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Evolution of microbial dynamics and headspace volatile profiles for predicting the freshness-to-spoilage transition in refrigerated cooked meatballs

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ABSTRACT

The present study investigates whether changes in headspace volatile organic compounds (VOCs) of refrigerated cooked meatballs (beef and pork) can predict the transition from freshness-to-spoilage driven by microbial growth. Hence, freshly prepared food samples were portioned into air-tight glass containers, stored at 6 °C, and analysed at regular intervals. Microbial growth was determined using culture-based techniques, while headspace VOCs were trapped on Tenax TA sorbent and analysed by thermal desorption heart-cut gas chromatography with flame ionisation and mass spectrometric detectors. Based on total viable counts (TVC) and the existing microbiological criteria, three food quality stages were defined: satisfactory ($\leq$ 4 log CFU/g), acceptable ($<$ 6 log CFU/g), and unacceptable ($\geq$ 6 log CFU/g). All samples remained in a satisfactory stage up to 3 days, entered the acceptable region from days 4–5, and reached the unacceptable zone from day 6. TVC showed a significant positive correlation with all the microbial groups ($r > 0.90$, $p < 0.0001$). VOC profiles of fresh samples were dominated by aldehydes emitted by cooking, whereas spoiled samples contained microbially derived alcohols, acetals, esters, and sulfides. Particularly, 1,1-diethoxyethane was associated with TVC, LAB, psychrotrophs, yeasts and moulds, whereas 1-hexanol, 2,3-butanediol, sulfides and other VOCs were primarily associated with *Pseudomonas*. Ratios of aldehydes/1,1-diethoxyethane and aldehydes/2,3-butanediol changed dramatically from days 3–6 ($Z = -22.43$, $p < 0.001$; $Z = 6.52$, $p < 0.001$, respectively), thus emerging as potential markers indicating imminent spoilage. In contrast, hexanal/1-hexanol ratio differentiated spoilage progression from days 9–12 ($Z = 52.72$, $p < 0.001$).

1. Introduction

Recent societal changes and increasingly busy lifestyles have significantly influenced domestic food practices. Time constraints associated with daily meal preparation often lead households to adopt convenience-oriented strategies. For instance, many households prefer to cook meals in bulk, portion them, and consume the leftovers for the following days (Ananda et al., 2025; Svartebekk et al., 2025; Van Rooijen et al., 2024). Although these practices improve convenience and reduce cooking frequency, they also contribute to increased household food waste. Consequently, households represent a major contributor to global food waste. Recent analyses also indicate that households account for approximately 54% of the total food waste generated in the European Union, estimated to be 72 kg of food waste per capita annually (Eurostat, 2025).

In many cases, refrigerated leftovers are discarded due to perceived spoilage rather than clear evidence of quality deterioration (Koppel et al., 2016). Although cooking greatly reduces microbial loads in food matrices, they can still be prone to post-cooking contamination during handling, slicing, portioning, and transfer to household storage

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containers. Moreover, inadequate cooking and cross-contamination from raw food items, kitchen surfaces, utensils and human contact can also introduce microorganisms into cooked food (Charles & Ifeoma, 2019; Iulietto & Evers, 2024). During the refrigerated storage, psychrotrophs and other cold-tolerant microorganisms can still proliferate (EFSA, 2025), which leads to overall deterioration in food quality, including nutritional losses and reduced shelf life. Moreover, a safe recommended refrigerator temperature for storing perishable foods is below 5 °C. However, a recent Europe-wide assessment involving 16 countries reported an average domestic refrigerator temperature of 6.4 °C, with considerable variability among households. Furthermore, they have shown that a substantial proportion of domestic refrigerators exceed the recommended safe temperature range mainly due to frequent door openings, overloading of food stuff, thermostat misadjustment, and consumer handling practices (Bonanno et al., 2024; Whitworth, 2025). Such deviations from ideal refrigeration conditions can accelerate microbial proliferation and spoilage progression in refrigerated foods.

Besides, previous research has mostly focused on classical culture-based microbiological techniques to analyse the growth patterns of spoilage microorganisms and their roles in food deterioration. Such techniques are labour-intensive and time-consuming, require specialised laboratory infrastructure, thereby limiting their applicability to rapid or routine food assessments, which are impractical for household leftovers. On the other hand, the analysis of the volatile organic compounds (VOCs) as a complement to microbial analysis, has attracted increasing research interest. Since VOCs are metabolic by-products of microbial activity they can serve as sensitive indicators of underlying biochemical changes in the food matrices. Furthermore, changes in VOC profiles provide insights into microbial metabolism and spoilage progression. In this context, most of the food spoilage research has mainly focused on raw, processed, and minimally processed simple food products (meat, seafood, dairy, vegetables and grains) rather than complex food matrices. Thus established links among the evolution of spoilage microbiota, elucidated mechanisms of food quality deterioration, and the formation of VOCs under controlled storage conditions (Acquaticci et al., 2024; Casaburi et al., 2015; Basdeki et al., 2025; Decimo et al., 2018; Zheng et al., 2021; Sun et al., 2025). Specifically, in raw meat systems, aldehydes generated through lipid oxidation have also been proposed as indicators of freshness and oxidative deterioration in meat, thus the ratio of aldehydes has shown significance in the freshness assessment (Karabagias, 2018).

