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Antimicrobial and Antioxidative Effects of Honey Marination on Beef Meat

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Antimicrobial, Antioxidant, Honey, Meat, Microbial count

ABSTRACT

The study was conducted to investigate the antimicrobial and antioxidant effects of honey marination on beef meat (*M. longissimus dorsi*). The samples were analyzed for moisture, fat, protein, glycogen, peroxide, thiobarbituric acid, total viable count and total coliform count after marination with 0, 10, 20, 30 and 100% concentrations of honey and after 1, 4, 8 and 12 days of storage (4 ℃). The moisture, fat and protein contents were moderately decreased on the 4, 8 and 12th days of storage in all the groups. The glycogen content showed a significant ($P < 0.05$) increase among the groups except for the control. Whereas peroxide and thiobarbituric acid values significantly ($P < 0.05$) decreased in all the groups, excluding control, which showed an increase. Moreover, the total viable and total coliform counts were markedly decreased at the 4, 8 and 12th days of storage in all the groups, except in group A. It concludes that the honey served as a natural preservative to reduce the lipid oxidation and microbial numbers due to its antimicrobial and antioxidative attributes. Hence, it prolongs the shelf life of beef meat without having any adverse effects on its quality.

INTRODUCTION

Beef is a powerhouse of essential nutrients obtained from animals, naturally rich in muscle-building protein and acts as a rich source of iron for energy. It is an edible postmortem component originating from animals that are used as food for humans. Approximately 73% water, 24% protein, 3.9% fat, 0.8% minerals and less than 1% carbohydrate-rich nutrients are present in beef meat (Brahmantiyo, 2000). Meat also contains other elements such as vitamin B12, niacin, vitamin B6, vitamin D, iron, zinc and phosphorus. Beef meat preserves an important role in human nutrition due to its distinct and high nutritional value (Williams, 2007). Naturally, beef is a highly perishable food commodity of animal origin, thus easily attacked by microbial flora which in turn causes reduced shelf life. The meat industry faces an important challenge to provide safe and wholesome meat and meat products to their consumers.

The availability of wholesome and safe food is a basic human right and is essential for human health. Among the factors affecting the shelf life of fresh meat, bacterial growth and metabolic activities are the most important causes of meat spoilage. The refrigerated meat spoilage was caused by microbial agents such as bacteria which brings undesirable changes in meat i.e., off-flavors, off odors, gas production, discoloration and slime production; such problems necessitate the usage of natural or artificial preservatives to increase the safety and shelf life of meat.

Various physical and chemical methods have been applied to decrease the contamination chances at various levels, raw meat and processing stages. Among them, marination is a method used to increase meat quality and preserve its nutritional value during storage. Moreover, due to the acidic or alkaline nature of the marinated solution, the shelf life of meat may be positively affected,

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and some marinade additives also produced antimicrobial and antioxidant activity. To prevent foodborne illness and increase shelf life, natural preservatives containing both antibacterial and antioxidant activities are desirable to be used.

Honey may serve as a natural food preservative. Honey is nectar collected from plants and processed by honeybees (*Apis mellifera*). This natural product is widely appreciated as the only concentrated form of sugar available worldwide (Krell, 1996) and is also used as a food preservative (Cherbuliez and Domerego, 2003). The natural honey is a viscous solution with a high sugar level of about 85% carbohydrate (mostly glucose and fructose), water ranges from 15–17%, low level of protein (0.1–0.4%), ash 0.2% and minute quantities of other substances i.e., minerals, amino acids, enzymes, lipids, organic acids, vitamins as well as phenolic antioxidants. Various components of honey-like tocopherol, phenolics and ascorbic acids flavonoids act as a preservative. Anti-bacterial and anti-oxidative properties of honey are due to various components naturally present in bee honey species obtained from different plant sources (Meda et al., 2005; Bertoncelj et al., 2007).

Honey inhibits the growth of microorganisms and fungi. Honey exhibited both bactericidal and bacteriostatic effects against various strains including *Escherichia coli, Streptococcus mutans, Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes, Salmonella enterica Ser: Typhimurium* and *Helicobacter pylori,* many of them are pathogenic. Honey antimicrobial activity is mainly attributed to its acidity (low pH 3.9), osmolarity, production of hydrogen peroxide, Maillard reaction products, phenolic compounds, aromatic acids, flavonoids, proteins and high sugar concentration (Aljadi and Kamaruddin, 2004; Cushnie and Lamb, 2005; Estevinho et al., 2008; Madeo et al., 2009; Montenegro et al., 2009; Alvarez-Saurez et al., 2010). Bactericidal effects have been produced by 5 to 50% concentrations of honey.

