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Synergistic anti-microbial effect of grilling and edible coating on spore forming *B. cereus* in beef fillets

[*]Nahla A. Abo EL-Roos, [**] Asmaa A. Hashhash, [*]Mohebat A. Abd El-Aziz, [*] El Sayed Abd El Aty

[*]Food hygiene Department, Animal Health Research Institute, Agriculture Research Center of Egypt, Shebin El koom Branch, Egypt.
[**]Faculty of Veterinary Medicine, Department of Food Hygiene and Control, Benha University, Qaluobia 13736, Egypt

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ABSTRACT

Quality and safety of refrigerated beef fillet represented a concern for consumers. Moreover, synergistic effect between two or more of anti-microbial protocols to control bacteria in food especially heat resistant spore forming bacteria is a demand for food safety. So, aim of this study was to evaluate the synergistic application of grilling (heat treatment), and CMC coated samples supported by lactoferrin and propolis on survival of Bacillus cereus in beef fillets. Results revealed an effective synergistic antimicrobial effect of LF and PR incorporated into the CMC edible coating in a combination with grilling against experimentally inoculated *B. cereus*.
So, grilling alone as antimicrobial can't eliminate *B. cereus* in beef fillets, while application of edible coating fortified with LF and PR enhanced the antimicrobial effect of grilling in a synergistic way to control *B. cereus*.

INTRODUCTION

B. cereus is largely linked to a variety of health un-wellness and it is an adaptable human pathogen connected to a number of severe infections, like endocarditis, periodontitis, necrotizing fasciitis, nosocomial acquired bacteraemia, osteomyelitis, severe eye infections and etc. **(Kumari and Sarkar, 2016).**

It is a Gram-positive, rod-shaped, spore-forming, facultative anaerobic bacterium widespread in nature and commonly isolated from soil, plants, water, as well as from very different type of foods, such as cereals, milk, spices, fruits and vegetables **(Choi and Kim, 2020).**

Although Foods contaminated with *B. cereus* do not usually show signs of spoilage because *B. cereus* does not change the appearance or taste of the food **(Tewari and Abdullah, 2015)**, they are very dangerous to human health because ingestion of a food containing as few as 10^4–10^5 CFU/g of *B. cereus* can cause foodborne illness **(MFDS, 2021).**

B. cereus is capable of forming endospores as a survival mechanism when faced with unfa-

Corresponding author: Nahla A. Abo EL-Roos, Food hygiene Department, Animal Health Research Institute, Agriculture Research Center of Egypt, Shebin El koom Branch, Egypt.
E-mail: Dr.nahlashawky@yahoo.com
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vorable environments not suitable for vegetative growth. These spores can protect it from food processing, pasteurization and heating **(Pereira and Sant'Ana, 2018).** So, it is practically hard to prevent *B. cereus* contamination in food due to the large presence of these spores **(Soleimani et al., 2018).**

Heat treatment is one of the most common methods used to eliminate bacteria in food, however, spores of bacteria can survive heat treatments and proliferate **(Almatroudi et al. 2018)**. This heat resistance cause limitation to control bacteria in food.

One of the novel applications that overcome this limitation is active packaging owing to the migration of active compounds from edible coating to food **(Nottagh et al. 2020)**.

Synergistic effect between two or more of antimicrobial protocols to control bacteria in food especially heat resistance spore forming bacteria is a demand for food safety.

So, aim of this study was to evaluate the synergistic application of grilling (heat treatment), and CMC coated samples supported by lactoferrin and propolis on survival of *B. cereus* in beef fillets.

MATERIALS AND METHODS

MATERIALS

CMC (DS = 0.9) was purchased from Sigma-Aldrich. Bovine Lactoferrin 20,000 IU/mg (Sigma-Aldrich, U.S.A.). Propolis powder was obtained from the apiaries of the faculty of agriculture at Cairo University. *B. cereus* strain (ATCC® 10876) was obtained from Media Unite, Food Hygiene Department, Animal Health Research Institute, Dokki, Giza, Egypt.

Sample preparation

Fresh beef fillets (tenderloin) were purchased from a local butcher's shop in Menofia Governorate (Egypt). Samples are immediately transferred to the laboratory in an iced box. Beef fillets were ultraviolet sterilized for 15 minutes on each side according to **Morsy et al. (2018)**.

