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RESEARCH

Impact of Red Wine Vinegar-Based Solution on Bacterial Communities of Squid and Shrimp Skewers: A Classic and Genomic Approach

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Abstract

Seafood is an essential component of a balanced and healthy diet, which increases its demand. However, its biological composition and high moisture content make these products extremely perishable. To prevent spoilage, and the consequent food waste and financial expenses throughout the seafood supply chain, new technologies have been successfully developed to inhibit bacterial growth, the main cause of seafood spoilage. This work aimed to test a shelf life extension technique for seafood skewers whilst maintaining an all-natural label using a financially feasible red wine vinegar treatment applied by immersion or pulverisation. Bacterial growth was monitored by classical methods and by 16S rRNA gene amplicon sequencing during the 5 days of storage. Immersion of samples in a vinegar-based solution effectively reduced *Pseudomonas* and Enterobacterales counts (by 2 log cfu/g), immediately after application and throughout storage. The overall structure and diversity of the bacterial community were analysed, and a strong reduction in bacterial diversity and impact on bacterial composition was observed immediately after immersion in the red wine vinegar solution. In untreated samples, Pseudomonadota (especially the Gammaproteobacteria class) was the principal phylum, whereas the microbiota of the treated samples was dominated by Bacillota (mainly the Bacilli class). Sensory analysis revealed a mild vinegar or vinaigrette flavour in treated samples; however, these characteristics were not unpleasant. Although applying a vinegar-based solution by immersion promoted a significant reduction in the growth of spoilage bacteria during the first days of storage, further tests are required to confirm the shelf life extension.

Keywords Whole genome sequencing · Food safety · Seafood · Spoilage

Introduction

Seafood is highly nutritious and important for a healthy diet (Anagnostopoulos et al., 2022). However, its perishable nature results in a short shelf life (Boziaris et al., 2013). While consumers increasingly value nutrition and healthy lifestyles (de la Caba et al., 2019), this short shelf life, combined with high prices, can reduce desirability. Consequently, significant seafood spoilage occurs, contributing to the food waste crisis (Fidalgo et al., 2018) and food insecurity by reducing the available nutrition for the population (de la Caba et al., 2019; FAO, 2011). This waste also results in financial losses for both the food industry and consumers (FAO, 2014).

Since microbial metabolism is the main cause of seafood spoilage (Anagnostopoulos et al., 2022), techniques to extend shelf life and improve safety must have antibacterial activity (Giarratana et al., 2016). To address this issue,

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various innovative technologies, such as high-pressure processing (Arnaud et al., 2018), hyperbaric storage (Fidalgo et al., 2018), ozonation (Brodowska et al., 2017), and irradiation (Yu et al., 2022), have been explored. While the efficiency of these techniques varies with product characteristics, such as the protein and lipid composition of the food, products processed with these innovative technologies have shown improved quality and stability (Arnaud et al., 2018). However, these technologies can be expensive, leading to increased manufacturing costs, reduced profit margins, and potentially increased prices for consumers (Wu et al., 2019). Simpler and more cost-effective methods, such as immersing or pulverising products with non-toxic, edible antibacterial solutions, are gaining popularity for extending shelf life (Ozogul et al., 2020). Pulverisation is especially popular because it is simple, easily automated, and widely used for the application of edible compounds (Teshome et al., 2022). The use of non-synthetic chemical preservatives is a promising innovation that addresses the demand for long-lasting, ready-to-cook foods while preserving their natural qualities (Olatunde & Benjakul, 2018). Moreover, these compounds impart an all-natural character to products, aligning with consumer preferences (Heu et al., 2010). This promising innovative approach has gained popularity, leading to the application of various antimicrobial compounds, such as limonene, chitosan, propolis, and numerous essential oils, to seafood with positive results (Alagawany et al., 2021; Han et al., 2020; Rezaei & Shahbazi, 2018). For example, the application of a chitosan-propolis coating to fresh Japanese threadfin bream extended shelf life by at least 5 days, reducing lipid oxidation and bacterial growth, and stabilising pH (Ebadi et al., 2019). Similarly, Raeisi et al. (2021) found that coating fresh shrimp with sodium alginate enriched with grapefruit seed extract inhibited bacterial growth, reduced melanosis, and regulated volatile nitrogen levels, confirming the treatment's effectiveness against chemical and microbiological decay. While essential oils and natural preservatives can extend shelf life by retarding bacterial growth, they often produce strong odours and flavours due to high volatile compound content, affecting seafood's smell, texture, and flavour (Casquete et al., 2016; Rezaei & Shahbazi, 2018). Therefore, selecting preservatives with traditional flavours can mitigate these effects. Vinegar, known for its preservative properties for thousands of years (Budak et al., 2014), is a promising candidate for seafood preservation due to its familiar sensory impact (Samad et al., 2016). Besides antimicrobial activity, vinegar has polyphenolic content and functional properties, including anti-diabetic, anti-tumour, and antioxidative activities (Budak et al., 2014; Chen et al., 2016). However, vinegar's composition and antibacterial activity vary significantly depending on its source materials, such as apples, figs, grapes, sherries, potatoes, and rice (Budak et al., 2014; Chen et al., 2016).

