



Bacterial species from retailed poultry eggs in Tshwane, South Africa: Implication for consumers

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DATES:

Received: 05 Aug. 2016

Revised: 07 Mar. 2017

Accepted: 11 June 2017

KEYWORDS:

Enterobacteriaceae; *Salmonella*; food safety; hens' eggs; pathogenic bacteria

HOW TO CITE:

Jambalang AR, Buys EM, Botha FS. Bacterial species from retailed poultry eggs in Tshwane, South Africa: Implication for consumers. S Afr J Sci. 2017;113(11/12), Art. #2016-0232, 7 pages. <http://dx.doi.org/10.17159/sajs.2017/20160232>

ARTICLE INCLUDES:

× Supplementary material

× Data set

FUNDING:

None

Food safety is an important public health issue and governments across the world are intensifying their efforts to improve the quantity, quality and the safety of national food supplies. Bacteria, especially *Salmonella* species, present in or on chicken meat and hens' eggs in particular are the most common causes of food poisoning and the major sources of human salmonellosis. Literature reveals little information on the risk factors for salmonellae infection in Africa. The aim of this study was to determine which, if any, bacteria, especially *Salmonella* species, are present in and on hens' eggs. Representative bacterial colonies were confirmed with Gram staining and then identified using the MALDI-TOF Biotyper assay. The genera identified were *Escherichia coli* (34%), *Enterococcus faecalis* (14%), *Proteus mirabilis* (9%), *Klebsiella pneumoniae* (7%), *Salmonella* Typhimurium (6%), *Enterobacter cloacae* (1%), *Stenotrophomonas maltophilia* (0.6%), *Salmonella* Dublin (0.6%) and *Salmonella* Braenderup (0.2%). Raw hens' eggs and products containing raw hens' eggs may contain pathogenic bacteria, thereby exposing a large number of consumers to the risk of contracting food poisoning when undercooked or uncooked hens' eggs are consumed.

Significance:

- Enterobacteriaceae counts are used as an indicator to evaluate the hygienic quality of food.
- The presence of *Salmonella* species and other Enterobacteriaceae in raw hens' eggs poses a health risk to consumers.
- Any product in which raw eggs are used must be provided with a conspicuous label stating that it may contain pathogenic bacteria.

Introduction

Enterobacteriaceae is a family of Gram-negative, facultative anaerobic rod-shaped bacteria. The genera include plant and human pathogens like *Escherichia*, *Klebsiella*, *Salmonella*, *Shigella* and *Yersinia*.^{1,2} *Escherichia coli* counts and the presence of coliforms are used as indicators to evaluate the 'hygienic' quality of raw foods.^{2,3}

Microbial contamination of egg shells is of increasing concern to farmers and consumers of hens' eggs and poultry products in general.⁴ In recent years, the 'farm to fork' approach to food safety has received considerable attention as a more complete method of ensuring food safety. *Salmonella* Enteritidis can be transmitted via trans-ovarian route or via faecal contamination through shell penetration to intact hens' eggs which can cause salmonellosis in consumers.⁵ Food safety therefore becomes everybody's responsibility and not just the processors'.⁶⁻⁸ Hens' egg farms or plants experience huge challenges in maintaining good hygienic conditions because of the high concentration of hens on the premises.⁴ Fomites, flies, dust, faeces and rodents serve as a vehicle for contamination, re-contamination or cross-contamination during collection, washing, sorting, transportation and packaging of eggs.^{2,8} Although great care is taken to maintain shell integrity, some eggs crack during transportation and the contents leak onto charts, providing good substrate for bacterial and fungal growth.⁴

Previous studies have found eggs and egg-processing environments to be contaminated with large numbers of Enterobacteriaceae and aerobic microorganisms. Identified isolates in eggs and on shells included *Citrobacter youngae*, *Enterobacter cloacae*, *Enterobacter sakazakii*, *Escherichia coli*, *Flavimonas oryzihabitans*, *Klebsiella pneumoniae*, *Kluyvera* spp., *Pantoea* spp., *Proteus* spp., *Providencia* spp., *Rahnella aquatilis*, *Salmonella* spp., *Serratia* spp., *Shigella* spp., *Xanthomonas maltophilia* and *Yersinia* spp.⁹⁻¹² Many members of this family are human pathogens, some are spoilage organisms, and others – like *Escherichia* and *Proteus* – cause various types of egg rot.⁹⁻¹²

Salmonellosis is a zoonotic food-borne bacterial disease that poses a major threat to public health and causes economic losses.^{13,14} Pathogenic *Salmonella* survive in water, soil and faeces, and contaminated food like raw eggs is one of the major sources of salmonellosis in humans.¹⁵ Treatment of salmonellosis has been complicated with the emergence of multidrug-resistant phenotypes among the *Salmonella* serotypes.¹⁶⁻¹⁸ In view of the annual estimated 1.3 billion human infections globally as a result of salmonellosis and socio-economic losses of about USD1.1 billion in the USA alone¹⁹, control measures should be put in place in order to safeguard life and contain the spread of the disease.

