Original article

Effect of Clove (Syzygium aromaticum) spice as microbial inhibitor of resistant bacteria and Organoleptic Quality of meat

Rebecca Tshabalala, Adia Kabelinde, Christ-DONALD KAPTCHOUANG TCHATCHEOUANG, Collins Njie Ateba, Madira Coutlyne Manganyi

Department of Microbiology, North West University – Mafikeng Campus, Private Bag X2046, Mmabatho 2735, South Africa
Food Security and Safety Niche Area, Faculty of Agriculture, Science and Technology, North-West University, Mafikeng Campus, Private Bag X2046, Mmabatho 2735, South Africa
Department of Biological and Environmental Sciences, Faculty of Natural Sciences, Walter Sisulu University PBX1, Mthatha 5117, South Africa

1. Introduction

Food-borne disease resulting from consumption of meat contaminated with pathogenic bacteria is currently one of the leading causes of morbidity. Cross contamination occurring within the premises, as well as no proper time-temperature control for heating, cooling and storage are considered as a main cause of contamination (Al-Kutby, 2012). Poor hygienic practices by personnel in the meat industry occurs frequently therefore precautions such as preservation of perishable foods such as meat is imperative. Perishable products have shelf life of a week or a month, respectively in the presence of high doses of chemical preservatives (Dhillon, 2011). The use of chemical preservation is becoming a major concern to human health. Therefore natural preservatives are coming up as an option. The International Standard Organization (ISO) define spices as “vegetable products or mixtures thereof, used for flavouring, seasoning, and imparting aroma in foods”. There has been a growing interest in natural compounds extracted from herbs and spices (Srinivasan, 2014) and derived extracts have been utilized since ancient times to improve sensory characteristics of food, as preservatives, for their nutritional and healthy properties and also as their antimicrobial effects (Martínez-Graciá et al., 2015). According to Monte et al. (2014); spices and herbs are rich sources of phytochemicals, bioactive compounds derived from plants that are capable to inhibit bacterial growth by damaging microbial membrane structures. These bioactive compounds consist of vitamins C, E, carotenoids and phenolic compounds, flavonoids,
plants (herbal spices) have been applied to functional foods or supplements due to their beneficial role in human health, and some extracts of polyphenol-rich spices have been associated with inhibitory effects on exotoxins. Polyphenols of various foods and herbs are gaining more importance due to their bioavailability. In addition, the safety of synthetic preservatives has been questioned. Nevertheless, it is considered that chemical preservatives are associated with carcinogenic and teratogenic effects on human health.

2. Materials and methods

2.1. Collection of meat samples

A total of twelve (n = 12) meat samples (raw steak, raw mutton, raw mince, and raw pork) were collected from three different retail supermarkets in North-West Province, South Africa. Four (N = 4) samples were collected from each supermarket in the area. Approximately 100 g of meat samples were purchased and transported to North-West University – Mafikeng, Molecular Microbiology laboratory for microbiological analysis.

2.2. Isolation of bacterial strains from meat sample

The target microbes in this study were Escherichia coli, Staphylococcus aureus and Enterococcus spp. and the samples were processed within 48 h of collection. Samples were cut aseptically into thin smaller pieces of 1 g using sterile scalpel. One (1) gram of meat samples was dissolved in 9 mL peptone water and homogenized by vortexing for 1 min. All samples were subjected to a 10-fold serial dilution (10^(-1)-10^(-6)). From the 10^(-4)-10^(-6) tubes an aliquot (100 l) of the diluent was spread-plated onto Nutrient agar (Merck, Germany) on a general media. Each diluent was prepared in triplicates. The plates were incubated aerobically at 37 °C for 18–24 h. Total microorganism count was counted and expressed in Colony Forming Unit per 100 mL (Bantawa et al., 2018). The diluent from the 10^-4 tube was further plated on selective media. For the isolation of Escherichia coli the rinsate was plated onto Eosin Methylene Blue agar (EMBA, Merck, Germany), Staphylococcus aureus, the rinsate was plated onto Mannitol Salt agar (MSA, Biolab, South Africa), Enterococcus spp., the sample rinsate was plated onto Bile Esculin Agar (BEA, Merck, Germany). Samples were incubated aerobically at 37 °C for 24 h. Appearance of black colonies on Bile esculin agar was presumptively identified as enterococci and green metallic sheen on Eosin Methylene Blue agar as E. coli, a single colony from each medium plate was sub-cultured for purification. Presumptive positive colonies were subjected to biochemical identification tests and Gram staining (Mulamattathil et al., 2014).

2.3. Bacterial identification tests

2.3.1. Gram-staining

Isolates were Gram-stained using standard techniques (Cruickshank, 1975), which differentiates bacterial species into Gram-positive and Gram-negative based on the chemical and physical properties of their cell walls. Colonies were retained for further identification (Akindolire et al., 2015).

2.3.2. Preliminary biochemical identification of isolates

Biochemical characterisation of the bacteria were conducted by performing specific tests such as catalase, oxidase, TSI, Esculin hydrolysis and 6.5% NaCl.