Skewers of raw shrimp and squid, known as "espetada," are a traditional Portuguese ready-to-cook food with a short shelf life (2 to 3 days). Being composed of shrimp and squid meat, two high-valued foods, this product is somewhat expensive to the common Portuguese consumer. This, associated with their perishability and high cost, reduces desirability, leading to food waste and financial losses. This work aimed to extend the shelf life of seafood skewers using a natural red wine vinegar-based treatment applied by pulverisation or immersion. This study evaluated the effect of the treatments on bacterial growth, product stability, and overall quality, while maintaining an all-natural label claim.

Materials and Methods

Application of a Red Wine Vinegar-Based Solution through Pulverisation or Immersion

The product used, an "espetada" composed mainly of raw squid (*Loligo duvauceli*) and shrimp (*Parapenaeopsis*, *Penaeus,* and *Metapenaeus* genera), was produced in Continente® (Portugal), packaged in plastic containers and transported to the laboratory in sealed styrofoam boxes under refrigeration conditions. Immediately after the reception, the product was pulverised or immersed in 50% (v/v) red wine vinegar solution (6% acidity, Continente®, Portugal), diluted in sterile deionised water.

This concentration was found to be the minimum bactericidal concentration that, within 10 min of exposure, could substantially reduce (by over 6 log cfu/g) the viability of *Serratia liquefaciens*, *Pseudomonas psychrophila*, and *Lactococcus gervieae* strains. These strains were previously isolated from the product and identified by Pinto de Rezende et al. (2022).

The product was carefully removed from the packaging under aseptic conditions before each treatment. Abundant pulverisation (four sprays), performed manually with a conventional hand-pumped sprayer, was attempted on all external surfaces of the product to guarantee a homogenous and all-encompassing treatment. Immersion was performed by completely submerging the product into the solution for 5 min. In addition to the pulverisation (PV) and immersion (IV) in the vinegar-based solution, control samples were prepared for comparison. These included untreated samples (control), samples pulverised with sterile deionised water (PW), and samples immersed in sterile deionised water (IW). A set of samples (control, PV, IV, PW, and IW) was immediately processed (T0), and two others were stored at 4 °C for two (T2) and five (T5) days. This last sample was processed after the due-by date of the product, set at 4 days after production.

Classical Microbiological Analysis

The efficiency of the treatment in inhibiting unwanted spoilage bacteria was analysed through bacterial enumeration using classical microbiological methods. The product (25 g) was homogenised in a Stomacher (Interscience, Saint Nom la Brèteche, France) in a proportion of 1:9 of sterile Buffered Peptone Water (Biokar Diagnostics, Beauvais, France). Three independent replicas of each sample were analysed.

Bacterial enumeration was performed using appropriate culture media according to corresponding ISO standards: mesophilic microorganisms on Plate Count Agar (ISO 4833–1:2013); Lactic Acid Bacteria on De Man, Rogosa and Sharpe agar (ISO 15214:1998); *Enterobacteriaceae* on RAPID'*Enterobacteriaceae* agar (ISO 21528–2:2017); and *Pseudomonas* spp. on *Pseudomonas* agar base supplemented with cetrimide, fusidate, and cephaloridine (ISO 13720:2010). While the ISO standard for the *Enterobacteriaceae* enumeration was followed, this work will follow the currently approved taxonomy proposed by Adeolu et al. (2016) and will henceforth refer to isolates and counts collected following this standard as members of the order Enterobacterales.

All colony types grown on *Pseudomonas* spp. culture medium were subjected to Gram staining (should be Gram-negative bacilli) and oxidase test (should be oxidase positive). Only those colonies showing the expected results for *Pseudomonas* spp. were considered.

Presumptive LAB colonies were confirmed by Gram staining (should be Gram-positive bacilli or cocci) and catalase test (should be catalase-negative).

After confirmation, the colony-forming units per gram (cfu/g) were calculated.

Bacterial Community Analysis

The bacterial communities were analysed by sequencing the 16S rRNA gene amplicons with Illumina MiSeq® (paired-end). Total DNA extraction was performed in triplicate, following the Qiagen DNeasy® mericon® food kit protocol, and the resulting DNA was pooled together to achieve a single sample. The hypervariable V3/V4 region was analysed. The raw reads were quality-filtered with PRINSEQ version 0.20.4 (Schmieder & Edwards, 2011) to remove sequencing adapters, trim low-quality bases (< Q25 in a window of 5), and eliminate reads with less than 150 bases. The paired-end reads were overlapped with AdapterRemoval version 2.1.5. (Schubert et al., 2016) The quality-controlled reads were analysed with the EZBioCloud 16S-based MTP pipeline (Yoon et al., 2017), which includes the chimeras removal with UCHIME and the definition of OTUs using a cut-off of 97% similarity. For alpha- and beta-diversity analysis the samples were normalised to 36,745 reads.

The sequences were submitted to the NCBI public database under the BioProject ID PRJNA1072673.

