among biogenic amines producing flora, HDB mainly dominated the flora by 15 % followed by LDB and ODB by 9 % (each) (Table 4). *S. warneri* showed the highest incidence of decarboxylation of histidine followed by decarboxylation of lysine and ornithine. *S. warneri* strains' abilities to decarboxylate histidine, lysine and ornithine in the current study agreed with Marino, Frigo, Bartolomeoli and Maifreni (2011) who found that 9 of 14 *S. warneri* strains decarboxylated histidine, lysine and ornithine. In fact, *Staphylococcus* sp. have been widely shown to have amino acid decarboxylation activities, mainly of histidine, lysine, ornithine and tyrosine; the main *Staphylococcus* sp. which showed decarboxylation activity of these amino acids include *S. xylosus, S. pasteuri*, *S. aureus*, *S. sciuri*, *S. warneri*, and *S. vitulinus* (Marino et al., 2011). Despite this potential, *Staphylococcus* sp. were found to be weaker formers of biogenic amines than certain Gram-negative species such as *Morganella morganii* (Rodriguez-Jerez, Mora-Ventura, López-Sabater & Hernandez-Herrero, 1994). Moreover, the reasonably low biogenic amine contents in the current study could indicate that these biogenic amines had been formed mainly by weaker producers of biogenic amines such as *Staphylococcus* sp.

Table 4: Amino acid decarboxylation potentials of the bacterial flora of dried anchovies

Bacteria	HD	LD	OD
Staphylococcus xylosus (10)	6		2
Micrococcus luteus (1)	1		
Staphylococcus warneri (37)	15	2	1
Streptococcus alactolyticus (1)	1		
Staphylococcus saprophyticus (1)	1		1
Kocurica kristinae (14)	3	2	2
Sphingomonas paucimobilis (8)	6	1	
Staphylococcus caprae (1)	1		
Staphylococcus hominis (3)	1		
Desemzia incerta (1)	1		
Sporosarcina aquimarina (1)	1		
Alloiococcus otitis (24)		7	11
Acinetobacter lwoffii (44)		5	3
Arthrobacter sp (2)		1	
Psychrobacter celer (6)		1	1
Psychrobacter sp.(6)		1	
Bhargavaea indica (1)		1	
Aeromonas salmonicida (3)		1	
Pseudomonas oryzihabitans (1)			1
Total	37	22	22

HD: histidne decarboxylation;
LD: lysine decarboxylation;
OD: ornithine decarboxylation

Table 5: Color values in dried anchovies during storage at 23±2^{o}C, 68% RH

Time, week	L value	a value	b value
0	47.2 ± 1.2^a	0.90 ± 0.11^a	6.2 ± 0.25^a
3	55.2 ± 2.8^b	1.1 ± 0.77^a	6.9 ± 0.22^a
6	39.0 ± 0.60^b	1.0 ± 0.19^a	6.5 ± 0.27^a
9	37.4 ± 0.50^b	0.88 ± 0.24^a	6.6 ± 0.46^a
12	35.7 ± 1.9^b	1.2 ± 0.49^a	6.6 ± 0.24^a

Each mean was compared with that of 0 week.
Means with different alphabetical superscripts in the same column are significantly different ($p< 0.05$), n = 6

3.3 Lipid changes

Peroxide value (PV), an indicator of lipid oxidation, was 0.19 ± 0.02 meq/kg at the beginning of storage and it did not significantly increase ($p > 0.05$) to 0.30 ± 0.11 meq/kg during ambient storage for 12 weeks. Peroxide values in the current study are not expected to induce any rancidity which is only noticeable at a PV of more than 10 meq/kg (Egan et al., 1981). Anchovy is a pelagic fatty fish which could be subjected to lipid oxidation, however, the low PV value in the current study indicated good dried anchovy's stability during 12 weeks of storage. The low PV value in the current study could be attributed to the effect of the high direct sun-drying temperature and ambient storage. The effect of processing temperature on the stability of lipids was reported by Ortiz et al. (2013) who found that drying at 60^{o}C resulted in the formation of more lipid oxidation products than drying at 40^{o}C. Whereas, the effect of ambient storage temperature was studied by Takiguchi (1996) who found that pulverized niboshi (boiled and dried anchovy) showed a decrease in PV during storage at 25^{o}C for 60 days compared with storage at -20^{o}C.
The PV value of the dried anchovy in the current study was lower than that found in some dried fish which might be explained by effects of some factors such as fish species, handling and conditions of traditional drying (Kakati et al., 2017). Lipids' stability in dried stored anchovies in the current study agreed with that found in *Stolephorus commersonnii* which was handled, dried and stored at similar conditions of dried anchovies (Patterson, Kailasam, Giftson & Immaculate, 2018). The color value of b (yellowness) has been found to increase with lipid oxidation via the interaction of oxidized products with amines in proteins (Thanonkaew, Benjakul, Visessanguan & Decker, 2006). In the current study, however, neither PV nor b value showed a significant change (Table 5).

3.4 Biogenic amines changes

The contents of histamine, cadaverine and putrescine in dried anchovies (Table 2) ranged from 52 ± 4.3, 85 ± 1.9 and 58 ± 0.6 mg/kg at the beginning of storage to 56 ± 3.8, 38 ± 1.0 and 38 ± 0 mg/kg at the end of storage; these changes were statistically non-significant ($p > 0.05$). In general, histamine levels in dried anchovy in our study did not exceed the FAO/WHO allowed limit for histamine of 200 mg/kg (Food and Agriculture Organization, 2012).
The contents of all biogenic amines in dried anchovy in the current study were found to be lower than those found in dried fish products such as flying fish, mahi-mahi and anchovy (Kung et al., 2015; Lin et al., 2014). This discrepancy may be attributable to different pre-drying conditions such as handling temperature and hygiene, to the different abilities of contaminating microbial flora to form biogenic amines and to different post-drying properties of the product such as water activity. The effects of these factors have been clearly elucidated in many studies (Kung et al., 2015; Lin et al., 2014). For instance, histamine reached 50 ppm in more than 12 h during on-board handling of mahi-mahi at 26^{o}C, whereas, this level was attained within 9 h at 35^{o}C (Staruszkiewicz et al., 2004). Certain bacteria such as *Enterobacter aerogenes* were found to produce more than 500 ppm histamine in trypticase soy broth supplemented with 1.0% L-histidine (Kung et al., 2015). All biogenic amines were stable during the 12-week ambient storage. This stability could be expected as the biogenic amine producers were inactive in the current study due to low water activity in dried anchovies. The stability of the biogenic amines in the current study is in agreement with that reported by Hwang et al. (2012).

4 Conclusions

This is the first study to show a high bacterial diversity in a dried fish product such as dried anchovy with 27 species belonging to 20 different genera with the dominancy of *Staphylococcus aureus*, *A. lwoffii* and *S. warneri*. Ambient storage (23±2^{o}C, 68% RH) for 12 weeks did not affect the bacterial load, levels of biogenic amines or PV. Traditional dried anchovy was found to be safe microbiologically and to retain good quality for 12 weeks at ambient temperature. The viab-

ility and absence of pathogens and good storage stability of traditional anchovies at ambient temperature can make this product a reliable source of animal proteins especially in poor developing countries lacking access to electricity and sea.

Acknowledgements

This project was funded by Sultan Qaboos University Internal Grand number: IG / AGR / FOOD / 11 / 03.

References

Al Bulushi, I. M., Poole, S., Deeth, H. C. & Dykes, G. A. (2008). Quantitative assessment of total and gram-positive aerobic bacteria in fresh and ambient-temperature-stored sub-tropical marine fish. *World Journal of Microbiology & Biotechnology*, *24*(9), 1867–1875. doi:10.1007/s11274-008-9687-5

Al Bulushi, I., Poole, S., Deeth, H. & Dykes, G. A. (2009). Biogenic amines in fish: Roles in intoxication, spoilage, and nitrosamine formation - a review. *Critical Reviews in Food Science and Nutrition*, *49*, 369–77. doi:10.1080/10408390802067514

Association of Official Analytical Chemists. (1990). Official Methods of Analysis of the Association of Chemists. Analysis of the Association of Chemists, Washington, DC., 223-225, 992-995.

Ayyash, M., Abushelaibi, A., Al-Mahadin, S., Enan, M., El-Tarabily, K. & Shah, N. (2018). In-vitro investigation into probiotic characterisation of streptococcus and enterococcus isolated from camel milk. *LWT-Food Science and Technology*, *87*, 478–487. doi:10.1016/j.lwt.2017.09.019

Baylis, C., Uyttendaele, M., Joosten, H. & Davies, A. (2011). The enterobacteriaceae and their significance to the food industry. *ILSI Europe Report Series*, 17–28.