Literature on the distribution of different types of bacteria on hens' eggshells in South Africa revealed limited information. Experiments and research conducted elsewhere in the world have evaluated eggshell microbial populations by simulating contamination under laboratory conditions.^{6-8,20}

Bacterial isolation and identification remains the gold standard to determine the presence of bacteria in food. However, a culture-based approach is not optimal because it is time consuming and not always specific.²¹ Molecular techniques, on the other hand, produce rapid, automated results with high sensitivity and specificity; however, they lack the ability to isolate the organism – which is the gold standard in microbial identification.²² Therefore,

in this study, a combination of a microbiological culture-based method (for the isolation and preliminary identification) and a rapid molecular technique (Matrix-assisted Laser Desorption Ionisation Time of Flight or MALDI-TOF) was used in the confirmatory identification (respectively) of selected bacterial species in retail hens' eggs.

The aim of this study was to determine which, if any, bacterial species, especially *Salmonella* species, are present in and on commercial hens' eggs in the Tshwane district, Gauteng Province, South Africa.

Materials

Unwashed hens' eggs of different egg brands were randomly purchased from retail outlets in Tshwane. The egg samples were transported in ice-boxes to the Phytomedicine Laboratory, Department of Paraclinical Sciences, Faculty of Veterinary Science, Onderstepoort, South Africa and tested on the day of arrival. The major public transport routes running from north to south and east to west of Tshwane were used as sample collection sites; this choice was because a high number of commercial activities usually take place along such routes and the chances of having a similar distribution of egg retail outlets along these routes was high. A total of 468 eggs representing 13 egg brands were purchased. To protect the brands' identity, codes AJ01–AJ13 were used for brands, and samples were identified as samples AJ01–AJ468.

Methods

Microbiological sampling

All the experimental work was carried out in a Class II Biological Safety Cabinet (ESCO, Singapore) and sterile hand gloves were used during the experiments to minimise contamination. The eggs were removed from their boxes and placed with the pointed end facing down on a plastic egg crate that was sterilised with 70% ethanol (Sigma Aldrich, Johannesburg, South Africa); each egg was individually labelled.

Shell

A plastic template sterilised with 70% ethanol was used to mark out a specific area (20 mm x 20 mm) on the egg shell (using a sterilised pencil), in order to standardise the area chosen and thereby prevent biased sampling.

A swab was taken from the marked area of each egg shell using a sterile cotton swab that had been dipped in buffered peptone water (BPW) and placed into a sterile, 10-mL, screw-capped bijoux bottle containing 9 mL BPW (Selecta-MEDIA, Johannesburg, South Africa).

Thereafter, the eggs were sprayed with 70% ethanol in order to disinfect the egg shell and prevent contamination of the egg content. The eggs were allowed to dry for 10 min.

Yolk and albumin

Using sterile scissors and thumb forceps, each egg was cracked open at the air sac end in order to avoid spillage. The albumin was aseptically separated from the yolk by gently decanting the albumin into sterile, wide-mouth, 30-mL, screw-capped, plastic centrifuge tubes. A sterile, 5-mL, single-channel pipette (GILSON, Villiers-le-Bel, France) was used to gently pipette out the remaining albumin, leaving behind the egg yolk. The egg yolk was carefully poured into sterile, 30-mL, screw-capped, plastic centrifuge tubes. The samples were homogenised according to the method previously described.¹⁰

Pre-enrichment for presumptive bacterial species

After homogenisation, 1 mL albumin and 1 mL yolk were separately pipetted into sterile bijoux bottles and pre-enriched in 9 mL BPW and incubated at 37 °C for 24 h.