Sensory Evaluation

The IV samples (immersed in a 50% (v/v) vinegar solution for 5 min) and the control (untreated) samples at time zero (T0) were cooked by grilling with no additional fat or oil. To check for sensory variation, sample preparation, cooking, and testing were performed in duplicate. A difference-from-control test was performed in a sensory evaluation session with 12 panellists (Meilgaard et al., 2016). Each panellist received a control sample presented marked "control" and three coded samples, two IV samples and a replicate of the control sample. Panellists were asked first to observe, smell, and taste the sample marked "control" and then the other samples, comparing them with the "control" sample and rating the size of the difference using anchored continuous scales (0 = no difference, 10 = very large difference). Panellists were also invited to comment on the differences found.

Statistical Analysis

All calculations were done using IBM SPSS Statistics software (version 27.0, IBM Corporation, Armonk, NY, USA). Significant differences in the microbial counts of each experiment were analysed using the non-parametric Kruskal–Wallis test. Comparisons between total bacterial communities were done with STAMP (Parks et al., 2014). Results from attribute difference-from-control tests were compared using the Wilcoxon signed-rank test, Friedman's test, and Nemenyi's procedure for multiple pairwise comparisons. A significance level of 0.05 was applied to all statistical procedures.

Results and Discussion

Classical Microbiological Analysis

Microbiological enumeration was performed from three independent samples from different batches immediately after reception (T0) and after two (T2) and five (T5) days of refrigerated storage. Results are presented in Fig. 1.

Significant ($p < 0.05$) increases in total viable counts (TVC; Fig. 1a), lactic acid bacteria (LAB; Fig. 1b), Enterobacterales (Fig. 1c), and *Pseudomonas* spp. (Fig. 1d) counts were observed throughout the 5 days of refrigerated storage in all batches and treatments.

Immediately after the treatments (T0) and throughout the storage period (T2 and T5), immersion and pulverisation with sterile deionised water (IW and PW) showed no significant variation ($P > 0.05$) in bacterial counts in comparison to the control (C) samples. It can therefore be assumed

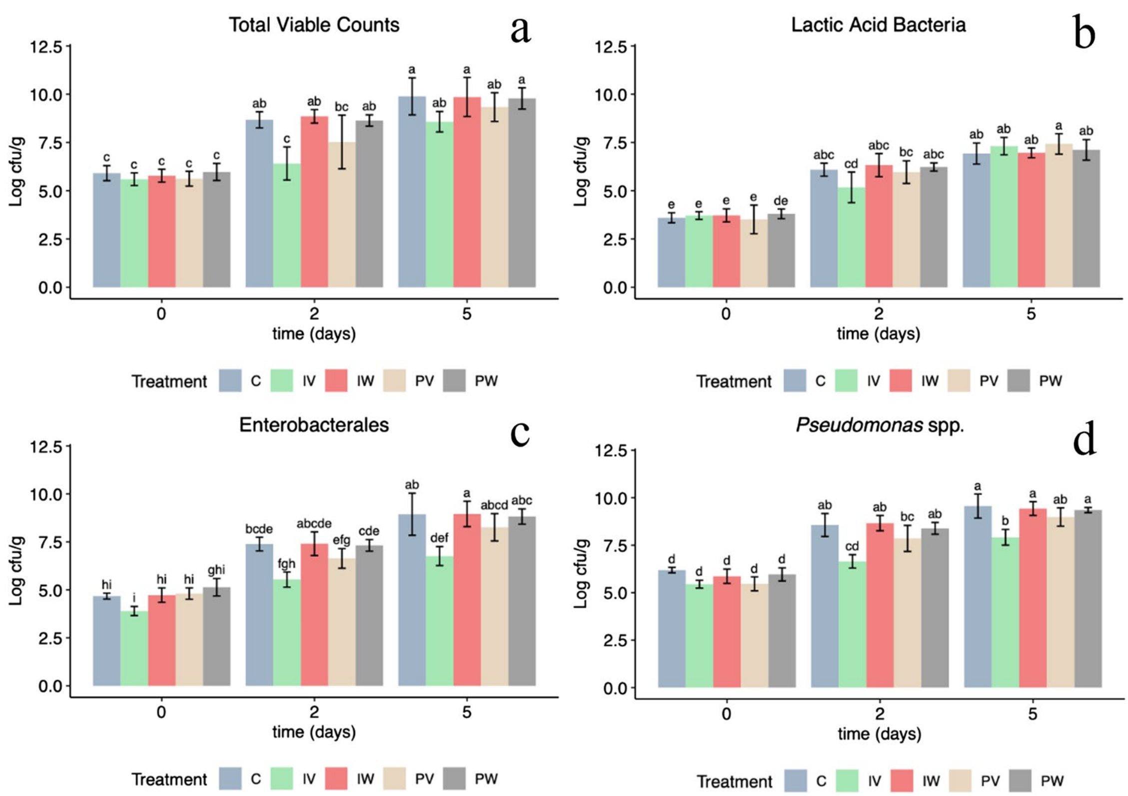

Fig. 1 Classical enumeration of total viable bacteria counts (**a**); lactic acid bacteria (**b**); Enterobacterales (**c**); and *Pseudomonas* spp. (**d**) of squid and shrimp skewers subjected to different treatments. Legend: C, No treatment; IV, immersed in a vinegar-based solution; PV, pulverised with a vinegar-based solution; IW, immersed in sterile deionised water; PW, pulverised with sterile deionised water

that the techniques applied (pulverisation and immersion) did not significantly alter the bacterial counts, and that the increase in humidity conferred by the solutions did not lead to a significant increase in bacterial growth during the period of analysis.

