Article

Optimization of Chinese Chive Juice as a Functional Feed Additive

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Featured Application: The optimized powder of Chinese chive products could potentially be applied in the animal feed industry.

Abstract: *Allium tuberosum*, commonly known as the Chinese chive (CC) is often used as a traditional medicine in East Asia for its health benefits. To explore the potential of CC as a functional feed additive, antibacterial and antioxidant assays, untargeted metabolomics, and a $2 \times 3 \times 3$ fractional factorial design (FFD) were conducted. In the present study, CC displayed stable DPPH radical scavenging activity with constant total phenolic content, however, the total flavonoid contents and the antibacterial activities were attenuated following heat treatment. The FFD results identified the solid content (SBM) as the main determinant of the antibacterial activity and moisture content of the CC products along with two other factors: drying time and temperature. Two CC products manufactured with 30% (*w/v*) SBM with 3 h drying at 80 °C and 20% (*w/v*) SBM with 8 h drying at 60 °C obtained the maximum antibacterial activity and least moisture content (<5%). Liquid chromatography-tandem mass spectrometry based multivariate analysis revealed 14 changed compounds in the non-heated and heated CC including flavonols, sinapinic acid, and lysophospholipids, which might affect the functionality. In conclusion, we propose an empirical approach to the pre-processing of CC juice that is suitable for blending in feed and simultaneously retaining its bioactivities.

Keywords: antibacterial activity; antioxidant activity; *Allium tuberosum*; fractional factorial design; Metabolites

1. Introduction

The overuse of in-feed antibiotics in farm animal nutrition is one of the main reasons for the rapid emergence of multi-drug resistant (MDR) bacterial strains and the recent advent of strains with reduced susceptibility to antibiotics, which adversely affects both humans and animals [1]. There has been increased interest in exploring the remedial antimicrobial agents derived either from natural products or their combinatorial forms [2,3]. *Allium* species, such as onions (*Allium cepa* L.), garlic (*Allium sativum* L.), leeks (*Allium ampeloprasum* L.), shallots (*Allium ascalonicum*), and chives (*Allium schoenoprasum* L.), have been shown to confer antimicrobial, antioxidant, antiviral, immune-enhancing, and cholesterol-lowering properties because they are rich in organosulfur

compounds, polyphenols, saponins, and oligosaccharides [4]. For instance, supplementation with garlic (*A. sativum* L.) essential oil (5 g/day) has been shown to increase the conjugated linoleic acid in the milk of dairy cattle [5]. Feeding dried garlic or onion (100 mg/day) improved the growth performance of broilers [6]. Dietary supplementation of garlic powder (6 g/kg/day) improved the immunity response and hepatic antioxidant activity in growing rabbits [7]. *A. tuberosum*, commonly known as the Chinese chive (CC), is native to Central Asia and Europe. It is widely used as an edible flavoring vegetable and spice. Aqueous CC extracts have exhibited antibacterial activity against a wide range of foodborne microorganisms, effectively inhibited *Campylobacter* species at a concentration of 2 mg/mL [8,9], and also reduced the cell count of penicillin-sensitive *Staphylococcus aureus* in rats supplemented with 400 mg/kg/day [10]. Recently, fermented CC juice was shown to have broad-spectrum antibacterial activity against poultry pathogens, and notable antioxidant activities [11,12]. So far, the application of CC as a functional feed additive is relatively less explored. To the best of our knowledge to date, only one study has reported the dietary supplementation of CC oil to reduce *Flavobacterium columnare*-based infections in Nile tilapia [13]. The high moisture content of CC is one of the main determinant factors that limits its further application in animal feed.

Dehydration is commonly used to preserve vegetables and fruits by reducing the water activity, thus hindering the perishability caused by autochthonous microorganisms and extending their shelf life [14]. However, traditional high-temperature drying may also adversely affect thermally labile bioactive compounds resulting in compromised nutritional or functional qualities [15]. In most cases, heat drying decreases the antioxidant content, which is generally attributed to the oxidation processes or thermal degradation [16]. Soybean meal (SBM) is a common feed ingredient and can be used as a good excipient for CC juice. Alam et al. [17] reported that the addition of SBM in oregano (*Origanum vulgare* L.) lippie seed oil extract effectively reduced the odorous compounds in swine in vitro. To minimize the influence of high-temperature drying on CC functionality, the drying temperature and duration, as well as the excipient ratio should be carefully tailored. Fractional factorial design (FFD) is used to identify the most influential factors along with their interactions [18], herein, FFD was used to obtain the optimal drying conditions for CC without significant loss of antibacterial activities. Metabolomics is a very useful tool for discovering the changes in specific compounds and to help us understand their correlation with the relevant functionality [12,19]. Here, we investigated the functional stability of CC with heat treatment and its optimized drying conditions based on antibacterial potential and changes in the relevant active compounds as profiled by untargeted metabolomic analysis.

