Regulation of acylated homoserine lactones (AHLs) in beef by spice marination

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Abstract Quorum sensing (QS) is a signaling mechanism used by bacteria to communicate each other through the release of auto-inducing signaling molecules. Despite the fact that bacteria regulate its phenotypes by QS mechanism, their potential role in meat spoilage is not yet elucidated. In the current study, beef samples were analyzed for its microbial association and for the presence of N-acyl-homoserine-lactone (AHLs) throughout the storage experiments. Isolates were screened for AHLs production and selected spices were screened for their quorum sensing inhibitory (QSI) activity. In addition, effect of spices on AHLs production of *Y. enterocolitica* was quantified through high performance thin layer chromatography (HPTLC). Outcome showed that microbial association of beef mainly consists of lactic acid bacteria (LAB) and *Enterobacteriaceae*. Samples stored at both aerobic and modified atmospheric packaging (MAP) exhibited higher counts whereas; marinated samples stored at MAP exhibited the lowest. It was found that out of 35 isolates *Y. enterocolitica* induced reporter strain CV026 and its cell-free supernatant contained 26.36 nM/100 ml of AHLs when compared to standard. Among the tested spices, *C. cyminum* exhibited pronounced results by significantly reducing the AHLs concentration up to 47.75 %. Findings revealed the presence of quorum molecules (AHLs) in beef meat throughout the spoilage process and spices can acts as quorum quenchers to influence the spoilage rate by reducing AHLs production.

Keywords Quorum quenching · N-acyl homoserine lactones (AHLs) · Marination · Microbiological spoilage · Meat storage

Introduction

Meat is a one of the highly consumable food product with short shelf life. Microbial growth and their metabolic activities are the most important among the factors affecting shelf life of fresh meat. Microbial spoilage may be manifested by visible growth of colonies, textural changes or off-odors and off-flavors (Borch et al. 1996; Gram et al. 2002). Microbial spoilage of meat is mainly associated with specific group of spoilage organism dominated by *Pseudomonas* spp., *Enterobacteriaceae*, *Br. thermosphacta* and lactic acid bacteria (Ercolini et al. 2006). Dominance and association of these organisms depends up on extrinsic factors including processing, transportation, storage temperatures, and packaging (Nychas et al. 2009). Most of these spoilage bacteria used to communicate each other by the release of small signal molecules called as autoinducers through quorum sensing (QS) to regulate their phenotypic characteristics based on their population density. Acylated homoserine lactones (AHLs) act as autoinducer molecule in Gram-negative bacteria, whereas peptides based molecules acts as autoinducers in Gram-positive bacteria (Gram et al. 2002; Novick et al. 1993). Presence of N-Acylated homoserine lactones (AHLs) were identified in many food sources like meat, fish and poultry products (Medina-Martinez et al. 2006). However, information on the potential role of these signaling molecules in food spoilage is very limited and the results were inconclusive (Nychas et al. 2009). Metabolic end products like proteolytic and pectinolytic enzymes

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related to meat spoilage were found to be regulated by quorum sensing (Smith et al. 2004). Hence, intervening the signaling mechanism between spoilage organisms would be of great strategy to avoid or minimize the spoilage reactions (Rasch et al. 2005). In the midst of all possibilities, use of anti-quorum compounds as quorum quenchers could be of immense potential to avoid bacterial infections (Rice et al. 2005; Adonizio et al. 2006).

So far, many synthetic and natural compounds have been investigated for its quorum sensing inhibitory (QSI) activity. These compounds include macrolides (Tateda et al. 2001), furanones (Givskov et al. 1996), ajoene (Jakobsen et al. 2012), ellagic acid (Sarabhai et al. 2013) and aspirin (Mowafy et al. 2014). However, limitations of these compounds to use in food and food industries have led to the search of food based compound which can be used in various applications. The process of coating or soaking meat with the mixture containing spices, organic acids, sauces, lemon juice and other essential oils to flavor and tenderize the meat products is called as marination (Bjorkroth 2005; Pathania et al. 2010). Shelf-life of the meat may be positively affected by this process. Marination ensures the shelf-life of the product due to the antimicrobial and antioxidant activity of some marinating spices due to its acidic or alkaline nature. Numbers of studies have been reported for improving delectableness and warmth of meat by the process of marination (Scanga et al. 2000; Dhanda et al. 2002; Burke and Monahan 2003; Sheard et al. 2005; Lunde et al. 2008). Even though few reports are available on the microbiological quality of meat during marination (Rhoades et al. 2009; Lia et al. 2011), investigations have been limited to the anti-bacterial or bactericidal effect of marinating agents (Tajkarimi et al. 2010). Study on the competence of spices to influence the bacterial interaction with food system may also be of good interest to manage the spoilage organisms.