However, to the best of our knowledge, the spoilage dynamics of complex cooked foods stored under household refrigeration conditions have so far not been systematically investigated. Such complex foods usually contain multiple ingredients and heterogeneous matrices, which can influence microbial behaviour and metabolite production in ways different from simpler food systems. Moreover, unlike raw products, the microbial ecology of cooked food is largely influenced by under-cooking, post-cooking contaminations and refrigerated storage practices while their VOC profiles are initially influenced by the cooking process and subsequently modified through microbial metabolism during storage. Therefore, spoilage indicators and quality assessment approaches developed for raw food products may not be directly applicable to cooked meals and leftovers stored in domestic environments.

Hence, in this study, a cooked meatball with almond sauce is chosen as a representative complex food since meatballs with different types of sauces are widely eaten; they are usually prepared in large quantities and are often kept as leftovers in household refrigerators. They consist of minced meat (beef and pork) with a large surface area and high nutrient availability, which can easily promote microbial growth. So far, research on meatballs has mainly focused on formulation, processing, and packaging methods, as well as evaluating their impact on microbiological and sensory parameters (Afidah et al., 2025; Meng et al., 2022). Also, only a few studies have explored the volatile profiles of various types and processes of cooked meatballs (Amalia et al., 2022; Geng et al., 2025; Qu et al., 2025; Su et al., 2025; Yılmaz, 2005; Zhao et al., 2025; Zhou et al., 2025).

Nevertheless, no previous research has systematically linked microbial dynamics with headspace VOC patterns to define freshness-to-spoilage transition stages of cooked meatballs mixed with almond sauce under realistic household refrigeration conditions. Establishing such relationships could enable the identification of potential VOC ratios that represent the freshness-to-spoilage transition and aid in assessing imminent spoilage of cooked food. This approach will be essential for the future development of VOC-based smart sensing systems for real-time freshness monitoring of refrigerated foods (Sabu et al., 2026).

Therefore, the present work aimed to investigate the shelf-life and freshness-to-spoilage transition of refrigerated cooked meatballs mixed with almond sauce by (i) quantifying the growth of spoilage microorganisms and pH changes at the storage temperature of 6 °C, (ii) analysing VOC profile dynamics in the headspace of the food matrix throughout the storage period, (iii) elucidation of relationships between microbial populations and VOCs associated with different stages of food storage, and (iv) identification of potential VOC ratios and analysing their evolution pattern associated with freshness-to-spoilage transition of the food sample to assess the imminent spoilage.

2. Materials and methods

2.1. Experimental design

In total, 2 kg of cooked meatballs with almond sauce (refer to the list of ingredients below) were ordered in advance. The food was freshly prepared in a nearby commercial kitchen to simulate a real kitchen environment, and the packed food was brought to the microbiology laboratory of the Faculty of Veterinary Science, University of Zaragoza. After homogenization of food samples, it was immediately portioned into air-tight glass containers, each weighing 90 ± 0.5 g. All samples were stored at 6 °C and analyses of microbial growth, pH changes, and headspace VOC evolutions were performed on days 0, 3, 6, 9, and 12. The experimental design and analyses carried out are illustrated in Fig. 1.

List of Ingredients: commercial meatballs 80% (pork, beef, grated corn bread, potato starch, salt, spices, sunflower oil, and food additives**), almond sauce 20% (**water, white leek, almond, onion, tomato sauce, beef stock, yeast extract, sugar, water, onion powder, veal broth, pepper spice extract, bread crumbs, wine, semi-skimmed milk, garlic, corn starch, extra virgin olive oil, sugar, salt, black pepper, parsley and food additives**).**

2.2. Microbial analysis

On each sampling point, three replicates ($n = 3$) were drawn and analysed. Around 10 ± 0.3 g of cooked meatballs with almond sauce were taken out from each container and diluted with 90 mL of buffered peptone water (Oxoid, CM0509, United Kingdom). Then, homogenization was performed at 300 rpm for 5 min using a stomacher (Seward Stomacher Lab Blender, United Kingdom). Based on microbial growth, decimal dilutions were performed using 0.1% (*w/v*) peptone water (Oxoid, CM0009, United Kingdom). The sample preparation and dilution protocols were followed according to ISO standards (ISO 6887-1, 2017) and the manufacturer's recommendations. The microbial groups, culture media, incubation conditions and their ISO standards were provided in Table 1.

