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Bacillus Cereus Control Using Lactoferrin and/or Propolis Incorporated Carboxymethyl Cellulose Edible Coating in Chilled Beef Fillets

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ABSTRACT

Background*: Bacillus cereus (B. cereus)* is a major food poisoning bacterium that resists high cooking temperatures. The aim of this study was to prepare Carboxymethyl Cellulose (CMC) edible coats incorporated with lactoferrin, propolis and mixtures of them and evaluate their effects on *B. cereu)* experimentally inoculated in beef fillets stored in the refrigerator till spoilage. **Methods:** Prepare Carboxymethyl Cellulose (CMC) edible coats incorporated with lactoferrin, propolis and mixtures of them and evaluate their effects on *bacillus cereus* that experimentally inoculated in beef fillets stored refrigerated till spoilage. **Results:** The results revealed a significant and gradual increase in *B. cereus* count from ~8 log CFU/g. In first day the count reached a population of 9.69±0.12 and 9.27±0.02 log CFU/gm. at 9th day of storage in un-coated group (BF/BC) and blank coated group (CMC/BF/BC), respectively. While coated groups showed a significant decrease in *B. cereus* count from ~8 log CFU/gm. in first day of inoculation to 5.80±0.1, 5.41±0.06, 5.11±0.02 in CMC/BF/BC/LF, CMC/BF/BC/PR and CMC/BF/BC/LF/PR groups, respectively. The groups with coated beef showed a significant decrease (P<0.05) in *B. cereus* count from approximately 8 log CFU/g. On the first day of inoculation to 5.80, 5.41, and 5.11 in the Lactoferrin fortified CMC edible coating beef fillet, propolis fortified CMC edible coating beef fillet, and Lactoferrin/propolis fortified CMC edible coating beef fillet groups, respectively, on the 21st day of refrigerated storage. A synergistic antimicrobial effect between Lactoferrin and propolis was shown against *B. cereus*. **Conclusion:** It was revealed that CMC fortified with LF/PR plus its anti-*B. cereus* effect increased the shelf life and enhanced sensory profile of beef fillets during the 21 days of storage.

Introduction

Food safety demand for public health and the economy has become a priority. About 1 in 10 suffers food poisoning as a result of consumption of contaminated food (Lee and Yoon, 2021). Quality, safety, and nutritional content of food application will become an obstacle in the next decades (EU, 2020, World Health Organization, 2021) with regard to hard application of both

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nutrition and safety in food system to satisfy consumer's health outcomes (World Health Organization, 2021).

One of the most common food poisoning threats is *Bacillus cereus (B. cereus)*. It has become a major food poisoning bacterium and survives a variety of food owing to its ability to form spores and biofilms (Etikala *et al.*, 2022). Its spores resist higher cooking temperatures. Hence, it leads to food poisoning (Kim *et al.*, 2020). Furthermore, *B. cereus* is resistant to lactam drugs due to the secretion of powerful lactamase enzymes (Bottone, 2010). These resistance results in higher morbidity and mortality and requires measures to combat these resistant strains (Marianne Frieri and Boutin, 2017).

Cold storage plays a role in controlling microbial growth and food oxidation; however, food packaging is considered an irreplaceable application in keeping meat products' quality during storage (Fang *et al.*, 2017, Ren *et al.*, 2022). Moreover, the direct addition of preservatives to food interferes with food components and lowers their efficiency, thus limiting their application in food system. One of the novel ways to overcome this limitation is active packaging owing to the migration of active compounds from edible coating to food (Nottagh *et al.*, 2020). Active packaging sustains safety and functionality of food. Edible coating economically decreases the cost of packaging by using less expensive packaging materials (Hassan *et al.*, 2018). It also enhances food quality.

The linear structure of cellulose in Carboxymethyl Cellulose Sodium (CMC) provides a flexible, tough, transparent, and stable coating (Arnon-Rips and Poverenov, 2018) with many applications in food industry. CMC considers main polysaccharides with the ability to produce odorless, tasteless, safe, transparent, water-soluble, and stable edible coatings with a powerful potential to carry active functional compounds (Panahirad *et al.*, 2021). Incorporation of a functional substance in an active edible coating is one of the novel ways for food preservation to enhance quality, safety, and shelf-life during storage (Panahirad *et al.*, 2021). Edible active coatings can be incorporated with natural additives, color enhancers, antimicrobials, and antioxidants (Dubey and Dubey, 2020).

