Foods 2021

MDPI

Proceedings Paper

Characterization of the Inclusion Complexes of Isothiocyanates with γ-Cyclodextrin for Improvement of Antibacterial Activities against *Staphylococcus aureus* †

Jianan Liu [1], Hongyan Wu [1], Xinying Ao [1], Hongshun Hao [2], Jingran Bi [1,3], Hongman Hou [1,3] and Gongliang Zhang [1,3,*]

[1] School of Food Science and Technology, Dalian Polytechnic University, No. 1, Qinggongyuan, Ganjingzi District, Dalian 116034, China; 1263778194@qq.com (J.L.); wuhongyan1908@hotmail.com (H.W.); 1979465645@qq.com (X.A.); bijingran1225@foxmail.com (J.B.); houhongman2011@hotmail.com (H.H.)

[2] Department of Inorganic Nonmetallic Materials Engineering, No. 1, Qinggongyuan, Ganjingzi District, Dalian 116034, China; beike1952@163.com

[3] Liaoning Key Lab for Aquatic Processing Quality and Safety, No. 1, Qinggongyuan, Ganjingzi District, Dalian 116034, China

* Correspondence: zhanggl78@gmail.com; Tel.: +86-411-8632-2020

†

Abstract: The aim of this study was to develop inclusions formed by γ-cyclodextrin (γ-CD) and benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC) and 3-methylthiopropyl isothiocyanate (MTPITC) to improve their controlled release for inhibition of *Staphylococcus aureus* (*S. aureus*). These inclusion complexes were characterized using X-ray diffraction, Fourier-transform infrared, thermogravimetry and scanning electron microscopy. The biofilm formation was less in *S. aureus* treated with γ-CD-BITC than that of BITC. The expression of virulence genes, including *sarA*, *agr*, *cp5D*, *cp8F*, *clf*, *nuc*, and *spa*, showed sustained downregulation in *S. aureus* treated with γ-CD-BITC. Moreover, the growth of *S. aureus* in cooked chicken breast treated with γ-CD-BITC and BITC was predicted by the Gompertz model. These results suggest that BITC has a more durable antibacterial effect against *S. aureus* after encapsulation by γ-CD.

Keywords: cyclodextrin; inclusion; isothiocyanates; *Staphylococcus aureus*; antibacterial activities

Citation: Liu, J.; Wu, H.; Ao, X.; Hao, H.; Bi, J.; Hou, H.; Zhang, G. Characterization of the Inclusion Complexes of Isothiocyanates with γ-Cyclodextrin for Improvement of Antibacterial Activities against *Staphylococcus aureus*. **2021**, *1*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s):

Published: date

1. Introduction

The Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) is one of the most frequent bacteria that causes food poisoning [1]. Therefore, certain steps were made to prevent *S. aureus* infection in order to maintain food safety, but it remains an issue that has to be addressed in the food business.

Isothiocyanates (ITCs), which are found in the Cruciferae family of plants, have long been used as antibacterial agents [2]. ITCs have strongly volatility, which diminishes their antibacterial activities and hinders the potential use as effective bioactive agents [3]. The use of carrier systems to encapsulate ITCs, thus achieving controlled release, is important to improve the inhibitory effect of ITCs on pathogenic bacteria. γ-cyclodextrin (γ-CD) is suitable for complexation, due to its wide cavity and water solubility [4]. However, no studies have been conducted to prepare ITCs inclusions using γ-CD as coating material and to investigate their controlled release and antibacterial activities.

In this study, an attempt was made to prepare ITCs inclusion complexes by the freeze-drying method using γ-CD as raw material. Also, the study was to improve the stability of ITCs and to increase the antibacterial time. In addition, the controlled release, antibiofilm activities, effect on virulence gene expression and antibacterial activities of ITCs and γ-CD-ITCs against *S. aureus* in the cooked chicken breast were investigated.

2. Methods

2.1. Chemicals and Bacterial Strains

ITCs, including MTPITC, PEITC, BITC, was selected from GB2760-2014 and purchased from Sigma, United States. The bacterium *S. aureus* ATCC 6538 was taken from the Dalian Polytechnic University Food Microbiology Laboratory (Dalian, China).