Considering above into account, the present study is designed to investigate the microbial profile and the presence of AHLs during storage. It was also designed to investigate the effect of different spices based marinating agents in regulating the signal molecules production.

Materials and methods

Sample collection and enumeration of bacteria

Fresh beef samples (<4 h after slaughtering) were collected from five different commercial markets in a sterile sample container and shifted to the laboratory within an hour. A piece of beef tissue from each sample was withdrawn periodically for microbiological analysis. Meat samples were homogenized in physiological saline and ten-

fold diluted for enumeration of (1) total viable count on nutrient agar, (2) lactic acid bacteria on Man-Rogosa-Sharpe (MRS) agar and (3) MacConkey agar for Enterobacteriaceae. Colonies were selected from all the plates by visual examination for typical colony and morphological characteristics. Selectivity was checked routinely by Gram staining and microscopical examination of smears. Total of 35 selected colonies were purified and stored as glycerol stocks at -40°C until further use.

Screening for AHL producing bacteria

Mutant strain of *Chromobacterium violaceum* (CECT 5999) which cannot produce its AHL but do respond to the exogenous AHLs is used as a reporter strain. Gram-negative bacterial colonies were screened for the production of quorum signal by induction method as described by Ravn et al. (2001). Briefly, test bacterial cultures were streaked against reporter strain on Luria-Bertani (LB) agar plates and incubated at 30°C . Production of AHLs was detected by induction of violacein pigment in CV026. *C. violaceum* MTCC 2656 was used as positive control whereas, bio-sensor strain CV026 itself was used as negative control.

For microtiter plate assay $360\ \mu\text{l}$ of reporter culture suspension grown in LB broth adjusted to 0.1 OD at 600 nm were transferred to 48 well microtiter plate and inoculated with $40\ \mu\text{l}$ of filter sterilized culture supernatant. Plates were then incubated at 30°C for 48 h. Reporter culture inoculated with synthetic HHL was used as positive control, whereas reporter strain itself was served as negative control.

Genotypic characterization

To identify the AHL-positive culture, total DNA was isolated using HiPURA bacterial genomic DNA purification kit (Himedia, India). Ribosomal RNA (16 s) was amplified by set of forward and reverse universal primer U1F-5'-AGAGTTTGATCCTGGCTCAG-3' and U1R-5'-GGTTACCTTGTTACGACTT-3' respectively. Thermocycling conditions were maintained as initial denaturation of 92°C for 2 min and 10 s followed by 35 cycles of 92°C for 1 min, 48°C for 30 s and 72°C for 2 min and 10 s and a final extension step of 72°C for 6 min and 10 s using mastercycler (Eppendorf, Germany). Amplified products were resolved on 1.5 % agarose, ethidium bromide gel. DNA sequencing was performed and subjected to BLAST analysis for determining nucleotide sequence similarities in NCBI. Identified isolate was deposited in NCBI (National Center for Biotechnology Information databases) and MTCC (Microbial Type Culture Collection) was assigned with accession number.

Spices extract

Marinating spices such as Clove (*Syzygium aromaticum*), Cumin (*Cuminum cyminum*), Fennel (*Foeniculum vulgare*), Garlic (*Allium sativum*), Ginger (*Zingiber officinale*), Pepper (*Piper nigrum*) and Turmeric (*Curcuma longa*) were purchased from a local departmental store (Pondicherry, India). Tray dried spices were ground to a fine powder and dissolved in 70 % methanol for overnight in a closed container at 150 rpm. Dissolved extracts were filtered through Whatman No. 1 filter paper, which were then concentrated and solidified by freeze-drying. Freeze dried powder was stored at -40°C until further use.

Determination of minimum inhibitory concentration

The MIC for spices was determined as per the guidelines of Clinical and Laboratory Standards Institute, USA. Stock solution was prepared by dissolving 100 mg of extracted spices powder in 1 ml of 70 % methanol in de-ionized water. One percentage of overnight test pathogens were added to appropriate growth medium supplemented with spices to attain the final concentrations ranging from (1 to 10 mg/ml). MTP plates were then incubated for 24 h. MIC was recorded as the lowest concentration which showed complete inhibition of visible growth. All further experiments in the present study were performed only at sub-MIC concentrations.