Many active functional compounds have been incorporated as antimicrobials in edible coating to create smart, active, and/or biodegradable packaging, such as lactoferrin (Bushra *et al.*, 2019, Padrao *et al.*, 2016, Tavassoli *et al.*, 2021) and propolis (Mehdizadeh and Langroodi, 2019). Although many studies were conducted to evaluate the antimicrobial effect of active edible coating fortified with natural functional compounds, no studies have been conducted on the control of *B. cereus* in meat products by propolis or even lactoferrin. Therefore, the aim of this study was the first to assess the effect of CMC edible coating incorporated with lactoferrin, propolis, and mixtures of them on *B. cereus* experimentally inoculated in beef fillets was stored in refrigerator till spoilage; and the second to evaluate the effect of bioactive CMC edible coating on shelf life and sensory attributes of beef fillets stored refrigerated till spoilage.

Materials and Methods

Materials

CMC (DS=0.9) was purchased from Sigma-Aldrich. Bovine Lactoferrin 20,000 IU/mg (Sigma-Aldrich, USA). 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, and Animal Health Research Institute, in Dokki, Giza, Egypt.

Sample preparation

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

Bacterial strain preparation

B. cereus strain (ATCC® 10876) was enriched in Tryptic Soy Broth,after that, it was cultured at 37℃ for 24 hrs on Tryptic Soy Agar ((TSB, and

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TSA ; Biolife Italiana SrI., Milan, Italy). The cultures were 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).

Bacterial inoculation

Previously prepared beef fillets were inoculated with a *B. cereus* population ~ 8 log CFU/g 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, 10% propolis, or a 1:1 mixture of lactoferrin and propolis. The concentration of propolis (10%) was performed according to Proiega (Pobiega *et al.*, 2020) and, the concentration of lactoferrin was performed according to Ombarak (Ombarak *et al.*, 2019).

Challenge study

Previously prepared beef fillet samples (inoculated with *B. cereus* 10^8 CFU/g) 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 with 2% edible coating on beef fillet experimentally inoculated with *B. cereus* (CMC/BF/BC); group 3: Lactoferrin with 2% fortified CMC edible coating on beef fillet experimentally inoculated with *B. cereus* (CMC/BF/BC/LF), group 4: Propolis with 10% fortified CMC coating on beef fillet experimentally inoculated with *B. cereus* (CMC/BF/BC/PR) and group 5: Mixture Lactoferrin/propolis (1:1) fortified with CMC edible coating on beef fillet experimentally inoculated with *B. cereus* (CMC/BF/BC/LF/PR). Control and coated beef fillet samples were packed in polyethylene plastic, vacuumed, and refrigerated at 2 °C for 21 days until spoilage. Samples were examined bacteriologically every 3 days for the remaining population of *B. cereus*. Three repetitions of this experiment were applied, and the mean values were statistically analyzed (n=3).

B. cereus count assessment

On each examination day, beef fillet samples from experimental groups were aseptically opened. Ten-fold serial dilutions of different samples were prepared. Then, *B. cereus* was counted on agar-based-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/g.

pH evaluation

pH values were estimated according to (Association of Official Analytical Chemists and (US), 2005) AOAC by a digital pH-meter (model P107, Consort, Belgium).

Sensory assessment of raw and cooked samples

The impacts of different CMC fortified edible coating on beef fillets' sensory attributes were applied to fresh coated beef fillets to evaluate consumer acceptability of CMC coating. A completely randomized assessment was applied under controlled conditions of temperature (28 ^{o}C), humidity 65% and light by 10 experienced panelists (male and female) who were selected according to (International Organization for Standardization, 2012). The panelists performed a descriptive sensory evaluation for coated and uncoated samples and gave comparative scores. Each sample contained a 50 g meat fillet, which was given to panelists in plastic containers, each containing 3 samples (2 identical samples and 1 different sample) in triangle form, randomly coded with four numbers and a work sheet to give the score for each point. The panel received a list of descriptors (texture, color, odor, and overall acceptability) to score on numerical and

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continuous scales from 0 to 10 according to (Cullere *et al.*, 2018).