Beardpegler, M. A., Stubbs, E. & Vickery, A. M. (1988). Observations on the resistance to drying of staphylococcal strains. *Journal of Medical Microbiology*, *26*(4), 251–255. doi:10.1099/00222615-26-4-251

Egan, H., Kirk, R. S. & Sawyer, R. (1981). Pearson's chemical analysis of foods. churchill livingstone. *Edinburgh/London/Melbourne/New York*.

Food and Agriculture Organization. (2012). Joint fao/who expert meeting on the public health risks of histamine and other biogenic amines from fish and fishery products. Retrieved from www . fao . org / state - of - fisheries-aquaculture

Food and Agriculture Organization. (2018). The state of world fisheries and aquaculture. Retrieved from www . fao . org / fileadmin / user_upload / agns / news . . . /Histamine_Final_Report.pdf

Frank, H. A., Baranowski, J. D., Chongsiriwatana, M., Brust, P. A. & Premaratne, R. J. (1985). Identification and decarboxylase activities of bacteria isolated from decomposed mahimahi (coryphaena-hippurus) after incubation at 0-degrees-c and 32-degrees-c. *International Journal of Food Microbiology*, *2*(6), 331–340. doi:10.1016/0168-1605(85)90023-6

Gencbay, G. & Turhan, S. (2016). Proximate composition and nutritional profile of the black sea anchovy (engraulis encrasicholus) whole fish, fillets, and by-products. *Journal of Aquatic Food Product Technology*, *25*(6), 864–874. doi:10 . 1080 / 10498850 . 2014 . 945199

Harrigan, W. F. (1998). *Laboratory methods in food microbiology*. Gulf Professional Publishing.

Hwang, C.-C., Lin, C.-M., Kung, H.-F., Huang, Y.-L., Hwang, D.-F., Su, Y.-C. & Tsai, Y.-H. (2012). Effect of salt concentrations and drying methods on the quality and formation of histamine in dried milkfish (chanos chanos). *Food Chemistry*, *135*(2), 839–844. doi:10.1016/j.foodchem.2012.05.035

Ingham, S. C., Searls, G. & Buege, D. R. (2006). Inhibition of Salmonella serovars, Escherichia coli O157 : H7 and Listeria monocytogenes during dry-curing and drying of meat: A case study with basturma. *Journal of Food Safety*, *26*(2), 160–172. doi:10 . 1111/j.1745-4565.2006.00040.x

International Commission on Microbiological Specifications for Foods. (1986). Microorganisms in foods. 2. sampling for microbiological analysis: Principles and specific applications. University of Toronto Press Toronto.

ISO. (2004). Microbiology of food and animal feeding stuffs-horizontal methods for the detection and enumeration of *Enterobacteriaceae*: Part 2: Colony-count method. Retrieved from https://www.iso.org/standard/34566.html

El-Jakee, J., Ata, N., Bakry, M., A. Zouelfakar, S., Elgabry, E. & A. Gad El-Said, W. (2008). Characteristics of staphylococcus aureus strains isolated from human and animal sources. *American-Eurasian J.Agric.and Environ.Sci. 4*, 221–229.

Jakhar, J., Kumar, A. & Vardia, H. K. (2015). Hygenicity and nutritional quality of traditional dried and smoked fishes at kawardha fish market, (chhattisgarh), india, 1099–1102.

Jawad, A., Heritage, J., Snelling, A. M., GascoyneBinzi, D. M. & Hawkey, P. M. (1996). Influence of relative humidity and suspending menstrua on survival of acinetobacter spp on dry surfaces. *Journal of Clinical Microbiology*, *34*(12), 2881–2887.

Kakati, B. K., Sharma, P. & Goswami, U. C. (2017). Quality evaluation of dried fish products commerce in assam, india. *International Journal of Advanced Biological Research*, *7*(3), 465–469.

Kung, H.-F., Huang, C.-Y., Lin, C.-M., Liaw, L.-H., Lee, Y.-C. & Tsai, Y.-H. (2015). The histamine content of dried flying fish products in taiwan and the isolation of halotolerant histamine-forming bacteria. *Journal of Food and Drug Analysis*, *23*(2), 335–342. doi:10.1016/j.jfda.2014.10.009

Lin, C.-S., Tsai, H.-C., Lin, C.-M., Huang, C.-Y., Kung, H.-F. & Tsai, Y.-H. (2014). Histamine content and histamine-forming bacteria in mahi-mahi (coryphaena hippurus) fillets and dried products. *Food Control*, *42*, 165–171. doi:10.1016/j.foodcont.2014.02.004

Marino, M., Frigo, F., Bartolomeoli, I. & Maifreni, M. (2011). Safety-related properties of staphylococci isolated from food and food environments. *Journal of Applied Microbiology*, *110*(2), 550–561. doi:10.1111/j.1365-2672.2010.04909.x

Milijašević, M., Babić Milijašević, J., Đinović-Stojanović, J., Vesković Moračanin, S. & Slobodan, L. (2017). Changes of pH and peroxide value in carp (cyprinus carpio) cuts packaged in modified atmosphere. *IOP Conference Series: Earth and Environmental Science*, *85*, 012041. doi:10.1088/1755-1315/85/1/012041

Montville, T. J. & Matthews, K. R. (2008). *Food microbiology: An introduction, 2nd (edn)*. United States of America: ASM Press, Washington, DC.

Moon, H.-J., Min, K.-J., Park, N.-Y., Park, H.-J. & Yoon, K.-S. (2017). Survival of staphylococcus aureus in dried fish products as a function of temperature. *Food Science and Biotechnology*, *26*(3), 823–828. doi:10.1007/s10068-017-0096-0

Musharrafieh, R., Tacchi, L., Trujeque, J., LaPatra, S. & Salinas, I. (2014). Staphylococcus warneri, a resident skin commensal of rainbow trout (oncorhynchus mykiss) with pathobiont characteristics. *Veterinary Microbiology*, *169*(1-2), 80–88. doi:10.1016/j.vetmic.2013.12.012

Ortiz, J., Lemus-Mondaca, R., Vega-Galvez, A., Ah-Hen, K., Puente-Diaz, L., Zura-Bravo, L. & Aubourg, S. (2013). Influence of air-drying temperature on drying kinetics, colour, firmness and biochemical characteristics of atlantic salmon (salmo salar l.) fillets. *Food Chemistry*, *139*(1-4), 162–169. doi:10.1016/j.foodchem.2013.01.037

Patterson, J., Kailasam, S., Giftson, H. & Immaculate, J. K. (2018). Effect of drying technologies on the biochemical properties of stolephorus commersonnii. *Food Quality and Safety*, *2*(3), 153–158. doi:10.1093/fqsafe/fyy010

Prester, L. (2011). Biogenic amines in fish, fish products and shellfish: A review. *Food Additives and Contaminants Part A-chemistry Analysis Control Exposure & Risk Assessment*, *28*(11), 1547–1560. doi:10.1080/19440049.2011.600728

Rahman, M. S., Al-Amri, O. S. & Al-Bulushi, I. M. (2002). Pores and physico-chemical characteristics of dried tuna produced by different methods of drying. *Journal of Food Engineering*, *53*(4), 301–313.

Ray, B. & Bhunia, A. (2014). Fundamental food microbiology. crc press. *Boca Raton, Florida pp*, *66*.

Reginald, W. B. & Gayle, A. L. (2016). FDA/BAM: Staphylococcus aureus. Bacteriological Analytical Manual, Chapter 12. Retrieved from https://www.fda.gov/food/laboratory-methods-food/bam-staphylococcus-aureus

Rodriguez-Jerez, J. J., Mora-Ventura, M., López-Sabater, E. & Hernandez-Herrero, M. M. (1994). Histidine, lysine and ornithine decarboxylase bacteria in spanish salted semi-preserved anchovies. *Journal of Food Protection*, *57*, 784–787. doi:10.4315/0362-028X-57.9.784

Staruszkiewicz, W. F., Barnett, J. D., Rogers, P. L., Benner, R. A., Wong, L. L. & Cook, J. (2004). Effects of on-board and dockside handling on the formation of biogenic amines in mahimahi (coryphaena hippurus), skipjack tuna (katsuwonus pelamis), and yellowfin tuna (thunnus albacares). *Journal of Food Protection*, *67*(1), 134–141. doi:10.4315/0362-028X-67.1.134

Stewart, C. M. (2003). Staphylococcus aureus and staphylococcal enterotoxins. *Foodborne Microorganisms of Public Health Significance*, 359–379.