Violacein inhibition assay

Overnight culture (10 μl) of *C. violaceum* (adjusted to 0.4 OD at 600 nm) was added into wells of sterile microtiter plates (MTP) containing 1 ml of LB broth and incubated with or without spices extract. These MTPs were incubated at 30°C for 24 h and observed for the reduction in violacein pigment production. Only the extract that exhibited potent QSI activity against *C. violaceum* MTCC 2656 alone was further considered for subsequent experiments in this study. Further, the quantitative analysis was performed in *C. violaceum* CV026, a violacein-negative mutant strain which requires AHL to induce violacein production (McClean et al. 1997). In quantitative analysis, CV026 was supplemented with 5 μM of HHL (Sigma) and cultivated in the presence and absence of test extract (Choo et al. 2006).

Kinetics of AHL production in positive strains

Yersinia enterocolitica isolated from meat and identified for its AHL production was used as a model organism in meat spoilage experiments. Kinetics of AHLs production in *Y. enterocolitica* was monitored in nutrient broth. AHL production was measured in a microtiter plate with CV026

by testing syringe filtered supernatant and comparing the violacein production with the same, produced by adding known concentrations of N-hexanoyl-DL-homoserine lactone (HHL).

Extraction and quantification of AHL from *Y. enterocolitica* isolated from beef

Overnight bacterial culture of *Y. enterocolitica* was inoculated into 100 ml of appropriate growth medium and incubated at 30°C . Spent culture supernatant was extracted with ethyl acetate and pooled extract was evaporated to dryness. Residues were resuspended in 100 μl of HPLC grade acetonitrile and stored at -40°C until further use (Ravn et al. 2001). HHL was used as a positive control in all the assays, when required.

High-performance thin layer chromatographic method was optimized for the quantitative evaluation of AHL profile in the culture broth of *Y. enterocolitica*. Two microliter of ethyl acetate extract + were applied onto C18 RP—Silica gel 60 F254 (10 $\times$ 10 Merck, Germany) using automatic TLC sampler 4 (ATS4) and the chromatograms were developed using methanol and water (60:40 v/v). Developed plate was air dried and overlaid with a thin film of soft agar seeded with reporter strain *C. violaceum* CV02, incubated for 24 h at 32°C . AHLs detected by blue pigmentation were scanned at 585 nm by TLC scanner (CAMAG, Switzerland) and results were recorded.

Effect of marinades on meat spoilage

A full factorial experiment (3 packaging conditions $\times$ 3 storage temperatures) was designed and performed. Freshly procured beef slabs from a same batch were divided into 70 pieces of approximately 2 $\times$ 2 cm each. Meat pieces were treated with one of the marinades (cumin, fennel, and pepper) except for control. Care was taken to ensure the immersion of meat pieces into the marinades and allowed to stand for 5 min. After the marination period, meat pieces were packed in aerobic and in modified atmospheric condition (40 % CO_2 /30 % O_2 /30 % N_2 and 50 % CO_2 /10 % O_2 /40 % N_2) in LDPE pouches and incubated at 0, 10 and 20°C . This time point was designated as day 0. Samples from each marinade and temperature were analyzed after 360, 240 and 120 h respectively. Changes in the bacterial density, and AHL content were determined.

Mathematical modeling

The experiment has been conducted with three factors each at three levels, namely temperature (0, 10 and 20°C), treatment (cumin, pepper and fennel) and microbial load (aerobic, MAP 1 and 2) with three replications. Since there

are three factors each at three levels, the appropriate statistical technique used is 3^3 factorial design with the following statistical model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \delta_l + \epsilon_{ijkl}$$

Here, Y_{ijkl} —response observed, μ —general mean effect, α_i — i th level of treatment, β_j — j th level of temperature, $(\alpha\beta)_{ij}$ —interaction effect between i th level of treatment and j th level of temperature, γ_k — k th level of microbial load, $(\alpha\gamma)_{ik}$ —interaction effect between i th level of treatment and k th level of microbial load, $(\beta\gamma)_{jk}$ —interaction effect between j th level of temperature and k th level of microbial load, $(\alpha\beta\gamma)_{ijk}$ is the interaction effect between i th level of treatment, j th level of temperature, and k th level of microbial load, δ_l — l th replicate effect, ϵ_{ijkl} —error term. The entire analysis has been carried out using IBM SPSS 19.0 version at 0.05 level of significance.