2.3. pH measurements

For each measurement, 10 ± 0.3 g (n = 3) of cooked meatballs with almond sauce was weighed, and 10 mL of deionised water was added to obtain a 1:1 (w/v) homogenate. Samples were homogenised using IKA T25 digital ULTRA-TURRAX dispersers (IKA, Germany) at 7200 rpm for

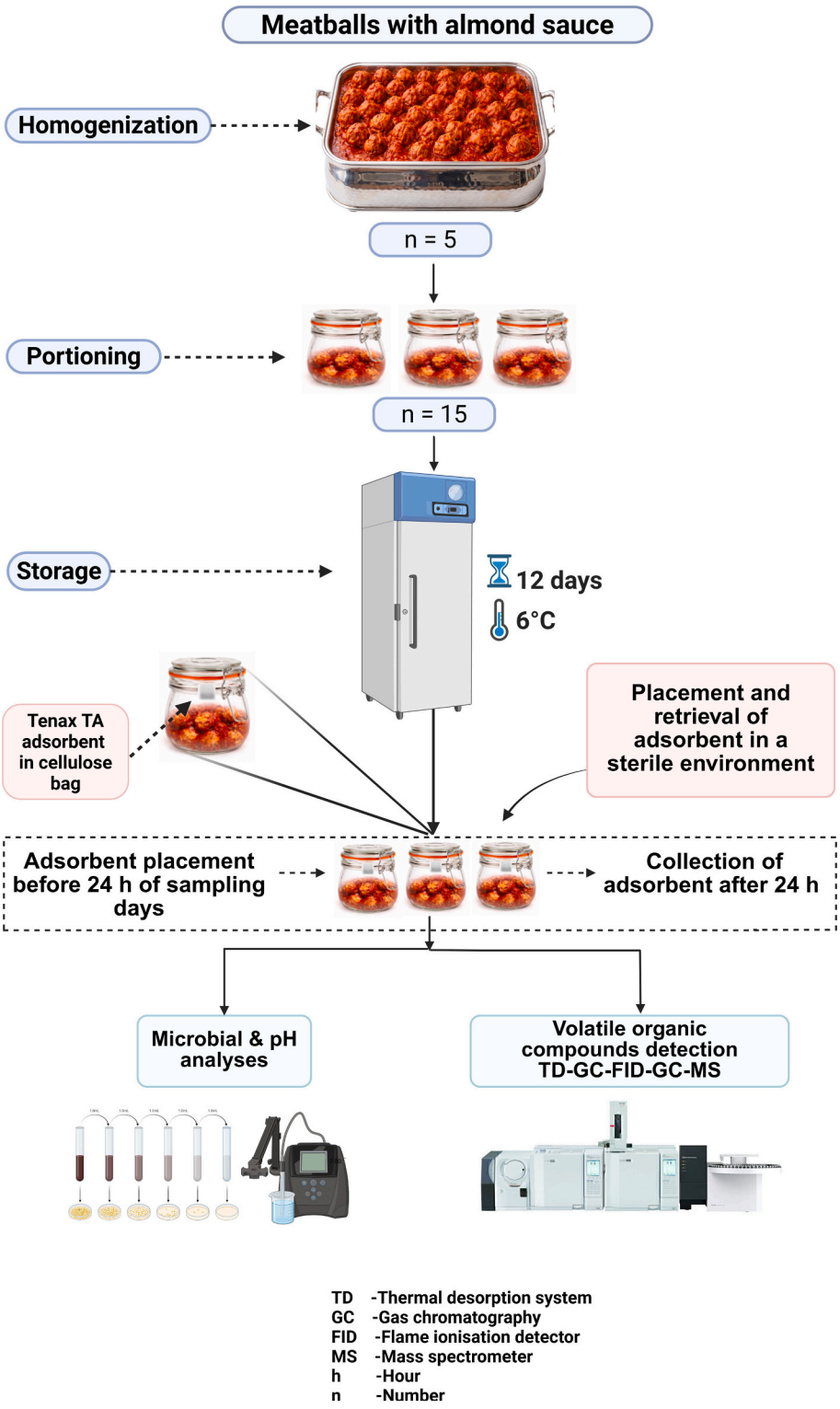

Fig. 1. The experimental design illustrates a storage trial of refrigerated (6 °C) cooked meatballs with almond sauce in order to predict the freshness-to-spoilage transition. Microbiological, pH and VOC analyses were carried out on days 0, 3, 6, 9, and 12. The image was created in BioRender. Gurusamy, R. (2026) https://BioRender.com/ppnn5j4.

Table 1
Microbial groups and the respective culture media used to analyse microbial growth in the refrigerated cooked meatballs with almond sauce.

S. No.	Microbial groups	Culture media	Incubation temperature & time	Manufacturer	Protocol references
1	Total viable counts (TVC)	Plate count agar (PCA)	30 ± 1 °C 72 ± 3 h	Sigma-Aldrich	(ISO 4833-1, 2013)
2	Psychrotrophs	Plate count agar (PCA)	6.5 ± 1 °C 10 days	Sigma-Aldrich	(ISO 17410, 2019)
3	*Pseudomonas*	Cephalothin-sodium fusidate-cetrimide (CFC) agar with *Pseudomonas* CFC selective supplement	25 ± 1 °C 44 ± 4 h	Sigma-Aldrich	(ISO 13720, 2010)
4	Lactic acid bacteria (LAB)	De Man, Rogosa, and Sharpe (MRS) agar	30 ± 1 °C 72 ± 3 h	Oxoid, CMO361B	(ISO 15214, 1998)
5	Enterobacterales	Violet red bile glucose (VRBG) agar	37 ± 1 °C 24 ± 2 h	Oxoid, CM1082	(ISO 21528, 2017)
6	Yeast and mould	Potato dextrose agar (PDA) with chloramphenicol supplement	25 ± 1 °C 5 days	Oxoid, CM0139; Sigma-Aldrich	(ISO 21527-1, 2008)