Honey serves as a natural antioxidant (Al-Mamary et al., 2002; Aljadi and Kamaruddin, 2004; Beretta et al., 2005; Kucuk et al., 2007). which play an important role in food preservation and human health by combating damage caused by oxidizing agents e.g., oxygen, namely reducing the risk of heart disease, cancer, immune system decline, cataracts, different inflammatory processes, etc (The National Honey Board, 2003). Antioxidant activity of honey is attributed to various substances like catalase, glucose oxidase (Schepartz, 1966; Iyorish, 1974), phenolic acids, alpha-tocopherol, organic acids, flavonoids, ascorbic acid, Maillard reaction products, proteins, amino acids and carotenoid derivatives (Blasa et al., 2006; Baltrusaityte et al., 2007; Brudzynski and Miotto, 2011). Generally, antioxidant power is directly related to phenolic contents of honey and these are more in darker color honey (Bertoncelj et al., 2007; Vela et al., 2007; Al-Marghitas et al., 2009). Honey as a source of antioxidant has been proven to be effective against deteriorative oxidation reactions in food, caused by light, heat and some metals, such as lipid oxidation in meat (Nagai et al., 2006).

Keeping in view the perishable nature of beef meat, public health hazards and the importance of honey as an antimicrobial and antioxidative agent, this study was designed to examine the antimicrobial and antioxidative activities of various honey concentrations on beef meat.

MATERIALS AND METHODS

Meat samples

One kg fresh beef meat (*M. longissimus dorsi*) was purchased from the local market of Tando Jam, Sindh Pakistan. The sample was aseptically collected in sterile plastic bags, which was then transported (at 4°C within 2-3 h) to the milk and meat chemistry and microbiological laboratory, Department of Animal Products Technology, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tando Jam for further processing and analysis.

Honey sample and its composition

Fresh honey was obtained from the local market and brought to the laboratory of Animal Products Technology, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tando Jam and stored at room temperature. Pure honey and its various concentrations (10%, 20% and 30%) in sterile distilled water were used for beef marination. Composition and physicochemical properties of honey used for present study contains 18 % moisture, 39% fructose, 3.5% sucrose, 1.15% protein, 0.35% minerals and a pH of 3.96.

Meat preparation and marination

Meat sample was sliced into small pieces and marinated with different honey concentrations (Table I). To ensure uniform marination, meat sample was left for 24 h at 4 ℃ and then vacuum packaged in polyethylene bags and refrigerated for storage (4 ℃). Finally, samples were analyzed on day 0 and after 1, 4, 8, 12 days of post storage for moisture content, total protein content and fat content according to methods reported by AOAC (2000), glycogen content according to Kemp and Van Heijningen (1954), peroxide value (POV) according to Cunniff (1999) and thiobarbituric acid (TBA) according to Schmedes and Hølmer (1989). Besides that, pour plate technique in triplicate per sample was used for enumeration of bacteria.

Table I. Different honey marinated beef meat groups.

Groups	Treatments	Meat sample (g)	Marination	Replicates
A	Control (No honey)	200	-	5
B	10% honey	200	20g of 10% honey	5
C	20% honey	200	20g of 20% honey	5
D	30% honey	200	20g of 30% honey	5
E	100% honey	200	20g of pure honey	5

Statistical analysis

The obtained values were analyzed through analysis of variance (ANOVA) on proximate analysis, colony counts and lipid oxidation from various honey concentrations and if significant differences noticed among the means, the least significant difference (LSD) was computed using statistical software (SPSS version 21.0).

RESULTS

Table II shows the effect of marination in different concentrations of honey on moisture, protein, fat, glycogen, peroxide, thiobarbituric acid, total viable count and total coliform counts of the beef meat.

The interactive influence of different honey treatments and days on the moisture content of beef meat was observed and statistical analysis showed that at day 1, the moisture content of all groups was found relatively similar ($P > 0.05$), while at day 4, 8 and 12, the moisture contents of groups D and E were significantly ($P < 0.05$) varied from groups A and B (Fig. 1A). Similarly, at days 4, 8 and 12, there was a significant ($P < 0.05$) difference in fat content between groups D and E compared to groups A and B (Fig. 1B). In case of protein content, the interactive influence of different honey treatments and days statistically showed similar values at day 1, while at day 4, 8 and 12, the protein contents of groups D and E were significantly ($P < 0.05$) varied from group A (Fig. 2A and Table II). Whereas the glycogen content of beef meat showed significant ($P < 0.05$) variation among the groups at day 1, 4, 8 and 12 (Fig. 2B). A positive relationship was observed between the honey concentrations and the glycogen level of beef meat. The interactive influence of different honey treatments and days on the peroxide and TBA value of beef meat was observed, and the statistical analysis showed that at day 1, the peroxide value of groups D and E was found significantly ($P < 0.05$) different from groups A and B, while at day 4, 8 and 12, the peroxide value of all the groups was significantly ($P < 0.05$) varied from one another (Fig. 3A).