Takiguchi, A. (1996). Changes in free amino acid composition caused by lipid oxidation in pulverized niboshi (boiled and dried anchovy) during storage. *Fisheries Science*, *62*(2), 240–245. doi:10.2331/fishsci.62.240

Thanonkaew, A., Benjakul, S., Visessanguan, W. & Decker, E. A. (2006). Development of yellow pigmentation in squid (loligo peali) as a result of lipid oxidation. *Journal of Agricultural and Food Chemistry*, *54*, 956–62. doi:10.1021/jf052107h

Tsai, Y. H., Kung, H. F., Lee, T. M., Chen, H. C., Chou, S. S., Wei, C. I. & Hwang, D. F. (2005). Determination of histamine in canned mackerel implicated in a food borne poisoning. *Food Control*, *16*(7), 579–585. doi:10.1016/j.foodcont.2004.06.019

Wu, V. C. H. (2008). A review of microbial injury and recovery methods in food. *Food Microbiology*, *25*(6), 735–744. doi:10.1016/j.fm.2008.04.011

Yamani, M. I. & Untermann, F. (1985). Development of a histidine-decarboxylase medium and its application to detect other amino-acid decarboxylases. *International Journal of Food Microbiology*, *2*(5), 273–278. doi:10.1016/0168-1605(85)90040-6

International Journal of Food Studies | IJFS April 2020 | Volume 9 | pages 251–263

Effects of Drying Temperature on Quality Parameters of Thai Fermented Fish Dip (Jaew Bong)

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Received: 8 May 2019; Published online: 18 April 2020

Abstract

Fermented fish dip is a popular condiment in Thailand and the Lao People's Democratic Republic. Thai fermented fish dip (TFFD) can be dried to increase its shelf life and ease of transportation. Dried TFFD can be rehydrated to return the powder to its original, paste-like form. Pre-cooked TFFD paste was dried at three different temperatures (40, 60, and 80 °C). Total plate count, yeast and mould count, CIE colour values (L*, a*, and b*), non-enzymatic browning, and sensory scores of the resulting powders were determined. The CIE colour values and sensory scores were also analysed for rehydrated TFFD. Increasing the drying temperature did not affect the total plate count or yeast and mould count. When dried at 80 °C , the L* value of TFFD powder was reduced, although the a* and b* values were unaffected compared with lower temperatures. All CIE colour values of rehydrated TFFD decreased as drying temperature increased. Drying temperature did not affect the sensory scores of dried TFFD powder. However, rehydrated samples that had been dried at 80 °C had significantly lower sensory colour scores than those dried at 40 or 60 °C . Overall preference rankings of dried and rehydrated TFFD dried at 40 and 60 °C were better than for those dried at 80 °C. Due to an undesirable colour change in the rehydrated product, 80 °C was deemed to be an unsuitable temperature for drying TFFD paste. In conclusion, both 40 and 60 °C are appropriate temperatures for drying the product.

Keywords: Drying temperature; Fermented fish dip powder; Dehydrated Thai chilli paste; Rehydrated quality; Chilli-based product; Thai condiment

1 Introduction

Fermented fish dip is a condiment that is popular in the north-eastern and central regions of Thailand. The product is also commonly consumed in Lao People's Democratic Republic (Lao PDR), a neighbouring country of Thailand. Fermented fish dip is known in the Thai language as Jaew Bong, Plara Bong, or Nam Prik Plara; and in the Lao PDR language as Jaew Pla Dak. Thai fermented fish dip (TFFD) is composed of fermented fish paste (Plara), dried chilli, and fresh herbs including galangal, lemon grass, red shallot, and garlic. Galangal and lemon grass are sliced and used fresh. Red shallot and garlic are pan-roasted, grilled, or baked and peeled prior to combination. The ingredients are combined and pounded into a paste (Thai Industrial Standards Institute, 2013), after which taste- and flavour-enhancing ingredients such as fermented fish sauce, tamarind paste, and sugar may be added (Duangsai, Srisataporn, Hauisan & Gawbor-

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isut, 2019; Posri, 2008). TFFD paste is typically served with glutinous rice, fried or roasted meat, and fresh or steamed vegetables. The viscous paste is able to stick well to the rice, meat, or vegetables, which balances the taste and heat of TFFD (Duangsai et al., 2019; Posri, 2008; Teaupun, 2009). In the past, TFFD was prepared in the home and served uncooked. At the present time, TFFD is primarily manufactured in small-scale factories, and the product is fully cooked to eliminate food-borne pathogens such as *Salmonella* spp. and *Staphylococcus aureus* (Thai Industrial Standards Institute, 2013). The quality of TFFD varies depending on the ratio of ingredients and the processing methods used (Posri, 2008). High-quality TFFD contains well-blended ingredients and a desirable herbal flavour (Thai Industrial Standards Institute, 2013). Poor-quality TFFD may show visible separation of liquid from the paste, or have offensive musty or sour flavours associated with spoilage (Thai Industrial Standards Institute, 2013). Colour is a vital element in assessing the quality of uncooked TFFD, because it can be indicative of the freshness and level of heat in the product (Posri, 2008; Ratchatachaiyos, 2007). Cooked TFFD can be expected to be darker in colour than the raw product due to non-enzymatic browning reactions that occur during the cooking process (Posri, 2008).

The pH of commercial TFFD ranges from 4.42 to 5.4 (Posri, 2008). The moisture content and water activity (a_w) of commercial TFFD are 48.61-64.40% and 0.82-0.86 respectively (Posri, 2008). Its medium/low acidity and intermediate moisture and a_w levels make TFFD prone to spoilage, therefore shortening its shelf life (Duangsai et al., 2019; Hiraga, Stonsaovapak, Sittipod & Mahakarnchanakul, 2008; Posri, 2008). Additionally, the high moisture content of the product means that liquid leakage may occur during transportation, and the transportation weight may be high (Duangsai et al., 2019).

Drying is a widely used method for preserving food. Reduction of the a_w to < 0.66 prevents microbial spoilage and prolongs the shelf life of food products (Ulloa et al., 2015). In the case of TFFD, the removal of moisture also eliminates the transportation issues described previously. Dried TFFD may be ground or pulverized, to be used as an instant powder. Appropriate amounts of water can be added to rehydrate the powder, returning the dried TFFD to the original paste-like form.

Utilization of the drying process is limited in foods containing heat-sensitive compounds (Ahmed, Shivhare & Ramaswamy, 2002). Chilli and herbs - key ingredients contributing to the freshness, heat, and flavour of TFFD - contain heat-sensitive colour pigments and volatile oils, respectively. Although the effects of the drying process on the quality of dried TFFD have not been reported, it is likely that these ingredients will be adversely affected by the heat of the process. The negative effects of dehydration on the colour of capsicums and chilli-based products have been found to be caused by non-enzymatic browning reactions and thermal degradation of colour pigments (Ahmed et al., 2002; Arslan & Ozcan, 2011; Kim, Lee, Park, Lee & Hwang, 2006; Topuz, Feng & Kushad, 2009; Vega-Galvez, Lemus-Mondaca, Bilbao-Sainz, Fito & Andres, 2008; Vega-Galvez et al., 2009). Using a proper drying temperature may minimize adverse effects and maintain the desired colour and flavour of dried TFFD. The aim of this research was to investigate the quality parameters of dried TFFD powder and rehydrated TFFD, after drying at different temperatures (40, 60, and 80 ^{o}C), in order to ascertain the appropriate temperature for drying TFFD.

2 Materials and Methods

2.1 Preparation of cooked TFFD

The TFFD was prepared in accordance with a protocol modified from Thathiwan (2017). Briefly, 6 g fermented tilapia paste, 480 g minced galangal, 360 g thinly sliced lemon grass, 900 g minced, peeled, and roasted shallot, 900 g minced, peeled, and roasted garlic, and 240 g tamarind paste were blended using a food processor (MCM 640660, Bosch, Bratislava, Slovakia). Then, 264 g chilli powder, 204 g sugar, and 72 g julienned kaffir lime leaves were hand-mixed into the blended herbs. Fermented tilapia paste was purchased from a local factory (Phetdam Foods Co. Ltd., Kalasin, Thailand) and

kept at ambient temperature as recommended by the manufacturer. Other ingredients were purchased from a local supermarket (Tesco Lotus, Khon Kaen, Thailand). The resulting TFFD was divided into three equal portions, each of which was weighed and cooked as recommended by the Thai Industrial Standards Institute (2013). This involved heating on the stovetop until the internal temperature reached 70 ^{o}C and then simmering for 15 min (Official Methods of Analysis of the Association of Official Analytical Chemists, 1990). The cooked TFFD portions were packed into polypropylene plastic bags, kept in a refrigerator at 4 ^{o}C, and used for the experiment within 3 h.