2 min. The homogenate was allowed to stand for 1–2 min to ensure that entrapped air bubbles dissipated before measurement. All measurements were conducted at standard laboratory temperature. Before measuring each sample, the benchtop pH meter (Hach, Sension+ pH 3 Tabletop pH Meter, Germany) was calibrated. The electrode was immersed in each homogenate, and the pH was recorded. The electrode was rinsed thoroughly between each replicate measurement to prevent contamination.

2.4. Microbiological criteria

At present, there are no established microbiological thresholds or criteria available to predict the microbiological quality and shelf life of cooked meals or leftovers stored under household refrigeration conditions. However, microbiological criteria for ready-to-eat (RTE) foods are well established in regulatory and advisory guidelines for industrial and commercial sectors, including those issued by the European Commission (EC, 2005), Food Standards Australia New Zealand (FSANZ, 2022), the New South Wales Food Authority (NSW, 2009), the Centre for Food Safety, Hong Kong (CFS, 2014) and the United Kingdom Health Security Agency (UKHSA, 2024). Therefore, the present study adopted these internationally established RTE food's microbiological criteria to provide practical guidelines for interpreting aerobic standard plate counts (SPC) as indicators of food quality and hygiene. The SPC is also known as the total viable count (TVC) or the aerobic plate count (APC), which reflects the total number of viable microorganisms that are capable of growing under aerobic or facultative incubation conditions. It is commonly used as a general indicator of the overall microbiological quality and hygienic conditions of the RTE food. Regulatory guidelines typically categorise TVC values below 4–5 log CFU/g as satisfactory, between 5 and 6 log CFU/g as marginal or acceptable, and above 6–7 log CFU/g as unsatisfactory or potentially hazardous, depending on the food type, preparation, handling and storage (CFS, 2014; FSANZ, 2022; NSW, 2009; UKHSA, 2024; EC, 2005).

Although most of the regulatory guidance does not define thresholds for specific spoilage organisms (SSO), such as *Pseudomonas*, lactic acid bacteria (LAB), yeasts and moulds, the European Food Safety Authority (EFSA) recognises that the signs of spoilage become apparent when SSO reach sufficiently high populations, often between 6 and 8 log CFU/g, owing to the production of microbial metabolites. These metabolites may serve as objective chemical indicators of food freshness or spoilage (EFSA et al., 2020). Further, the UKHSA RTE guidelines pointed out that high TVC loads above 6 log CFU/g in food matrices represent the dominant microbial flora and are responsible for the organoleptic consequences of the food matrix. For example, lactic acid bacteria may dominate in refrigerated meat products and contribute to spoilage by producing organic acids at higher growth (~ 9 log CFU/g), whereas gram-negative spoilage bacteria such as *Pseudomonas* spp. may generate off-odours at even lower population levels (~ 7 to 8 log CFU/g). Yeasts may also contribute to spoilage by producing acids and gases at approximately 6 to 7 log CFU/g (UKHSA, 2024). Nevertheless, several studies on refrigerated raw meat or cooked meat have reported that TVC values above 6 log CFU/g can be associated with the quality deterioration, spoilage development, or reduced acceptability, which might be food matrix-dependent, influenced by storage temperature, packaging atmosphere, and SSO (Blanco – Lizarazo et al., 2022; Chmiel et al., 2025; Talukder et al., 2025; Triki et al., 2018). Considering this variability, a more stringent classification was applied in the present study. For instance, the food is considered satisfactory at microbial loads of ≤ 4 log CFU/g, acceptable at > 4 to < 6 log CFU/g and unacceptable at ≥ 6 log CFU/g. This approach provides a conservative and consistent microbiological quality threshold in the absence of household-level standards to assess imminent spoilage rather than establishing absolute regulatory spoilage limits. Accordingly, the classification adopted in the present study was intended solely as a microbiological quality framework to interpret microbial dynamics, along with VOC profiles to assess the freshness-to-spoilage transition in cooked food, and it was not intended to define the sensory spoilage or consumer rejection.

Therefore, although several microbial groups (psychrotrophs, LAB, Enterobacterales, *Pseudomonas*, yeasts and moulds) were enumerated individually in this study, TVC was selected as the primary microbiological indicator of the freshness-to-spoilage transition, as it consistently exhibited the most abundant counts and closely followed the temporal behaviour of the dominant microbial group throughout storage.