At T0, TVC at 30 °C was ca. 6 log cfu/g (Fig. 1a). The observed values were anticipated, taking into account the inclusion of raw squid and undeveined shrimp. As previously reported (Erkmen & Bozoglu, 2016; Leroi & Joffraud, 2011), high levels of bacteria, ranging from 7 to 9 log cfu/g, can be found in the skin, gills, and gastrointestinal tract of fresh seafood. In the study by Jeya et al. (2010), bacterial enumeration of squid tubes drew similarly high bacterial loads. Nonetheless, these results are considerably higher than those observed in shrimp samples at the time of reception by Farajzadeh et al. (2016), in which TVC was 2.5 log cfu/g. Similarly, in the study by Narasimha et al. (2018), squid meat samples frozen immediately after being caught had TVC below 6 log cfu/g after 11 days of storage. While there is no current regulation regarding TVC in seafood, the International Commission on Microbiological Specifications for Foods (ICMSF) previously recommended a maximum limit of 10^7 cfu/g for fresh seafood (ICMSF, 1986). Although this guideline has been extensively revised (ICMSF and Swanson, 2011), shifting focus towards specific spoilage and pathogenic microorganisms rather than TVC, the proximity of the observed TVC to this historical value justifies the short shelf life of the analysed shrimp and squid skewers.

Regarding TVC during refrigerated storage, no inhibition was observed immediately after immersion (IV) or pulverisation (PV) with the vinegar solution. However, after 2 days of refrigerated storage, samples treated by immersion (IV) showed a significant reduction ($p < 0.05$) of around 2 log cfu/g (Fig. 1a). After 5 days of storage, this effect was reverted, with no significant differences observed between samples. This means that after two days of refrigerated storage, IV samples maintained bacterial levels similar to those at the beginning of the storage period, with no significant growth ($P > 0.05$). While this suggests a generalised bacterial susceptibility to the solution, the inhibition was insufficient to maintain TVC below the previously recommended 10^7 cfu/g after 5 days of storage. This indicates that the product exceeded the threshold for microbial acceptability at the end of its shelf life, regardless of the treatment applied (ICMSF, 1986). Although the vinegar solution effectively inhibited total bacterial growth, its application through pulverisation, did not significantly inhibit bacterial growth.

No reduction in LAB counts was observed (Fig. 1b). This result may be due to the acidophilic properties of LAB (Menconi et al., 2014) and is also in line with the trend observed for TVC (Fig. 1a). Given their tolerance and ability to grow in acidic environments, it is understandable that the effectiveness of acetic acid in vinegar may not effectively inhibit the growth of LAB. While LAB are common members of the seafood microbiota (Gram, 2009) and are not the main spoilage organisms of these products (Françoise, 2010), their presence and metabolic activity in seafood, namely the lactic acid fermentation, can contribute to microbial deterioration if not controlled (Gänzle, 2015). As the treatment fails to inhibit LAB proliferation, it is expected that the spoilage activity of these bacteria will continue, leading to the acidification and deterioration of the product.

Significant reductions ($p < 0.05$) in Enterobacterales counts were observed exclusively in the samples immersed in 50% (v/v) vinegar (IV samples, Fig. 1c). Over 2 log cycles of inhibition were observed throughout storage in the IV samples compared to the control. While growth of some Enterobacterales was observed in the solution-immersed samples, counts at T2 were similar to those observed in the untreated samples (C) at the beginning of the experiment. This 2-day growth delay persisted throughout storage, as the IV samples at T5, well past the expiry date, had bacterial counts similar to the untreated samples at their expiry date (T2). This inhibition represents an important step in ensuring a longer-lasting product. The metabolism of amino acids by members of the order Enterobacterales, such as *Hafnia* spp. and *Morganella* spp., results in the production of biogenic amines and a reduction in product shelf life (Ramos-Vivas et al., 2022; Yamaki et al., 2018). Besides being a source of significant sensory alterations, these amines, such as cadaverine and putrescine, which cause rot-like odours and unpleasant flavours (Leroi & Joffraud, 2011), are also cytotoxic compounds (del Rio et al., 2019). Similarly, high concentrations of the biogenic amine histidine (also known as scombrotoxin) can cause serious allergic reactions and food poisoning (Comas-Basté et al., 2020). Therefore, by inhibiting the growth of strains of the order Enterobacterales, the red wine vinegar solution and immersion treatment tested appear to provide a product with a potentially longer shelf life and improved safety for the consumer. No significant Enterobacterales inhibition ($P > 0.05$) was observed in samples treated by pulverisation (PV).