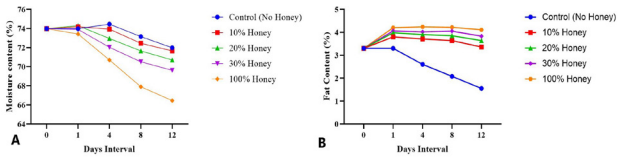

Fig. 1. Interactive influence of different treatments of honey and days interval on moisture percentage (A) and fat content (B) of beef meat.

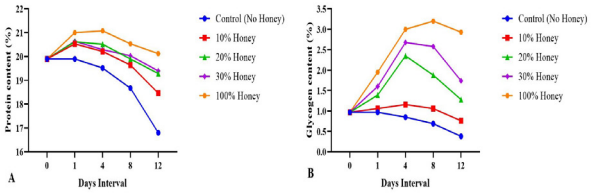

Fig. 2. Interactive influence of different treatments of honey and days interval on protein content (%) (A) and glycogen content (B) of beef meat.

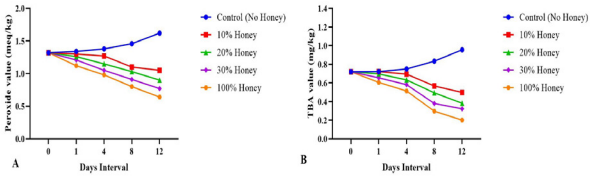

Fig. 3. Interactive influence of different treatments of honey and days interval on POV (meq/kg) (A) and TBA (mg/kg) value (B) of beef meat.

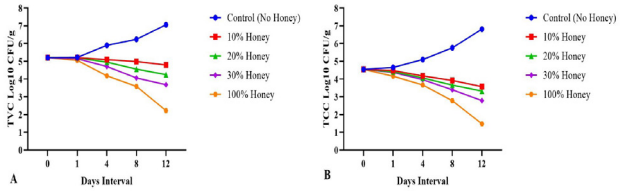

Fig. 4. Interactive influence of different treatments of honey and days interval on TVC (Log_{10} CFU/g) (A) and TCC (Log_{10} CFU/g) (B) of beef meat.

The influence of various honey treatments and storage days on the total viable count (TVC) and total coliform count (TCC) of beef meat showed similar TV and TC counts at day 1, except group E which showed significant ($P < 0.05$) decrease in TCC. while significant ($P < 0.05$) difference been observed between the groups at day 4, 8 and 12 for TV and TCC (Fig. 4A and B).

Table II. Interactive influence of different treatments of honey and days interval on various parameters of the present study.