Microbiological analysis

Samples from each variation were collected after experimental period and analyzed for its bacterial load as follows. Beef pieces were homogenized, and subsequent decimal dilutions were performed in sterile saline. Desired dilutions were spread plated on nutrient agar plates and incubated at 37 °C for 24 h before enumeration.

Preparation of cell-free meat extracts

After the experimental period, samples exhibited pronounced results from each variation was collected, minced and homogenized in sterile PBS. Homogenized samples were centrifuged at 4500 g at 4 °C for 20 min to obtain CFME. Cell-free supernatants were filter sterilized through 0.2 μ m filters and stored at −40 °C until further use.

Extraction and quantification of quorum signals

Signal molecule was extracted from the cell free meat extract (CFME) as described by Ravn et al. (2001). Briefly, 10 ml of CFME was extracted with the equal volume of ethyl acetate (acidified with 0.5 % formic acid). After vigorous shaking for 30 min, fractions were allowed to separate. The whole extraction process was repeated three times before the separation of ethyl acetate fraction, which was then dried under nitrogen flow. Obtained residue was reconstituted with 2 ml of acidified ethyl acetate and stored at −40 °C until further use. The extracted compound was also tested for the confirmation of pro. AHL content in the beef samples was quantified using HPTLC as described earlier.

Statistical analysis

All the experimental data represents the mean of triplicate values. Differences among control and test samples were analyzed by one way ANOVA.

Results

Quorum sensing mechanism is involved in the regulation of phenotypic characteristics like biofilm formation, pectinolytic and chitinolytic activities that are often related to food spoilage (Liu et al. 2006). Presence of N-Acylated homoserine lactones (AHLs) have been reported in many food sources like meat, fish and poultry products (Martínez-García et al. 2007). However, information on the potential role of these signaling molecules on food spoilage is very limited, and the results were inconclusive in one of the earlier reports (Nychas et al. 2009). Several bio-actives from plant sources like quercetin (Gopu et al. 2015) and various native compounds (El-Mowafy et al. 2014) were proved to have anti-quorum properties but application of these compounds in food and food processing industries may be highly reactive.

Microbiological analysis

Microbiological analysis of beef samples exhibited total viable count in the range of 3.45–9.65 log CFUg^{−1}, LAB were at the range of 1.95–7.90 log CFUg^{−1} and Enterobacteriaceae members were at the range of 1.15–5.30 log CFUg^{−1}. As isolation time increases, elevated levels of total aerobic counts were observed with lactic acid bacteria being dominated in the population followed by Enterobacteriaceae being the least in number. Pervasiveness of these bacteria may happen during post-slaughter processing (Bruhn et al. 2004), our results are also in accordance with Tsigarida and Nychas (2001) who reported the predominant level of *Pseudomonas* spp. in beef samples.

Screening and identification

Among the beef isolates screened for AHLs production, PUFSTb04 alone induced continuous production of violacein in the reporter strain. Efficiency of the strain to produce AHLs was tested both in induction as well as in MTP assay with CV026. This does not rule out the remaining cultures are not producing signal molecules, as this may be due to the reporter strain (*C. violaceum* CV026) used in this study responds only to the short chain AHLs. These limitations can be overcome by using bio-sensors that respond to the wide range of signaling molecules.

Partial sequencing of 16S rDNA nucleotide determined in this report has been compared with GenBank databases using BLASTN. The strain was identified as *Yersinia enterocolitica* which shared 99 % similarity in the local alignment search. Even though, *Pseudomonads* predominated the total microflora of beef, detectable level of AHLs were not produced. Ferrocino et al. (2009), stated that *Pseudomonas fragi* isolated from meat did not produce sufficient level of AHLs in bioassays and production of signaling molecules by *Enterobacteriaceae* isolated from food sources are common. This is line with our results.

AHL bioassay

Table 1 represents the qualitative determination of spices to inhibit violacein production in MTCC 2656. Among seven spices screened, extracts of *C. cyminum*, *F. vulgare* and *P. nigrum* showed consistent and significant inhibition than the remaining spices. *S. aromaticum*, *Z. officinale* showed weak and inconsistent inhibition. Whereas, *A. sativum* and *C. longa* showed no inhibition of violacein production. In qualitative analysis of spice extract to inhibit violacein production, cumin, fennel and pepper exhibited significant results among the others. Hence *C. cyminum*, *F. vulgare*, and *P. nigrum* were selected for further studies.