Similar to Enterobacterales, *Pseudomonas* spp. counts were significantly lower in the samples treated by immersion in vinegar solution compared to the control after 2 days of storage (Fig. 1d). The inhibition was significantly higher ($p < 0.05$) on the second day of storage (around 2 log cycles), but this inhibitory effect of the vinegar solution was still evident on the fifth day of refrigerated storage. The genus *Pseudomonas* includes the most important specific spoilage microorganisms in seafood (Bolívar et al., 2016). Given the initial level (6 log cfu/g) and favourable growth conditions, any shelf life extension technique must focus on inhibiting these bacteria. In this study, the immersion of the product in the vinegar solution effectively slowed *Pseudomonas* spp. population growth. After 5 days of storage, immersed samples showed an increase of 1.5 log cfu/g, compared to the 3 log cfu/g increase observed in control samples (Fig. 1d). On the contrary, as seen for TVC, LAB, and Enterobacterales, the application of the solution through pulverisation was ineffective in inhibiting the growth of *Pseudomonas* spp.

In summary, the immersion of the product in the 50% (v/v) red wine vinegar solution effectively reduced TVC, Enterobacterales, and *Pseudomonas* spp. According to Leroi and Joffraud (2011), the neutral pH of seafood products, usually above 6.0, provides excellent growth conditions for these microorganisms. It is therefore suggested that the immediate and continuous acidification of the product as a result of the contact with an acidic solution, as used here, was the main inhibitor to these spoilage-inducing microorganisms. However, significant antibacterial activity was only observed in the IV samples. This is assumed to be a consequence of the characteristics of the pulverisation technique, as the contact time of the product with the solution, as well as the percentage of the surface area exposed to the solution activity, is substantially shorter than by immersion. Although this could be overcome by increasing the concentration of red wine vinegar, it is believed that the sensory characteristics would be significantly affected.

The results obtained in this study are in accordance with Laranjo et al. (2018), who found that the application of a white-wine vinegar solution to a meat product through immersion resulted in a significant reduction of around 4.0 and 1.5 log cfu/g, of *Enterobacteriaceae* and total viable counts, respectively. Similarly, Roila et al. (2022) reported inhibition of aerobic bacteria growth during the storage of wild boar carcasses following the application of aromatic vinegar with an equivalent concentration of acetic acid. Adding to this, acetic acid, and consequently vinegar, was found by Halstead et al. (2015) to have significant inhibitory and bactericidal activity against *Pseudomonas aeruginosa*, even suppressing the formation or eradicating pre-existing *P. aeruginosa* biofilms.

Total Bacterial Community Analysis

Aware of the limitations of analysis based solely on cultivable bacteria, the effect of the treatments tested was also analysed on the overall structure and diversity of the bacterial community by sequencing 16S rRNA gene amplicons. All analysed samples presented a Good's coverage of the library higher than 99.76% (Table 1), enabling a statistically significant analysis of the bacterial diversity. Control samples (C) showed a decay in species richness during the 5 days of incubation. A similar trend was also observed in samples treated with the vinegar-based solution. Conversely, an increase in the species richness was observed for the samples treated with sterile water. This could be attributed to the increased moisture content without any stressor for bacterial growth, unlike the samples treated with vinegar.

Regarding bacterial diversity index, with the exception of the IV samples, some small variations were observed during the incubation period (Table 1). These slight fluctuations, similar to those observed in cultivable bacteria counts (Fig. 1), are presumed to be a natural consequence of some variability in skewer composition and microbial colonisation of the meat. However, samples treated by immersion in the vinegar-based solution (IV samples) showed a significant reduction in bacterial diversity immediately after treatment. This clearly demonstrates that the red wine vinegar solution affected the bacterial diversity, with effects persisting for at least two days of storage (Table 1). After 5 days (T5) of refrigerated storage, the bacterial diversity of the IV samples returned to values similar to the control. This could indicate a decrease in the antibacterial activity of the solution in the product, possibly due to progressive dilution of the solution by the natural water content of the squid and shrimp skewer, subsequentially enabling bacterial growth.

As anticipated from the differences in Shannon indexes of bacterial diversity, immersion of samples in the vinegar-based solution (IV) significantly altered the composition of their bacterial communities. This effect is clearly observed in the principal component analysis (Fig. 2), where samples IV_T0 and IV_T2 cluster apart from the other samples.

Table 1 Coverage, richness, and diversity indexes obtained for the 16S rRNA gene sequencing

Sample	Good's coverage of library (%)	OTUs (richness)	Shannon (diversity)
C_T0	99.76	238	2.25
C_T2	99.87	159	2.16
C_T5	99.93	113	2.46
IV_T0	99.89	226	1.16
IV_T2	99.89	180	1.25
IV_T5	99.87	135	2.52
PV_T0	99.92	110	2.57
PV_T2	99.91	102	2.30
PV_T5	99.94	84	2.08
IW_T0	99.95	81	2.02
IW_T2	99.89	113	2.12
IW_T5	99.85	133	2.14
PW_T0	99.93	98	2.32
PW_T2	99.91	141	2.45
PW_T5	99.90	128	2.61

All the samples were normalized to 36,745 reads

Similar to the findings of Parlapani et al. (2018), Huang et al. (2018), and Cao et al. (2020), Pseudomonadota (mainly the class Gammaproteobacteria) was the predominant phylum, together with Bacillota (mainly the class Bacilli), representing more than 98% of the total bacterial diversity (Fig. 3).