S/N.	Days	A (0%)	B (10%)	C (20%)	D (30%)	E (100%)	LSD=0.05
1.	**Moisture percentage (%)**						
	Day 0 value (Raw meat) = 73.88						
	1	$73.98 \pm 1.33^{a-c}$	74.18 ± 1.05^{ab}	74.3 ± 0.90^{ab}	$73.9 \pm 0.98^{a-c}$	$73.42 \pm 1.18^{a-d}$	1.1658
	4	74.45 ± 1.82^{a}	$73.94 \pm 1.27^{a-c}$	$72.96 \pm 1.01^{c-f}$	$72.03 \pm 1.48^{c-g}$	70.7 ± 1.29^{hi}	
	8	$73.16 \pm 1.53^{b-e}$	$72.45 \pm 0.75^{d-g}$	71.66 ± 0.60^{gh}	70.52 ± 0.73^{hi}	67.9 ± 3.23^{j}	
	12	71.98 ± 1.41^{fg}	71.66 ± 1.17^{gh}	70.71 ± 1.16^{hi}	69.62 ± 1.00^{i}	66.46 ± 2.15^{k}	
2.	**Fat percentage (%)**						
	Day 0 value (Raw meat) = 3.3						
	1	3.3 ± 0.49^{g}	$3.8 \pm 0.73^{c-e}$	$3.98 \pm 0.84^{a-d}$	$4.06 \pm 0.84^{a-c}$	4.2 ± 0.86^{ab}	0.3321
	4	2.6 ± 0.34^{h}	3.71 ± 0.79^{de}	$3.9 \pm 0.84^{b-e}$	$4.02 \pm 0.89^{a-d}$	4.24 ± 0.91^{a}	
	8	2.08 ± 0.32^{i}	3.64 ± 0.71^{ef}	$3.85 \pm 0.81^{c-e}$	$4.05 \pm 0.85^{a-c}$	4.22 ± 0.84^{ab}	
	12	1.55 ± 0.27^{j}	3.36 ± 0.69^{fg}	3.64 ± 0.63^{ef}	$3.83 \pm 0.70^{c-e}$	$4.11 \pm 0.82^{a-c}$	
3.	**Protein percentage (%)**						
	Day 0 value (Raw meat) = 19.9						
	1	$19.9 \pm 2.48^{a-d}$	$20.53 \pm 1.94^{a-c}$	20.62 ± 1.86^{ab}	20.64 ± 2.19^{ab}	21.0 ± 1.98^{a}	1.3326
	4	$19.52 \pm 3.24^{b-e}$	$20.21 \pm 2.34^{a-c}$	$20.52 \pm 2.90^{a-c}$	$20.29 \pm 3.42^{a-c}$	21.08 ± 2.98^{a}	
	8	18.67 ± 2.67^{de}	$19.64 \pm 2.85^{b-e}$	$19.9 \pm 2.33^{a-d}$	$20.03 \pm 3.23^{a-c}$	$20.54 \pm 2.24^{a-c}$	
	12	16.8 ± 3.04^{f}	18.46 ± 2.44^{e}	$19.29 \pm 3.10^{c-e}$	$19.4 \pm 2.16^{b-e}$	$20.12 \pm 2.18^{a-c}$	
4.	**Glycogen percentage (%)**						
	Day 0 value (Raw meat) = 0.97						
	1	0.97 ± 0.13^{kl}	1.06 ± 0.11^{jk}	1.39 ± 0.15^{h}	1.60 ± 0.14^{g}	1.95 ± 0.08^{e}	0.1710
	4	0.85 ± 0.16^{lm}	1.16 ± 0.10^{ij}	2.35 ± 0.17^{d}	2.68 ± 0.13^{c}	3.0 ± 0.21^{b}	
	8	0.69 ± 0.13^{m}	1.06 ± 0.16^{jk}	1.88 ± 0.28^{ef}	2.58 ± 0.23^{c}	3.2 ± 0.19^{a}	
	12	0.38 ± 0.10^{n}	0.76 ± 0.20^{m}	1.28 ± 0.38^{hi}	1.74 ± 0.54^{fg}	2.93 ± 0.13^{b}	
5.	**Peroxide value (meq/kg)**						
	Day 0 value (Raw meat) = 1.32						
	1	1.34 ± 0.19^{cd}	$1.30 \pm 0.20^{c-e}$	1.26 ± 0.19^{ef}	1.21 ± 0.19^{fg}	1.12 ± 0.13^{hi}	0.0766
	4	1.38 ± 0.20^{c}	$1.27 \pm 0.23^{d-f}$	1.15 ± 0.16^{gh}	$1.05 \pm 0.16^{i-k}$	0.98 ± 0.11^{kl}	
	8	1.46 ± 0.13^{b}	$1.10 \pm 0.15^{h-j}$	1.03 ± 0.15^{jk}	0.91 ± 0.10^{lm}	0.80 ± 0.09^{n}	
	12	1.62 ± 0.13^{a}	$1.05 \pm 0.15^{i-k}$	0.90 ± 0.14^{m}	0.77 ± 0.09^{n}	0.64 ± 0.06^{o}	
6.	**Thiobarbituric acid value (mg/kg)**						
	Day 0 value (Raw meat) = 0.72						
	1	0.72 ± 0.02^{d}	0.72 ± 0.02^{d}	0.70 ± 0.02^{e}	0.66 ± 0.02^{f}	0.61 ± 0.02^{h}	0.0204
	4	0.75 ± 0.03^{c}	0.70 ± 0.03^{e}	0.63 ± 0.03^{g}	0.58 ± 0.01^{i}	0.51 ± 0.03^{j}	
	8	0.83 ± 0.03^{b}	0.57 ± 0.02^{i}	0.49 ± 0.03^{j}	0.38 ± 0.01^{k}	0.30 ± 0.02^{m}	
	12	0.96 ± 0.03^{a}	0.50 ± 0.04^{j}	0.38 ± 0.02^{k}	0.32 ± 0.04^{l}	0.20 ± 0.02^{n}	
7.	**Total viable count (Log_{10} CFU/g)**						
	Day 0 value (Raw meat) = 5.21						
	1	5.22 ± 0.05^{d}	5.21 ± 0.04^{d}	5.20 ± 0.05^{d}	5.15 ± 0.06^{de}	5.06 ± 0.04^{de}	0.2355
	4	5.90 ± 0.04^{c}	5.09 ± 0.02^{de}	$4.95 \pm 0.02^{e-f}$	4.71 ± 0.04^{gh}	4.18 ± 0.03^{i}	
	8	6.20 ± 0.02^{b}	$4.99 \pm 0.02^{d-f}$	4.56 ± 0.04^{h}	4.06 ± 0.02^{i}	3.59 ± 0.04^{j}	
	12	7.06 ± 0.02^{a}	4.80 ± 0.05^{fg}	4.25 ± 0.08^{i}	3.68 ± 0.04^{j}	2.22 ± 1.18^{k}	
8.	**Total coliform count (Log_{10} CFU/g)**						
	Day 0 value (Raw meat) = 4.55						
	1	4.66 ± 0.04^{d}	4.48 ± 0.03^{e}	4.42 ± 0.07^{e}	4.38 ± 0.06^{e}	4.17 ± 0.02^{f}	0.1221
	4	5.10 ± 0.02^{c}	4.18 ± 0.02^{f}	4.06 ± 0.02^{fg}	3.97 ± 0.02^{gh}	3.67 ± 0.04^{i}	
	8	5.76 ± 0.04^{b}	3.93 ± 0.03^{h}	3.67 ± 0.05^{i}	3.40 ± 0.06^{j}	2.78 ± 0.03^{k}	
	12	6.81 ± 0.03^{a}	3.58 ± 0.05^{i}	3.32 ± 0.08^{j}	2.79 ± 0.03^{k}	1.47 ± 0.59^{l}	