2.2 Drying and rehydration of cooked TFFD

The three portions of cooked TFFD were spread on silicon baking mats to a thickness of 2 mm and dried in a hot-air oven at either 40, 60, or 80 ^{o}C until the a_w reached 0.66 or lower (Ulloa et al., 2015). The a_w assessment was conducted using an Aqua Lab Series 4TEV water activity meter (Aqua Lab, Pullman, WA, USA). Each sample was then aseptically removed from the mat with a sterilized spatula, weighed, and the microbial content analysed. The samples were pulverized prior to assessment of CIE colour values, non-enzymatic browning, and sensory acceptability. Dried TFFD powder samples were rehydrated with hot water. The rehydrated pastes were also analysed for CIE colour values and sensory acceptability. All experiments were repeated in triplicate using three lots of fermented fish.

2.3 Analysis of dried TFFD

Microbial content

Analysis of microbial content involved measurement of the total plate count (TPC) and yeast and mould count (YM). The TPC was determined using a published protocol (Al-Harbi & Uddin, 2005) after culturing on standard plate count agar (BBL, Sparks, MD, USA) at 30 ± 0.1 ^{o}C for 48 h. The YM was determined after culturing on acidified potato dextrose agar (BBL, Sparks, MD, USA) at 23-25 ^{o}C for 5 days (Mislivec & Stack, 1989). All microbial counts are expressed as log CFU g^{-1} sample.

CIE colour values

The samples were analysed for CIE colour values (L*, a*, and b*) using a Konica Minolta CM-2600d spectrophotometer (Konica Minolta, Inc., Japan). A D65 artificial daylight bulb and 10^o standard angle observer were used to illuminate the samples. The L* value indicates lightness, while a* and b* are the red/green and yellow/blue coordinates respectively.

Non-enzymatic browning

Non-enzymatic browning was assessed using the method described by Dissaraphong, Benjakul, Visessanguan and Kishimura (2006). Briefly, 5 g of dried TFFD powder was combined with 50 mL of ethanol 50% (v/v), stirred continuously for 60 min, and filtered with Whatman No. 1 filter paper. The absorbance of the filtrate was determined at 420 nm using a Spectronic-15 spectrophotometer (Thermo Scientific, Thermo Fisher Scientific India Pvt. Ltd., Nasik, India).

Sensory score evaluation

Sensory acceptability (colour, odour, texture, flavour, and overall acceptability) was evaluated using a nine-point hedonic scale according to Meilgaard, Civille and Carr (1991) (1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely). Prior to evaluation, samples were randomly assigned a three-digit number and presented to 45 panellists acquainted with TFFD. The panel was composed of 18 females and 17 males with ages between 20-50 years. All sensory evaluations were conducted in an air-conditioned room at 25 ^{o}C. A score of 5 was considered the limit of acceptability for all sensory parameters. Overall preference ranking according to Lu (2017) was also evaluated, with a ranking of '1' meaning most preferred. Any undesirable sensory characteristics noted by the panellists were recorded.

2.4 Analysis of rehydrated TFFD

Samples of dried TFFD powder were rehydrated with hot water (temperature > 90 ^{o}C). The amount of water added to the powder was calculated according to equation (1). The samples were allowed to absorb the water for 30 min and then stirred continuously for 2 min. CIE colour values and sensory tests were then evaluated as described in section 2.3.

$$WT_{reh} = W_{uncook} - W_{dried} \quad (1)$$

where WT_{reh} is the amount of hot water used for rehydrating the sample (g), W_{uncook} is the weight of uncooked TFFD (g), and W_{dried} is the weight of dried TFFD (g).

2.5 Statistical analysis

The experiment was carried out using a randomized complete block design (RCBD). Data were analysed using SAS University Edition (SAS Institute Inc., Cary, NC) with a 95% confidence level. The calculated means were compared using the least significant difference (LSD) test. Overall preference rankings were compared using Friedman's test as recommended by Meilgaard, Carr and Civille (2006).

3 Results and Discussion

3.1 Analysis of dried TFFD

Microbial content

The calculated TPC and YM values of dried TFFD samples are shown in Fig. 1. TPC and YM showed no significant difference ($p > 0.05$) between the three drying temperatures. The results indicated that increasing the drying temperature did not promote microbial destruction. It has been reported that some microorganisms are destroyed in the drying process. However, many microorganisms - such as bacterial endospores, yeasts, moulds, and several Gram-negative and Gram-positive bacteria - are resistant to dehydration (Jay, 2000). Our results indicated that the tested TFFD may have contained microorganisms that were able to withstand drying temperatures of $\leq$ 80 ^{o}C . Herbs and spices contain microorganisms indigenous to the soil and plants where they are grown, often including heat-resistant spore-forming bacteria which are able to survive the drying process (Farkas, 2001; Fellows, 2000). It is likely that the heat-resistant microorganisms in the TFFD originated from the herbal ingredients in the product.
These results were in agreement with Orphanides, Goulas, Botsaris and Gekas (2017) who found that increasing drying temperature from 40 to 70 ^{o}C did not affect the TPC of dried spearmint samples. Bourdoux, Li, Rajkovic, Devlieghere and Uyttendaele (2016) suggested that the complex structures and compositions of fruits, vegetables, herbs, and spices may explain the high variability in survival rates of microorganisms during the drying process. Complex structures of the herbal ingredients included in TFFD may therefore provide protection for microorganisms, explaining the unchanged TPC and YM values of the dried samples.

CIE colour values

The CIE colour values of dried TFFD are shown in Fig. 2. The results showed that increasing the drying temperature to 80 ^{o}C significantly reduced the L* value of dried TFFD, compared with 40 and 60 ^{o}C ($p < 0.05$) (Fig. 2 a). However, L* values showed no significant difference ($p > 0.05$) between 40 and 60 ^{o}C . The three temperatures had no effect on the a* and b* values ($p > 0.05$) (Fig. 2 b and c). The reduction of L* value may have resulted from increased non-enzymatic browning at higher temperatures. To our knowledge, the effect of drying temperature on the colour values of dried TFFD has not yet been reported. In both uncooked and cooked TFFD, non-enzymatic browning has been reported to be a cause of colour change (Posri, 2008). Changes in the colour values of dried jumbo squid and Atlantic salmon fillets are reported to be due to non-enzymatic browning, and are more prominent in samples exposed to high drying temperatures of 50 ^{o}C or more (Ortiz et al., 2013; Vega-Galvez et al., 2011). Additionally, the colour changes in dried salted cod (*Gadus morhua*) that occur at higher temperatures have been found to be due to contraction of

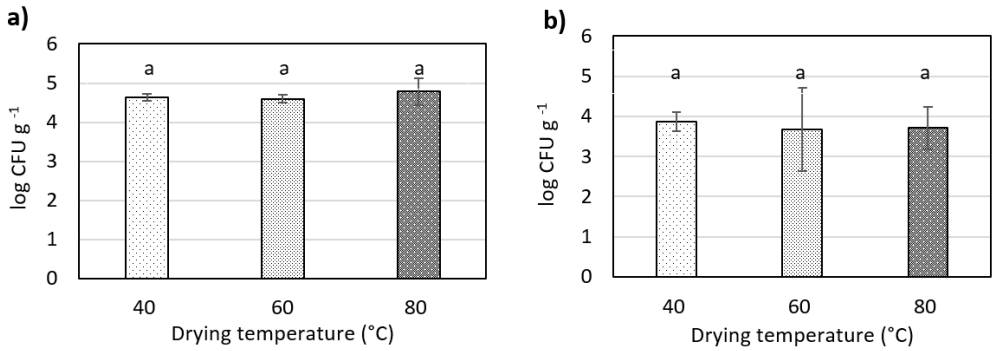

Figure 1: Total plate count (a) and yeast and mould count (b) of dried Thai fermented fish dip as affected by drying temperature. Identical letters above the bars within each parameter indicate counts that are not significantly different at a confidence level of 95%.