Immersion in the vinegar-based solution resulted in a drastic reduction in the relative abundance of Pseudomonadota (from ~ 94 to 13%), compensated by a significant increase in the relative abundance of Bacillota (from ~ 6 to 85%), compared to the control. This effect diminished progressively, and after 5 days of refrigerated storage, the bacterial community of the IV samples was similar to that of the other samples (Fig. 3).

While bacteria of the family *Pseudomonadaceae*, mostly represented by the genus *Pseudomonas*, dominated the microbiota of control samples (~ 59%), this family and genus appeared to be substantially inhibited in IV samples (T0 and T2) and PV samples (T0) (Fig. 4).

Nonetheless, this inhibition appeared to be prolonged and intensified in the immersed samples, as observed in the classical enumeration (Fig. 1d), where a reduction of up to 2 log cfu/g of *Pseudomonas* spp. was found. Given the spoilage activity of this genus (Raposo et al., 2017), inhibiting *Pseudomonas* spp. may be beneficial in extending the product shelf life. Other bacterial families were also significantly reduced by the immersion treatment in vinegar-based solution: *Vibrionaceae*, *Yersiniaceae*, *Hafniaceae*, *Moraxellaceae*, and *Pseudoalteromonadaceae*. Members of these families are well known for their role in seafood spoilage (Anagnostopoulos et al., 2022; Erkmen & Bozoglu, 2016; Kuuliala et al., 2018; Olatunde & Benjakul, 2018; Ramos-Vivas et al., 2022).

The pulverisation with the vinegar-based solution had no significant effect on the microbial composition of the product, except for a slight reduction in *Pseudomonas* dominance immediately after treatment (Fig. 4).

Vibronaceae, mainly *Vibrio* spp., were detected in some PW and PV samples, as well as in the untreated control samples. Due to the pathogenicity of some species, such as *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*, and the increasing impact of foodborne diseases caused by these pathogens, inhibition of *Vibronaceae* is imperative to guarantee a safe product (Bintsis, 2017; Bonnin-Jusserand et al., 2019). Curiously, these bacteria were highly abundant in pulverised samples (PV and PW) and in control samples (C) but significantly reduced after five days of storage (Fig. 4). Samples treated by immersion, both with water and the vinegar-based solution, showed a low relative abundance (< 1%) of these taxa.