2.5. Volatile organic compounds (VOCs) analysis using a heart-cut gas chromatography (GC-GC) system

2.5.1. Sample preparation

VOCs sample collection was carried out in parallel with microbial analysis. Headspace VOCs were collected using Tenax TA 60/80 sorbent (Sigma-Aldrich, Netherlands). Prior to use, the sorbent was conditioned in a TC-20 tube conditioner (Markes International, United Kingdom) at 320 °C for 8 h under a continuous flow of high-purity nitrogen. The conditioning and operational temperatures of the sorbent were selected based on the technical specifications provided in the manufacturer's datasheet (Sigma-Aldrich, Netherlands). Before each microbial sampling point, 50 ± 5 mg of conditioned sorbent was packed into cellulose bags and hung inside the air-tight glass containers containing food samples to adsorb headspace VOCs. After 24 h of exposure, the VOC-enriched Tenax sorbents from each container were collected and stored in a clean vial, which was sealed and stored at 4 °C for 12 days. Prior to gas chromatography (GC) analysis, the VOC-enriched Tenax sorbents were transferred into thermal desorption glass tubes (Sigma-Aldrich, United States of America) and immediately sealed with technical-grade glass wool (PanReac AppliChem, Spain).

2.5.2. Chromatographic system specification

VOCs were analysed using a heart-cut gas chromatography (GC-GC) system (Shimadzu, Tokyo, Japan) combined with a thermal desorption unit (TD-30R). The system consists of a first-dimension GC coupled with a Dean's switch connected to a flame ionisation detector (GC–FID) with a 30 m × 0.25 mm × 0.25 μm ZB-FFAP column (Phenomenex, Spain). A second-dimension GC interfaces with a QP2010 Plus quadrupole mass spectrometer (GC–MS) fitted with a 30 m × 0.25 mm × 1 μm ZB-5MSplus column (Phenomenex, Spain).

2.5.3. Thermal desorption (TD) of VOCs

At first, VOC enriched Tenax samples were thermally desorbed on the TD unit at 300 °C for 15 min with a helium flow rate of 50 mL/min, and then transferred to an internal Tenax TA trap maintained at −15 °C. Then, the trapped VOCs were heated to 300 °C for 10 min and subsequently transferred to the first-dimension column via a heated transfer line maintained at 250 °C. A 1:5 split was used to accelerate transference.

2.5.4. GC-GC analytical procedure

The first-dimension oven was maintained at 40 °C for 5 min, then increased at 4 °C/min to 145 °C, later at 8 °C/min to 240 °C and finally maintained at 240 °C for 20 min. The second-dimension oven programme also began at 40 °C, held for 52.40 min, heated at 4 °C/min to 145 °C, then increased at 8 °C/min to 240 °C, and finally at 10 °C/min to 300 °C, held for 5 min. The pressure in the first column was 220 kPa, and the auxiliary pressure was 150 kPa, respectively. The heart-cutting programme was optimised using samples with the longest storage times, as maximum VOCs production was expected in these samples. These samples were first analysed exclusively using GC-FID to obtain the complete chromatogram. Chromatographic peaks with intensities above 100,000 μV in the first-dimension were left in that dimension, while the remaining chromatographic regions were cut and transferred to the second-dimension, in which the remaining compounds were analysed using the most sensitive and selective MS detector. The cut times were as follows: 2.4–3.25 min; 4.0–4.92 min; 5.29–11.29 min; 11.93–13.26 min; 13.63–16.64 min; 17.25–52.30 min. The upper time limit was determined by the presence of contaminants detected in an exhaustive study of blanks (thermal desorption glass tubes, tubes containing Tenax sorbent, tubes with glass wool, and tubes containing exposed Tenax sorbent stored in cellulose bags) as shown in **Fig. S1** (supplementary material). Once the cutting programme was optimised, all the samples were analysed using the same procedure. This strategy protects the MS from the most concentrated VOCs, extending its lifetime, preventing it from contamination, and ensuring a clean MS signal while maintaining the ability to measure peaks. Importantly, due to difficulties associated with accurate introduction of internal standards in the VOC adsorbed Tenax material, quantification of VOCs relied on the absolute areas of the selected ions. Although the instrumental system is robust, in order to avoid any bias caused by drifts in the instrumental sensitivity, all the samples were analysed in a single long batch over a 5-day period in a completely random order. This ensured that biological replicates were evenly distributed throughout the analytical period.

2.5.5. Flame ionisation detector (FID) and mass spectrometer (MS) conditions

The FID in the first-dimension was maintained at 270 °C. The quadrupole mass spectrometer (MS) in the second-dimension was operated in scan mode (35 to 350 *m/z*). The ion source and quadrupole temperatures were kept at 200 °C, and the MS transfer line was maintained at 240 °C.

2.5.6. Peak selection, identification and semi-quantification

The chromatograms from both dimensions were analysed using LabSolutions software version 4.45 (Shimadzu, Tokyo, Japan). Peak selection was performed by visual comparison of overlapping chromatograms ($n = 3$) obtained on day 0 and day 12. Peak areas showing consistent changes (i.e., increases or decreases across replicates) between days 0 and 12 were chosen for further analysis. For each target peak, the individual MS ions were carefully visually examined to verify whether they originated from the compound of interest or from co-eluting interferences. The compound identification was performed by selecting the mass spectrum that best represented each analyte, usually obtained from the sample in which the compound showed the highest abundance. Spectra were extracted from the region of the chromatographic peak least affected by co-elution, while avoiding saturated signals. Tentative identification was based on spectral similarity to the National Institute of Standards and Technology (NIST) mass spectral library, version 2.3 (United States of America), supported by the relative retention order of compounds and comparison with literature/NIST-reported linear retention index (LRI) values. The semi-quantitative data were derived from the integrated peak areas of selected ions, which were calculated for all analysed samples and used as the basis for subsequent data analysis.