Selective enrichment for *Salmonella*

After pre-enrichment, 1 mL of the pre-enriched broth was used to inoculate 9 mL of Muller–Kaufmann Tetrathionate Broth (Selecta-MEDIA) for *Salmonella* selective enrichment and the broth mixture was incubated at 37 °C for 24 h. The remaining pre-enriched BPW was used

for the isolation of other bacterial species on several agars like Xylose Lysine Deoxycholate agar (XLD), McConkey agar and Nutrient agar (Sigma-Aldrich). XLD agar is both a selective and differential medium for the isolation, cultivation and differentiation of *Salmonella* and *Shigella* species in particular, and most Gram-negative enteric microorganisms. Colonies of the different genera were distinguished based on morphology of the bacteria on different agar, change in colour of the agar and Gram stain reaction.

Isolation of *Salmonella* on selective agar plates

Using a calibrated inoculating loop (Sigma-Aldrich), 10 µL of the tetrathionate broth was streaked on XLD and McConkey agars (MERCK, Darmstadt, Germany) and incubated at 37 °C for 24 h. This procedure was also repeated on XLD, McConkey and nutrient agars for the isolation of bacteria other than *Salmonella* that may have been present in the enriched broth. Isolated colonies were purified and placed on ceramic beads in microbank cryoprotective media (Pro-Lab, Texas, USA) and stored at -80 °C until further analyses were performed.

MALDI-TOF assay

After the preliminary bacterial isolation and identification, all *Salmonella* and other representative presumptive bacterial isolates were subjected to the MALDI-TOF assay – a protein fingerprinting technique for confirmatory identification and biotyping to species level. The MALDI Biotyper System identifies microorganisms using MALDI-TOF mass spectrometry to measure highly abundant proteins that are found in all microorganisms. The characteristic patterns (or 'fingerprints') of these highly abundant proteins are used to reliably and accurately identify a particular microorganism by matching the respective pattern with an extensive database to determine the identity of the microorganism.²³ After the acquisition of the spectral data had been completed, a run results report was generated. The resultant report for each sample shows the best match along with the respective matching score.

The MALDI-TOF assay was done in the Department of Microbiology and Plant Pathology, University of Pretoria, using a modified method previously described by Mellmann et al.²⁴ The method is briefly described here.

A bacterial colony was added into an Eppendorf tube (Oxoid, Basingstoke, England) containing 300 µL deionised water; the contents of the tube were vortexed and 900 µL electrophoresis-grade ethanol (Sigma-Aldrich) was added to the bacteria-water mixture and the tube was centrifuged at 15 000 g for 2 min. Then 10 µL of 70% formic acid (Sigma-Aldrich) was added to the bacterial pellet and auto vortexed; 10 µL acetonitrile (Sigma-Aldrich) was also added to this mixture and the mixture was then centrifuged at 15 000 g for 2 min. A volume of 1 mL of supernatant containing the bacterial extract was spotted on a MALDI-TOF steel target plate and allowed to dry at room temperature. The material was next overlaid with 1 µL of a saturated solution of α -cyano-4-hydroxycinnamic acid in 50% acetonitrile, 2.5% trifluoroacetic acid (Sigma-Aldrich) within 1 h and allowed to dry at room temperature. The steel target plate was inserted into a Bruker Microflex Daltoniks MALDI Biotyper (Bruker Microflex MALDI Biotyper, Bremen, Germany) and the results were read. For bacterial identification, the spectrometer was set at linear positive mode, 60 Hz laser frequency, 20 kV acceleration voltage, 16.7 kV IS2 voltage, 170 ns extraction delay and 2000–20 137 m/z range. MALDI-TOF Biotyper 3.0 Real Time Classification by Bruker Daltoniks was used to analyse spectra for the newly investigated bacteria against the default reference settings of the Biotyper databank directly.²⁴ It took about 3 h to identify 96 samples from preparation to species identification and approximately 10 min for a single sample.²⁴

Dendrograms of identified bacteria were generated from repeat profiles and calculated based on simple matching similarity coefficient and complete linkage from the generated and stored data using Pearson product moment correlation and gel view (BioNumerics 7.5). Ward's clustering method was used, which is a hierarchical agglomerative method whose main objective is to create clusters that give minimum increase in the total within group error sum of squares.²⁵

Results

A total number of 13 different egg brands and 468 egg samples were analysed. The results indicate that 73% of the egg samples had bacterial contamination distributed on the shell, albumin and yolk as follows: *Escherichia coli* 159 (34%), *Enterococcus faecalis* 66 (14%), *Proteus mirabilis* 42 (9%), *Klebsiella pneumoniae* 33 (7%), *Salmonella* serotype Typhimurium 28 (6%), *Enterobacter cloacae* 5 (1%), *Stenotrophomonas maltophilia* 3 (0.6%), *Salmonella* serotype Dublin 3 (0.6%) and *Salmonella* serotype Braenderup 1 (0.2%). (Figure 1). However, 128 (27%) samples were not contaminated (Figure 1). Some broth enrichments had one isolate while others had more than one isolate.