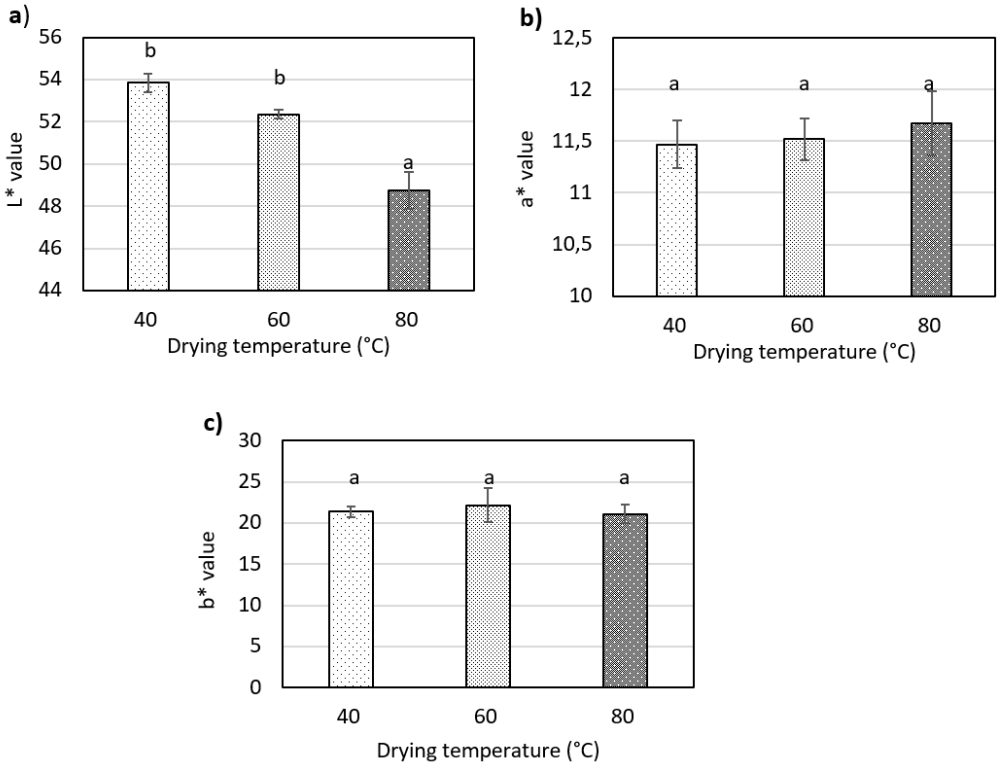

Figure 2: L* (a), a* (b), and b* (c) values of dried Thai fermented fish dip as affected by drying temperature. Identical letters above the bars within each parameter indicate values that are not significantly different at a confidence level of 95%.

muscle myotomes caused by protein aggregation (Ozuna, Gomez Alvarez-Arenas, Riera, Carcel & Garcia-Perez, 2014). Similar contraction of the fish muscle in TFFD at increased drying temperatures may contribute to reduction of the L* value.

Colour changes in dried red pepper and chilli are reported to be caused by non-enzymatic browning and the thermal degradation of colour pigments (Ahmed et al., 2002; Arslan & Ozcan, 2011; Kim et al., 2006). These reports showed that high drying temperatures cause more brown pigments to form, contributing to discolouration of the dried products. Discolouration of dried chilli in TFFD may have occured at 80 ^{o}C , thus reducing the L* value of the dried product. From these studies, it can be hypothesized that non-enzymatic browning of the fish in TFFD contributed to the reduction in the L* value of TFFD dried at 80 ^{o}C . To prevent colour changes in TFFD caused by non-enzymatic browning of chilli, drying temperatures of 40 and 60 ^{o}C are more appropriate than 80 ^{o}C .

Non-enzymatic browning

The results of the assessment for non-enzymatic browning of dried TFFD samples are shown in Fig. 3. The lowest value (0.76) was detected in the sample exposed to 40 ^{o}C , while the highest value (1.97) was found in the sample exposed to 80 ^{o}C . The rate of browning increased significantly as the drying temperature increased ($p < 0.05$). Non-enzymatic browning involves the reaction of carbonyl compounds with amino groups. In muscle-based foods, the carbonyl compounds mainly originate from carbohydrates, in the form of glycogen, reducing sugars, and nucleotides. Amino groups are readily available from the muscle protein (Vega-Galvez et al., 2011). The rate of non-enzymatic browning is reported to be highly sensitive to heat, increasing 2-3-fold for each 10 ^{o}C rise (Gögüs, Fadıloglu & Soysal, 2009).

It has been reported that non-enzymatic browning reactions reduce the colour quality of uncooked TFFD (Posri, 2008), but the effect of drying temperature on the non-enzymatic browning of dried TFFD has not, to our knowledge, been previously reported. Heat from the cooking process increased the rate of browning in TFFD, resulting in the cooked product displaying a darker colour (Posri, 2008). Drying temperatures of 50-90 ^{o}C have been shown to increase non-enzymatic browning reactions in both dried and rehydrated jumbo squid (*Dosidicus gigas*) (Vega-Galvez et al., 2011). Furthermore, Atlantic salmon (*Salmo salar* L.) fillets dried at 60 ^{o}C exhibit more colour change due to non-enzymatic browning compared with those dried at 40 or 50 ^{o}C (Ortiz et al., 2013).

The results led to the conclusion that fermented fish may influence the non-enzymatic browning of TFFD. This ingredient is composed of fish, salt, and carbohydrates such as rice bran or roasted rice, fermented for at least 6 months at ambient temperatures. During the fermentation process, proteolytic enzymes - naturally present in the guts and muscle of fish and those produced by microorganisms - break down the muscular protein into amino acids. Amylases and lipases produced by the microorganisms also cause chemical changes in the product, by releasing sugars from the rice bran or roasted rice (Krusong, 2004). Fermented fish in TFFD may therefore supply significant amounts of free amino acids and reducing sugars, which could contribute to excessive non-enzymatic browning of the product. This theory is supported by the fact that squid - which is also rich in free amino acids - is susceptible to excessive browning. This poses a significant quality problem in dried squid products, especially during the drying process and subsequent storage (Vega-Galvez et al., 2011).

Sensory score evaluation

The sensory acceptability scores of dried TFFD are shown in Fig. 4. Although a slight decrease in the scores was noted as the drying temperature increased, statistical analysis revealed that there was no significant difference in the sensory scores ($p > 0.05$). All samples were rated > 7 (like moderately), higher than the cut-off score of 5. Therefore, dried TFFD powder was considered acceptable.

Overall preference rankings of dried TFFD are illustrated in Fig. 5. There were no significant differences in the scores for TFFD dried at 40 and 60 ^{o}C ($p > 0.05$). The results show that the

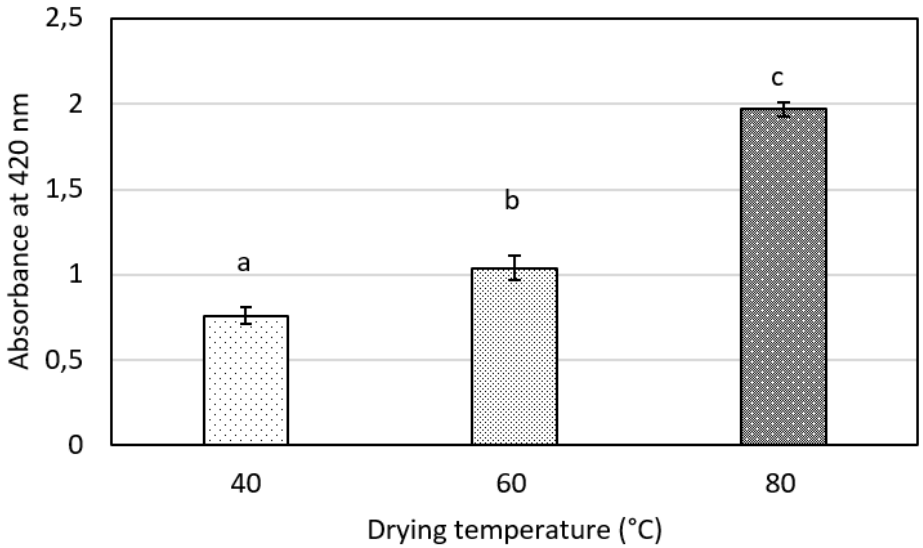

Figure 3: Non-enzymatic browning of dried Thai fermented fish dip as affected by drying temperature. Identical letters above the bars indicate values that are not significantly different at a confidence level of 95%.