Minimum inhibitory concentration

Minimum inhibitory concentrations of three spices were determined against the biosensor strain *C. violaceum* and *Y. enterocolitica*. The MIC value of cumin, pepper and fennel was found to be 3.5, 4.5 and 3.5 mg/ml respectively against *C. violaceum*; 3.5, 4.5 and 4.0 mg/ml for *Y. enterocolitica* respectively.

Quantitative inhibition of violacein production

Figure 1 shows the quantitative inhibition of violacein production by three selected spices. All the spices at tested

Fig. 1 Inhibition of violacein production in *C. violaceum* (CECT5999) by selected spices at different concentration (1–3 mg/ml). Vertical bars represent the mean values of triplicates with standard deviation. Same letters in the column are not significantly different ($p < 0.05$)

concentration (1.0–3.0 mg/ml) showed a significant drop in violacein content without inhibiting bacterial growth. At the concentration of 3 mg/ml, cumin inhibited 85.04 % of violacein production, whereas pepper inhibited only 49.28 %. Concentration dependent increase in the inhibitory activity was observed with all the spices, no significant difference was observed between cumin and fennel seeds. Among all tested spices, cumin exhibited pronounced results when compared with other two (Fig. 1). In quantitative determination of violacein inhibition by the selected spices against CV026 cumin exhibited pronounced inhibition when compared to fennel and pepper. This corroborates well with the findings of Bodini et al. (2009) and Choo et al. (2006) who reported the inhibition of violacein production by *Allium sativum* and *Vanilla planifolia* respectively. Studies below the minimal inhibitory concentration would not expect to impose selective pressure on the test organism to develop resistance which was evidenced by growth curve studies.

Kinetics of AHL production in *Y. enterocolitica*

Assessment of AHL production by *Y. enterocolitica* was performed by measuring the violacein zone induced in CV026. *Y. enterocolitica* started producing AHLs when the cell density reached 0.46 at OD₆₀₀. This result was in accordance with the findings of Liu et al. (2006) who stated the earliest detection of signaling molecules in *pseudomonads* and *Enterobacteriaceae*. Maximum induction of violacein pigment was observed when the cell density reached 1.914 at OD₆₀₀. AHLs production was found to be independent of cell numbers. Even though growth of the test bacteria stopped, production of AHLs was found to be increased for further days. *Y. enterocolitica* produced

Table 1 Quorum sensing inhibitory activity of selected spices against *C. violaceum* CV2656

Spices	Common name	Violacein inhibition
<i>Allium sativum</i>	Garlic	–
<i>Cuminum cyminum</i>	Cumin	+++
<i>Curcuma longa</i>	Turmeric	–
<i>Foeniculum vulgare</i>	Fennel	+++
<i>Piper nigrum</i>	Pepper	+++
<i>Syzygium aromaticum</i>	Clove	+
<i>Zingiber officinale</i>	Ginger	+

‘+’ moderate activity; ‘+++’ pronounced activity; ‘–’ no activity

AHLs to the maximum level of 5 μM compared with that of violacein induced by HHL in CV026 (Data not shown).

Extraction and quantification of AHL from *Y. enterocolitica* isolated from beef

AHLs in the culture broths of *Yersinia enterocolitica* strain recovered from beef were extracted and quantified using HPTLC. Ethyl acetate extract of *Y. enterocolitica* indicated the presence of AHL compounds which induced violacein pigmentation in the reporter strain CV026. Sharp, well-resolved peaks were obtained at R_f value of 0.43 compared with that of N-Acyl homoserine lactone (HHL) used as a standard. It was calculated that cell-free supernatant of *Y. enterocolitica* contained signaling molecule at the concentration of 83.16 $\mu\text{g/ml}$ (Fig. 2). To best of our knowledge, AHL production by bacterial isolate has not been quantified using HP-TLC so far. Hence, we could not compare the result obtained in the present study with others for its efficacy. Though studies were there for the detection of signaling molecules using HPLC, results suggest that this is an extremely perceptive method for precise detection and quantification as compared to other conventional methods.