Bacterial strain preparation

B. cereus strain (ATCC® 10876) was enriched in Tryptic Soy Broth then cultured at 37℃ for 24 hrs on Tryptic Soy Agar ((TSB, and TSA ; Biolife Italiana SrI., Milan, Italy). The cultures are suspended in 0.1% sterile peptone water to obtain a suspension of ~ 8 log10 CFU/mL. A Final bacterial population count was enumerated and verified by TSA **(Chen et al. 2007)**.

Previously prepared beef fillets were inoculated with a *B. cereus* population ~ 8 log CFU/gm by surface spreading. Inoculated beef fillet samples were kept for cell attachment at room temperature for 15 min.

Edible coating preparation

A blank and fortified CMC edible coating was prepared with a concentration of 2% CMC at 80 ◦C using magnetic stirring for 2 hrs. After complete dissolving, glycerol at 10% was added to the mixture**.** Different CMC edible coatings were fortified with 2% propolis **(Pobiega et al. 2020)**, 10% propolis **(Ombarak et al. 2019)**, or mixture of lactoferrin and propolis.

Challenge study

Previously prepared beef fillet samples (inoculated with *B. cereus* 10^8 cfu/gm) were completely immersed in the blank and fortified CMC edible coating solution for about 5 seconds. Coated samples were left to dry at room temperature for 15 minutes. This was repeated twice. Samples were divided randomly into 5 groups as follows; group 1: beef fillet experimentally inoculated with *B. cereus* without coating (BF/BC); group 2: blank CMC 2% edible coating to beef fillet experimentally inoculated with *B. cereus* (CMC/BF/BC); group 3: Lactoferrin 2% fortified CMC edible coating to beef fillet experimentally inoculated with *B. cereus* (CMC/BF/BC/LF), group 4: propolis 10% fortified CMC coating to beef fillet experimentally inoculated with *B. cereus* (CMC/BF/BC/PR) and group 5: mixture Lactoferrin/propolis (1:1) fortified CMC edible coating to beef fillet experimentally inoculated with *B. cereus* (CMC/BF/BC/LF/PR). Control and coated beef fillet samples were packed in poly-

ethylene plastic, vacuumed, and refrigerated at 4 °C for 21 days until spoilage. Samples were examined bacteriologically every 3 days for the remaining population of *B. cereu*s. Three repetitions of this experiment were applied, and the mean values were statistically analyzed (n = 3).

Heat treatment

In the experiment a traditional grilling method was done. The beef fillets are subjected to heat treatment in a double sheet metal grill, remaining on it for 5 min after the temperature had reached 80°C at the geometric center of the fillets according to the method recommended by the American Public Health Association **(APHA, 2001)**.

Bacillus cereus count assessment

B. cereus was counted in each group before and after grilling as following; beef fillets Samples from experimental groups were aseptically opened then grilled. Ten-fold serial dilutions of different samples were prepared. Then, *B. cereus* was counted on Agar Base-MYP (BC-MYP, Biolife) supplemented with polymyxin B sulphate supplement (Code 4240001) and egg yolk emulsion (Code 42111601) according to **ISO (2004)**. After 24 hours of incubation at 37 °C, the colonies were counted and expressed as log10 CFU/gm. Variation between counts of *B. cereus* in the same group, before and after grilling, was evaluated.

RESULTS

Table (C1) Effect of grilling (without coating) on *B. cereus* counts experimentally inoculated in beef fillet

Storage period / Groups	BF/BC	BF/BC grilled	P Value
Zero day	8.30± 0.06****	6.30± 0.05****	0.0008
3rd day	8.86± 0.18**	6.90± 0.18**	0.017
6th day	9.1±0.05***	7.64±0.05***	0.002
9th day	9.69±0.12***	7.71±0.11***	0.007
12th day	R	R	
15th day	R	R	
18th day	R	R	
21th day	R	R	