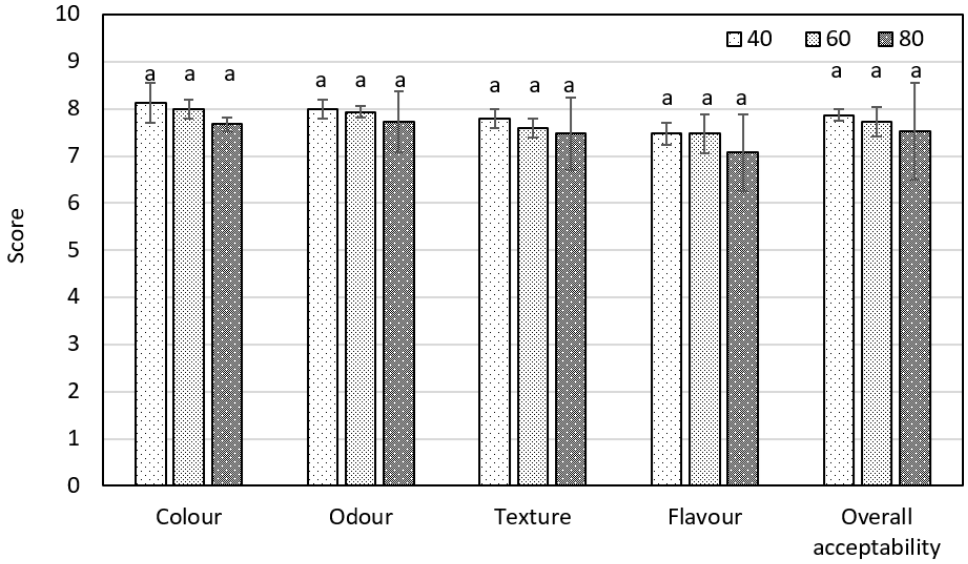

Figure 4: Sensory acceptability scores of dried Thai fermented fish dip as affected by drying temperature. Identical letters above the bars within each attribute indicate scores that are not significantly different at a confidence level of 95%.

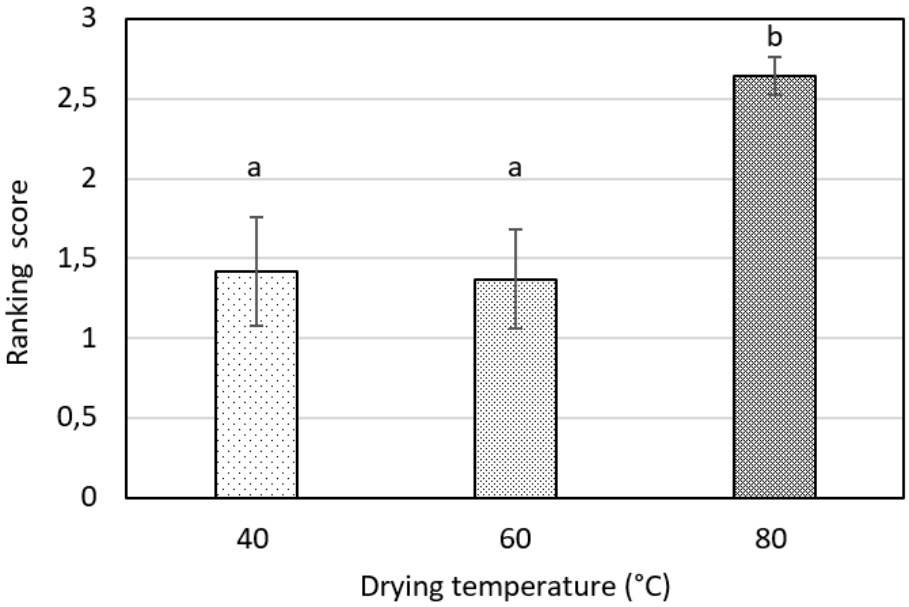

Figure 5: Overall preference ranking of dried Thai fermented fish dip as affected by drying temperature. Identical letters above the bars indicate rankings that are not significantly different at a confidence level of 95%.

average rankings of TFFD exposed to 40 and 60 ^{o}C were significantly better (close to 1) than of those dried at 80 ^{o}C ($p < 0.05$). Undesirable dark colour of TFFD exposed to 80 ^{o}C , recorded by panellists, may cause a worse rank (close to 3). It was clear that treatment at 40 and 60 ^{o}C gave better rankings compared to 80 ^{o}C treatment. Based on these results, drying temperatures of 40 and 60 ^{o}C were considered to be more appropriate for drying TFFD than 80 ^{o}C .

3.2 Analysis of rehydrated TFFD

CIE colour values

The CIE colour values of rehydrated TFFD are presented in Fig. 6. The results show that the L*, a*, and b* values of rehydrated TFFD decreased significantly as drying temperature increased ($p < 0.05$). Non-enzymatic browning may play an important role in the colour changes of rehydrated TFFD, as previously described for dried TFFD. The results revealed that drying at 80 ^{o}C significantly reduced the redness (indicated by the a* value) of the rehydrated TFFD samples. Redness plays an important role in the colour quality of TFFD, because it reflects the freshness and level of heat of the product (Posri, 2008). Drying at a temperature of 80 ^{o}C is therefore not appropriate for TFFD, due to the obvious changes in CIE colour values, particularly the a* value, that occur at this temperature. Several studies have investigated the colour values of rehydrated food products, most commonly products that are intended to be consumed after rehydration (Ulloa et al., 2015; Vega-Galvez et al., 2008; Vega-Galvez et al., 2009; Vega-Galvez et al., 2011). The effects of drying temperature on the colour values of rehydrated fish products have been investigated by Vega-Galvez et al. (2011). As the drying temperature increased from 50 to 90 ^{o}C , the L*, a*, and b* values of

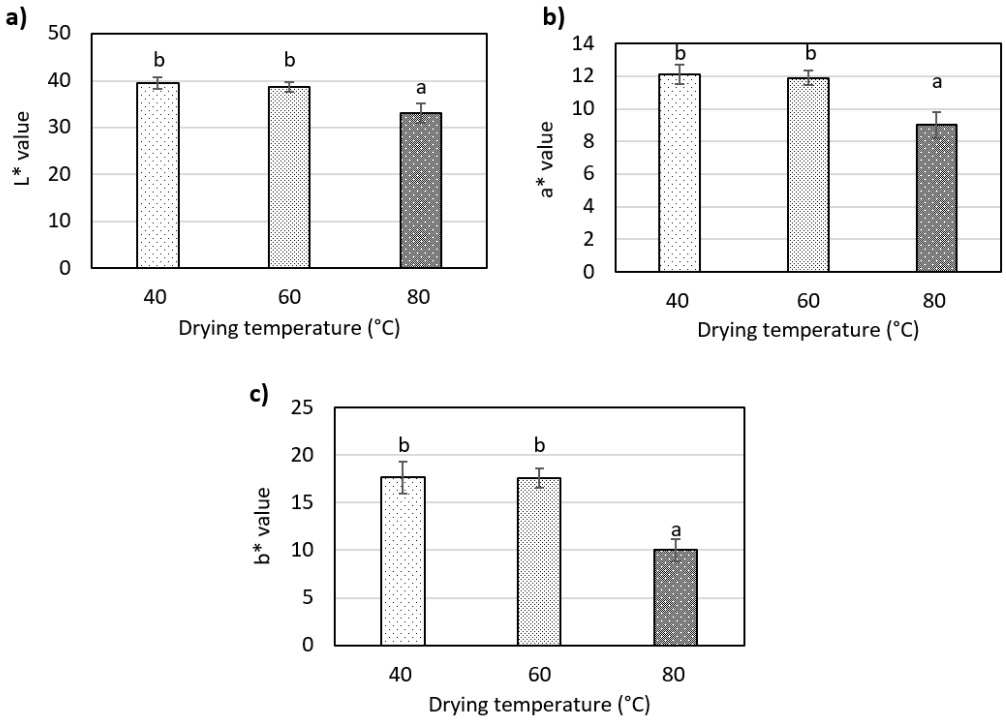

Figure 6: L* (a), a* (b), and b* (c) values of rehydrated Thai fermented fish dip as affected by drying temperature. Identical letters above the bars within each parameter indicate values that are not significantly different at a confidence level of 95%.

rehydrated jumbo squid decreased (Vega-Galvez et al., 2011). Non-enzymatic browning was suggested to be the cause of the colour changes observed in the product.