2.2. Preparation of the Inclusion Complex (γ-CD-ITCs)

γ-CD and ITCs were mixed at mass ratio of 1:1, and the γ-CD-ITCs inclusion complexes were obtained by freeze-drying.

2.3. Characterization of the Inclusion Complexes

The characterization of γ-CD-ITCs was determined by an Fourier Transform Infrared (FTIR) spectrum (PerkinElmer, Norwalk, CT, Japan), thermogravimetric analyzer (TGA 550, TA Instruments, USA), diffractometer (XRD-6100, Shimadzu, Kyoto, Japan), and scanning electron microscopy (SEM) (Quanta 450, Waltham, MA, USA), respectively.

2.4. Antibacterial Assays

The antibacterial activities of ITCs were studied using agar diffusion method. On the plate, 100 mL of *S. aureus* suspension and 1 μmoL ITCs and γ-CD-ITCs (ITCs = 1 μmoL) were added. The antimicrobial activities were compared by measuring the diameter of the inhibition zone (DIZ). The minimum inhibitory concentration (MIC) was determined by using the broth microdilution technique.

2.5. Effects of BITC and γ-CD-BITC on Formation of S. aureus Biofilm

The *S. aureus* suspensions were mixed with 0, 1/8MIC, 1/4MIC, 1/2MIC BITC and γ-CD-BITC (based on BITC concentration) and incubated for 48 h at 37 °C. As for the biofilm, *S. aureus* biofilm samples were examined under a microplate reader (SpectraMax M2, Molecular Devices, USA) and an SEM (Quanta 450, Waltham, MA, USA).

2.6. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

S. aureus was cultured with 1/4MIC BITC and γ-CD-BITC (basing on BITC concentration) for 24 h. qRT-PCR was performed on SYBR® AdvancedTM II (Dalian Tara, China). Seven virulence genes, including accessory gene regulator (*agr*), accessory gene regulator protein A (*sarA*), capsular polysaccharide biosynthesis protein *(cp5D*), capsular polysaccharide synthesis enzyme (*cp8F*), thermonuclease (*nuc*), clumping factor (*clf*), and protein A (*spa*) in *S. aureus* strain were studied. The 16S rRNA gene was used as the endogenous gene, and the $2^{-\Delta\Delta Ct}$ method was used to assess gene expression levels [5].

2.7. Primary Modeling

The data of *S. aureus* in the cooked chicken breast under 0.25 mmol/L BITC and γ-CD-BITC were modeled using the modified Gompertz model [6].

2.8. Secondary Modeling

Temperature, maximum specific growth rate (SGR) and lag time (LT) were used in secondary modeling with the polynomial model equation. The bias factor (Bf), accuracy factor (Af), and correlation coefficient (R^2) were utilized in this study to evaluate the performance of the projected models.

2.9. Statistical Analysis

An OriginPro 8.5 software (OriginLab Corporation, Northampton, MA, USA), SPSS version 2.0 and GraphPad Prism 8.0.2 were used to perform the data analysis.

3. Results and Discussion

3.1. Characterization of the ITCs Inclusion Complexes with γ-CD

Figure 1a shows the FTIR spectra of γ-CD and γ-CD-ITCs. The characteristic broad peak in the FTIR spectra of ITCs at 2140-2040 cm^{-1} attributed to the N=C=S group [3]. Although characteristic benzene peaks (between 1345 cm^{-1} and 1600 cm^{-1}) [3] vanished in the spectrum of γ-CD-PEITC and γ-CD-BITC, the vibration bands of N=C=S groups still existed. The γ-CD-MTPITC, γ-CD-BITC and γ-CD-PEITC peaks were not significantly different. Therefore, these results suggested that the N=C=S groups exposed out the cavity of γ-CD, while benzene ring of PEITC and BITC, and carbon chain structure of MTPITC were entrapped in γ-CD. Besides, it has been reported that ITCs was volatile compounds and had mass loss from 80 °C to 165 °C [7]. As shown in Figure 1b, the γ-CD and γ-CD-ITCs inclusion complexes decomposed in two step mechanisms. The first step was to dehydrate the γ-CD sample from 30 °C to 132 °C. In fact, due to the impact of γ-CD, phase mass loss of the γ-CD-ITCs inclusion complexes showed evident slope between 132 °C and 300 °C, which delayed its volatilization. Therefore, the elevated temperature for complete decomposition of ITCs (165 °C) further confirmed the formation of ITCs inclusion complexes with γ-CD (300 °C).