Means with different superscripted alphabets $^{(a, b, c)}$ in same row/column varied significantly ($P \leq 0.05$) from one another.

DISCUSSION

The moisture content of meat indicated an inverse relationship with honey concentrations as to increase the level of honey in beef meat lowers the moisture content. which is correlated with the previous results (Gandotra et al., 2012; Jouki and Khazaei, 2011), who also observed a significant decrease ($P < 0.01$) in the moisture content of marinated meat samples up to 21 days of storage. A similar trend of loss in water content of meat with an increase in honey concentration and storage was noted (Alabdulkarim, 2012). Dissimilarity was also observed in results of current investigations for moisture content and concluded that to some extent the moisture content of meat was increased initially by the addition of honey and then reduced before baking (Hashim et al., 1999). Similarly, non-significant variation in moisture percent was also noticed before and after storage in fish meat (Arannilewa et al., 2006). Fat contents of meat indicated a positive relationship with honey concentrations, as to increase the level of honey reduces the loss of fat content in beef meat. Present study results are not in agreement with the previous studies (Gandotra et al., 2012) who observed reduction of the fat content of meat during storage for 21 days. In another study, the highest fat was observed in the case of fresh meat ($9.72 \pm 0.25\%$) and the least fat value ($7.20 \pm 0.19\%$) was recorded for sixty days of stored meat (Arannilewa et al., 2006). However, non-significant impact of marinade containing 10% and 20% honey on the fat content of chicken meat was also observed. The fat percentage in chicken patties reduced significantly with the addition of honey and treatment of samples with (10%) honey had minimum fat content as compared to samples treated with (0 and 5%) honey (Alabdulkarim, 2012). These results of protein content showed a positive relationship with honey concentrations and as the loss of protein content in beef meat was reduced due to an increase in the level of honey. However, the storage period inversely affects the protein content of beef meat at 4ºC. Present findings were agreed with previous research (Gandotra et al., 2012), who indicated a significant decrease ($P < 0.01$) in protein content in fish meat during storage at 4 ± 1ºC. The storage time had also a significant impact on the formation of carbonyl substances (protein oxidation) as it was increased over a while in vacuum-packaged meat samples, resulted in a significant decrease in protein content of beef (Popova et al., 2009). An increase in storage duration results in the reduction of protein level, initially it was recorded as $60.65 \pm 2.40\%$ in fresh meat samples, while it reduced up to $43.70 \pm 1.17\%$ at the 60th day of storage (Arannilewa et al., 2006).