Effect of marinades on meat spoilage

Table 2 shows the microbiological analysis of beef stored at various temperatures of incubation under aerobic and modified atmospheric conditions. After the experimental period changes in the bacterial population were recorded by plate counting method in both selective and non-selective agar media. Aerobic storage of meat at all three tested

temperature, allowed total viable counts to reach higher levels up to 9.49 $\log \text{CFUg}^{-1}$ at 20 °C. Lactic acid bacteria had the highest level of growth rate at the range of 8.05 $\log \text{CFUg}^{-1}$ while Enterobacteriaceae were the smallest component being isolated reaching the maximum level of 5.39 $\log \text{CFUg}^{-1}$ at 20 °C. Growth rate of all group of organism were found to be decreased at 0 °C as expected, whereas at increasing temperature growth rate were also found to be increased. It was reported that increase in the microflora was always found to be associated with time and temperature (Skandamis and Nychas 2002), in our study highest number of pseudomonads and Enterobacteriaceae were recorded at 20 °C.

Beef samples stored under modified atmospheric conditions were monitored for the changes in the density of microflora. Microbial analysis revealed that, storage under modified atmospheric condition delayed the growth of all groups of microbial association when compared with aerobic storage. Initial LAB load in the control sample was 8.05, 7.38 and 6.94 $\log \text{CFUg}^{-1}$ for aerobic and MAP conditions respectively. Whereas, the count on cumin treated samples lowered to the level of 5.88, 4.21, and 4.93 logs respectively when compared with control ($p < 0.05$). Throughout the MAP storage, a significant drop in the all groups of microorganism was observed when compared with aerobic storage. It is noteworthy that, no significant differences were found between the microbial loads of beef sample stored at modified atmospheric condition 1 and 2. Obtained results are in accordance with the fact that plant essential oils affect bacterial growth inhibition (Szabó et al. 2010). In 3^3 factorial designs it was observed that spices treatment has not exhibited any significant changes in the total viable count, which may be due to the treatments at

Fig. 2 HPTLC analysis graph showing the AHL profile of *Y. enterocolitica* isolated from beef developed using the reporter strain CV026. Lane 1: Blank; Lane 2: *Y. enterocolitica*-PUFSTb04; Lane 3: N-hexanoyl-homoserine lactone (HHL) standard

Table 2 Microbiological analysis of beef samples stored at 0, 10 and 20 °C stored at aerobic and modified atmospheric conditions

Temperature (°C)	Time (h)	Treatment	Microbial load (Log CFUg ⁻¹)								
			Aerobic			MAP1			MAP2		
			TVC	LAB	Entero	TVC	LAB	Entero	TVC	LAB	Entero
20	120	Control	9.49	8.05	5.39	7.83	7.38	3.41	8.12	6.94	4.05
		Cumin	6.29	5.88	4.22	5.09	4.21	2.83	5.29	4.93	3.20
		Pepper	8.30	7.25	5.08	6.80	6.02	3.53	7.13	5.91	3.29
		Fennel	6.87	5.46	4.69	6.22	4.96	3.14	6.14	6.54	4.09
10	240	Control	7.93	6.56	3.89	6.49	6.07	2.78	7.26	6.31	2.84
		Cumin	5.71	5.12	2.01	4.81	4.28	1.95	5.98	4.74	2.10
		Pepper	6.88	6.38	3.02	6.16	5.44	2.39	7.24	5.56	2.57
		Fennel	5.36	5.64	2.25	5.02	4.81	1.71	6.16	4.44	1.63
0	360	Control	4.80	5.12	2.23	4.18	3.70	1.70	3.89	2.53	1.15
		Cumin	2.36	2.85	1.41	2.62	1.97	1.26	2.18	1.10	0.47
		Pepper	3.59	4.95	2.00	4.07	3.08	1.59	3.02	2.46	0.87
		Fennel	2.95	3.56	1.57	2.78	2.10	1.34	1.57	1.21	0.59

TVC total viable count, LAB lactic acid bacteria, MAP modified atmospheric condition 1(40 % CO₂/30 % O₂/30 % N₂) and 2 (50 % CO₂/10 % O₂/40 % N₂)

sub-MIC level. Except in total viable count expected results were observed in all other interactions and replicates as well.