2. Materials and Methods

2.1. CC Juice Preparation

Chinese chives, *A. tuberosum* Rottl, were purchased from the local market (Lotte, Seoul, Korea). The leaves and tubers of CC (1 kg) were cleaned using tap water and placed in a ventilated place for 2 h at room temperature. The CC leaves and tubers were squeezed using a juicer (Angeljuicer, Busan, Korea), then the fresh CC juice was collected by separating it from the solid particles using four-layered gauze. The fresh CC juice was centrifuged (10,000× g, 5 min, and 4 °C) and the supernatant was collected and designated as fresh CC juice. The supernatant was then treated at 60 °C and 80 °C for different time periods and designated as heated CC juice. The fresh and heated CC juice were stored at deep freezing conditions (−80 °C) until further analyses.

2.2. Antibacterial Activity of CC Juice

The inocula of *Clostridium perfringens* Type E SK870, Enterotoxigenic *Escherichia coli* O157:H7 (ETEC), *Pantoea agglomerans* SK876, *Haemophilus parasuis* SK890, *Haemophilus somnus* SK891, *Burkholderia* sp. SK1450, *Salmonella gallinarum* SK3359, *Salmonella pullorum* SK3360 obtained from the Korean National Veterinary Research and Quarantine Service (NVRQS) were prepared in Luria-Bertani (LB) medium by overnight culture at 37 °C with 120 rpm shaking. The inocula of

Vibrio ichthyenteri KCCM40870, *Vibrio harveyi* KCCM40866 and *Photobacterium damsela* subsp. *damsela* KCTC2734 were prepared in marine broth (MB) by 24 h of culturing at 26 °C and 120 rpm shaking. The inocula of KCCM40698 and KCTC3657 were prepared in brain heart infusion medium by 24 h culture at 30 °C with 120 rpm shaking. *Edwardsiella tarda* SK3946 obtained from the Kunsan National University was prepared in nutrient broth (NB) by 24 h culture at 37 °C with 120 rpm shaking. The antibacterial activity of the CC juice (fresh and heated) was determined using the agar well diffusion method. Briefly, 100 µL of the samples was loaded into the wells (6 mm) of LB agar plates that were inoculated by spreading the pathogens grown overnight and they were subsequently examined for inhibition zones after 24 h of incubation at 37 °C.

2.3. Antioxidant Activity and Antioxidant Compound Contents of CC Juice

2.3.1. DPPH Free Radical Scavenging Assay

DPPH (2-diphenyl-2-picrylhydrazyl) radical scavenging activity was determined according to a previous method with some modifications [20]. The fresh and heated CC juice (100 µL) were mixed with 500 µL of DPPH (0.06 mg/mL, *w/v*) in ethanol solution. The mixture was kept in a dark place for 15 min and gently agitated. The absorbance was measured using a microplate reader (Synergy 2, BioTek, USA) at 515 nm using ethanol (99.99%, *v/v*) as a blank. The DPPH radical scavenging activity was expressed as the inhibition percentage (%) and calculated using the following Equation (1).

$$\text{DPPH free radical scavenging activity (\%)} = (\text{Abs control} - \text{Abs sample}) / (\text{Abs control}) \times 100 \quad (1)$$

Total antioxidant compounds (µg/mL) in 100 µL of the fresh and heated CC juice was expressed as the ascorbic acid equivalent antioxidant content (AEAC) as well as the quercetin equivalent antioxidant content (QEAC). The AEAC and QEAC were determined via measuring the DPPH free radical scavenging activity of ascorbic acid (0–40 µg/mL) or quercetin (0–15 µg/mL) using the calculation $y = 2.0234x - 1.986$ or $y = 5.2237x - 0.3673$, respectively (y is the scavenging activity, and x is the concentration). The CC equivalents were converted based on the DPPH scavenging activity.

2.3.2. Determination of Total Phenolic Content

The total phenolic content (TPC) of the fresh and heated CC juice was determined using the Folin–Ciocalteu method with some modifications [21]. An aliquot (100 µL) of each sample, standard or blank was mixed with Folin–Ciocalteu (FC) reagent (10%, *v/v*) and vortexed thoroughly, followed by the addition of 800 µL of Na₂CO₃ (700 mM) solution. The mixture was vortexed again and allowed to stand for 2 h at room temperature. The reaction absorbance was measured at 765 nm using a microplate reader after transferring 200 µL of the mixture to a 96-well plate. TPC was calculated using gallic acid (0–250 µg/mL) as the standard and expressed as the gallic acid equivalent (GAE) (µg/mL).

2.3.3. Determination of Total Flavonoid Content

Total flavonoid content (TFC) of the fresh and heated CC juice was determined according to a previous study [22]. The assay was performed by adding distilled water (100 µL), 5% (*w/v*) sodium nitrite (10 µL) and sample or standard (25 µL) into the wells of the microplate, and the reaction mixture was allowed to stand for 5 min. Then, 15 µL of aluminium chloride solution (10%, *w/v*) was added to the reaction mixture and the reaction was incubated for 6 min. Finally, 1M of NaOH solution (50 µL) and 50 µL of distilled water were added to each reaction mixture in the microplate wells. The plate was shaken for 30 s prior to measurement of the absorbance at 510 nm. The TFC was calculated using quercetin (50–500 µg/mL) as the standard and expressed as quercetin equivalents (QE).