R: rejected

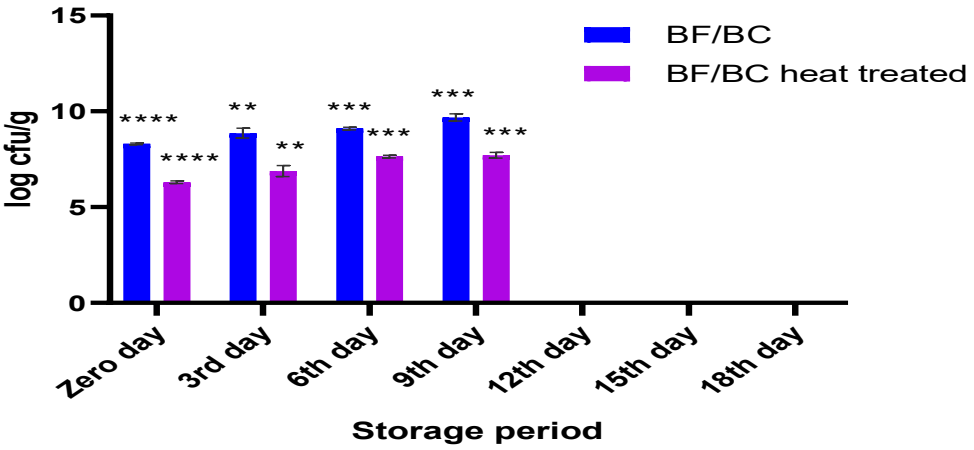

Figure (C1) Effect of grilling (without coating) on *B. cereus* counts experimentally inoculated in beef fillet.

Table (C2) Effect of grilling and/or plank CMC edible coating on *B. cereus* counts experimentally inoculated in beef fillet stored at 4ºC till spoilage.

Storage period Groups	CMC/BF/BC	CMC/BF/BC grilled	P Value
Zero day	8.24± 0.05^{a} ****	6.24± 0.03****	0.0001
3rd day	8.52±0.03^{ab} ***	6.60±0.03***	0.0007
6th day	8.82±0.04***	6.86±0.04***	0.0003
9th day	9.27±0.02****	7.02±0.04****	0.0001
12th day	R	R	
15th day	R	R	
18th day	R	R	
21th day	R	R	

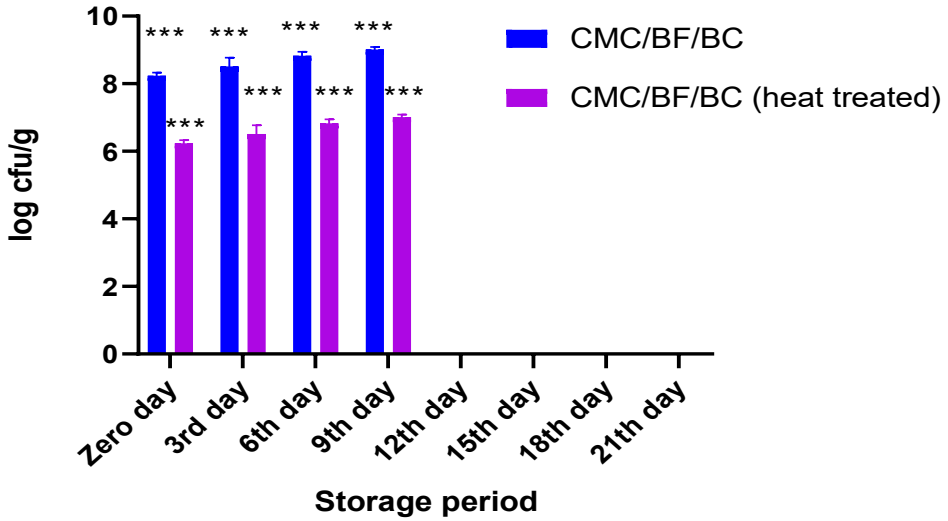

Figure (C2) Effect of grilling and/or plank CMC edible coating on *B. cereus* counts experimentally inoculated in beef fillet stored at 4ºC till spoilage.

Table (C3) Effect of grilling and/or CMC edible coating fortified with lactoferrin on *B. cereus* counts experimentally inoculated in beef fillet stored at 4ºC till spoilage.