2.4. Molecular identification

2.4.1. Genomic DNA extraction

Deoxyribonucleic acid (DNA) extraction was performed using the boiling method as described by Mauger et al. (2004). Fresh pure colonies were suspended in nutrient broth and incubated at 37 °C for 24 hrs. After incubation, samples were transferred into 1.5 mL Eppendorf tubes and were vortexed and further transferred into centrifuge tubes and centrifuged (Tomos, Singapore) at 15,000g for 15 min. The supernatant was eliminated and the pellet was re-suspended in molecular biology-grade water (Eppendorf, Hamburg, Germany) and centrifuged at 15,000g for 10 min. The supernatant was eliminated and the pellet was re-suspended in 200 l of sterile nuclease-free water, subjected to boiling at 100 °C in a heating block for 10–15 min using MS2 a Dri-Block DB.2A (Tech, South Africa) and centrifuged at 13,500g for 5 min.
before storing at −20 °C. Aliquots (5 μL) of template DNA was used for PCR amplification.

2.4.2. Agarose gel electrophoresis of DNA extraction

The presence of DNA extracts was confirmed by electrophoresis on a 1% (w/v) agarose gel in 1X TAE buffer. The gel separation was done at 80 V for 45 min according to protocol and the electrophoretic pattern was analysed on a UV illuminator (Bio-Rad Laboratories, USA) in the Molecular Microbiology laboratory at the North-West University Mafikeng.

2.4.3. PCR amplification of 16SrRNA gene fragments

Amplification of targeted genes was performed using Polymerase chain reaction (PCR) analysis. For the amplification, 1 μL of DNA was added to 12.5 μL of master mix (2X DreamTag Green Buffer, dATP, dCTP, dGTP, and dTTP, 0.4 mM each, and 4 mM MgCl2) (Thermo Scientific, USA), 0.5 μL (0.2 μM) of respective oligonucleotide primers and the reaction volume were made up with 11 μL nuclease free water. The final volume of 25 μL, PCR was performed in a thermal cycler (Bio-Rad Laboratories, USA). The amplification cycles consisted of an initial DNA denaturation at 95 °C for 1 min, followed by 25 cycles of denaturation at 94 °C for 1 min, primer annealing at 55 °C, for 1 min, extension at 68 °C for 2 min, and a final single elongation at 72 °C for 10 min.

2.4.4. Identification of E. coli by PCR analysis

The identities of a total 20 presumptive E. coli isolates were determined through amplification of the uidA gene fragments (Anbazhagan et al., 2011). Amplifications were performed using DNA thermal cycler (model- Bio-Rad C1000 Touch TM Thermal Cycler). The PCR reactions were performed using the oligonucleotide primer sequences in Table 1. The reactions were prepared in 25 μL volumes that constituted 12.5 μL of 2X DreamTag Green Master Mix, 11 μL RNase free distilled water, 0.5 μL mixture of both the forward and reverse primers and 1 μL of template DNA. The PCR reagents were obtained from the Inqaba Biotechnical Industry Ltd, Sunnyside, Pretoria, South Africa. PCR conditions for E. coli: 1 cycle of 10 min at 95 °C, 35 cycles of 45 s at 95 °C, 30 s at 59 °C, 1 min 30 s at 72 °C; 1 cycle of 10 min at 72 °C.

2.4.5. PCR analysis for specific identification of Enterococcus spp

Species specific identification of Enterococcus spp was performed using specific primers that are shown in Table 1 (Jackson et al., 2004). Amplifications were performed using DNA thermal cycler (model- Bio-Rad C1000 Touch TM Thermal Cycler). The PCR reactions were performed using the oligonucleotide primer sequences in Table 1. The cycling conditions utilised were as follows: initial denaturation of 95 °C for 4 min and 30 cycles of 94 °C for I minute, 55 °C for 1 minute and 72 °C for 1 minute. A final elongation step was performed at 72 °C for 10 minutes and samples were held at 4 °C.

2.4.6. Agarose gel of PCR amplicons

The positive PCR amplicons were displayed on the electrophoresis with a 1% (w/v) agarose gels (Sambrook et al., 1989). Gels were stained with ethidium bromide (1.0 μL) and electrophoresis was conducted using a horizontal Pharmacia biotech equipment system containing 1X TAE buffer for an hour at 70 V. A 1000 kb DNA molecular weight Ladder (#N04675) obtained from New England Biolabs Ltd (UK) was included in each gel in order to confirm the sizes of the amplicons. Gels were viewed under the UV light Trans illuminator (Lasec, South and Africa) (Sambrook et al., 1989), and images were captured using a Bio-Rad imaging system (Model Bio-RAD ChemiDocTM MP Imaging System, UK) using Gene Snap software (version 6.0.02).

2.5. Preparation of spice extracts

2.5.1. Extraction method of spices

Approximately 10 g of spices were grinded with mortar, pestle, and were dissolved in 90 mL of sterile distilled water to make 100 mL of aqueous extract (11%w/v). The mixture was placed at room temperature for 24 h in sterile flasks and were filtered through sterilized Whatman no.1 filter paper (Lasec, South Africa). After filtration, the extracts were evaporated in water bath (Monitoring and control Laboratories, Lyndhurst, South Africa) until a final volume of 50 mL extract was reached (Upadhyaya et al., 2018).