Pronounced changes were observed in the microbial loads of beef samples stored at MAP condition with the presence of spices extract. Microbial counts were recorded ever lower than the samples stored at aerobic and MAP conditions. Initial load of Enterobacteriaceae in the control sample stored at 0 °C was at the range of 2.23, 1.70 and 1.15 log CFUg⁻¹; cumin treated samples exhibited Enterobacteriaceae count in the range of 1.41, 1.26 and 0.47 in aerobic and MAP conditions respectively when compared with control ($p < 0.05$). Among the spices screened, treatment with cumin was found to be efficient throughout the experiment under all tested conditions. Fennel exhibited comparable results with cumin followed by pepper with least activity (Table 2).

Table 3 depicts the F-value and corresponding significance value for total viable count, lactic acid bacteria and Enterobacteriaceae. In total viable count, there is no effect due to replications and the interaction between treatment $\times$ microbial load is also insignificant. Where as all other main effects and interactions are observed to be significant indicating that they have some impact in evoking response in the micro-organisms. Shelf-life study was conducted to understand the potential role of AHLs in meat spoilage and the effect of spice-based marinades on its regulation. Samples which exhibited pronounced results in microbiological analysis were only selected and estimated for its AHLs content. Role of quorum sensing in meat spoilage and slimy appearances of biofilm formation

was already established by Jay et al. (2003). Observations of the current study confirmed the presence of signal molecules in all the tested samples. Even samples stored at 0 °C which harbored aerobic total count of 2.18 log CFUg⁻¹ exhibited AHLs at detectable level which was believed to be produced by Enterobacteriaceae which was at the level of 0.47 log CFUg⁻¹. This is in accordance to the fact that interaction between Enterobacteriaceae and lactic acid bacteria may result in spoilage of meat (Bruhn et al. 2004). This was in contrast to the report of Szabó et al. (2010) who stated the presence of oregano essential oil has not modified the QS system. However, our results are in agreement with the fact that the presence of volatile compounds and essential oils has inhibited not only the bacterial growth but also its QS.

Further, similar kind of analysis is carried out for lactic acid bacteria and Enterobacteriaceae. Results showed that there was a significant outcome in all main and interaction effects as well as in replicates. Thus expected response was clearly observed in lactic acid bacteria and Enterobacteriaceae groups than the total viable count.

AHL content

AHL profiles of selected CFME were determined using HP-TLC, optimized to detect and quantify the levels of AHLs extracted from the samples at final storage periods. On scanning the plate overlaid with soft agar at 585 nm well-resolved peaks were observed at R_f value of 0.41 (Fig. 3). Results showed the presence of AHLs in all tested meat extract, indicated by the induction of violacein

Table 3 3^3 factorial design showing the F value and significance value of treatments and replicates

Effects	TVC		LAB		Enterobacteriaceae	
	F	Sig.	F	Sig.	F	Sig.
Replicates	0.636	0.533 ^{NS}	3.738	0.030*	4.005	0.024*
Temp	2876.341	0.000*	2202.770	0.000*	1592.338	0.000*
Treatment	419.428	0.000*	374.482	0.000*	73.691	0.000*
Microbial load	56.503	0.000*	346.264	0.000*	194.095	0.000*
Temp × treatment	16.241	0.000*	6.963	0.000*	16.083	0.000*
Temp × microbial load	85.058	0.000*	68.268	0.000*	52.142	0.000*
Treatment × microbial load	2.507	0.053 ^{NS}	9.676	0.000*	6.419	0.000*
Temp × treatment × microbial load	6.256	0.000*	21.211	0.000*	5.715	0.000*

* Significance; NS not significant at 0.05 level

Fig. 3 HPTLC graph showing the presence of signaling molecule in the cell free meat extract packed at modified atmospheric condition II. N-Hexanoyl-homoserine lactone standard (1); AHL extracted from samples stored at 20 °C [Control (2), treated (3)]; AHL extracted from samples stored at 10 °C [Control (4), treated (5)]; AHL extracted from samples stored at 0 °C [Control (6), treated (7)]

pigment in CV026. On comparing treated and untreated samples 51.50, 43.57 and 23.92 % of reduction in AHL content was observed in cumin marinated samples stored at 20, 10 and 0 °C respectively. About 47.75 % of reduction in AHLs content was noticed in untreated samples stored at 20 and 0 °C, only 18.04 % of reduction was observed between cumin marinated samples.

Conclusion

To this end, we evidenced the presence of quorum sensing signals throughout the storage period of beef invariable to packaging conditions and spice based marinating agents can inhibit the AHLs production leading to lower microbial load and delayed spoilage.

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