The GC-FID compound identification follows two stages: (i) GC-FID chromatograms were obtained using a polar ZB-FFAP column in the first-dimension and were first evaluated to define the retention time (R_t) ranges of peaks, and (ii) a narrow R_t window around the apex point of the selected peaks was given as a cut time in the second-dimension programme with a non-polar ZB-5MSplus column, and VOC samples were run using the same second-dimension programme as mentioned above. Hence, with the GC–MS chromatograms, compound identification was performed based on the spectral similarity to the NIST library. Further, the chromatographic elution order of those compounds in ZB-FFAP polar column LRI was obtained from reference databases like NIST and PubChem. Since GC-FID and GC–MS analyses were performed on columns with different stationary phases, the peaks from the GC–MS chromatograms serve as a reference for compound identification, while the R_t from the FID chromatograms and LRI of those compounds (identified using GC–MS) on the polar ZB-FFAP column help to determine the elution order of the compounds in the first-dimension.

2.6. Statistical analysis

All microbiological and physicochemical data were statistically analysed using XLSTAT advanced 2025.2.0 (Addinsoft, Paris, France). The microbiological data were log-transformed, and replicate values were used in the statistical analysis. All data were normalised before carrying out statistical analysis and the significance level was set at $\alpha = 0.05$. A two-way ANOVA and Tukey's post hoc test were used to evaluate the effect of storage time on the microbial growth and their interactions. Additionally, Pearson's correlation analysis was used to correlate the linear relationships among the variables. One-way ANOVA and Tukey's post hoc test were used to analyse the significance of changes in VOCs over time. MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/) was used for Partial Least Squares Discriminant Analysis (PLS-DA) analysis to evaluate sample discrimination with VOC abundance as X-variables and storage days as Y-variables. Hierarchical clustering analysis was carried out to interpret relationships and relative changes in the microbial counts, pH, and VOC profiles. The analysis was conducted using the options Euclidean distance as the similarity metric and Ward's linkage for cluster formation. The data were normalised using Pareto-scaling before analysis to ensure equal contribution of all variables, regardless of their absolute abundance.

3. Results and discussion

3.1. Evolution of microbial growth and pH changes

The microbial growth and pH changes in cooked meatballs with almond sauce during the refrigerated storage trial are depicted in Fig. 2. The results revealed that microbial growth was primarily driven by

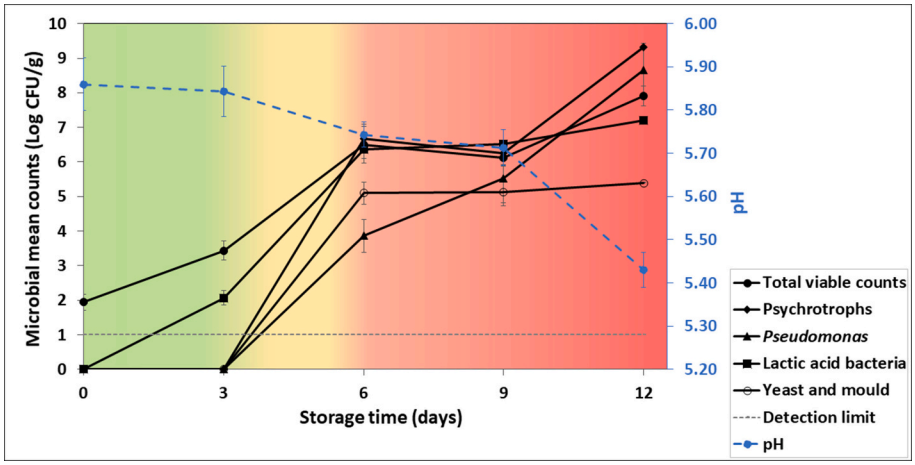

Fig. 2. Changes in the microbial populations and pH of cooked meatball samples during the refrigerated storage at 6 °C. The X-axis represents storage time (days), while the primary Y-axis shows microbial mean counts (log CFU/g), and the secondary Y-axis shows pH. Each sampling point indicates the average microbial count of three triplicates. Error bars represent the standard error of the mean. Distinct markers represent different microbial groups: total viable counts (—●—), psychrotrophs (—◆—), *Pseudomonas* (—▲—), lactic acid bacteria (—■—), yeasts and moulds (—○—), and pH (—●--). The background colour gradient denotes the progression of the samples from satisfactory (green) through acceptable (yellow), and to the unacceptable (red) stages of food as in the microbiological criteria section 2.4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

storage time (two-way ANOVA, F = 416.45, $p < 0.0001$). Throughout the storage period, the Enterobacterales group was not detected. On day 0, the freshly cooked meatballs had an initial pH of 5.86 ± 0.06. The microbial load of fresh samples was below the detection limit (< 1 log CFU/g) for all microbial groups except TVC (1.94 ± 0.24 log CFU/g), which is within the limits suggested for the heat processed food by the regulation of Food Standards Australia New Zealand (FSANZ, 2022). On day 3, the TVC and LAB counts increased to 3.44 ± 0.27 log CFU/g and 2.07 ± 0.22 log CFU/g, respectively, while the pH value was relatively stable (5.84 ± 0.06). At this point, the microbial load was within the satisfactory limit (green zone).