The pie chart presented in Figure 2 shows the percentage distribution of 47 presumptive bacterial species from an initial 468 egg samples confirmed by MALDI-TOF analysis. The egg shell had the highest bacterial contamination at 43%, followed by the yolk at 30% and albumin at 27%. Within the scope of this study, only bacteria that cause gastrointestinal disorders were confirmed; the others were not further investigated.

In the dendrogram in Figure 3, the high discriminatory power of MALDI-TOF was used for the identification of different bacterial isolates with a high level of confidence. MALDI-TOF results are expressed as log (score) values ranging from 0 to 3 (0–100% pattern match). The higher the log (score) value, the higher the degree of similarity to a given organism in the reference database. A log (score) value of ≥ 2.00 can be considered an excellent probability for test organism identification at the species level; a value in the range 1.700–1.999 indicates probable genus identification and one in the range 0.000–1.6999 is not reliable for identification.^{23,24} All 47 isolates had log (score) values of ≥ 2.00 , which is the minimum threshold for secure species identification after MALDI-TOF spectral comparisons (Figure 3). None of the 47 isolates log (score) value was below 2.00. The protein profiles in normalised gel of confirmed bacteria showed the isolates' relationships with different egg brands, percentage relationship, grouping (from a–h) and the part of the egg from where they were isolated (Figure 3). The similarity pattern exhibited by the different bacterial species in each genus indicates that they are closely related with only slight variation in their protein profile that separates them into strains, as seen in their gel pattern. All the strains that have been grouped in the same cluster are assumed to belong to the same species (Figure 3).²⁶

Three different serotypes of *Salmonella* were identified from 47 bacterial isolates that were previously identified by MALDI-TOF (Figure 3). *Salmonella* ser. Typhimurium represents 60% (28), *Salmonella* ser. Dublin 7% (3), and *Salmonella* ser. Braenderup 2% (1) of the identified isolates. The *Salmonella* species were present on the shells and in the albumin and yolk of eggs from different egg brands (Figure 3).

Discussion

The latest outbreak of *Salmonella* in Europe²⁷ emphasises the importance of this study. Consumers and health officials should be aware that hens' eggs may contain pathogenic bacteria. Isolating different bacteria in this study suggests that eggs sold by retailers in South Africa may be infected or contaminated with potential pathogenic bacteria; similar findings were previously reported by other researchers.¹⁰ However, 27% of the eggs were uncontaminated, indicating that some retailers or producers practise hygienic measures to ensure the distribution of wholesome eggs to the public.

All the *Salmonella* isolates and representatives of the other bacteria that were subjected to MALDI-TOF analysis were present on the shell, in the albumin and in the yolk. This finding may suggest that these organisms may spread from contamination on the outside to the edible inside of the egg.⁵

Escherichia coli was isolated from samples AJ47 and AJ46 (marked as group 'f') and the relatedness in their protein profiles can be seen in Figure 3. This bacterium is a common enteric pathogen present in poultry^{28,29} and it was isolated from only the shell surfaces of Brand 10 in this study. The shell is the most exposed part of the egg and *E. coli*, which is an enteric commensal, may have been transferred to the egg during the laying process or by trans-shell contamination with faecal material.^{28,29}

Proteus mirabilis was isolated from the shell, albumin and yolk of egg in Brand 4 and the protein profiles in Figure 3 marked as 'c' of AJ35, AJ36 and AJ34 showed related bands. *P. mirabilis* contamination can be waterborne and this contamination may occur when the eggs are laid in a wet or damp environment because *P. mirabilis* is known to thrive in wet or damp environments and is also widely distributed in soil.²⁹⁻³¹

Isolating *Klebsiella pneumoniae* from two different egg brands during this study is noteworthy. The protein profile of isolates from Samples AJ19 and AJ45 marked as 'b' in Figure 3 show that these bacteria are similar even though they are from two different egg brands. Intestinal infection caused by *Klebsiella* species can spread through the oral-faecal route. The presence of *Klebsiella* on eggs may be from more than one source because it easily spreads between hens kept in close contact in a hatchery via the respiratory system, faeces, fomites or even caretakers.^{2,9} It is known that antibiotic resistance is very common in *Klebsiella* infections³²⁻³⁴, making the present finding important to note so that appropriate measures can be taken to prevent disease occurrence.