XRD diffraction patterns of γ-CD, γ-CD-MTPITC, γ-CD-PEITC and γ-CD-BITC complexes were further investigated. As shown in Figure 1c, 2θ of 5.84–5.90 °, 10.28–10.36 °, 11.80–11.84 °, 15.74–15.90 ° and 21.80–21.86 ° were the similar sharp peaks of the three inclusions. At 14.21°, sharp peaks appeared in the XRD patterns of γ-CD-BITC and γ-CD-PEITC, but not γ-CD-MTPITC, which may be a crystal morphology unique to the inclusion complexes containing a benzene ring structure [8]. On the other hand, Figure 1d showed the morphological images of crystals, γ-CD exhibited different sizes of irregularly blocky crystal structures with small particles attached to the surfaces of crystals, while γ-CD-MTPITC appeared as regular striped structures, the difference was that γ-CD-PEITC and γ-CD-BITC showed regular rectangular structures. Similar results have been reported [9,10]. Therefore, the different structures, crystal morphologies and high thermal stability of γ-CD and γ-CD-ITCs inclusion complexes further confirmed the formation of ITCs with γ-CD inclusion complexes.

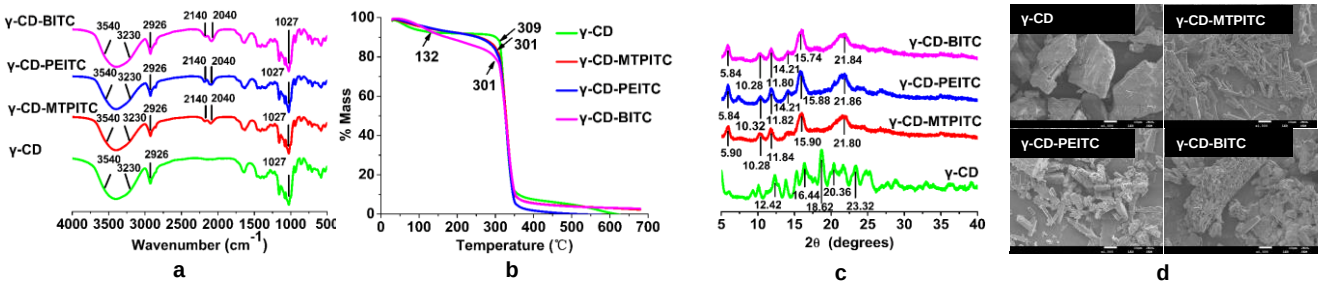

Figure 1. (**a**): FTIR spectra of γ-CD and γ-CD-ITCs. (**b**): TGA curves for γ-CD and γ-CD-ITCs. (**c**): X-ray diffraction patterns of γ-CD and γ-CD-ITCs. (**d**): The morphological images (×1000) of γ-CD and γ-CD-ITCs.

3.2. Antibacterial Activities of ITCs and γ-CD-ITCs against S. aureus

The antimicrobial activities of the different ITCs and γ-CD-ITCs were preliminarily evaluated by agar diffusion. At the first day, the inhibition zone sizes of the three ITCs against *S. aureus* were 7.9 ± 0.4, 10.8 ± 0.4, 16.9 ± 0.7 mm, following the order of BITC > PEITC > MTPITC. For BITC, its antibacterial activity lasted until the third day. After being encapsulated by γ-CD, the antimicrobial activity was still stronger on day 10. Although the antibacterial activities still showed a gradual decrease with time for the γ-CD-ITCs inclusions, the ability of controlled release was significantly improved after encapsulation, in comparison with free ITCs. These results are consistent with previous study [11]. Furthermore, BITC had a MIC of 0.5 mmol/L against *S. aureus*. In view of the good bacterial

inhibitory ability of BITC and the good sustained release of its inclusions, therefore, we selected BITC and γ-CD-BITC in the following studies.