A major transition occurred from days 3 to 6, and on the day 6, the TVC, LAB, and psychrotrophs counts surged to 6.49 ± 0.53 log CFU/g, 6.36 ± 0.26 log CFU/g, and 6.67 ± 0.42 log CFU/g, respectively. These observations indicate that the food has entered into the unacceptable stage (red zone) as per the defined threshold (≥ 6 log CFU/g). Besides, the pH value of the food decreased to 5.74 ± 0.03 on day 6. This drop may be associated with the metabolic activity of spoilage microbiota, specifically LAB, which are known to proliferate under refrigerated, oxygen-limited meat storage conditions and may produce organic acids by metabolising carbohydrate (Bleicher et al., 2022). In the present study, the cooked meatballs additionally contained carbohydrate sources in the almond sauce formulation and were stored in air-tight containers, which might have developed an oxygen-limited environment as storage progressed. Additionally, LAB populations exceeding 6 log CFU/g represented an important transition stage in refrigerated cooked meat storage, while spoilage characteristics might be more evident when LAB levels approach or exceed 7 log CFU/g (Kreyenschmidt et al., 2010; Nychas et al., 2008; Patsias et al., 2006). Therefore, the observed pH decline in the present study likely reflects fermentative microbial metabolism during the freshness-to-spoilage transition of refrigerated cooked meatballs. By contrast, the growth of *Pseudomonas,* yeasts and moulds reached 3.86 ± 0.48 and 5.00 ± 0.31 log CFU/g, respectively, remaining significantly lower than the other microbial groups.

Between days 6 and 9, the levels of microbial populations remained relatively stable, forming a plateau phase during which levels of LAB, psychrotrophs, and TVC remained between 6 and 6.7 log CFU/g, with *Pseudomonas* reaching 5.52 ± 0.70 log CFU/g. As days passed, microbial proliferation intensified. On day 12, the TVC and LAB counts gradually reached 7.90 ± 0.28 log CFU/g and 7.20 ± 0.03 log CFU/g, respectively. In contrast, the growth of psychrotrophs and *Pseudomonas* surged to 9.31 ± 0.04 log CFU/g and 8.66 ± 0.78 log CFU/g, respectively. However, yeast and mould stabilised between 5 and 5.4 log CFU/g from days 6 to 12. Besides, the overall microbial growth coincided with a further sharp decline in pH, which reached to 5.43 ± 0.03 at the end of the storage trial.

The early rise of LAB and the steep increase of psychrotrophs between days 3 and 6 may be due to cross-contamination from other food items (including raw food and dairy products) through the kitchen aids like cutting boards, knives, utensils, storage containers and human handling (Charles & Ifeoma, 2019; Iulietto & Evers, 2024) or from undercooked meatball and sauce ingredients. However, at the end of the storage trial, psychrotrophs and *Pseudomonas* became dominant. On the other hand, pH values showed a range of negative correlations ($r = -0.69$ to -0.86), which indicates an inverse relationship with all the microbial growth. Specifically, the sharp decrease in the pH after day 9 coincided with the growth of *Pseudomonas* ($r = -0.86$, $p < 0.0001$) and psychrotrophs ($r = -0.81$, $p < 0.001$). Specifically, psychrotrophic bacteria such as *Pseudomonas* can adapt to refrigerator temperatures and tend to become dominant over time, making them one of the main contributors to meat spoilage, even under oxygen-limited conditions (Casaburi et al., 2015). As expected, TVC counts were higher from the initial days and closely followed the temporal pattern of the dominant microbial groups, which also showed significant positive correlations with LAB ($r = 0.95$, $p < 0.0001$), psychrotrophs ($r = 0.93$, $p < 0.0001$), *Pseudomonas* ($r = 0.91$, $p < 0.0001$), yeast and mould ($r = 0.92$, $p < 0.0001$), indicating a strong association with growth pattern. Hence, in this study, the above observations support the practical use of TVC to monitor the freshness to spoilage transition of refrigerated cooked meatballs.

3.2. Evolution of volatile organic compounds (VOCs)

The VOCs present in the headspace of refrigerated food samples were trapped on Tenax sorbents, which were subsequently desorbed into the thermal desorption unit and analysed using a GC-GC–MS system (refer to section 2.5). Several hundred VOCs were separated by the chromatography system, with approximately 100 showing clearly distinguishable signals in the FID and total ion chromatograms. The 27 out of those 100 compounds, showing consistent changes throughout storage were retained for data analysis. The list of compounds, including their CAS numbers, retention times (R_t), reference linear retention indices (LRI), NIST match score, diagnostic ions, and the significance of peak areas over the storage days is shown in Table 2. Levels of these VOCs changed

Table 2
A list of selected VOCs (2 VOCs from FID and 25 VOCs from MS) is provided, along with their compound names, CAS numbers, retention times (R_t), reference linear retention indices (LRI), NIST match, diagnostic ions, and peak areas over the storage days (D0, D3, D6, D9, and D12).