2.6. Antimicrobial sensitivity testing for the isolates

Antimicrobial sensitivity testing was performed using the Kirby Bauer’s disc diffusion method. Nutrient broth was prepared and pure colonies from nutrient agar were inoculated into it and the suspension was incubated overnight, an aliquot (100 μL) of the bacterial suspension was spread-plated and a lawn was performed on the surface of Mueller-Hinton agar (MHA, Biolab, Wadeville, South Africa). Diffusion discs were impregnated with spice extracts and were performed in triplicates on the surface of inoculated plates. The plates were incubated at 37 °C for 16–24 h. After incubation, the zone diameter was observed and measured using a ruler. Thereby the zone of inhibition was interpreted as susceptible (S), Intermediate (I) or resistant (R).

2.7. Organoleptic assay

Organoleptic properties of the meat samples were evaluated in the laboratory of Molecular Microbiology in the North-West University, Mafikeng.

2.7.1. Preparation and evaluation of meat samples

Approximately 1 kg of beef steak was obtained from a local supermarket and transported to the laboratory. Upon arrival the meat was cut into six (6) equal pieces and two (2) pieces were untreated; one piece was used for bacterial load count, the other

Table 1

| Species/target gene | Primers Sequence (5’ – 3’) | Amplicon Size | Ref. |
|---------------------|----------------------------|--------------|------|
| E. coli 16S rRNA    | Forward 27F                | AGACTTTGTACATGGTGCAG | 1420 bp | Korzeniewska and Hamez, 2013 |
|                     | Reverse 1492R              | GGTACCAGTACGACCG |         |                             |
| UidA                | Forward                    | AAAACGGCAAGAGATAGAGAGAG | 147 bp | Anbazhagan et al., 2011     |
|                     | Reverse                    | ACCGTTGCTTATGAGGTG |         |                             |
| Enterococci 16S rRNA| Forward                    | TTAGAAGACCCGCTGCC | 356 bp | Kariyama et al., 2000       |
|                     | Reverse                    | GCACCTGACAAACGCC |         |                             |
| Enterococci ddi     | Forward                    | ATGCTGACTGACCAG | 475 bp |                             |
served as a control. The remaining four (4) pieces were treated with two (2) different spices at varying masses. Five (5) samples were refrigerated for seven (7) days at 4°C. Results were observed on day one (1), day three (3) and day seven (7). A six-point hedonic scale was used, where six was extremely desirable while one was extremely undesirable (Siham, 2015).

2.7.2. Bacterial load count

One (1) gram from the untreated sample was subjected to a 10-fold serial dilution (10^-1-10^-3). From the 10^-1-10^-3 tubes an aliquot (100 μL) was plated on three (3) different media, the Plate Count Agar (PCA) (Merck, Germany), Eosin Methylene Blue Agar (EMBA) for the presence of E. coli spp and Enterococcus spp. The plates were incubated aerobically at 37°C for 18–24 hrs. Total microorganism count was measured and expressed in Colony Forming Unit per 100 mL (Bantawa et al., 2018). After the seventh (7th) days; microbial counts were repeated on both treated and untreated samples. Samples were incubated aerobically at 37°C for 24 hrs and the bacterial load was recorded.

3. Results and discussions

3.1. Isolation of bacteria from meat sample

A total of twelve (n = 12) meat samples consist of mincemeat, pork, wors and steak were collected from three (n = 3) different supermarkets in the area of Mmabatho North-West Province and screened for bacterial load, the presence and the prevalence of Escherichia coli, Enterococcus species and Staphylococcus aureus in Table 1; shows the results obtained. A total viable count and prevalence was reported under Section 4.2.

3.2. Determination of bacterial load and the prevalence of pathogens in the meat samples

The results shown in Table 1, indicates that mincemeat from shop Sm had the highest mean value of 300 cfu/mL as compared to other supermarkets, followed by Wors at 293 colonies counted from shop Sw. The steak from shop Ps had 46 colonies and followed by pork from shop Pp at a value of 83 cfu/mL, which was the least number of colonies isolated. A total of twelve (n = 12) meat samples comprising of mincemeat, pork, wors and steak were collected form three (n = 3) different supermarkets in the area of Mmabatho North-West Province and screened for bacterial load, the presence and the prevalence of Escherichia coli, Enterococcus species and Staphylococcus aureus in Table 1; shows the results obtained. These include a mean total viable count of meat samples that were collected from these various supermarkets and the number in percentages (%) for the prevalence of E. coli and Enterococci species. However, no S. aureus was detected in any of the samples.

TVC = Total viable count; TCC = Total coliform count

3.3. Determination of prevalence of Escherichia coli in the meat samples

The overall prevalence of E. coli was 60% (186.1/333) of the total samples. At this level, considering hygiene and sanitary quality, the presence of E. coli indicate that consumers are at risk of exposure to a food-borne disease. This prevalence of E. coli in meat samples from various supermarkets in Mmabatho compare to other studies was, lower than that showed by previous study of Bantawa et al., 2018, yet higher than that of (Al-Mutairi, 2011) sampled from various supermarkets in Giza governorate, Egypt. The meat sample that showed the highest contamination by E. coli was pork from Pp shop at 17% and followed by pork from shop Fp, mincemeat from shop Pm and Fm all at 15%. The shop that indicated lower contamination by E. coli was Shop Sw at only 5% documented in Fig. 1a.