Storage period Groups	CMC/BF/BC/LF	CMC/BF/BC/LF grilled	P Value
Zero day	8.19±0.09***	6.19± 0.04***	0.0011
3rd day	8.04± 0.02 ****	6.08±0.02****	0.0001
6th day	7.89±0.09****	5.77±0.09****	0.0001
9th day	7.27±0.3***	5.19±0.13c ***	0.0002
12th day	6.94±0.1***	4.94±0.11***	0.0009
15th day	6.74±0.11***	4.8±0.11***	0.00047
18th day	6.02±0. 2****	4.04±0. 2****	0.0001
21th day	5.80±0. 1***	3.80±0. 1***	0.00136

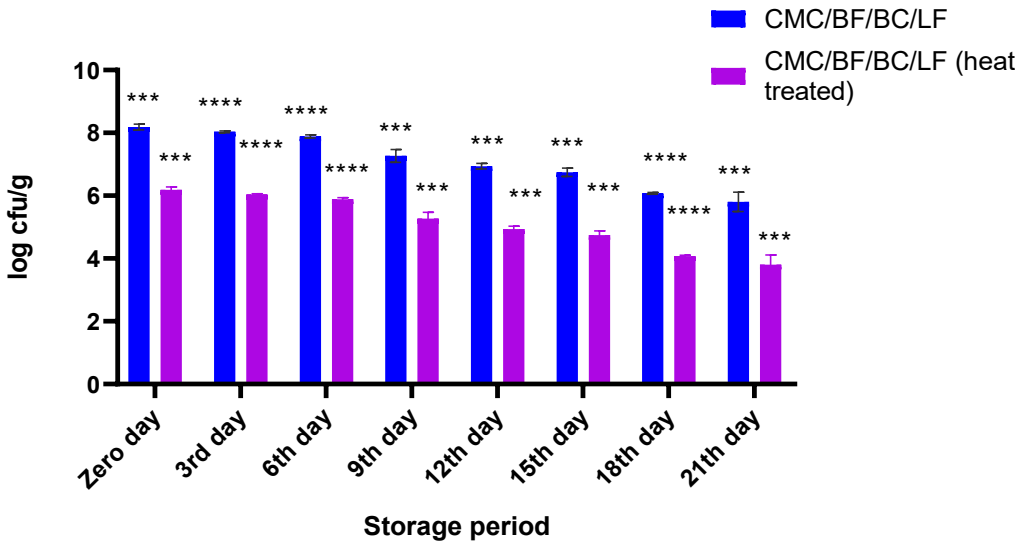

Figure (C3) Effect of grilling and/or CMC edible coating fortified with lactoferrin on *B. cereus* counts experimentally inoculated in beef fillet stored at 4ºC till spoilage.

Table (C4) Effect of grilling and/or CMC edible coating fortified with propolis on *B. cereus* counts experimentally inoculated in beef fillet stored at 4ºC till spoilage.

Storage period Groups	CMC/BF/BC/PR	CMC/BF/BC/PR grilled	P Value
Zero day	8.20± 0.01***	6.15± 0.05***	0.0003
3rd day	7.87±0.11****	5.79±0.11***	0.0006
6th day	7.23±0.02****	5.33±0.02***	0.0079
9th day	7.02±0.06***	5.12±0.03***	0.0007
12th day	6.65±0.11d ***	4.59±0.1***	0.0012
15th day	6.41±0.15***	4.51±0.15***	0.0018
18th day	6±0.04****	4.01±0. 04****	0.00027
21th day	5.41±0.06***	3.41±0. 06***	0.00041