Enterobacter cloacae is an organism present in the intestines of hens and may be transferred to the eggs during the egg laying process or by contamination of the egg shell with faeces.²⁹ If the eggs are kept in environments littered with contaminated faeces before and after packaging, this organism may penetrate through the shell to the albumin and yolk.²⁹ *E. cloacae* isolates from Samples AJ08, AJ09, AJ07, AJ21 and AJ20 grouped in group 'd' showing close grouping and relatedness although they were from two different egg brands (Brands 7 and 8).

Stenotrophomonas maltophilia is a pathogen of importance that can be found in water, soil and sewage or very humid conditions and may be spread to the chickens if they are kept in humid conditions.^{29,35} *Stenotrophomonas maltophilia* that was isolated from the shell, albumin and yolk of an egg in Brand 7 marked as samples AJ12, AJ11 and AJ10 marked 'a' in Figure 3 had related bands in their protein profiles. The presence of this organism on the shell and inside the egg suggest that the organism may have been transmitted to the inside of the egg through faecal contamination of the shell.²⁹ Isolation of *S. maltophilia* from only one egg brand suggests that this infection may be limited to the farm from where these eggs were sourced.

The mass protein profile of the different *Salmonella* isolates was used to group them in three main clusters that look very similar with only a slight variation in the band pattern (Figure 3). All the strains that are grouped in the same cluster of the sequenced one are assumed to belong to the same species.²⁶

Salmonella Typhimurium isolates were grouped into two separate clusters. The first cluster consists of *Salmonella* Typhimurium isolates from samples with code numbers AJ01–AJ06, AJ17–AJ18, AJ22–AJ31, AJ41, AJ43 and AJ44, which were grouped together as one cluster because of the similarity in their band patterns. The second cluster of *Salmonella* Typhimurium is made up of isolates with code numbers AJ13–AJ15, AJ31–AJ33 and AJ37 while another group that is made up of *S. Dublin* and *S. Braenderup* isolates with code numbers AJ38–AJ40 and AJ42, respectively, were all grouped together into the third separate cluster because of their similarity pattern (Figure 3). This result shows that all 32 *Salmonella* isolates were correctly identified at the genus to species level with a high level of confidence.

Egg Brands 7–11 were from a particular big chain supermarket that had several group stores with different brand names under which it retails its eggs. It is highly suggestive that eggs from this outlet may have been supplied by the same producer who packed the eggs under different brand names before distributing them to the smaller sales outlets as its marketing strategy.

Although the bacteria were widely distributed on the eggs, there was a higher percentage contamination on the egg shell, which is not surprising because the shell is the most exposed part of the egg which makes it most vulnerable to contamination. Contamination was next highest in the yolk and then in the albumin. The wide distribution of these bacteria on different parts of the egg may help to enhance their survival in the face of different environmental conditions.