3.4. Determination of prevalence of Enterococcus spp. in meat samples

Fig. 1b indicates the overall prevalence of Enterococcus spp as 51% (179.8/353) for all the samples as compare to other studies which was much lower than those reported by Hayes et al. (2003), McGowan et al. (2006) and Koluman et al. (2009) who found, respectively, 99%, 73.5% and 80% of raw meat contaminated by enterococci. Nineteen (19%) of the microorganism was isolated from the mincemeat in shop Pm which at this stage was the highest contaminated and the least being the steak from shop Ss at 9%.

3.5. Presumptive identification of isolates

Pure isolation was achieved with Bile esculin agar and Eosin methylene blue agar that facilitates growth of organisms belonging to the genus spp and E. coli, respectively. Enterococcus species were presumptively identified by the presence of black colonies that
hydrolysed the esculin incorporated into the media and *E. coli* identified by a green metallic sheen colour which indicate lactose fermentation, although most of the colonies were identified as pink/lavender. According to literature, pink/lavender colonies on Eosin Methylene Blue Agar indicate non-lactose fermenters. With their ability to hydrolze esculin in the presence of bile, enterococci possess a morphological appearance of cocci that grow in chains or in singles following Gram staining. Fourteen (14) of the twenty isolates satisfied the morphological characteristics of a Gram positive cocci for enterococci (Montwendi, 2013).

### 3.5.1. Biochemical assay

In TSI stabbing, the suspected *E. coli* of the 20 samples, 8 showed yellow butt (acid) and red slant (alkaline) with gas production and only three showed yellow butt and yellow slant without gas production. None of the isolates showed any blackening there-

### Table 2

Determination of antimicrobial activity of spice water extract against pathogens by disc diffusion method.

| Bacterial Samples | Thyme | Rosemary | Parsley | Turmeric | Basil | B. Cumin | Ginger | W. pepper | Garlic | Cinnamon | Cayenne pepper | Clove |
|-------------------|-------|---------|---------|----------|-------|----------|--------|-----------|--------|-----------|----------------|-------|
| ECPrS1            | -     | -       | -       | -        | -     | ++       | -      | ++        | ++     | +         | +              | ++    |
| ECPrL1            | -     | +       | +       | -        | -     | ++       | -      | -         | -      | ++        | ++             | +     |
| ECPrS1            | -     | -       | -       | -        | -     | -        | -      | ++        | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | ++       | -      | +         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | +        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | +        | -      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | +        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | +        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | +        | -      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | +        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | +        | -      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | +        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | +        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | +        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | +        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | ++       | +      | -         | -      | -         | -              | +     |
| ECPrS1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |
| ECPrL1            | -     | -       | -       | -        | -     | -        | -      | -         | -      | -         | -              | +     |

Zone of inhibition: – no activity; + slight activity (>5mm); ++ good activity (6–10 mm); +++ very good activity (>10 mm).
fore depicting no production of hydrogen sulphide. All the 20 Gram positive cocci and 20 Gram negative rods were subjected to the catalase test and results are shown in Table 2. A small proportion 15 (75%) of the Gram positive isolates grown on BEA were able to breakdown hydrogen peroxide despite the fact that a few cata-
lase negative strains were observed. This observation suggests that some \textit{E. faecium} isolates are usually catalase negative whereas \textit{E. faecalis} are frequently catalase positive (Montwendi, 2013) as most literature would point out. All suspected \textit{E. coli} isolates tested positive. The suspected Enterococci spp were tested for their abil-
ity to grow on 6.5% NaCl, majority of the isolates satisfied the find-
ing. About 10% proved otherwise, therefore the results prove that the microorganisms could be Enterococci and for the fact that they did hydrolyse bile on BEA. The identities of presumptive \textit{E. coli} iso-
lates were also tested for the production of cytochrome \textit{c} oxidase enzyme. A large portion of the isolates proved to belong to the genus and spp.

3.6. Molecular identification

3.6.1. Extraction of DNA from presumptive \textit{E. coli} isolates

DNA was extracted from all presumptive \textit{E. coli} and Enterococci spp. isolates. DNA extraction was successful in the study, which was confirmed by electrophoresis on a 1% (w/v) agarose gel and DNA was of high quality.

3.6.2. The 16SrRNA gene analysis of presumptive \textit{E. coli} and Enterococci spp.

Bacterial 16S rRNA gene fragments were amplified from all the 20 presumptive isolates of \textit{E. coli} and 20 presumptive isolates for Enterococci spp. All the isolates were positive for the universal 16S rRNA gene PCR analysis. A 1% (w/v) agarose gel image of bact-

erial 16S rRNA gene fragments amplified during the study. PCR amplicons possessed the expected size (1420 bp) and all these iso-

lates were subjected to an \textit{E. coli} and Enterococci species-specific PCR assay.