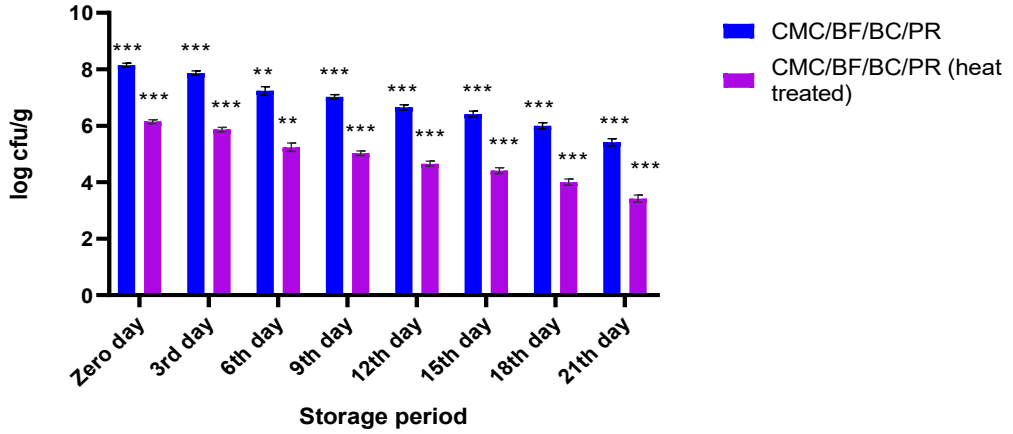

Figure (C4) Effect of grilling and/or CMC edible coating fortified with propolis on *B. cereus* counts experimentally inoculated in beef fillet stored at 4ºC till spoilage

Table (C5) Effect of grilling and/or CMC edible coating fortified with lactoferrin and propolis on *B. cereus* counts experimentally inoculated in beef fillet stored at 4ºC till spoilage.

Storage period Groups	CMC/BF/BC/LF/PR	CMC/BF/BC/LF/PR grilled	P Value
Zero day	8.18± 0.05***	6.09± 0.07***	0.001
3rd day	7.65±0.15****	5.7±0.11***	0. 001
6th day	7.07±0.04****	5.11±0.05***	0.001
9th day	6.72±0.22***	4.78±0.13***	0.0009
12th day	6.11±0.15 ***	4.31±0.15***	0.0004
15th day	6.01±0.03***	4.06±0.03***	0.001
18th day	5.59±0.08****	3.60±0.08****	0.001
21th day	5.11±0.02***	3.22±0.02***	0.001

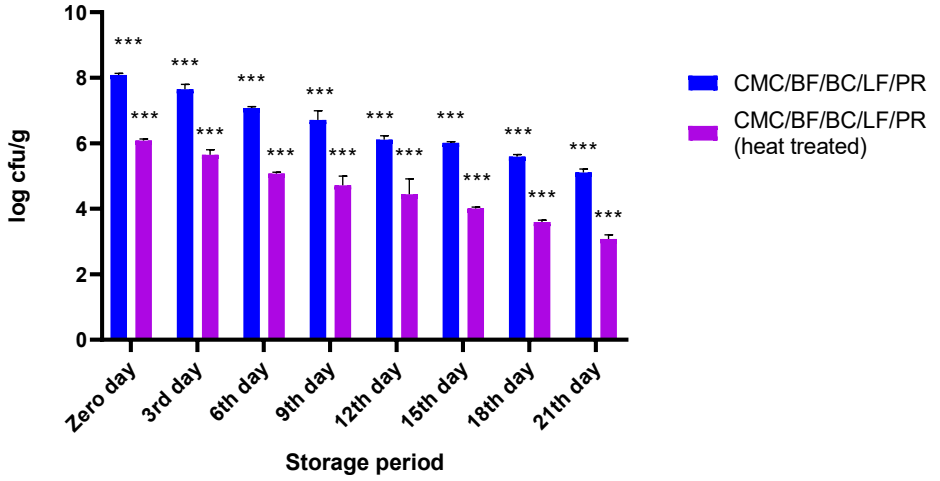

Figure (C5) Effect of grilling and/or CMC edible coating fortified with lactoferrin and propolis on *B. cereus* counts experimentally inoculated in beef fillet stored at 4ºC till spoilage.

DISCUSSION

Thermal processing remains the principal and traditional method of microbial inactivation and reduction **(Wang et al. 2017),** however its limitation to control spore-forming bacteria, increases demand to be combined with another antimicrobial mean of preservation.

The results in table (C1) evaluated the antimicrobial effect of grilling (heat treatment) on experimentally inoculated *B. cereus* in beef fillet stored refrigerated at 4 °C for 21 days. Results revealed a significant ($p \leq 0.05$) decrease in *B. cereus* count from 8.30± 0.06 log CFU/gm. in first day to reach a population of 6.30± 0.05 and from 9.69±0.12 log CFU/gm. at 9th day of storage (rejected by naked eye) to7.71±0.11 in un-coated group (BF/BC).