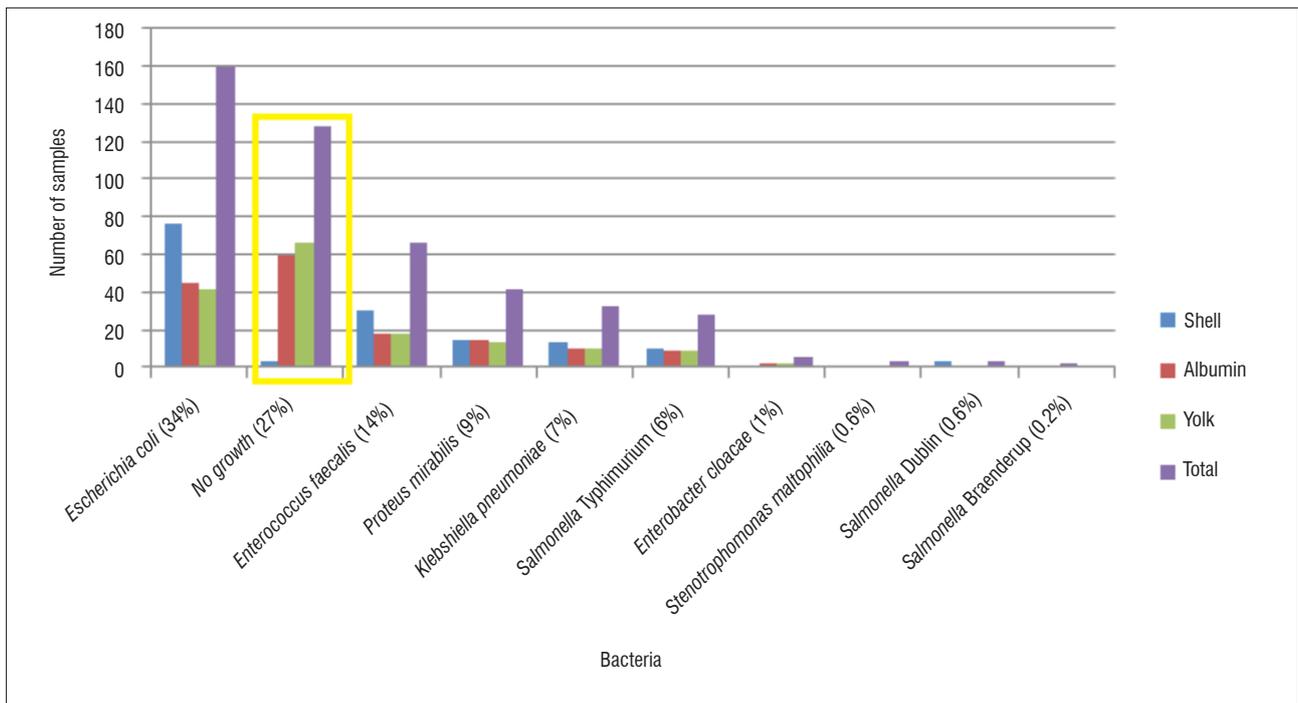


Figure 1: Distribution of different presumptive bacterial species isolated from the shell, albumin and yolk of retail hens' eggs ($n=468$).

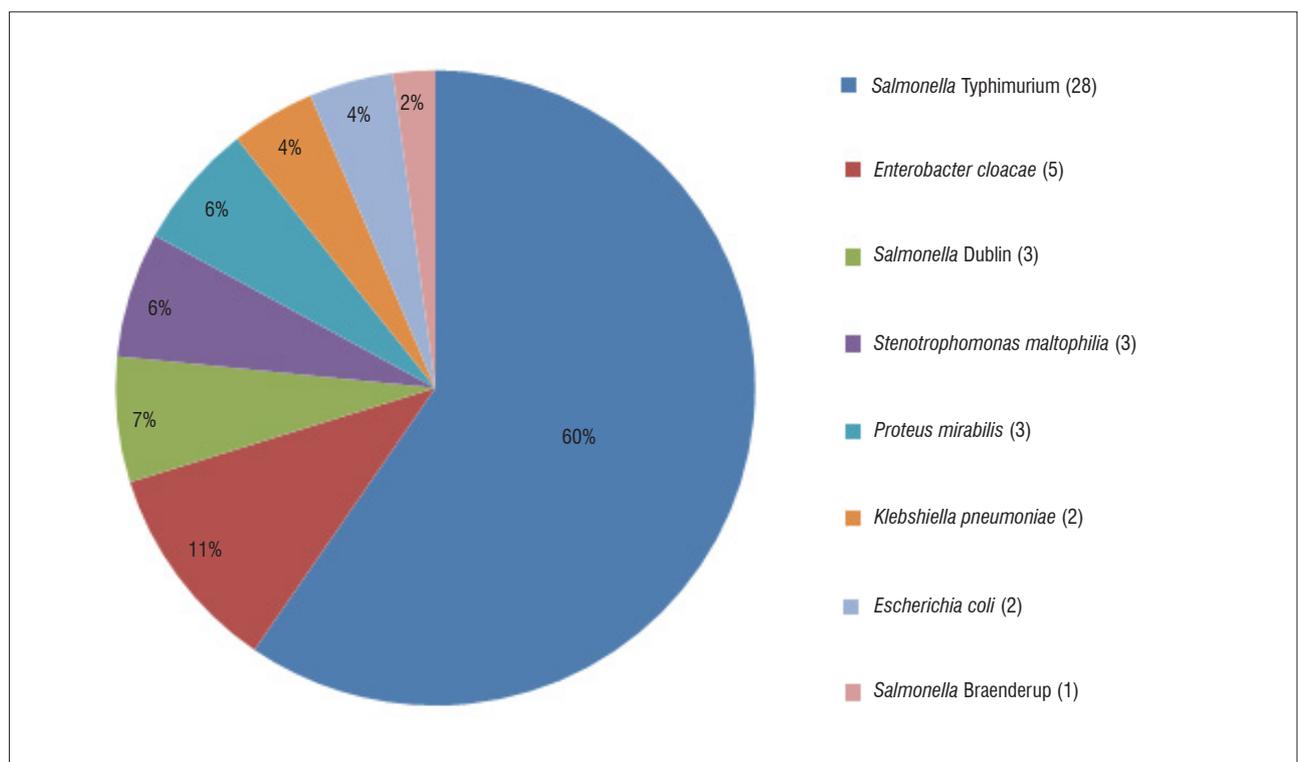


Figure 2: Distribution of presumptive bacterial species identified and confirmed by a Matrix-assisted Laser Desorption Ionisation Time of Flight (MALDI-TOF) analysis.