A negative relationship was seen between honey concentrations, peroxide and TBA values of beef meat. Minimum POV with an increase in honey concentration; 7.5% honey added sample showed lowest POV than respective sample treated with 0.0, 2.5 and 5.0% of honey (Mohammed et al., 2013). Honey treatment significantly reduced the Thiobarbituric acid reactive substances (TBARS) of lipids, samples treated with 10% honey had minimum TBARS during storage than the rest of the samples treated with (0 and 5%) honey (Alabdulkarim, 2012). Present findings are following the concept that honey and other natural antioxidants protected chicken meat from oxidation by lowering the hexanal values (Sampaio et al., 2012). Honey marinated treatments showed reduced TBA and POV after the storage period as to that of control (Istrati et al., 2011). It is further highlighted that the oxidative stability of meat was increased by adding honey, as showed a decreased in hexanal content, TBA values and oxidative stability index (Antony et al., 2006). Similarly, clover honey (CH), wildflower honey (WH) and buckwheat honey delayed the lipid oxidation and after 12 days of refrigerated storage, all were equally effective in decreasing the TBARS, lipid hydroperoxides (LOOH) and heterocyclic aromatic amine (HAA) formation in beef (Johnston et al., 2005; Shin and Ustunol, 2006). It was also noticed that honey produced greater antioxidative effects to decrease the TBA and hexanal values as the amount of added honey was increased (Antony et al., 2000, 2002).

Present findings indicated a negative relationship between honey concentrations and TVC because of the antimicrobial activity of honey. Least TVC was enumerated in meat treated with honey than control (Mohammed et al., 2013). Regardless, during the storage period by increasing the addition of bee honey concentrations in sausage, there was a decrease in the TVC. Another previous study revealed that bacterial load found within acceptable limits at day 10 (6.04 ± 0.11 log CFU/g), after that, further bacterial contamination leads to deteriorating the quality of meat and unfit for consumption (Gandotra et al., 2012). It is fact that natural honey (NH) provided significantly less ($P < 0.05$) number of bacteria compared to artificial honey (AH) as well as manuka honey 1 and 2 at different concentrations (12.5, 25 and 50%) (Badet and Quero, 2011; Nassar et al., 2012). Further it is indicated that manuka honey and different dilutions of honey (20% to 100%) prevent the growth of *S. pyogenes* and *P. aeruginosa*, respectively (Maddocks et al., 2012; Shenoy et al., 2012). It has been observed that both gram negative as well as gram positive bacteria were inhibited by honey (Alvarez-Saurez et al., 2010; Yucel et al., 2005). It is confirmed from the literature that honey is a mixture of higher sugar compounds, hydrogen peroxide generation and the presence of proteinaceous substances inhibit the

bacterial growth and concluded that various spoilage and pathogenic microbial agents can be controlled by using various concentrations of honey (Mundo et al., 2004; Taormina et al., 2001) and all honey samples showed significant levels of antimicrobial activities against standard organisms (Nzeako and Hamdi, 2000).

These results showed a negative relationship between honey concentrations and TCC. An increase in the amount of added honey reduces the number of TCC in beef meat due to the antimicrobial activity of honey which is confirmed from the results of previous study where beef sausages treated with bee honey had the lowest ($P < 0.05$) TCC than control and TC bacteria decreased with the increase of bee honey concentration (Mohammed et al., 2013). Selective growth inhibitory effects of honey against *E. coli* has been observed due to the presence of H_2O_2 in honey (Brudzynski, 2006). TC count $3.0x10^3$ was increased up to $7.5x10^6$ in control samples with the increase of storage duration (Arannilewa et al., 2006). All-natural honey as well as artificial commercial honey, can inhibit the growth of *E. coli* and *S. typhimurium* growth and high concentrations of honey produced more antibacterial effects (Badawy et al., 2004; Shamala et al., 2002).

CONCLUSIONS

The moisture content, peroxide value, TBA value, TVC and TCC of honey marinated beef meats were lower as compared to control and it was inversely proportional to honey concentration. Whereas fat, protein and glycogen contents were found to be higher in honey marinated beef meats. It concludes that the honey served as a natural preservative to reduce the lipid oxidation and microbial number due to its antimicrobial and antioxidative attributes. Hence, prolong the shelf life of beef meat without producing any adverse effects on its quality.

Statement of conflict of interest

The authors have declared no conflict of interest.

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