2.4. Fractional Factorial Design (FFD)

A total of 18 runs with $2 \times 3 \times 3$ FFD were employed. Three factors were used as variables: drying temperature ($\times 1$, 2 levels, 60 and 80 °C), duration ($\times 2$, 3 levels, 3, 6 and 9 h) and SBM content ($\times 3$, 3 levels, 20, 30 and 50% (*w/v*) based on the literature and preliminary experiments. A total of 20 g of CC and SBM mixture was prepared for each run according to the experimental design. Drying was carried out in drying oven (WFO-600SD, EYELA, Tokyo, Japan) for the designated time. The dried mixture was ground using a mortar and pestle, and then analyzed for its moisture content and antibacterial activity. The moisture content was measured by drying in a dry oven (105 °C) for 12 h [23]. For the antibacterial assay, CC mixture powder was extracted with aqueous methanol (95%, *v/v*) in the ratio of 1:9 with overnight shaking (20 °C, 150 rpm) and the suspension was filtered through four-layered gauze. The CC product extract (CCPE) was concentrated using a rotary evaporator (RE111, Buchi, Flawil, Switzerland) and then re-suspended in methanol to a working concentration of 100 mg/mL. Enterotoxigenic *E. coli* and *S. pullorum* were selected as the indicator strains for the antibacterial activity of CCPE due to their pathogenicity for causing diseases in farm animals and their multi-drug-resistant properties [24,25].

2.5. UHPLC-LTQ-Orbitrap-MS/MS Analysis

Freeze-dried fresh and heated CC juice samples (5 g) derived from the optimal heating conditions (60 °C for 8 h, CC60 and 80 °C for 3 h, CC80) were extracted with 50 mL of 80% (*v/v*) methanol followed by the sonication for 20 min. Then the mixture was centrifuged (10,000 $\times$ g, 4 °C, 10 min) and the collected supernatants were dried using a rotary vacuum evaporator at 45 °C followed by freeze-drying. The extracts were re-dissolved in 80% methanol at a concentration of 10 mg/mL (*w/v*) and chloramphenicol (2.5 mg/mL) was used as an internal standard (IS) for ultra-high-performance liquid chromatography coupled to a linear trap quadrupole-orbitrap-tandem mass spectrometry (UHPLC-LTQ-Orbitrap-MS/MS) analysis. Five microliters of sample were injected into a UHPLC system equipped with a Vanquish binary pump H system (Thermo Fisher Scientific, Waltham, MA, USA), auto-sampler, and column compartment. Chromatographic separation was performed on a Phenomenex KINETEX® C18 column (100 mm $\times$ 2.1 mm, 1.7 μ m particle size; Torrance, CA, USA). The column temperature was 40 °C and the flow rate was 0.3 mL/min. The mobile phase consisted of 0.1% formic acid in water (*v/v*, solvent A) and 0.1% formic acid in acetonitrile (*v/v*, solvent B). The 14 min gradient run was carried out by 5% solvent B for 1 min followed by a linear increase to 100% solvent B over 9 min and maintained for the next 1 min, and then re-equilibrated to the initial condition (5% solvent B) over 3 min. The total run time was 14 min. The MS data were collected by using an Orbitrap Velos Pro™ system, which was equipped with an ion-trap mass spectrometer and HESI-II probe. Mass spectra were acquired over the range of 100–2000 *m/z*. The MS parameters were: probe heater at 300 °C, capillary temperatures at 350 °C, and capillary voltage of 2.5 KV in negative mode.

2.6. Statistical Analysis

All experiments were performed in triplicate and the results were expressed as the mean with the standard error of mean (SEM). The results of the antibacterial activity, antioxidant activity, and antioxidant compounds (TPC and TFC) were analyzed using a general linear model with two-way analysis of variance (ANOVA) and validated with Duncan's multiple comparison test at 5% significant level (SPSS version 24.0, New York city, NY, USA). The data obtained from the FFD experiments were analyzed using Minitab® 14 software (Minitab Inc., State College, PA, USA). UHPLC-LTQ-MS data were acquired with Xcalibur software (version 2.00, Thermo Fisher Scientific, Waltham, MA, USA) and converted into netCDF format (*.cdf) using the Xcalibur software. Peak detection, retention time correction, and alignment were conducted using the MetAlign software package (<http://www.metalign.nl>). The resulting data were exported to an Excel file format (Microsoft; Redmond, WA, USA). Multivariate statistical analysis including principal component analysis (PCA)

and partial least square-discriminant analysis (PLS-DA) was performed using SIMCA-P+12.0 software (Umetrics; Umeå, Sweden). The data sets were auto-scaled (univariate scaling) and mean-centered in a column-wise fashion. The quality of the model was evaluated by R²_Y, and Q² which indicated the fitness and prediction accuracy of the model. In addition, the quality of the model was evaluated by the *p*-value derived from cross-validation analysis. The significantly different metabolites between experimental groups were selected based on variable importance projection (VIP) values, and significance was tested by one-way analysis of variance (ANOVA) and Students *t*-test using PASW Statistics 18 software (SPSS, Inc., Chicago, IL, USA). Selected metabolites were tentatively identified by comparing mass spectra, retention time, mass fragment patterns, and elemental compositions derived from UHPLC-LTQ-Orbitrap-MS/MS analyses considering standard compounds, in-house library, and published references. The box and whisker plots were constructed using the relative peak area by STATISTICA 7 software (StatSoft Inc., Tulsa, OK, USA).