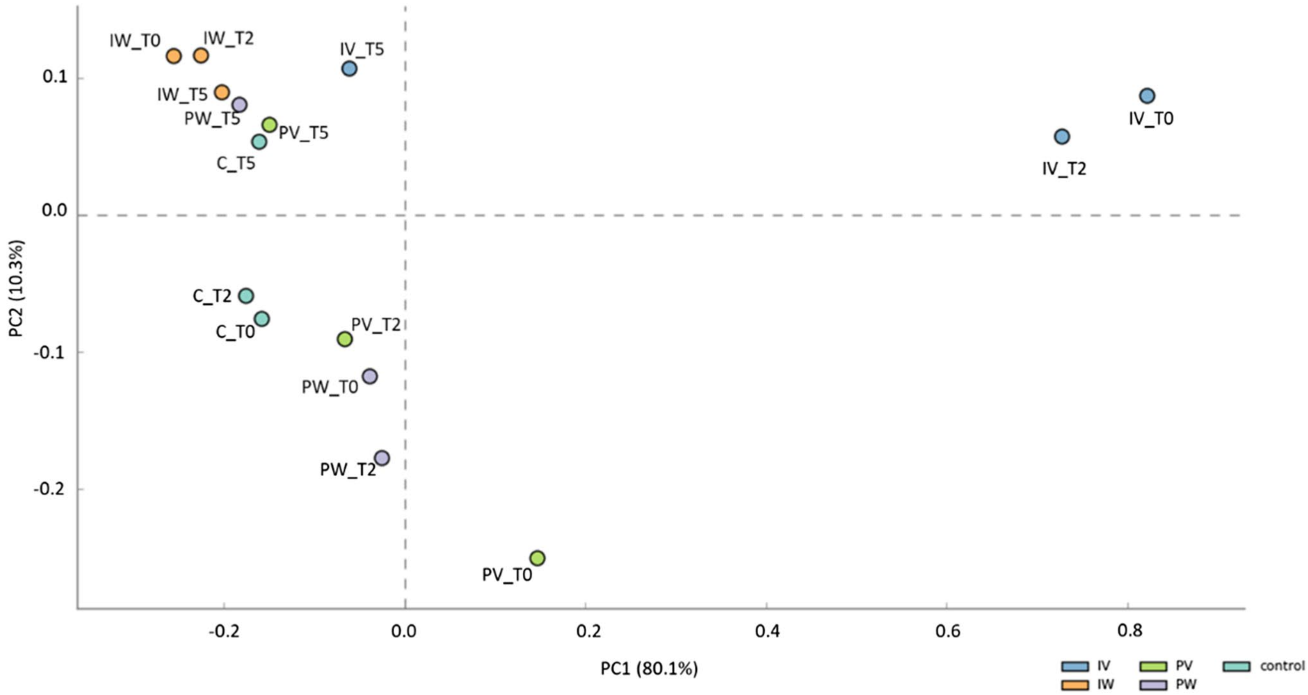

Fig. 2 Principal component analysis (PCA) of the bacterial community composition considering the identification at the family level

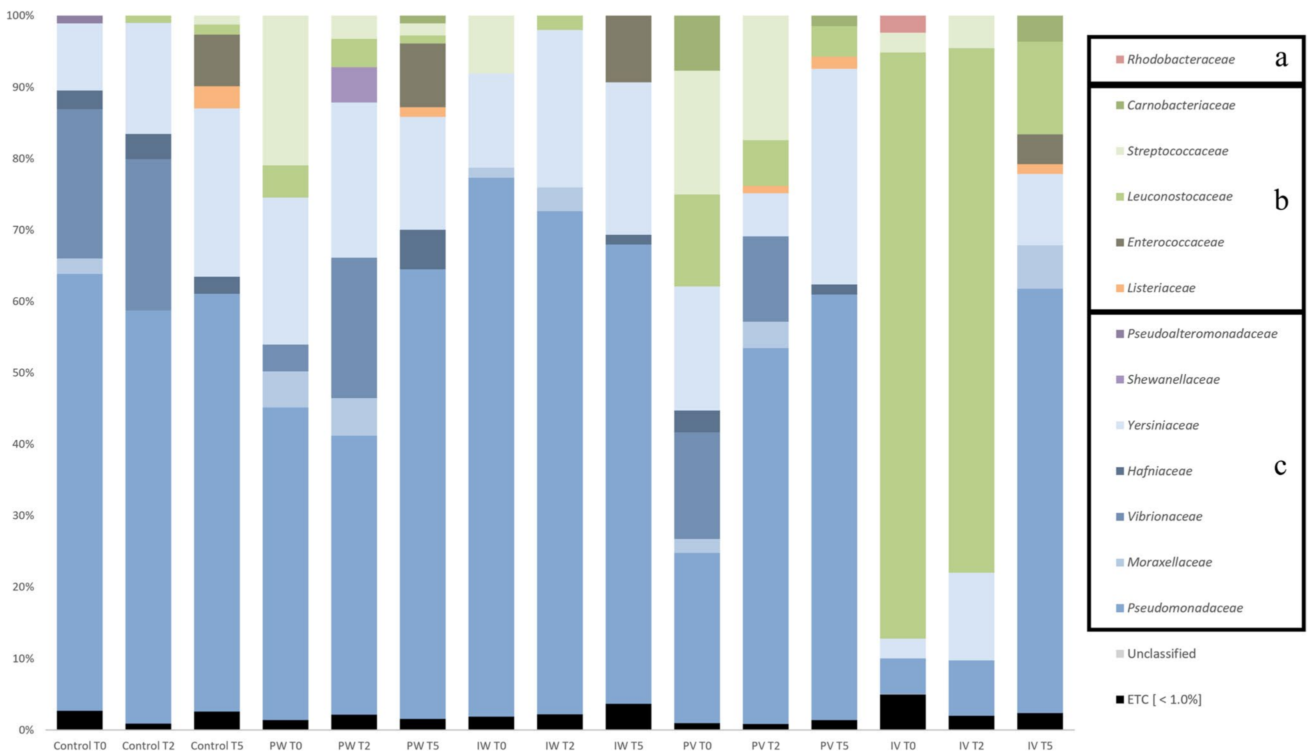

Fig. 3 Taxonomic composition of samples at the family level. Legend: Alphaproteobacteria (**a**); Bacilli (**b**); Gammaproteobacteria (**c**)