3.6.3. Proportion of isolates confirmed as \textit{E. coli} through PCR amplification of uid\textit{A} gene

Escherichia coli species specific PCR was performed on all iso-

lates that were positive for 16S rRNA gene by amplifying the uid\textit{A} \textit{E. coli} housekeeping gene. Isolates containing the uid\textit{A} gene frag-

ment and were therefore confirmed as \textit{E. coli} isolates. The expected amplicon size of 147 bp was obtained. Fig. 1c indicates a 1% (w/v) agarose gel image of uid\textit{A} gene fragments amplified from \textit{E. coli} isolates and the \textit{E. coli} positive control strain.

3.6.4. Proportion of isolates confirmed as Enterococcus spp. through PCR amplification of ddl gene

Enterococci spp. strains were confirmed using ddl gene. After running the 1% (w/v) agarose gel, the bands were measured at 475 bp.

3.7. Antimicrobial profile of isolates

3.7.1. Antibacterial activity of spice extracts

In the present study, the antimicrobial activities of twelve spice extracts were examined against food borne bacterial pathogens. Results obtained by agar well diffusion technique, as a qualitative method, were summarized in Table 3. The spice extracts were found to have potent antimicrobial activity against some of the Gram-positive and Gram-negative microorganisms tested. Clove represented in Fig. 2 and black cumin (\textit{B. cumin}) extracts proved to be the most inhibitors against all tested bacteria. Results

Table 3

| Time / temp. | Source | Control mean value (cfu/mL) | \textit{B. cumin} mean value (cfu/mL) | \textit{Clove} mean value (cfu/mL) |
|--------------|--------|----------------------------|------------------------------------|-----------------------------------|
|              |        | Single                      | Double dose                        | Single                           | Double dose                      |
| DAY 1        | Ec     | $1.3 \times 10^2 \pm 6.5 \times 10^1$ | –                                  | –                                 | –                                |
| 4 \degree C  | Ent    | $2.3 \times 10^2 \pm 5.4 \times 10^1$ | –                                  | –                                 | –                                |
| DAY 7        | Ec     | $4.4 \times 10^5 \pm 3.4 \times 10^2$ | $1.5 \times 10^5 \pm 2.8 \times 10^5$ | $1.1 \times 10^5 \pm 2.3 \times 10^5$ | $5.4 \times 10^6 \pm 4.4 \times 10^2$ | $9.1 \times 10^6 \pm 2.1 \times 10^2$ |
| 4 \degree C  | Ent    | $2.2 \times 10^5 \pm 3.6 \times 10^4$ | $1.9 \times 10^5 \pm 1.4 \times 10^5$ | $1.0 \times 10^5 \pm 1.5 \times 10^4$ | $1.7 \times 10^5 \pm 4.1 \times 10^4$ | $8.9 \times 10^5 \pm 2.1 \times 10^2$ |

Ec = \textit{E. coli}; Ent = Enterococci spp. Time / temp. Source Control mean value (cfu/mL) \textit{B. cumin} mean value (cfu/mL) single/ double dose \textit{Clove} mean value (cfu/mL) single/ double dose.

Table 4

| Storage temperature | Organoleptic parameters | Preservation time (days) with \textit{B. cumin} | Preservation time (days) with \textit{Clove} | Control preservation time (days) |
|---------------------|-------------------------|-----------------------------------------------|---------------------------------------------|---------------------------------|
|                     | Days                    |                                               |                                             |                                 |
|                     |                         | 0     | 1     | 3     | 7     | 0     | 1     | 3     | 7     | 0     | 1     | 3     | 7     |
| 4 \degree C         | Colour                  | 6     | 6     | 4     | 3     | 6     | 6     | 4     | 3     | 6     | 6     | 5     | 2     |
|                     | Texture                 | 6     | 6     | 4     | 4     | 6     | 6     | 4     | 5     | 6     | 6     | 4     | 1     |
|                     | Odour                   | 6     | 6     | 5     | 5     | 6     | 6     | 6     | 6     | 6     | 6     | 4     | 1     |
|                     | pH                      | 3     | 3     | 4     | 3     | 3     | 3     | 4     | 3     | 3     | 3     | 4     | 5     |
|                     | Shape                   | 6     | 6     | 3     | 5     | 6     | 6     | 3     | 4     | 6     | 6     | 5     | 5     |
|                     | Overall acceptability   | 6     | 6     | 6     | 5     | 6     | 6     | 6     | 5     | 6     | 6     | 4     | 1     |

Overall acceptability.
obtained for clove and cumin extract in the present study were found to be similar to those reported by Nanasombat and Lohasupthawee (2005), in their findings clove and cumin also exhibited (>10 mm) against the same pathogens tested in this study. According to Saeed et al. (2016) clove has vital role as a spice because of its high value for its therapeutic activity. The phenylpropene eugenol compound in clove is a well-known aromatic compound. Eugenol is the main necessary component of clove that has significantly higher antimicrobial properties against food micro-organisms. On the other hand, the spice extracts of cinnamon and thyme showed weak antibacterial activities against most of the tested samples. Our results correlates with Nzeako et al. (2006) in which they found thyme extract could only inhibit S. aureus and therefore concluded thyme to be an extract with limited or narrowed antimicrobial activity. The attribute the poor performance of spice water extracts to maybe evaporation during boiling. (Table 4).