3. Results

3.1. Antibacterial Stability of CC Juice under Heat Treatment

The fresh CC juice displayed broad-spectrum antibacterial activities against a wide range of pathogens except *L. garvieae* and *V. harveyi*, hence, these two strains were excluded from further experiments in this study (Table 1). Considering that CC drying is an essential prerequisite to producing powdered products, the effects of different heat treatment (60 °C and 80 °C for 3 h) on the antibacterial activity of CC juice were examined (Table 2). We observed that the antibacterial activities of CC juice were significantly decreased following the increase in heating temperature (*p* < 0.01) and time (*p* < 0.01). However, the interactive effects of heating temperature and time did not significantly impact the antibacterial effects of CC against *E. coli*, *P. agglomerans*, *H. somnus* and *Burkholderia* sp., but showed a decreasing trend against *S. iniae* and *S. gallinarum*. The antibacterial activity of CC juice was partially impaired by the heat treatments, but was still effective after 3 h of heating at 80 °C.

Table 1. Antibacterial activity of the fresh Chinese chive (CC) juice against pathogens.

Pathogens	Antibacterial Activity ⁽¹⁾
<i>Clostridium perfringens</i> Type E	++
<i>Streptococcus iniae</i>	+++
<i>Lactococcus garvieae</i>	-
Enterotoxigenic <i>E. coli</i> O157:H7	++
<i>Pantoea agglomerans</i>	++
<i>Haemophilus parvis</i>	++
<i>Haemophilus somnus</i>	+++
<i>Burkholderia</i> sp.	+++
<i>Salmonella gallinarum</i>	++
<i>Salmonella pullorum</i>	+++
<i>Edwardsiella tarda</i>	+++
<i>Photobacterium damsela</i> subsp. <i>damsela</i>	+++
<i>Vibrio ichthyenteri</i>	+++
<i>Vibrio harveyi</i>	+

⁽¹⁾ Antibacterial activity (clear zone diameter): -, Not detected; +, 0–10 mm; ++, 10–20 mm; +++, 20–30 mm.

Table 2. Variations in the antibacterial activity of the fresh and heated CC juice subjected to heat treatment.

Pathogens	Heating Time at 60 °C (h)				Heating Time at 80 °C (h)				SEM	p-Value		
	0	1	2	3	0	1	2	3		Temp ⁽¹⁾	Time	Temp * Time
	———— Zone of inhibition (mm) ————											
<i>C. perfringens</i>	16.2 ^a	14.5 ^{a,b}	14.3 ^b	13.5 ^b	15.2 ^a	12.3 ^b	9.8 ^c	10.7 ^c	0.45	<0.01	<0.01	0.01
<i>S. iniae</i>	21.7 ^a	23.7 ^b	19.5 ^c	18.0 ^c	20.7 ^a	20.7 ^a	16.0 ^b	14.0 ^c	0.63	<0.01	<0.01	0.09
<i>E. coli</i> O157:H7	18.7 ^a	18.8 ^a	16.7 ^b	16.5 ^b	16.0 ^a	14.5 ^b	13.3 ^c	12.3 ^d	0.47	<0.01	<0.01	0.14
<i>P. agglomerans</i>	14.3 ^a	13.0 ^{a,b}	11.7 ^{b,c}	11.0 ^c	12.3 ^a	11.3 ^a	10.5 ^{a,b}	8.7 ^b	0.36	<0.01	<0.01	0.71
<i>H. parsuis</i>	22.5 ^a	22.5 ^a	21.0 ^b	20.0 ^b	19.8 ^a	17.3 ^b	15.0 ^c	13.7 ^c	0.66	<0.01	<0.01	<0.01
<i>H. somnus</i>	28.5 ^a	29.7 ^a	27.3 ^{a,b}	24.5 ^b	25.3 ^a	24.3 ^a	22.7 ^{a,b}	20.2 ^b	0.66	<0.01	<0.01	0.65
<i>Burkholderia</i> sp.	30.7 ^a	31.0 ^a	28.7 ^b	26.5 ^c	28.5 ^a	25.0 ^b	23.2 ^b	18.2 ^c	0.91	<0.01	<0.01	0.11
<i>S. gallinarum</i>	16.5 ^a	15.0 ^b	13.8 ^c	13.8 ^c	14.5 ^a	13.0 ^a	11.2 ^b	10.0 ^b	0.42	<0.01	<0.01	0.06
<i>S. pullorum</i>	20.7 ^a	20.7 ^a	18.3 ^{a,b}	17.0 ^b	21.3 ^a	17.3 ^b	14.7 ^c	11.8 ^d	0.67	<0.01	<0.01	<0.01
<i>E. tarda</i>	25.8 ^a	23.8 ^b	23.8 ^b	22.5 ^c	29.8 ^a	21.5 ^b	20.3 ^b	16.5 ^c	0.79	<0.01	<0.01	<0.01
<i>P. damsela</i>												
subsp. <i>damsela</i>	24.8 ^a	23.0 ^b	23.3 ^b	23.5 ^b	24.6 ^a	22.9 ^b	13.0 ^c	12.9 ^c	0.96	<0.01	<0.01	<0.01
<i>V. ichthyenterii</i>	27.3 ^a	22.8 ^b	23.0 ^b	20.5 ^c	25.8 ^a	20.3 ^b	16.5 ^c	15.8 ^c	0.80	<0.01	<0.01	<0.01

⁽¹⁾ Temp, Temperature. Means with different superscripts (a, b, c, d) in the same row within same temperature differ significantly ($p < 0.05$). * means interactive effects of temperature and time; SEM: Standard error of mean.