3.3. Effect of BITC and γ-CD-BITC against S. aureus Biofilm Formation

The inhibitory effects of BITC and γ-CD-BITC at 0-1/2 MIC (basing on BITC concentration) on biofilm formation of *S. aureus* were investigated. As shown in Figure 2a, the untreated *S. aureus* showed that the biofilm was highly dense, with numerous bacteria wrapped in the biofilm structure. In contrast, for both BITC and γ-CD-BITC groups, the biofilm was thinner and less bacteria were wrapped as the BITC concentration increased (Figure 2b–g). More importantly, both biofilm and bacteria obviously reduced, compared to BITC at the same concentration (Figure 2b–g). Furthermore, as shown in Figure 2h, biofilm formation of *S. aureus* reduced as BITC concentration increased as measured by the crystal violet quantitative assay. Biofilm formation of *S. aureus* with γ-CD-BITC was obviously less than that of BITC at each concentration. This phenomenon is due to the high volatility of BITC, while the controlled release of the γ-CD-BITC inclusion significantly attenuates this weakness. Previous findings were consistent with ours [12]. Therefore, BITC encapsulated by γ-CD was more effective than free BITC against *S. aureus* biofilm activities.

3.4. Effect of BITC and γ-CD-BITC Virulence-Related Genes Expression in S. aureus

As shown in Figure 2i, γ-CD-BITC showed stronger inhibitory effects on these virulence genes in *S. aureus* than BITC. The relative expression of genes *sarA*, *agr*, *cp5D*, *cp8F*, *clf*, *nuc*, and *spa* was 12.69-37.42% and 35.62-63.18%, respectively, for γ-CD-BITC and BITC. Therefore, due to the controlled release effect of the γ-CD-BITC inclusion, it was more effective than free BITC in inhibiting virulence genes. Our studies were consistent with previous findings [13].