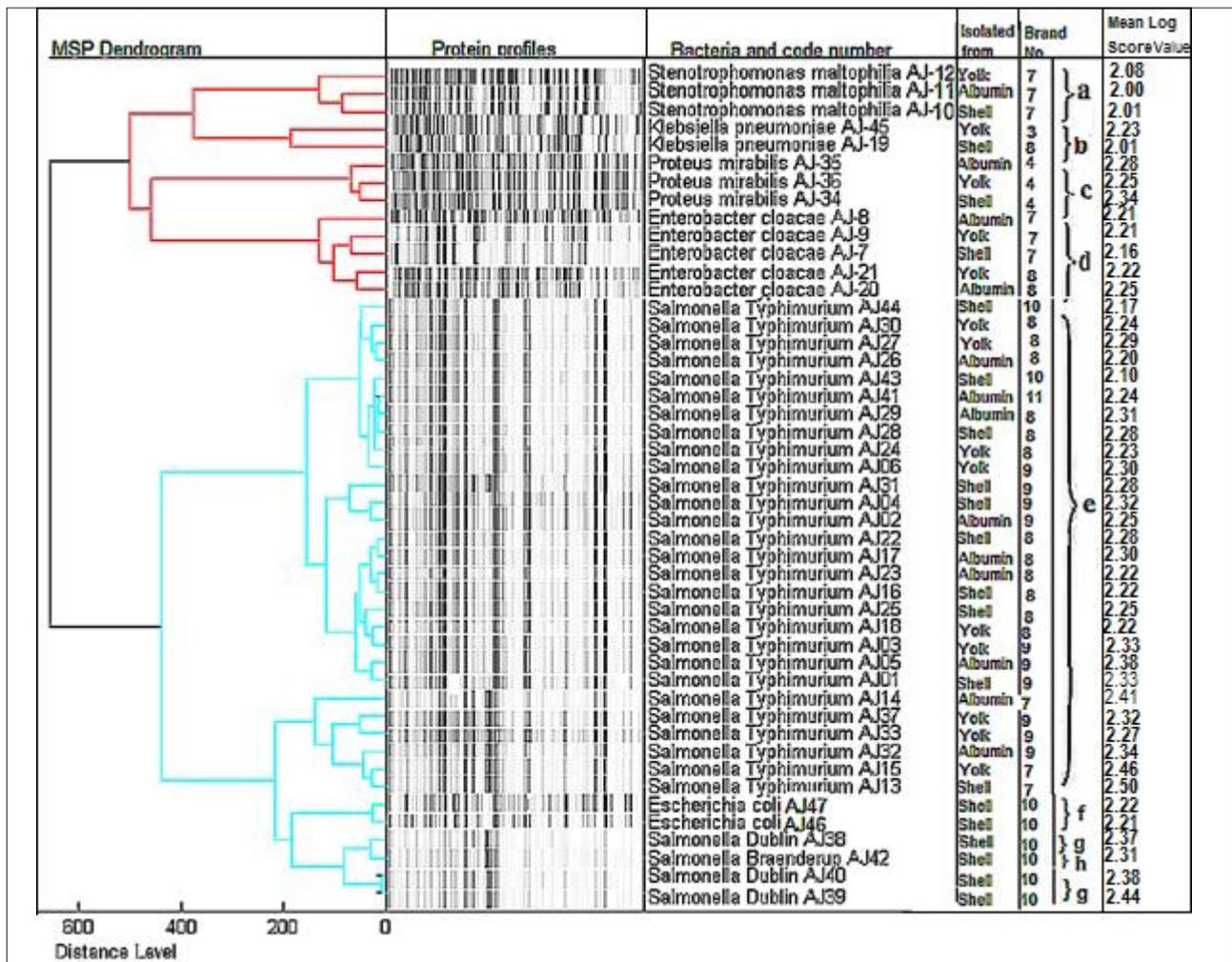


Figure 3: Dendrogram of mass spectral protein profiles in normalised gel of confirmed bacterial species showing log score values, isolates' relationships with different egg brands, percentage relationship grouping (from a-h) and the portion of the eggs from where they were isolated.