3.2. Antioxidant Stability of CC Juice under Heat Treatment

The antioxidant stability of the CC juice treated with different heating conditions is presented in Table 3. All CC juice samples showed strong DPPH radical scavenging activity (~70%) equivalent to 35 µg/mL of AEAC and 13 µg/mL of QEAC. The DPPH scavenging activity of heated CC juice was significantly increased at both 60 °C and 80 °C after 24 h treatments. The TPC and TFC of CC juice were determined in the concentration range of 360 ± 8.1 to approximately 380 ± 7.5 µg/mL of GAE and 111 ± 3.1 to approximately 165 ± 7.0 µg/mL of QE, respectively. None of the heating temperatures ($p = 0.38$), heating times ($p = 0.50$) or the interactive effects ($p = 0.46$) had a significantly effect on the TPC of the CC juice. However, the TFC showed a decreasing trend ranging from 164.6 ± 7.0 to 110.9 ± 3.1 µg/mL of QE (60 °C) and from 164.6 ± 7.0 to 127.1 ± 2.4 µg/mL of QE (80 °C) after 24 h and 6 h, respectively. Intriguingly, the DPPH scavenging activity was significantly increased whereas the TFC was significantly decreased following heat treatments. The heat treatment reduced the TFC, but the CC juice still exhibited very stable antioxidant activity even after being treated at 80 °C for 24 h.

Table 3. Variations in the antioxidant activity of the CC juice subjected to heat treatment.

Items ⁽¹⁾	Heating Time at 60°C (h)							Heating Time at 80°C (h)							SEM	<i>p</i> -Value		
	0	1	2	3	6	9	24	0	1	2	3	6	9	24		Temp ⁽²⁾	Time	Temp * Time
DPPH inhibition (%)	69.4 ^a	69.3 ^{a,b}	71.0 ^{a,b,c}	70.4 ^{a,b}	71.1 ^{a,b,c}	72.0 ^{b,c}	73.2 ^c	69.4 ^a	70.3 ^{a,b}	71.5 ^{b,c}	71.1 ^b	71.9 ^{b,c,d}	72.7 ^{c,d}	72.9 ^d	0.2	0.42	<0.01	0.97
AEAC (µg/mL)	35.3 ^a	35.2 ^{a,b}	36.1 ^{a,b}	35.8 ^{a,b}	36.1 ^{a,b}	36.6 ^{b,c}	37.1 ^c	35.3 ^a	35.7 ^{a,b}	36.3 ^{b,c,d}	36.1 ^{a,b}	36.5 ^{b,c,d}	36.9 ^{c,d}	37.0 ^d	0.1	0.35	<0.01	0.96
QEAC (µg/mL)	13.4 ^{a,b}	13.3 ^a	13.7 ^{b,c,d}	13.6 ^{a,b,c}	13.7 ^{b,c,d}	13.9 ^{c,d}	14.1 ^d	13.4 ^a	13.5 ^{a,b}	13.8 ^{b,c}	13.7 ^{a,b,c}	13.8 ^{b,c}	14.0 ^c	14.0 ^c	0.0	0.15	<0.01	0.88
TPC (GAC, µg/mL)	375.5	360.0	366.1	371.1	373.1	371.1	366.8	375.5	373.5	373.1	373.7	372.0	379.9	371.9	6.8	0.38	0.50	0.46
TFC (QE, µg/mL)	164.6 ^c	129.0 ^b	112.8 ^a	115.3 ^a	112.8 ^a	115.3 ^b	110.9 ^a	164.6 ^a	142.8 ^b	129.0 ^c	131.5 ^c	127.1 ^c	127.8 ^c	129.6 ^c	2.7	<0.01	<0.01	0.03

⁽¹⁾ Items: AEAC, Ascorbic acid equivalent antioxidant content; QEAC, quercetin equivalent antioxidant content; TPC, Total phenolic contents; TFC, Total flavonoid contents; GAC, Gallic acid equivalent; QE, quercetin equivalents. ⁽²⁾ Temp, Temperature. The mean with different superscripts in the same row within the same temperature significantly different ($p < 0.05$); * means interactive effects of temperature and time; SEM: Standard error of mean.