Data analysis

The experiment was designed in a completely randomized design in a 4×8 factorial design; 4 treatments (control group, 2% lactoferrin, 10% propolis and mixture of 2% lactoferrin + 5% propolis during 8 sampling days (zero day, 3rd, 6th, 9th, 12th, 15th, 18th and 21st day) at refrigerated storage (2 °C). In this experimental model, fixed factors were counted for inoculated bacteria (*B. cereus*) and natural antibacterial substances used for treatments as well as a cofactor for sample size, sampling day, analyst and environmental conditions during refrigerated storage, sampling, and analysis, and for sensory analysis, the panelists' age, gender, training, and experience. Random factors were arranged according to their relation to meat samples collected from different random sources, mean values of triplication, and sensory panel (rounds per session, random coding of samples and panelist's number).

All datasets were subjected to analysis of variance (ANOVA) using SPSS program for Windows (Version 22) (SPSS Inc. Chicago, IL, USA). F-values at P-value<0.05 were indicated as significantly different. Duncan's multiple range test is a post-hoc test used for measuring specific differences between pairs of means (Duncan, 1955). The mean ± standard error from triplication of the design was conducted to remove diversity of results in order to evaluate the effect of natural antibacterial substances with different concentrations on the reduction of *B. cereus* counts and enhancement of shelf life time.

Results

The results in **Table 1** evaluated antimicrobial effect of CMC edible coating fortified with LF and/or PR against experimentally inoculated *B. cereus* in beef fillet stored refrigerated at 2 °C for 21 days. Results revealed a significant (*P*<0.05) and gradual increase in *B. cereus* count from ~ 8 log CFU/g on the first day to reach a population of 9.69±0.12 and 9.27±0.02 log CFU/g at 9th day of storage in un-coated group (BF/BC) and blank coated group (CMC/BF/BC), respectively. While coated groups showed a significant decrease (*P*<0.05) in *B. cereus* count from ~ 8 log CFU/g in the first day of inoculation to 5.80±0.1, 5.41± 0.06, 5.11±0.02 in CMC/BF/BC/LF, CMC/BF/BC/PR and CMC/BF/BC/LF/PR groups, respectively in the 21st day of refrigerated storage.

Results in **Table 2** showed pH increase in the un-coated group (BF/BC) and the blank coated group (CMC/BF/BC), but pH values of CMC/BF/BC/LF, CMC/BF/BC/PR, and CMC/BF/BC/LF/PR groups remained within the range until the 21st day of storage.

Results in **Figure 1** revealed the variation of sensory attributes (overall acceptability) in coated and uncoated beef fillets and the effect of coating on shelf-life time and consumer acceptability. BF/BC was accepted till the 9th day of storage, while plane coated CMC/BF/BC was accepted till the 12th day of storage.

Discussion

Food poisoning caused by *B. cereus* strains leads to gastrointestinal diseases, emesis, and diarrhea due to certain toxins (Lin *et al.*, 2022). It has become an emerging source of serious health risks owing to the fact that most of its strains show resistance to most antibiotics, and this makes it difficult to control its contamination in food (Khameneh *et al.*, 2016). Moreover, consumers are looking forward to safe and healthier products that reflect the orientation of a new coating technique in food industry (Sapper and Chiralt, 2018). A novel CMC edible coating fortified with natural bioactive compounds such as LF and PR was conducted in a trial to control *B. cereus* ,experimentally inoculated in beef fillet stored refrigerated till its spoilage.

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Table 1. Anti-*B. cereus* evaluation of edible CMC coating fortified with LF and PR in beef meat fillet stored at 4 ºC till spoilage (n=3).

Group	Zero day	3rd day	6thday	9th day	12th day	15th day	18th day	21st day
BF/BC	8.30±0.06^{a}	8.86±0.18^{b}	9.1±0.05^{b}	9.69±0.12^{e}	Refused	Refused	Refused	Refused
CMC/BF/ BC	8.24±0.05^{a}	8.52±0.03^{ab}	8.82±0.04^{ab}	9.27±0.02^{e}	Refused	Refused	Refused	Refused
CMC/BF/ BC/LF	8.19±0.09^{a}	8.04±0.02^{ab}	7.89±0.09^{ab}	7.27±0.30^{c}	6.94±0.1^{d}	6.74±0.11^{f}	6.02±0.20^{f}	5.80±0.10^{h}
CMC/BF/ BC/PR	8.20±0.01^{a}	7.87±0.11^{ab}	7.23±0.02^{ab}	7.02±0.06^{c}	6.65±0.11^{d}	6.41±0.15^{g}	6.00±0.04^{h}	5.41±0.06^{i}
CMC/BF/ BC/LF/PR	8.18±0.05^{a}	7.65±0.15^{ab}	7.07±0.04^{ab}	6.72±0.22^{ab}	6.11±0.15^{f}	6.01±0.03^{g}	5.59±0.08^{h}	5.11±0.02^{j}