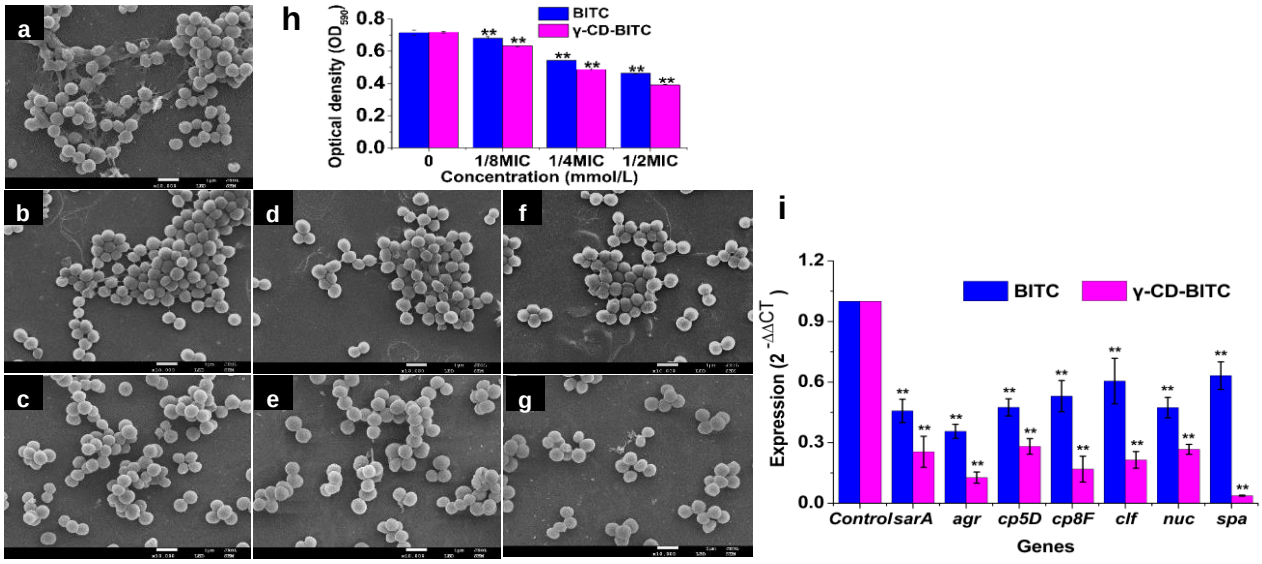

Figure 2. Effect of BITC and γ-CD-BITC on biofilm formation and virulence-related genes expression in *S. aureus*. SEM images (×10,000) of *S. aureus* biofilm (**a**), control; (**b**), 1/8MIC BITC; (**d**), 1/4MIC BITC; (**f**), 1/2MIC BITC; (**c**), 1/8MIC γ-CD-BITC; (**e**), 1/4MIC γ-CD-BITC; (**g**), 1/2MIC γ-CD-BITC. (**h**): crystal violet assay. (**i**): *sarA*, *agr*, *cp5D*, *cp8F*, *clf*, *nuc*, *spa* relative expression compared to 16S rRNA normalized to one control. Each bar represents the mean ± SD of three independent experiments, ** $p < 0.01$ versus the control group.

3.5. Primary Model for the Inhibitory of BITC and γ-CD-BITC on S. aureus Growth in Cooked Chicken Breast

Figure 3 presents the growth for *S. aureus* in cooked chicken breast with the presence or absence of BITC and γ-CD-BITC. The R^2 of the modified Gompertz models were 0.9783–

0.9863, 0.9816–0.9915 and 0.9816–0.9916, for control, BITC and γ-CD-BITC at 10, 15, 20, and 25 °C. Bacterial growth of chicken breast at different storage temperatures showed a similar pattern to that of *S. aureus* in pork [14]. Therefore, the modified Gompertz model predicted well the growth of *S. aureus* with and without BITC and γ-CD-BITC in cooked chicken breast.

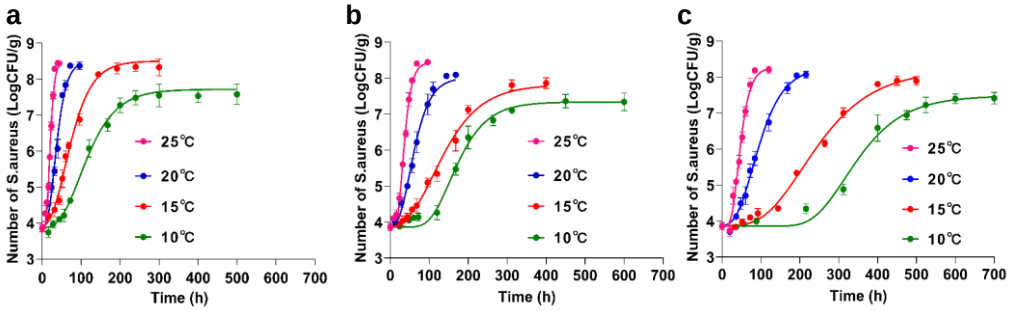

Figure 3. The Primary model for the inhibitory of BITC and γ-CD-BITC on *S. aureus* growth in cooked chicken breast stored at 10, 15, 20, and 25 °C. (**a**) Control; (**b**) BITC; (**c**) γ-CD-BITC.