3.3. Fractional Factorial Design for Optimized CC Products

FFD was conducted to identify optimized CC products based on the moisture content and antibacterial activities against the two dedicated pathogens. A total of 18 runs of the experiment were used to investigate the optimal drying conditions of solidified CC (Table 4). The moisture content (Y1) of each run varied from 1.6 to 28.0%, and decreased steeply during 3 to 6 h of drying but no notable changes were observed with the extended heating treatments. The moisture content was reduced with the increase in SBM content (20% to 50%). The antibacterial activities of CCPE against *E. coli* (Y2) and *S. pullorum* (Y3) varied in the range of 8.7 ± 0.5 to approximately 14.0 ± 1.4 mm and 9.3 ± 0.6 to approximately 15.0 ± 0.8 mm, respectively. The antibacterial activity was significantly decreased with the increase in the drying temperature, drying time, and SBM content. Analysis of the major effects of the variables (Figure 1) showed that the SBM content was the main determinant factor to impact both the moisture content and antibacterial activity against *E. coli* and *S. pullorum*. Moreover, the moisture content and antibacterial activity against *S. pullorum* were additionally impacted by the drying time ($p < 0.01$) and drying temperature ($p < 0.05$), respectively. Considering these results, an SBM content of 20% and drying temperature at 60 °C were selected for further analysis (Table 5). The moisture content was linearly reduced with extended heating time and was less than 5% within 8 h. No significant effect on antibacterial activities of CCPE was observed along with the drying time. Taken together, the results suggested that CC juice with 30% SBM content and 3 h drying at 80 °C or with 20% SBM content and 8 h drying at 60 °C achieve high antibacterial activity with less than 5% moisture content.

Table 4. Fractional factorial design (FFD) for evaluation of the effect of critical factors (drying temperature, time, and the solid content (SBM) on antibacterial activities and moisture contents of the CC products.

Runs	Variables ⁽¹⁾			Responses ⁽²⁾		
	X ₁	X ₂	X ₃	Y ₁	Y ₂ (<i>E. coli</i>)	Y ₂ (<i>S. pullorum</i>)
1	60	3	20	28.69 ± 9.51	14.00 ± 0.81	15.00 ± 0.47
2	60	3	30	18.97 ± 6.61	11.33 ± 0.54	12.00 ± 0.94
3	60	3	50	9.56 ± 2.12	8.67 ± 0.27	10.17 ± 0.59
4	60	6	20	8.82 ± 1.62	10.33 ± 0.98	12.67 ± 0.72
5	60	6	30	4.45 ± 0.31	10.33 ± 0.27	12.00 ± 0.00
6	60	6	50	3.59 ± 0.18	8.83 ± 0.36	9.50 ± 0.24
7	60	9	20	3.78 ± 0.17	11.50 ± 0.70	12.83 ± 0.89
8	60	9	30	3.41 ± 0.18	9.33 ± 0.72	10.33 ± 0.72
9	60	9	50	3.17 ± 0.18	9.00 ± 0.47	9.33 ± 0.36
10	80	3	20	28.34 ± 4.77	10.33 ± 0.72	12.00 ± 1.25
11	80	3	30	5.73 ± 0.16	9.67 ± 0.72	11.00 ± 0.41
12	80	3	50	3.22 ± 0.19	9.00 ± 0.47	9.17 ± 0.36
13	80	6	20	3.93 ± 0.39	10.67 ± 1.18	11.67 ± 1.09
14	80	6	30	2.47 ± 0.09	10.33 ± 0.95	10.33 ± 0.59
15	80	6	50	1.74 ± 0.03	9.00 ± 0.47	9.33 ± 0.27
16	80	9	20	2.23 ± 0.10	11.17 ± 1.30	11.33 ± 1.38
17	80	9	30	1.60 ± 0.12	10.00 ± 0.94	9.50 ± 0.85
18	80	9	50	1.83 ± 0.05	10.17 ± 0.89	9.67 ± 0.72

⁽¹⁾ Variable: X₁, temperature (°C); X₂, drying time (h); X₃, SBM content (%). ⁽²⁾ Response: Y₁, Moisture (%); Y₂, antibacterial activity against *E. coli* and *S. pullorum* (mm). The data expressed as mean ± SEM.

Figure 1. Effect of different drying conditions on moisture content and antibacterial activity of CC products.

Table 5. Effect of different drying durations (h) at 60 °C on moisture content and antibacterial activity of the CC products with 20% SBM content.

Drying Time (h)	Moisture Content (%)	Zone of Inhibition (mm)	
		<i>E. coli</i>	<i>S. pullorum</i>
6	25.71 ^c	12.2	14.0
7	14.96 ^b	13.7	15.0
8	4.68 ^a	12.7	13.8
9	5.51 ^a	12.0	13.0
10	2.70 ^a	11.7	13.8
SEM	2.45	0.27	0.24
<i>p</i> -value	<0.001	0.122	0.118

Different superscripts in the same column mean they are significantly different ($p < 0.05$). SEM: Standard error of mean.