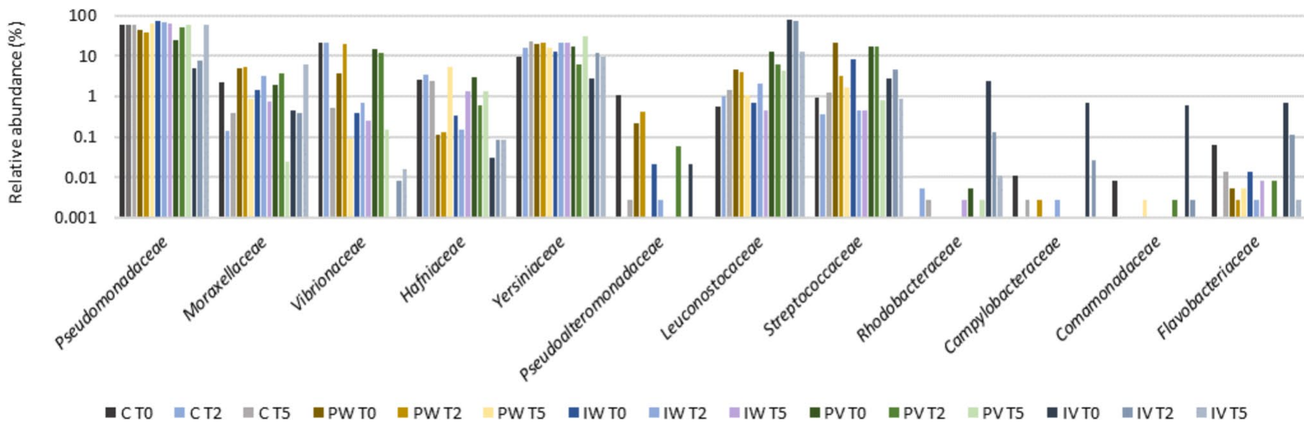

Fig. 4 Variance in the relative abundance for the families with the most significant differences ($p < 1e-15$) among samples and for which was observed the strongest effect when treated by immersion in vinegar-based solution (IV T0) in comparison to the control (C T0)

Members of the family *Hafniaceae* were found in all the samples, although their relative abundance was significantly reduced after vinegar immersion treatment (Fig. 4). Bacteria such as *Hafnia alvei* are known to alter the sensory characteristics of foods by producing biogenic amines (Ramos-Vivas et al., 2022). Therefore, inhibition of members of *Hafniaceae* family may reduce the rate and impact of bacterial spoilage in this squid and shrimp skewers.

While *Listeria* spp. and specifically the pathogen *Listeria monocytogenes* (Selvaganapathi et al., 2018) were not identified, other members of the *Listeriaceae* family, such as the genus *Brochothrix*, have been detected. Although bacteria within this genus are not known to be pathogenic to humans, *Brochothrix thermosphacta*, identified in most samples, is a significant spoilage organism. Its metabolic activity leads to the production of intense volatile compounds (Illikoud et al., 2019).

Lastly, the *Moraxellaceae* family, mainly represented by the genus *Psychrobacter*, appears inhibited in IV samples immediately after vinegar treatment. Despite *Psychrobacter* spp. (Bowman, 2006) being regarded as opportunistic pathogens and minor food spoilage organisms, infections caused by these bacteria can lead to serious health concerns. Consequently, inhibiting this genus enhances both the safety and shelf life of the product.

Overall, immersion in the vinegar-based solution significantly altered the bacterial composition of the treated samples (IV). After 5 days of storage, although spoilage bacteria became dominant, bacterial levels in treated samples were similar to those observed in control samples on the second day of storage. Given that the control samples on day two are within the product shelf life, these alterations suggest improvements in both food safety and a potentially extension of shelf life.

Sensory Evaluation

Samples were subjected to sensory analysis immediately after treatment (T0) to investigate the impact on their sensory characteristics.

No significant differences in appearance and flavour between IV samples and the control were found using the difference-from-control test ($p = 0.75$ and $p = 0.55$, respectively). Significant differences were found for odour between the control and IV samples for both replicates ($p < 0.05$, median of difference 2 out of 10, corresponding approximately to “slight difference” and described as acid/vinegar). For texture, a significant difference from the control was found only for one of the IV replicates ($p < 0.05$, median of difference 2 out of 10, corresponding approximately to “slight difference”), but not for the other IV sample ($p = 0.12$).

Conclusion

Immersion of a seafood skewer in a vinegar-based solution significantly changed the composition of the bacterial community composition of the product, promoting a drastic reduction in the bacterial diversity and the relative abundance of Pseudomonadota (mainly the class Gammaproteobacteria). While this effect was less for cultivable bacteria, a significant reduction in TVC, LAB, Enterobacterales, and *Pseudomonas* spp. was observed during the first days of storage. Some bacterial groups significantly reduced immediately after treatment, play an important role in seafood spoilage (e.g. *Hafniaceae*).

Red wine vinegar, being a traditional condiment in Portuguese and Mediterranean gastronomy often added to seafood and other foods, produced a perceivable but not unpleasant sensory impact. Adding to this, the significant inhibition of major spoilage microorganisms (e.g. *Pseudomonas* and Enterobacterales) in treated samples suggests that immersion of squid and shrimp skewers in a red wine vinegar solution has the potential as a preservation and shelf life extension technique. Nonetheless, although microbiological metabolism is strongly correlated with food spoilage, the impact of the treatment on the spoilage characteristics of the product needs further investigation to guarantee shelf life extension.

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Author Contribution L.P.R. designed the study, carried out the experiments, and wrote the article. J.B.B. designed and supervised the study and carried out the experiments. T.B.C. carried out the experiments and helped in their formal analysis. I.V.M. helped in the formal analysis and validation of the results. M.J.M. designed the study and helped in the formal analysis and validation of the results. P.T. designed and supervised the study and helped in their formal analysis. All the authors reviewed the manuscript.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Conflict of Interest The authors declare no competing interests.

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