### 3.7.2. Comparison of the sensitivity of bacterial strains to antimicrobial spice extracts

In the present study, isolates from different sample differed in their antimicrobial resistance patterns and similar observations have been reported. The results reflected great variation in the sensitivity of bacterial strains against spice extracts (Fig. 2), generally Gram-positive bacteria were found to be more sensitive than Gram-negative bacteria (Table 2). Most studies are in line with our results Al-Kutby (2012) E. coli (EcFmS1 and EcFpL1) was considered the most resistant bacterial strain being inhibited only by two of twelve spices (clove and B. cumin) and the least resistant being the Enterococci spp. (EnFmL1) inhibited by eight of twelve spices.

### 3.8. Organoleptic assay

#### 3.8.1. Bacterial load from the analysis

The bacterial load was determined at day 1 and on the final day (7th) while refrigeration at 4 °C (Table 3). The highest bacterial load observed was from the control sample on day seven (7) when compared to the bacterial load at the beginning (bacterial load increased). These numbers show that bacteria might climates to the environment, temperature in this instance and replicate with changes in pH being the main factor (Grámatina et al., 2017). The lowest amount of bacterial load was recorded from the samples that were treated with a single dose of clove and black seed cumin spices. The findings of this study are in agreement with the work of Shadrack et al. (2012) in which they found rosemary spice to be effective at low doses, they attribute the effectiveness to bactericidal effects of rosemarinic acid.

#### 3.8.2. Evaluation of organoleptic parameters

Meat samples were also evaluated based on the attributes such as colour, texture, odour, pH, shape and overall acceptability. Different levels of the attributes were chosen based on a 6-points hedonic scale from extremely bad (1) to extremely good (6) as shown in Table 5. Observations were done on the first and the last day of refrigeration. The freshness of meat samples change as proteins and fats start to break down, during storage. The morphological structure of muscle tissue also changes: meat secretes juice, the surface colour changes and unpleasant odour develops. Such changes

| Scale | Colour | Texture | Odour | pH | Shape | Overall acceptability |
|-------|--------|---------|-------|----|-------|----------------------|
| 6     | Extremely desirable | Extremely hard | Extremely pleasant | Extremely alkaline | Raised | Extremely acceptable |
| 5     | Very desirable | Hard | Very pleasant | Alkaline | Slightly raised | Acceptable |
| 4     | Moderately desirable | Moderately hard | Moderately pleasant | Neutral | Flat | Slightly acceptable |
| 3     | Moderately undesirable | Moderately slimy | Moderately smelly | Acidic | Slightly flat | Slightly unacceptable |
| 2     | Very undesirable | Very slimy | Very smelly | Acidic | Very flat | Unacceptable |
| 1     | Extremely undesirable | Extremely slimy | Extremely smelly | Extremely acidic | Extremely flat | Discard |

Fig. 3. Meat samples on the first day (a) control, (b) meat treated with double the volume of black cumin, (c) meat sample treated with a single dose of black cumin, (d) meat sample treated with double the volume of clove, (e) meat sample treated with single dose of clove for day 1 and 7.
meat is unfit for human consumption (Grämatina et al., 2017). For the control sample, the first unpleasant changes in sensory parameters were detected on day 3 of storage, but for the meat samples with spice extract changes were observed on day 7 (the last day). Fig. 3 represents the overall appearance of the organoleptic assay on day 1 and 7.

In terms of colour, all the samples (control and treated) from day 0 to day 3 appeared to be extremely desirable. However, on day 7, the treated samples were moderately undesirable while the untreated sample was very undesirable. The texture of the meat on day 1 to 3, all sample were soft and tender under cold storage. However, on the last day under the same conditions, treated samples appeared to be hard with the double dose being extremely hard. While the untreated sample was very slimy. The odour on day 0 to day 1, all meat sample had an extremely pleasant smell. And day 3, the smell began to change from being extremely pleasant to very pleasant for the samples treated with black cumin and moderately pleasant for the untreated sample. The sample treated with clove maintained the extremely pleasant smell. The pH from day 0 to 3, the pH of the meat samples was acidic and began changing to neutral for the treated samples and to alkane to the day 0 to 3, the pH of the meat samples was acidic and began changing to neutral for the treated samples. And to alkane to the day 0 to 3, the pH of the meat samples was acidic and began changing to neutral for the treated samples.

4. Conclusion

As the world is aiming at a more sustainable and eco-friendly approaches, the use of natural preservatives has gained popularity because of its effectiveness and tremendous health benefits. Spices have been used since ancient times for combating food spoilage and contaminants, hence securing food safety. The findings in this study demonstrate that the meat in the retail supermarkets in Mafikeng, Mmabatho are highly contaminated with pathogenic bacteria. Clove and cumin have exhibited excellent antibacterial activity as compared to other spices such as rosemary, parsley, ginger, cayenne pepper and white pepper that were tested. Another advantage of using these herbs as antimicrobial agent is that, there is no harmful effect on body recorded so far and there is less chance of development of resistance in bacteria against these herbs (Shalheen et al., 2015). In case of the evaluation of organoleptic parameters, the treated meat samples yielded better results in terms of the colour, odour, texture, shape, during the observation period compared to the control samples. Therefore, our data strongly supports the use of spices in reducing the pathogenic bacteria on meat and meat products. In conclusion, the results show the effect of herbal spices as natural preservatives to replace completely or partially the chemical preservatives (sodium nitrite etc.). The use of chemical preservation is becoming a major concern to human health therefore natural preservatives are coming up as an option. Additionally, this study and other similar studies endorse the usage of spices as natural preservatives with antimicrobial properties in meat and establish their organoleptic effectiveness.