The results showed a relation between the L* values of dried TFFD and those of rehydrated TFFD. Similar decreasing trends in L* values of dried and rehydrated TFFD were observed as drying temperature decreased (Fig. 2 a and Fig. 6a). However, a* and b* values did not show any relation between dried and rehydrated samples. No reduction of a* and b* values was detected in dried samples (Fig. 2b and c), but reductions were found in rehydrated samples (Fig. 6 b and c). Addition of water to rehydrate TFFD may help the product gain a more homogenous structure, thus able to display more colour variation. Therefore, measurement of colour values in rehydrated TFFD along with dried TFFD is recommended.

Sensory score evaluation

The sensory acceptability scores recorded for rehydrated TFFD are shown in Fig. 7. Odour, texture, flavour, and overall acceptability scores were not significantly different between the drying temperatures ($p > 0.05$). However, the sensory colour score of the samples dried at 80 ^{o}C was significantly lower than those dried at 40 and 60 ^{o}C ($p < 0.05$; Fig. 7). Examination of panellists' records revealed that TFFD dried at 80 ^{o}C exhibited a darker colour, which was deemed to be less acceptable compared with those dried at 40 or 60 ^{o}C . Excessive brown pigments caused by non-enzymatic browning reactions during high-temperature drying may have contributed to a darker colour. The sensory colour scores correlated well with the CIE colour values of rehydrated TFFD (Fig. 6), in which the colour values decreased significantly when TFFD was exposed

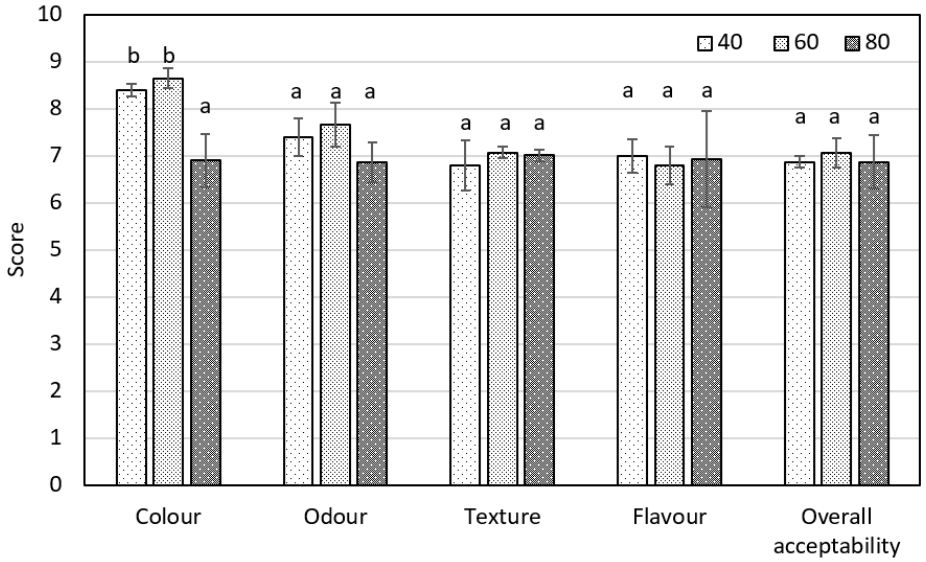

Figure 7: Sensory acceptability scores of rehydrated Thai fermented fish dip as affected by drying temperature. Identical letters above the bars within each attribute indicate scores that are not significantly different at a confidence level of 95%.

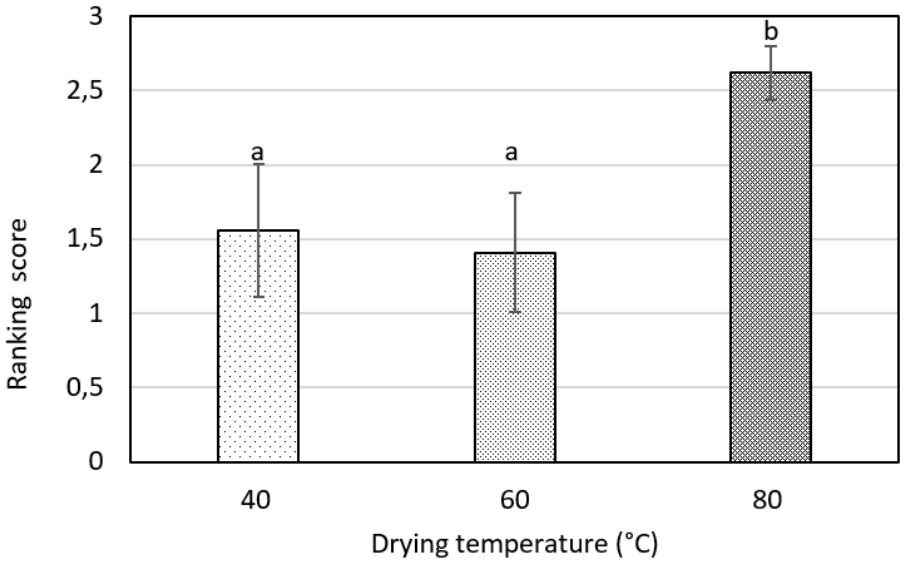

Figure 8: Overall preference ranking of rehydrated Thai fermented fish dip as affected by drying temperature. Identical letters above the bars indicate rankings that are not significantly different at a confidence level of 95%.

to 80 ^{o}C drying temperature. Drying temperatures of 40 and 60 ^{o}C were not significantly different in term of sensory scores ($p > 0.05$; Fig. 7). Overall preference rankings of rehydrated TFFD samples are presented in Fig. 8. The samples exposed to 40 and 60 ^{o}C had rankings of 1.56 and 1.41, respectively. These scores represented better ranks (close to 1) compared to drying at 80 ^{o}C which produced a score of 2.62. Panellists' records confirmed that 80 ^{o}C caused an unpleasant dark colour in rehydrated TFFD. The rankings of rehydrated TFFD (Fig. 8) showed a similar trend to those of dried TFFD (Fig. 5). Therefore, it is clear that 40 and 60 ^{o}C are more appropriate temperatures for drying TFFD than 80 ^{o}C .

4 Conclusions

The microbial content, CIE colour values, and sensory acceptability scores did not differ significantly between TFFD dried at 40 ^{o}C and that dried at 60 ^{o}C . Drying at 60 ^{o}C resulted in increased non-enzymatic browning compared with drying at 40 ^{o}C , but this did not have an adverse effect on sensory colour scores. Drying at a temperature of 80 ^{o}C caused significant changes in the CIE colour values and sensorial colour score, which were particularly evident when rehydrated product was examined. Overall preference rankings of dried and rehydrated TFFD confirmed that 40 and 60 ^{o}C gained better ranks than 80 ^{o}C . Therefore, both 40 and 60 ^{o}C are appropriate temperatures for drying TFFD, but at 80 ^{o}C there was an unacceptable amount of undesirable changes in the product and so that temperature should not be used for drying cooked TFFD.
Based on this study, optimization of drying factors such as time, temperature, and thickness of TFFD for commercial preparation using a mathematic model of data with multivariate analysis should be further investigated. Although dried TFFD is intended to be used as an instant powder, which should be rehydrated prior to consumption, it was found that the powder needed a rehydrating period of 30 min, due to its poor water absorption. Rehydrating for less than 30 min caused solid particles to form at the bottom of the container and separate from the liquid portion. To solve this problem, the addition of food binding or thickening agents to dried TFFD powder should be further explored.

Acknowledgements

The research was supported by project no. 114235 of Khon Kaen University, Thailand.

References

Ahmed, J., Shivhare, U. S. & Ramaswamy, H. S. (2002). A fraction conversion kinetic model for thermal degradation of color in red chilli puree and paste. *LWT- Food Science and Technology*, *35*(6), 497–503. doi:10.1006/fstl.897

Arslan, D. & Ozcan, M. M. (2011). Dehydration of red bell-pepper (*Capsicum annuum* L.): Change in drying behavior, colour and antioxidant content. *Food and Bioproducts Processing*, *89*(C4), 504–513. doi:10.1016/j.fbp.2010.09.009

Bourdoux, S., Li, D., Rajkovic, A., Devlieghere, F. & Uyttendaele, M. (2016). Performance of drying technologies to ensure microbial safety of dried fruits and vegetables. *Comprehensive Reviews in Food Science and Food Safety*, *15*(6), 1056–1066. doi:10.1111/1541-4337.12224

Dissaraphong, S., Benjakul, S., Visessanguan, W. & Kishimura, H. (2006). The influence of storage conditions of tuna viscera before fermentation on the chemical, physical and microbiological changes in fish sauce during fermentation. *Bioresource Technology*, *97*(16), 2032–2040. doi:10.1016/j.biortech.2005.10.007

Duangsai, P., Srisataporn, A., Hauisan, K. & Gawborisut, S. (2019). Quality parameters of instant dried thai fermented fish dip as affected by levels of guar gum. *Khon Kaen Agriculture Journal*, *47*(2). doi:10.14456/kaj.2019.37

Farkas, J. (2001). Food irradiation principles and applications. In R. A. Molins (Ed.), (Chap. Radiation decontamination of Spices, Herbs, Condiments, and Other

Dried Food Ingredients, pp. 291–303). New York: John Wiley & Sons.