The isolation of different bacteria in retail poultry eggs in this study may be an indication that some eggs that are sold to the public in Gauteng for consumption are not always of good quality. Some of these bacteria are members of coliforms, and coliform counts are used as indicators to evaluate the hygienic quality of raw foods.^{2,3}

Researchers have previously isolated *Enterobacter*, *Klebsiella*, *Salmonella* and other bacterial species from egg shells in other places, that were similar with those isolated in this study.⁹⁻¹¹ This finding is important to note so that preventive measures can be put in place because of public health concerns.

Of a total of 13 egg brands analysed in this study, 5 egg brands were found to be contaminated by *Salmonella* species. *E. coli* was isolated from all the egg brands, which was not surprising because of its ubiquitous nature. The presence of *Salmonella* in eggs raises serious public health concerns and there may be a need to introduce hygienic regulations for producers and retailers regarding the hygienic quality of retail eggs. Findings in this study also highlight the extent to which hens' eggs in South Africa are infected with different potentially pathogenic bacteria – information which has hitherto been very limited; our findings therefore address this knowledge gap.

Using contaminated unpasteurised eggs in different products poses serious health risks to consumers and could lead to multiple infections especially in immunocompromised persons such as those infected with the human immunodeficiency virus, further worsening the disease burden and contributing to an increase in mortality rates.^{36,37} Therefore, it is advisable to use uncontaminated, preferably pasteurised, safe and wholesome eggs.

In South Africa, unlike in the USA, the EU and Canada³⁸⁻⁴⁰, no law exists to regulate the content of raw eggs sold to consumers. Results from this study further emphasise the urgent need to introduce regulations on egg contents in South Africa.

The World Health Organization (WHO) recognises that control of *Salmonella* infection from poultry products can take place through public education, improvement of hygiene and control of infection in the birds themselves.⁴¹ These measures can be applied to the other pathogens isolated in this study as a general control measure. Control can be achieved by observing strict biosecurity in the hatchery, breeding farm, environs, feed and water and during processing, which will ultimately protect the consumer.^{41,42} WHO encourages the education of farmers and training of food handlers and consumers in food safety as the pivotal point of preventing salmonellosis. WHO and the Food and Agricultural Organization jointly encourage and enhance national, regional and provincial laboratories in the monitoring and surveillance of *Salmonella* transfer between food animals and humans, and in the coordination and response to outbreaks.⁴³

A technical report was released by the European Food Safety Authority and the European Centre for Disease Prevention and Control on 27 October 2016 on a multi-country outbreak of *Salmonella* Enteritidis linked to unpasteurised eggs.²⁹ Confirmed and probable cases were reported in this outbreak; isolates of 112 of the confirmed and 148 of the probable cases belonged to two distinct genetic clusters. A fatal case linked to the outbreak was also reported. The eggs originated from a packing centre in Poland and were distributed to other countries in Europe. Restrictive measures to withdraw and stop orders for implicated eggs in the market were introduced while investigations to eliminate the source are ongoing.²⁷

Conclusion

This study shows that some hens' eggs that are retailed in the Tshwane district of Gauteng Province, South Africa may contain potential pathogenic bacteria that may have public health consequences if the eggs are eaten undercooked, uncooked or used unpasteurised to prepare products containing raw hens' eggs. More efficient monitoring measures and even laws for public health concerns should be put in place in order to ensure that only uncontaminated, preferably pasteurised, safe and wholesome eggs are sold to consumers.

Acknowledgements

We acknowledge the staff of the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science and the Department of Plant Pathology and Microbiology of the University of Pretoria for assisting with laboratory work and the National Veterinary Research Institute, Vom, Nigeria, for granting leave to A.R.J. to enrol for his PhD at the Faculty of Veterinary Science, University of Pretoria, South Africa. Funding of the project was through the research funds of F.S.B. and E.M.B.

Authors' contributions

A.R.J. was the main researcher, and was responsible for planning and executing the field experiments, the interpretation of the results and the write-up. E.M.B. co-supervised A.R.J., provided advice during the experimental work and the interpretation of results and edited the manuscript. F.S.B. supervised A.R.J., developed some of the methods executed by A.R.J., and assisted in the planning of the experimental work and the interpretation of results and edited the manuscript.

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