3.4. Biochemical Changes with Optimized Drying Conditions

In order to evaluate the heating effect on the composition of the active compounds in the non-heated and heated CC juice, untargeted metabolite profiling using UHPLC-LTQ-Orbitrap-MS/MS platform was performed. The PCA score plot presented a clearly separate pattern for the non-heated (CC) and heated CC (CC60 and CC80) metabolomic datasets across PC1 (40.6%) and PC2 (26.1%) (Figure 2A). Similar clustered patterns were observed among the datasets in supervised PLS-DA for PLS1 (40.6%) and PLS2 (26.1%) coupled with high predictive ability ($Q^2 = 0.942$), high predictive variations ($R^2X = 0.667$ and $R^2Y = 0.999$), and a considerable significance metric ($p = 0.0235$) (Figure 2B). Overall, there were fifteen different metabolites tentatively identified in the CC juice extracts, which were mainly composed of flavonoids and lipids (Table 6). Based on the VIP (variable importance for projection) value (>0.7) and $p < 0.05$, significantly discriminated metabolites among the groups were selected, and a total of 14 metabolites were annotated (Figure 3). In the fresh CC juice extract, the relative content of quercetin-glucosylgalactoside-glucoside (or quercetin-triglucoside), kaempferol-di-glucoside (or kaempferol-sophoroside), kaempferol-sophoroside-glucoside, feruloyl-galactaric acid, kaempferol diglucoside-(feruloylglucoside), lysoPE(18:2), lysoPC(18:2), lysoPE(16:0), and lysoPC(16:0) was significantly higher than that in the heated CC juice extracts. Conversely, the relative content of several metabolites such as N-(1-deoxy-1-fructosyl) leucine, quercetin-di-glucoside, sinapinic acid-O-glucuronide isomer, quercetin-hexoside, and kaempferol-glucoside was significantly higher in the CC60 group compared to that of other groups.

Figure 2. Multivariate statistical analysis of LC-MS datasets for fresh and heated CC juice extracts with dedicated conditions. (A) Principal component analysis (PCA) score plot. (B) Partial least squares discriminant analysis (PLS-DA) score plot. ■ CC, fresh CC juice; ■ CC60, CC juice heated at 60 °C for 8 h; ■ CC80, CC juice heated at 80 °C for 3 h.

Figure 3. Box and whisker plots of metabolites that were significantly different between the fresh and heated CC juice with dedicated conditions analyzed by UHPLC-LTQ-Orbitrap-MS/MS. The Y-axis of the box and whisker plots indicates the peak area of each metabolite (Square, mean; box, standard error; whisker, standard deviation). (A) N-(1-Deoxy-1-fructosyl)leucine, (B) Quercetin-glucosylgalactoside-glucoside or Quercetin-triglucoside, (C) Kaempferol-di-glucoside or Kaempferol-sophoroside, (D) Feruloyl-galactaric acid, (E) Kaempferol-sophoroside-glucoside, (F) Quercetin-di-glucoside, (G) Sinapinic acid-O-glucuronide isomer, (H) Kaempferol diglucoside-feruloylglucoside, (I) Quercetin-hexoside, (J) Kaempferol-glucoside, (K) LysoPE (18:2), (L) LysoPC (18:2), (M) LysoPE (16:0), and (N) LysoPC (16:0).

Table 6. Tentatively identified metabolites in CC juice based on the UHPLC-LTQ-Orbitrap-MS/MS analyses.

No.	Tentatively Identified Metabolites ⁽¹⁾	RT ⁽²⁾ (min)	MW ⁽³⁾	Measured Mass	MS/MS Fragments	Class of Compounds
				Negative Mode (<i>m/z</i>)		
1	N-(1-Deoxy-1-fructosyl)leucine	1.00	293	292.1377 [M – H] [–]	292 > 274/202/172/130	Amino acid
2	Quercetin-glucosylgalactoside-glucoside or Quercetin-triglucoside	3.35	788	787.1852 [M – H] [–]	787 > 625/463/301	Flavonol
3	Kaempferol-di-glucoside or Kaempferol-sophoroside	3.59/4.05/4.37/4.50	610	609.1404 [M – H] [–]	609 > 447/285/489/581	Flavonol
4	Feruloyl-galactaric acid	3.94	386	385.0739 [M – H] [–]	385 > 191/209/367	Hexaric acid
5	Kaempferol-sophoroside-glucoside	3.65/4.01	772	771.1522 [M – H] [–]	609 > 429/447/489	Flavonol
6	Quercetin-di-glucoside	4.39	626	625.1349 [M – H] [–]	625 > 463/300/445/505/607	Flavonol
7	Sinapinic acid-O-glucuronide isomer	4.55	400	399.0897 [M – H] [–]	399 > 381/355/223/205/187	Phenolic acid
8	Kaempferol diglucoside-(feruloylglucoside)	4.55/4.80	948	947.2362 [M – H] [–]	947 > 623/785/447/609/285	Flavonol
9	Quercetin-hexoside	4.81	464	463.0843 [M – H] [–]	463 > 301	Flavonol
10	Kaempferol-glucoside	5.04	448	447.0893 [M – H] [–]	447 > 284/255	Flavonol
11	LysoPE(18:2)	8.22/8.34	477	476.2734 [M – H] [–]	476 > 279/402/214	Lipid
12	LysoPC(18:2)	8.28/8.40	519	564.3246 [M + FA – H] [–]	564 > 504/279	Lipid
13	LysoPE(16:0)	8.61	453	452.2737 [M – H] [–]	452 > 255/214/323/378	Lipid
14	LysoPC(16:0)	8.68	495	540.3253 [M + FA – H] [–]	540 > 480/255/391/224	Lipid

⁽¹⁾ Tentatively identified metabolites based on variable importance projection (VIP) analysis with cutoff value of 0.7 and a *p*-value <0.05. ⁽²⁾ RT: Retention time. ⁽³⁾ MW: Molecular weight. LysoPE: lysophosphatidylethanolamine; LysoPC: lysophosphatidylcholine.