Fellows, P. J. (2000). *Food processing technology: Principles and practice, second edition*. Woodhead Publishing in food science and technology. Taylor & Francis. Retrieved from https://books.google.pt/books?id=w6nJo2ZUi4MC

Gögüs, F., Fadıloglu, S. & Soysal, Ç. (2009). Biological oxidations: Enzymatic and non-enzymatic browning reactions and control mechanisms. *Advances in food biochemistry*, 341–373.

Al-Harbi, A. H. & Uddin, M. N. (2005). Microbiological quality changes in the intestine of hybrid tilapia (*Oreochromis niloticus x· Oreochromis aureus*) in fresh and frozen storage condition. *Letters in Applied Microbiology*, *40*, 486–490. doi:10.1111/j.1472-765X.2005.01716.x

Hiraga, C., Stonsaovapak, S., Sittipod, S. & Mahakarnchanakul, W. (2008). The development of processing and packaging for moist ready to eat/cook nam prik products. Retrieved from http://www3.rdi.ku.ac.th/exhibition/48/Project/index_72.htm

Jay, J. M. (2000). *Modern food microbiology*. Gaithersburg: Aspen Publishers Inc.

Kim, S., Lee, K. W., Park, J., Lee, H. J. & Hwang, I. K. (2006). Effect of drying in antioxidant activity and changes of ascorbic acid and colour by different drying and storage in korean red pepper (*Capsicum annuum*, L.) *International Journal of Food Science and Technology*, *41*(1), 90–95. doi:10.1111/j.1365-2621.2006.01349.x

Krusong, W. (2004). Industrialization of indigenous fermented foods. In K. H. Steinkraus (Ed.), (Chap. Production of Thai fermented fish: Plara, Pla-som, Som-fak, pp. 707–718). New York: Marcel Dekker.

Lu, X. (2017). *Sensory quality of atlantic salmon as affected of fish size and fillet part* (Master's thesis, Norwegian University of Life Sciences, Ås).

Meilgaard, M. C., Carr, B. T. & Civille, G. V. (2006). *Sensory evaluation techniques, fourth edition*. Food science & technology. Taylor & Francis. Retrieved from https://books.google.pt/books?id=F%5C_A-YtWXF3gC

Meilgaard, M., Civille, G. V. & Carr, B. T. (1991). *Sensory evaluation techniques*. CRC Press. Retrieved from https://books.google.pt/books?id=bwMpAQAAMAAJ

Mislivec, P. B. & Stack, M. E. (1989). Mycotoxin prevention and control in food grains. (Chap. Enumeration of yeasts and moulds and production of toxins). Retrieved from http://www.fao.org/docrep/X5036E/x5036E0o.htm#Enumeration%20%5C%20of%5C%20yeasts%5C%20and%5C%20moulds%5C%20and%5C%20production%5C%20of%5C%20toxins

Official Methods of Analysis of the Association of Official Analytical Chemists. (1990). *Official methods of analysis of the association of official analytical chemists*. Arlington: Association of Official Analytical Chemists.

Orphanides, A., Goulas, V., Botsaris, G. & Gekas, V. (2017). Influence of air-drying on the quality characteristics of spearmint: Effects of air temperature and velocity. *Journal of Food Processing and Preservation*, *41*(2), e12817. doi:10.1111/jfpp.12817. eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/jfpp.12817

Ortiz, J., Lemus-Mondaca, R., Vega-Galvez, A., Ah-Hen, K., Puente-Diaz, L., Zura-Bravo, L. & Aubourg, S. (2013). Influence of air-drying temperature on drying kinetics, colour, firmness and biochemical characteristics of atlantic salmon (*Salmo salar* L.) fillets. *Food Chemistry*, *139*(1-4), 162–169. doi:10.1016/j.foodchem.2013.01.037

Ozuna, C., Gomez Alvarez-Arenas, T., Riera, E., Carcel, J. A. & Garcia-Perez, J. V. (2014). Influence of material structure on air-borne ultrasonic application in drying. *Ultrasonics Sonochemistry*, *21*(3), 1235–1243. doi:10.1016/j.ultsonch.2013.12.015

Posri, W. (2008). *The study of extrinsic and intrinsic cues in fermented fish in chili paste to develop consumer acceptance in the North-East and the South of Thailand*. Bangkok, Thailand: Thailand Research Fund. Retrieved from http://

elibrary.trf.or.th/project_content.asp?PJID=MRG4680179

Ratchatachaiyos, K. (2007). *Consumer test and segmentation using multi-item attitude scales on chili paste product (jaew bong)* (Doctoral dissertation, Khon Kaen University, Khon Kaen, Thailand). Retrieved from https://tdc.thailis.or.th/tdc/browse.php?option=show&browse_type=title&titleid=178678&query=%A1%D1%AD%AD%D2%20%C3%D1%AA%B5%AA%D1%C2%C2%C8&s_mode=any&d_field=&d_start=0000-00-00&d_end=2563-03-21&limit_lang=&limited_lang_code=&order=&order_by=&order_type=&result_id=2&maxid=2

Teaupun, P. (2009). *Extension of chili paste shelf-life using essential oil from holy basil and a good manufacturing practice (gmp)* (Master's thesis, Naresuan University, Phitsanulok, Thailand). Retrieved from https://tdc.thailis.or.th/tdc/browse.php?option=show&browse_type=title&titleid=205101&query=%C0%D2%C7%D4%B3%D5%20%E0%B7%D5%E9%C2%C7%BE%D1%B9%B8%EC&s_mode=any&d_field=&d_start=0000-00-00&d_end=2563-03-21&limit_lang=&limited_lang_code=&order=&order_by=&order_type=&result_id=1&maxid=2

Thai Industrial Standards Institute. (2013). Thai Community Products Standard No. 123/2556: Plara Bong. Bangkok, Thailand: Thai Industrial Standards Institute, Ministry of Industry..

Thathiwan, J. (2017). Esan food: Jaew bong. Retrieved from https://bit.ly/2IZXYgg

Topuz, A., Feng, H. & Kushad, M. (2009). The effect of drying method and storage on color characteristics of paprika. *LWT- Food Science and Technology*, *42*(10), 1667–1673. doi:10.1016/j.lwt.2009.05.014

Ulloa, J. A., Ibarra-Zavala, S. J., Ramírez-Salas, S. P., Rosas-Ulloa, P., Ramírez-Ramírez, J. C. & Ulloa-Rangel, B. E. (2015). Chemical, physicochemical, nutritional, microbiological, sensory and rehydration characteristics of instant whole beans (*Phaseolus vulgaris*). *Food Technology and Biotechnology*, *53*(1), 48–56. doi:10.17113/ftb.53.01.15.3663

Vega-Galvez, A., Lemus-Mondaca, R., Bilbao-Sainz, C., Fito, P. & Andres, A. (2008). Effect of air drying temperature on the quality of rehydrated dried red bell pepper (var. lamuyo). *Journal of Food Engineering*, *85*(1), 42–50. doi:10.1016/j.jfoodeng.2007.06.032

Vega-Galvez, A., Di Scala, K., Rodriguez, K., Lemus-Mondaca, R., Miranda, M., Lopez, J. & Perez-Won, M. (2009). Effect of air-drying temperature on physico-chemical properties, antioxidant capacity, colour and total phenolic content of red pepper (*Capsicum annuum*, L. var. Hungarian). *Food Chemistry*, *117*(4), 647–653. doi:10.1016/j.foodchem.2009.04.066

Vega-Galvez, A., Miranda, M., Claveria, R., Quispe, I., Vergara, J., Uribe, E., ... Di Scala, K. (2011). Effect of air temperature on drying kinetics and quality characteristics of osmo-treated jumbo squid (*Dosidicus gigas*). *LWT- Food Science and Technology*, *44*(1), 16–23. doi:10.1016/j.lwt.2010.06.012