ANOVA and Duncan's multiple range tests were used to compare mean values. Mean values with different superscripts in the same columns are significantly different at P<0.05;Results expressed as mean±SE; ***BF****: Beef fillet;* ***BC****: B. cereus;* ***CMC****: Carboxymethyl cellulose;* ***PR****: Propolis;* ***LF****: Lactoferrin;.*

Table 2. pH evaluation of edible CMC coating regarding beef meat fillet, fortified with LF and PR during storage at 2 ºC every 3 days all over the storage period.

Groups	Zero day	3rd day	6thday	9th day	12th day	15th day	18th day	21st day
BF/BC	5.66±0.06^{a}	6.01±0.02^{ab}	6.33±0.04^{c}	6.62±0.06^{c}	6.87±0.03^{e}	7.20±0.10^{e}	7.40±0.33^{e}	7.60±0.16 ^{e}
CMC/BF/B C	5.66±0.06^{a}	5.85±0.04^{ab}	6.05±0.08^{b}	6.40±0.08^{b}	6.51±0.02^{e}	7.10±0.20^{e}	7.2±0.04 ^{e}	7.52±0.23 ^{e}
CMC/BF/B C/LF	5.66±0.06 ^{a}	5.72±0.02^{ab}	5.09±0.02^{b}	5.99±0.01^{d}	6.10±0.04^{d}	6.23±0.01^{f}	6.33±0.06^{f}	6.75±0.03^{f}
CMC/BF/B C/PR	5.66±0.06^{a}	5.7±0.06^{ab}	5.84±0.03^{b}	5.91±0.01^{d}	5.99 ±0.03^{d}	6.20±0.04^{f}	6.28±0.02^{f}	6.73±0.05^{f}
CMC/BF/B C/LF/PR	566±0.06^{a}	5.69±0.01^{ab}	5.81±0.02^{b}	5.88±0.03^{d}	5.96 ±0.01^{d}	6.1±0.03^{f}	6.19±0.01^{f}	6.69±0.00^{g}

ANOVA and Duncan's multiple range tests were used to compare mean values. Mean values with different superscripts in the same columns were significantly different at P<0.05; Results expressed as mean±SE; ***BF****: Beef fillet;* ***BC****: B. cereus;* ***CMC****: Carboxymethyl cellulose;* ***PR****: Propolis;* ***LF****: Lactoferrin.*

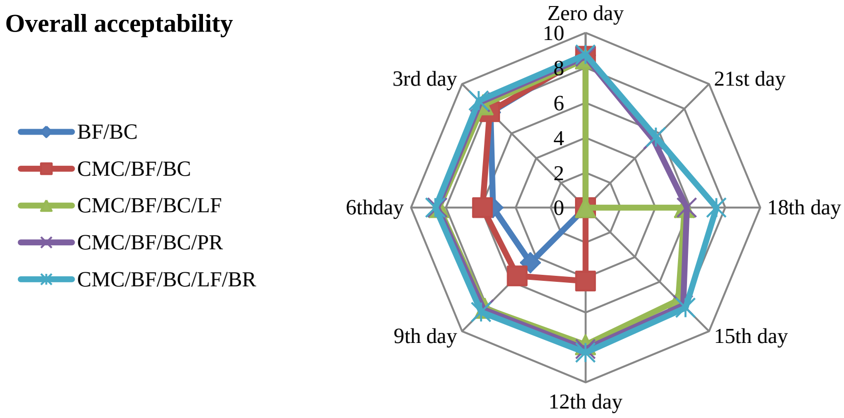

Figure 1. The effect of edible CMC coating fortified with LF and PR regarding sensory attributes (overall acceptability) of raw beef meat fillet stored at 4 ºC till spoilage; Results expressed as mean± SE; **BF**: Beef fillet; **BC**: *B. cereus;* **CMC**: Carboxymethyl cellulose; **PR**: Propolis; **LF**: Lactoferrin.