4. Discussion

Chinese chives are rich in substantial active compounds that provide it with effective antimicrobial and antioxidant activities. In our study, CC displayed broad-spectrum antibacterial activities against 12 pathogenic bacteria responsible for diseases in swine, poultry, horse, and aquaculture. This was consistent with previous studies that have reported the inhibitory effects of CC on a variety of Gram-positive and Gram-negative bacteria [8–11]. To the best of our knowledge, to date very few studies have explored the use of CC as a functional additive in animal feed. Commercial phytogetic feed additives (PFA) generally need to be in a powder form with stable functional properties. The antibacterial stability of CC was primarily investigated under common drying temperature (60 °C and 80 °C) within 3 h. The attenuated antibacterial activity of CC with heat treatment might be attributed to the specific compositional changes. The organosulfur compounds are considered as the main antibacterial agents in CC [26,27], however they are heat-labile and easily degraded under heating conditions [28]. Lee et al. [9] reported that the antibacterial activity of *Allium* extracts against *Campylobacter* species was reduced when subjected to 75 °C for 30 min. Conversely, Mau et al. [8] reported that CC extracts showed thermal stability with heat pre-processing up to 100–121 °C for 15 min, and considerable antibacterial activity was retained against diverse food-borne pathogens. Flavonoids are another important compound in CC that are responsible for both antibacterial and antioxidant activities [29]. In line with our TFC result, the flavonoids in kale and raspberry were reported to decrease by 2–92% after air-drying as compared with the fresh products [16]. However, the TPC was not significantly affected by the heating treatments in this study, signifying that the flavonoid constituents are closely related to the antibacterial activity of CC. Kim et al. [30] reported that phenolic acids and flavonoid constituents in garlic (*A. sativum*) varied following different thermal processing conditions, herein, the synthesis and degradation of these compounds in CC during heating displayed relatively constant TPC and reduced TFC. SBM was used as an excipient to solidify the CC juice with optimized drying conditions. The high moisture content (80%) of CC juice may lower shelf-life due to microbial contamination, which may lead to the reduction of biofunctionalities in CC-based products. Low moisture content (<5%) in post-harvest products is desirable as this can extend their shelf life, for example, if an environment that is unsuitable for microbial proliferation is created this prevents deterioration in the product [31]. Hence, the optimal drying conditions are very important in order to make high quality products while simultaneously retaining their biological activities. FFD is widely used to optimize the functional parameters, mass production of end-products, and growth of the target phenotypes [32–34]. Among the three variable factors, solid content (SBM) ratio was found to be the most critical factor impacting the antibacterial activity of CC against the selected pathogens.

Untargeted metabolomics using a UHPLC-LTQ-Orbitrap-MS/MS platform was carried out to analyze the metabolites' disparity in fresh and heated CC samples. Among different flavonoid groups, flavonols mainly consist of kaempferol, quercetin, and their glycosides derivatives in *Allium* species with potent antibacterial and antioxidant activities [35,36]. Quercetin and its glucoside derivatives have been reported to exhibit bacteriostatic effects due to their damage to cell wall and membrane, anti-quorum sensing and anti-biofilm properties [37,38]. The decreased levels of quercetin and/or kaempferol glycosides in heated CC might have resulted in the reduced antibacterial activity. Sinapinic acid and its derivatives, commonly found in fruits, vegetables and some spices, has also been reported to have antioxidant and antimicrobial activities [39]; therefore, the increased level of sinapinic acid in this study might be correlated with the slightly increased DPPH radical scavenging activity. CC is traditionally eaten after cooking or fermentation because fresh CC has been reported to have toxic effects in liver and kidney cells [40]. Lysophosphatidylcholine (LysoPC) has been reported to induce cell apoptosis in endothelial and vascular smooth muscle cells, which can be associated with atherogenesis [41]. Thereby, the lowered level of LysoPC in this study might reduce the toxicity of CC. Very few studies have investigated the antioxidant and antimicrobial effects of these lysophospholipids (LPLs). Meylaers et al. [42] reported the antimicrobial activity of 1-lysophosphatidylethanolamine

(C16:1) isolated from a housefly. The LPLs are heat-labile, but whether their decrease is correlated to the changed functionality is still unclear. Advanced untargeted metabolomic analysis can aid in understanding the correlation between the changed metabolites and functionality, although it is significantly limited by low-quality spectra, incomplete databases, and the ambiguous annotation of unknown compounds [43]. Thus, further studies are needed to validate these results with the focus on organosulfur compounds and flavonoids using standard compounds.

5. Conclusions

In conclusion, the results of this study suggest that CC products manufactured with 30% (*w/v*) solid content with 3 h drying at 80 °C or 20% solid content with 8 h drying at 60 °C achieve significant antibacterial activities with less moisture content (<5%). Our study also indicates that manipulating the drying conditions can influence the overall availability of antimicrobial and antioxidant compounds in CC. Future animal studies will be carried out to evaluate the *in vivo* efficacy of CC products.

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