Declaration of Competing Interest

All authors declare that there is no conflict of interest.

Acknowledgements

This work was supported by the North West University (NWU), School of Biological Sciences, Department of Microbiology and the National Research Fund (NRF, nGAP program) for the financial support.

References

Akindolire, M., Babalola, O., Ateha, C., 2015. Detection of antibiotic resistant Staphylococcus aureus from milk: A public health implication. Int. J. Environ. Res. Public Health. 12, 10254–10275. https://doi.org/10.3390/ijerph120910254.
Al-Kutby, S., 2012. Applications of spice extracts and other hurdles to improve microbial safety and shelf-life of cooked, high fat meat products (doner kebab). University of Plymouth, England. https://pearl.plymouth.ac.uk/bitstream/handle/10026.1/1184/2012AlKutby10311520phd.pdf.pdf?sequence=1&isAllowed=y.
Al-Mutairi, M.F., 2011. The incidence of Enterobacteriaceae causing food poisoning in some meat products. Adv. J. Food. Sci. Technol. 3, 116–121.
Anbazhagan, D., Mui, W.S., Mansor, M., Yan, G.O., Yusof, M.Y., Sekaran, S.D., 2011. Development of conventional and real-time multiplex PCR assays for the detection of nosocomial pathogens. Braz. J. Microbiol. 42, 448–458. https://doi.org/10.1590/S1517-83822010000200006.
Ateha, C.N., Mochiawa, B., 2014. Use of inva gene specific PCR analysis for the detection of virulent Salmonella species in beef products in the North West Province, South Africa. J. Nutr. Res. 2, 294–300. https://doi.org/10.1209/1-nfr-2-6-5.
Bantawa, K., Rai, K., Limbu, D.S., Khanal, H., 2018. Food-borne bacterial pathogens in marketed raw meat of Dharan, eastern Nepal. BMC. Res. Notes. 11, 618. https://doi.org/10.1186/s13104-018-3722-x.
Bisholol, K.Z., Ghuman, S., Haffeejee, F., 2018. Food-borne disease prevalence in rural villages in the Eastern Cape, South Africa. Afr. J. Prim. Health Care Med. 10, 1796. https://doi.org/10.4102/ajphm.v10i1.1796.
Costa, K.A.D., Moura, R., Miller, A.F., 2019. Antimicrobial and antibiofilm activity of Cymbopogan flexuosus essential oil microemulsions. Rev. Ceres, Viçosa 66, 372–379. https://doi.org/10.1590/0034-737x20196605006.
Cruickshank, R., 1975. Medical microbiology: The practice of medical microbiology. Churchill Livingstone, New York.
Dhillon, G.K., 2011. Utilization of Spices and Herbs for Improvement of Quality and Shelf Life of Bakery Products. Punjab Agricultural University Ludhiana, Asia.
Grämatin, I., Sazonova, S., Kriuna, Z., Skudra, L., Priecina, L., 2017. Herbal Extracts for Ensuring Pork Meat Quality during Cold Storage. Proc. Latv. Acad. Sci. Section B. Natural, Exact, and Applied Sciences. De Gruyter Open, 453–460.
Hayes, J.R., English, L.L., Carter, P.J., Proescholdt, T., Lee, K.Y., Wagner, D.D., White, D. G., 2003. Prevalence and antimicrobial resistance of Enterococcus species isolated from retail meats. Appl. Environ. Microbiol. 69, 7135–7160. doi.org/10.1128/AEM.69.12.7135-7160.2003.
Jackson, C.R., Fedorka-Cray, P.J., Barrett, J.B., 2004. Use of a genus- and species-specific multiplex PCR for identification of enterococci. J. Clin. Microbiol. 42, 3558–3565. https://doi.org/10.1128/JCM.42.8.3558-3565.2004.
Kariyama, Y., Mitsuhashi, R., Chow, J.W., Clewell, D.B., Kmon, H., 2000. Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. J. clin. microbiol. 38, 3992–3995. https://doi.org/10.1128/JCM.38.3992-3995.2000.
Keisam, S., Tuikhar, N., Ahmed, G., Jayaram, K., 2019. Toxigenic and pathogenic potential of enteric bacterial pathogens prevalent in the traditional fermented foods marketed in the Northeast region of India. Int. J. Food microbiol. 296, 21–30. https://doi.org/10.1016/j.jfoodmicro.2019.02.012.
Koluman, A., Akam, L.S., Čakiroglu, F.P., 2009. Occurrence and antimicrobial resistance of enterococci in retail foods. Food Control 20, 281–283. https://doi.org/10.1016/j.foodcont.2008.05.007.
Korzenevski, E., Harnisz, M., 2013. Beta-lactamase-producing Enterobacteriaceae in hospital effluents. Environ. manage. 123, 1–7. https://doi.org/10.1016/j.jenvman.2013.03.024.
Li, A.L., Li, G.H., Li, Y.R., Wu, X.Y., Ren, D.M., Lou, H.X., Wang, X.N., Shen, T., 2019. Activity of selected phytochemicals against Escherichia coli and Staphylococcus aureus and their biofilms. Pathogens 3, 473–498. https://doi.org/10.3390/pathogens3020473.
Montwendi, M.G., 2013. Phenotypic and Genotypic characterization vancomycin resistance determinants in Enterococcus faecalis isolated from groundwater in Mafikeng North West Province. North West University, Mafikeng, South Africa.