3.6. Secondary model for Growth Factors of the Inhibitory of BITC and γ-CD-BITC on S. aureus in Cooked Chicken Breast

As shown in Figure 4a, the SGR of both BITC and γ-CD-BITC was significantly decreased compared to the control at each temperature. In the γ-CD-BITC, the SGR was significantly lower than BITC at 20 °C and 25 °C. Besides, LT all showed a tendency to decrease as the experimental temperature was increased with or without BITC and γ-CD-BITC (Figure 4b). And the LT of γ-CD-BITC was 1.3–2.4 times longer than those of BITC at 10, 15, 20, and 25 °C, this phenomenon was due to the high volatility of BITC [3], while the controlled release of γ-CD-BITC prolonged its LT. The validation findings for the *S. aureus* growth factor of the secondary model were also investigated. The Bf, Af, and R^2 of the secondary models were 0.94–1.02, 1.00–1.23, and 0.90–0.99, respectively. For the description of growth factors in *S. aureus* under BITC and γ-CD-BITC compared with other study findings [14,15], the validation results for secondary model were acceptable. Therefore, γ-CD-BITC also demonstrated a controlled release effect against *S. aureus* in chicken systems.

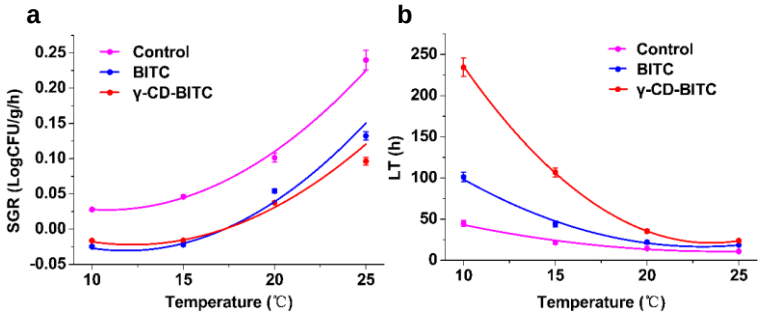

Figure 4. The secondary model for growth factors of the inhibitory effects of BITC and γ-CD-BITC on *S. aureus* in cooked chicken breast stored at 10, 15, 20, and 25 °C. (**a**) SGR, maximum specific growth rate; (**b**) LT, lag time.

4. Conclusions

The present work showed the successful entrapment of BITC in γ-CD, overcoming the lacunas of the high volatility of BITC. Compared with BITC, γ-CD-BITC showed more sustained inhibition against *S. aureus*. Both the biofilm formation and the expression of

virulence genes were less in *S. aureus* treated with γ-CD-BITC than those of BITC. In addition, this study predicted the growth of *S. aureus* in cooked chicken breast with or without BITC and γ-CD-BITC using a modified Gompertz model. The results suggested that γ-CD was a suitable excipient for increasing the stability of BITC, and the γ-CD-BITC inclusion complex could improve the controlled release for inhibition of *S. aureus*.

Author Contributions: Conceptualization, G.Z. and J.L.; methodology, J.L. and H.W.; software, X.A. and J.B.; validation, J.B.; formal analysis, J.L. and G.Z.; investigation, H.W.; resources, X.A. and H.W.; data curation, J.L. and H.W.; writing—original draft preparation, J.L.; writing—review & editing, J.L. and G.Z.; visualization, H.H. (Hongshun Hao); supervision, H.H. (Hongman Hao) and H.H. (Hongshun Hou); project administration, H.H. (Hongman Hao) and G.Z.; funding acquisition, G.Z. All authors discussed the results and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Key R and D Program of China (2019YFC1605902), the Innovative Talent Support Program for Colleges and Universities of Liaoning Province (LR2019009), and the Liaoning Provincial Natural Science Foundation of China (2019-MS-021).

Institutional Review Board Statement:

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available within the article.

Acknowledgments: The authors gratefully acknowledge laboratory colleagues for their helps.

Conflicts of Interest: All authors declare no conflict of interest.