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Discussion

Food poisoning caused by *B. cereus* strains leads to gastrointestinal diseases, emesis, and diarrhea due to certain toxins (Lin *et al.*, 2022). It has become an emerging source of serious health risks owing to the fact that most of its strains show resistance to most antibiotics, and this makes it difficult to control its contamination in food (Khameneh *et al.*, 2016). Moreover, consumers are looking forward to safe and healthier products that reflect the orientation of a new coating technique in food industry (Sapper and Chiralt, 2018). A novel CMC edible coating fortified with natural bioactive compounds such as LF and PR was conducted in a trial to control *B. cereus*, experimentally inoculated in beef fillet stored refrigerated till its spoilage.

In this study, results in **Table 1** revealed that coating of beef fillets with CMC incorporated with LF/or PR showed an antimicrobial effect against *B. cereus*, one of the most resistant bacteria in food (spore-forming, thermophiles). The antimicrobial effect of LF incorporated in edible coating was in line with antimicrobial study by Tavassoli *et al.*'s study (Tavassoli *et al.*, 2021). This, owing to the ability of LF to save the iron needed for microbial growth (Bushra *et al.*, 2019), affect the permeability of bacterial membrane by conjugation with lipopolysaccharide, leading to cell membrane damage (González-Chávez *et al.*, 2009).

Moreover, PR demonstrated an antimicrobial effect, which was consistent with another studies (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 is regarded 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 membrane (Przybyłek and Karpiński, 2019). Results revealed a synergistic antimicrobial effect of LF and PR incorporated into CMC edible coating against experimentally inoculated *B. cereus.*

Results in **Table 2** evaluated pH value which is one of the indicators for meat freshness over the storage period to detect incipient spoilage of beef fillets. There was a significant variation in pH level between the groups. A marked increase in pH level in un-coated group (BF/BC) and blank coated group (CMC/BF/BC) indicated the spoilage of BF on 9th day of storage even in the blank CMC group. The pH values of CMC/BF/BC/LF, CMC/BF/BC/PR, and CMC/BF/BC/LF/PR groups, on the other hand, remain within the range until the 21st day of storage. It was found that edible coating fortified with antioxidants increased the shelf-life of products. These results were in line with the anthers (Nor and Ding, 2020, Zhou *et al.*, 2022) who proved the positive effect of edible coating on shelf-life. Moreover, delay in pH of the coated products was previously proved by Pirsa (Pirsa and Shamusi, 2019) in fresh chicken thigh meat and Khezrian (Khezrian and Shahbazi, 2018) in minced camel meat. This relates to delayed unfavorable oxidative reactions in the product (Arroyo *et al.*, 2020), which leads to a prolongation of shelf-life.

Results in **Figure1** indicated that fortification of CMC edible coating with bioactive compounds positively affected sensory attributes and shelf-life of chilled beef fillets, and this was confirmed when PR and LF were fortified with CMC edible coating.

CMC/BF/BC/PR and CMC/BF/BC/LF/PR showed more acceptability till 21st day of storage compared with those fortified with LF (CMC/BF/BC/LF) which was accepted till 18th day of storage. This may be due to the fact that tightly closed CMC edible coating provided favorable aroma, oxygen, and oil barrier properties to the products (Saxena *et al.*, 2020). Moreover, because of migration of fortified bio active compounds in the edible coating to the product and delayed unfavorable changes that may occur (Kumar and Sethi, 2018). These bioactive compounds enhance sensory attributes, nutritional values, and delayed spoilage (Arroyo *et al.*, 2020) by its antimicrobial and antioxidant effects of these bioactive compound (Dubey and Dubey, 2020).This illustrated the previous results in **Tables 1**, **2**, and **Figure 1**.

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Conclusion

CMC edible coating incorporated with bioactive compounds as LF and/or PR showed anti-*B.cereus* effect in beef fillets stored at 4̊c for 21days. Moreover, application of bioactive edible coating enhanced shelf-life of beef fillets to reach 21days compared to 9 days in uncoated fillets with a marked enhancement in sensory attributes of beef fillets.

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Authors' contributions

The conceptualization and design of the study were supported by all the authors. Material preparation, data collection, and analysis were performed by Rasha Elsabagh, Nahla Abo EL-Roos, Mohebat Abd El-Aziz, and Asmaa Hashhash . The first draft of the manuscript was written by Asmaa Hashhash, and all the authors read and approved the final manuscript.

Conflict of interest

The authors declared no conflict of interests.

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