These results disagree with those by **Gueŕin (2017)** who eliminate vegetative *B. cereus* by a mild heat treatment.

The results in table (C2) evaluated the antimicrobial effect of grilling against experimentally inoculated *B. cereus* in beef fillet with CMC edible coat stored refrigerated at 4 °C for 21 days. Results revealed a significant ($p \leq 0.05$) decrease in *B. cereus* count from 8.24± 0.05 log CFU/gm. in first day to reach a population of 6.24± 0.03 and from 9.27±0.02 log CFU/gm. at 9th day of storage to 7.02±0.04 in (BF/BC) group.

Although, edible coating with CMC showed antibacterial effect, but this effect enhanced when it is incorporated with natural antimicrobials.

The results in table (C3) evaluated the antimicrobial effect of grilling against experimentally inoculated *B. cereus* in beef fillet coated with CMC and treated with lactoferrin stored refrigerated at 4 °C for 21 days. Results revealed a significant ($p \leq 0.05$) decrease in *B. cereus* count from 8.19±0.09 log CFU/gm. in first day to reach a population of 6.19± 0.04 and from5.80±0. 1 log CFU/gm. at 21th day of storage to 3.80±0.1 in (BF/BC /LF) group. The antimicrobial effect of LF incorporated in edible coating agrees with **Tavassoli et al. (2021).** This, owing to the ability of LF to scave iron needed for microbial growth **(Bushra et al., 2019)**, affect the permeability of the bacterial membrane by conjugation with lipopolysaccharide, leading to cell membrane damage **(González-Chávez et al. 2009).** So, application of CMC edible coating fortified with LF enhanced the antimicrobial effect of grilling against spore forming bacteria with enhancement of shelf-life time of beef fillets.

The results in table (C4) evaluated the antimicrobial effect of grilling against experimentally inoculated *B. cereus* in beef fillet coated with CMC and treated with propolis stored refrigerated at 4 °C for 21 days. Results revealed a significant ($p \leq 0.05$) decrease in *B. cereus* count from 8.20± 0.01 log CFU/gm. in first day to reach a population of 6.15± 0.05 and from 5.41±0.06 log CFU/gm. at 21th day of storage to 3.41±0. 06in (BF/BC /PR) group.

From results, fortification of PR in combined with grilling significantly reduce bacterial load of *B. cereus*. This regards to that PR showed an antimicrobial effect, that agree with **Mahdavi-Roshan et al. (2022); Mehdizadeh and Langroodi (2019).** PR has powerful antibacterial, antioxidant, antifungal, antiviral, anti -tumor, and anti-inflammatory properties **(Huang et al. 2014).** The antimicrobial effect of PR with regards to phenolic, flavonoids and aromatic acids such as benzoic acid, galangin, pinocembrin, coumaric acid **(Sheikhi Koohsar et al. 2018).** These bioactive compounds cause damage to bacterial cell membrane by increasing the permeability of the membrane.

The results in table (C5) evaluated the antimicrobial effect of grilling against experimentally inoculated *B. cereus* in beef fillet coated with CMC fortified with lactoferrin and propolis stored refrigerated at 4 °C for 21 days. Results revealed a significant ($p \leq 0.05$) decrease in *B. cereus* count from 8.18± 0.05 log CFU/gm. in first day to reach a population of 6.09± 0.07 and from 5.11±0.02 log CFU/gm. at 21th day of storage to 3.22±0.02 in (BF/BC / LF/PR) group.

Results revealed an effective synergistic antimicrobial effect of LF and PR incorporated into the CMC edible coating in a combination

with grilling against experimentally inoculated *B. cereus*. This agrees with previous study by **Condón-Abanto et al. (2016)** who combined ultrasonic waves under pressure with heat treatment as a synergistic antimicrobial effect against spores but do not agree with **Evelyn and Silva (2016)** who noticed a low effect of thermal treatment in the deactivation of microbial spores of *B. cereus*.

CONCLUSION

Results concluded that grilling alone as antimicrobial can't eliminate *B. cereus* in beef fillets, while application of edible coating fortified with LF and PR enhanced the antimicrobial effect of grilling in a synergistic way to control *B. cereus*.

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