References

1. Kostoglou, D.; Protopappas, I.; Giaouris, E. Common Plant-Derived Terpenoids Present Increased Anti-Biofilm Potential against Staphylococcus Bacteria Compared to a Quaternary Ammonium Biocide. *Foods* **2020**, *9*, 697.
2. Pate, J.; Yin, H.B.; Bauchan, G.; Mowery, J. Inhibition of *Escherichia coli O157:H7* and *Salmonella* enterica virulence factors by benzyl isothiocyanate. *Food Microbiol.* **2020**, *86*, 1–6.
3. Uppal, S.; Sharma, P.; Kumar, R.; Kaur, K.; Bhatia, A.; Mehta, S.K. Effect of benzyl isothiocyanate encapsulated biocompatible nanoemulsion prepared via ultrasonication on microbial strains and breast cancer cell line MDA MB 231. *Colloids Surf. A Physicochem. Eng. Asp.* **2020**, *596*, 124732.
4. Nguyen, T.V.A.; Yoshii, H. Release behavior of allyl sulfide from cyclodextrin inclusion complex of allyl sulfide under different storage conditions. *Biosci. Biotechnol. Biochem.* **2018**, *82*, 848–855.
5. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* **2001**, *25*, 402–408.
6. Guo, L.; Wang, Y.; Bi, X.; Duo, K.; Sun, Q.; Yun, X.; Zhang, Y.; Fei, P.; Han, J. Antimicrobial activity and mechanism of action of the Amaranthus tricolor crude extract against Staphylococcus aureus and potential application in cooked meat. *Foods* **2020**, *9*, 359.
7. Li, W.; Liu, X.; Yang, Q.; Zhang, N.; Du, Y.; Zhu, H. Preparation and characterization of inclusion complex of benzyl isothiocyanate extracted from papaya seed with β-cyclodextrin. *Food Chem.* **2015**, *184*, 99–104.
8. Yuan, H.N.; Yao, S.J.; Shen, L.Q.; Mao, J.W. Preparation and Characterization of Inclusion Complexes of β-Cyclodextrin-BITC and β-Cyclodextrin-PEITC. *Ind. Eng. Chem. Res.* **2009**, *48*, 5070–5078.
9. Xiao, Z.; Hou, W.; Kang, Y.; Niu, Y.; Kou, X. Encapsulation and sustained release properties of watermelon flavor and its characteristic aroma compounds from γ-cyclodextrin inclusion complexes. *Food Hydrocoll.* **2019**, *97*, 105202.
10. Phunpee, S.; Ruktanonchai, U.R.; Yoshii, H.; Assabumrungrat, S.; Soottitantawat, A. Encapsulation of lemongrass oil with cyclodextrins by spray drying and its controlled release characteristics. *J. Agric. Chem. Soc. Jpn.* **2017**, *81*, 718–723.
11. Wilson, A.E.; Bergaentzlé, M.; Bindler, F.O.; Marchioni, E.; Lintz, A.; Ennahar, S.D. Invitro efficacies of various isothiocyanates from cruciferous vegetables asantimicrobial agents against foodborne pathogens and spoilage bacteria. *Food Control* **2013**, *30*, 318–324.
12. Anjum, M.; Patel, K.K.; Pandey, N.; Tilak, R. Development of Anacardic Acid/hydroxypropyl-β-cyclodextrin inclusion complex with enhanced solubility and antimicrobial activity. *J. Mol. Liq.* **2019**, *296*, 112085.
13. Liu, L.; Shen, X.F.; Yu, J.Y.; Gao, X.W.; Zhan, Q.; Guo, Y.J.; Yu, F.Y. Subinhibitory Concentrations of Fusidic Acid May Reduce the Virulence of S. aureus by Down-Regulating *sarA* and *saeRS* to Reduce Biofilm Formation and α-Toxin Expression. *Front. Microbiol.* **2020**, *11*, 25.

14. Choi, W.S.; Son, N.; Cho, J.I.; Joo, I.S.; Han, J.A.; Kwak, H.S.; Hong, J.H.; Suh, S.H. Predictive model of *Staphylococcus aureus* growth on egg products. *Food Sci. Biotechnol.* **2018**, *28*, 913–922.
15. Baranyi, J.; Ross, T.; Mcmeekin, T.A.; Roberts, T.A. Effects of parameterization on the performance of empirical models used in 'predictive microbiology'. *Food Microbiol.* **1996**, *13*, 83–91.