Mulamattathil, S.G., Bezuidenhout, C.C., Mbewe, M., Ateba, C.N., 2014. Isolation of environmental bacteria from surface and drinking water in Mafikeng, South Africa, and characterization using their antibiotic resistance profiles. J. Pathog. https://doi.org/10.1155/2014/371208. Article ID 371208.

Nanasombat, S., Lohasupthawee, P., 2005. Antibacterial activity of crude ethanolic extracts and essential oils of spices against Salmonella and other enterobacteria. 5, 527–538.

Nisar, M., Ahmad, M.U.D., Mushtaq, M.H., Shehzad, W., Hussain, A., Nasar, M., Nagaraja, K.V., Goyal, S.M., 2018. Occurrence of Campylobacter in retail meat in Lahore, Pakistan. Acta Trop. 185, 42–45. https://doi.org/10.1016/j.actatropica.2018.04.030.

Nzeako, B., Al-Kharousi, Z.S., Al-Mahrooqui, Z., 2006. Antimicrobial activities of clove and thyme extracts. Sultan Qaboos Univ. Med. J. 6, 33–39 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3074903/pdf/squmj-06-33.pdf.

Pal, M., Dana, F.D., Ayele, Y., 2018. Microbiological and hygienic quality of Meat and Meat Products. Rev. Food World. 45, 21–27.

Saeed, M., Yasin, I., Khan, M.I., Nadeem, M., Shabbir, M.A., Azam, M., 2016. Herbs and spices as a potential antimicrobial agents for food application. Pak. J. Food Sci. 26, 153–160.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Shadrack, O., Konyole, S.O., Ngala, S.N., 2012. Effects of Rosemary Spice (Rosmarinus Officinalis L.) and Nitrite Picking Salt Combination on Keeping and Organoleptic Quality of Beef Sausages 2, 4008–4015.

Shaheen, A.Y., Sheikh, A.A., Rabbani, M., Aslam, A., Bibi, T., Liaqat, F., Muhammad, J., Rehman, S.F., 2015. Antibacterial activity of herbal extracts against multi-drug resistant Esherichia coli recovered from retail chicken meat. Pak. J. Pharm. Sci. 28, 1295–1300.

Sibanyoni, J.J., Tshabalala, P.A., Tabit, F.T., 2017. Food safety knowledge and awareness of food handlers in school feeding programmes in Mpumalanga, South Africa. Food Control 73, 1397–1406. https://doi.org/10.1016/j.foodcont.2016.11.001.

Siham, A.A., 2015. A comparative study of chemical composition and quality Attributes of fresh and processed meat of calf, camel meat and goat meat PhD. Thesis Sudan University of Science and Technology, Sudan.

Sokamte, T.A., Mbougueng, P.D., Tatsadjieu, N.L., Sachindra, N.M., 2019. Phenolic compounds characterization and antioxidant activities of selected spices from Cameroon, S. Afr. J. Bot. 121, 7–15. https://doi.org/10.1016/j.sajb.2018.10.016.

Sinivasan, K., 2014. Antioxidant Potential of Spices and Their Active Constituents. Crit. Rev. Food Sci. Nutr. 54, 352–372. https://doi.org/10.1080/10408398.2011.585525.

Taguri, T., Tanaka, T., Kouno, I., 2004. Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. Biol. Pharm. Bull. 27, 1965–1969. https://doi.org/10.1248/bpb.27.1965.

Upadhyaya, S., Yadav, D., Chandra, R., Arora, N., 2018. Evaluation of antibacterial and phytochemical properties of different spice extracts. Afr. J. Microbiol. Res. 12, 27–37. https://doi.org/10.5897/AJMR2017.8731.

Wyness, L., 2016. The role of red meat in the diet: nutrition and health benefits. Proc. Nutr. Soc. 75, 227–232. https://doi.org/10.1017/S0029665115004267.

Further Reading

Alamin, S.A., 2015. Sensory Evaluations of Different Types of Red meat in Sudan. Bulletin of Environment, Pharmacology and Life Sciences Bull. Environ. Pharmacol. Life Sci. 4, 45–48.

Liu, S.-N., Han, Y., Zhou, Z.-J., 2011. Lactic acid bacteria in traditional fermented Chinese foods. Food Res. Int. 44, 643–651. https://doi.org/10.1016/j.foodres.2010.12.034.

Suman, U., Divya, Y., Ram, C., Naveen, A., 2018. Evaluation of antibacterial and phytochemical properties of different spice extracts. Afr. J. Microbiol. Res. 12, 27–37. https://doi.org/10.5897/AJMR2017.8731.