S. No.	Compound name	CAS No.	Retention times (min)	LRI	NIST match (%)	Diagnostic ions (*m/z*)	Significance*	Peak area (μV)				
								D0	D3	D6	D9	D12
List of selected VOCs from GC-FID												
1	Acetone	67–64-1	13.39	500	97	43, 58, 42	<0.0001	$(8.74 \pm 0.61) \times 10^{5b}$	$(5.32 \pm 0.03) \times 10^{5c}$	$(1.05 \pm 0.01) \times 10^{6ab}$	$(9.20 \pm 0.59) \times 10^{5b}$	$(1.20 \pm 0.08) \times 10^{6a}$
2	1-undecene	821–95-4	16.85	1091.3	97	43, 55, 56, 70, 41, 69, 83, 97, 111, 154	0.015	$(5.75 \pm 0.14) \times 10^{5b}$	$(5.03 \pm 0.00) \times 10^{5b}$	$(6.78 \pm 0.04) \times 10^{5b}$	$(5.39 \pm 0.36) \times 10^{5b}$	$(9.01 \pm 4.95) \times 10^{6a}$
List of selected VOCs from GC–MS												
3	Pentane	109–66-0	3.93	500	92	42, 43, 57, 72	0.361 /0.035	$(5.08 \pm 1.41) \times 10^{6a}$	$(5.01 \pm 0.42) \times 10^{6a}$	$(6.64 \pm 1.65) \times 10^{6a}$	$(9.97 \pm 5.32) \times 10^{6a}$	$(1.07 \pm 0.25) \times 10^{7a}$
4	Dimethyl sulfide	75–18-3	4.6	505	95	62, 47, 61	0.092 /0.004	$(2.38 \pm 0.65) \times 10^{6a}$	$(3.19 \pm 0.58) \times 10^{6a}$	$(2.95 \pm 0.36) \times 10^{6a}$	$(1.05 \pm 0.60) \times 10^{7a}$	$(1.58 \pm 0.40) \times 10^{7a}$
5	Carbon disulfide	75–15-0	4.72	568	86	76, 44, 78, 77	0.014	$(3.87 \pm 3.13) \times 10^{6b}$	$(4.60 \pm 0.19) \times 10^{6b}$	$(2.22 \pm 0.51) \times 10^{6b}$	$(1.57 \pm 0.91) \times 10^{7ab}$	$(2.44 \pm 0.14) \times 10^{7a}$
6	3-methylbutanal	590–86-3	8.32	641	97	44, 41, 43, 58, 71, 86, 85	<0.0001	$(3.63 \pm 0.19) \times 10^{6a}$	$(4.55 \pm 0.07) \times 10^{6a}$	$(4.54 \pm 0.35) \times 10^{6a}$	$(3.59 \pm 0.28) \times 10^{6a}$	$(2.87 \pm 0.00) \times 10^{5b}$
7	2-methylbutanal	96–17-3	8.49	650	94	88, 73, 45, 61, 90	<0.0001	$(2.29 \pm 0.06) \times 10^{7a}$	$(1.85 \pm 0.14) \times 10^{7ab}$	$(2.19 \pm 0.29) \times 10^{7ab}$	$(1.75 \pm 0.21) \times 10^{7b}$	$(5.63 \pm 3.52) \times 10^{5c}$
8	Allyl methyl sulfide	10152–76-8	10.35	693	94	88, 73, 45, 61, 90	<0.0001	$(5.72 \pm 0.37) \times 10^{6c}$	$(4.76 \pm 0.33) \times 10^{6c}$	$(1.01 \pm 0.03) \times 10^{7b}$	$(1.04 \pm 0.11) \times 10^{7b}$	$(2.15 \pm 0.08) \times 10^{7a}$
9	1,1-diethoxyethane	105–57-7	10.57	734	90	45, 73, 103, 61	<0.0001	$(4.21 \pm 0.47) \times 10^{7c}$	$(2.80 \pm 0.06) \times 10^{7d}$	$(8.77 \pm 0.46) \times 10^{7b}$	$(1.01 \pm 0.04) \times 10^{8a}$	$(8.24 \pm 0.86) \times 10^{7b}$
10	Isoamyl alcohol	123–51-3	20.62	736	96	55, 42, 70	<0.0001	$(7.59 \pm 1.09) \times 10^{5b}$	$(7.64 \pm 7.93) \times 10^{5b}$	$(9.01 \pm 2.95) \times 10^{5b}$	$(5.20 \pm 0.84) \times 10^{5b}$	$(1.84 \pm 0.19) \times 10^{6a}$
11	Hexanal	66–25-1	20.95	776	91	44, 56, 41, 43, 57, 72, 82, 67	<0.0001	$(5.60 \pm 0.35) \times 10^{7ab}$	$(6.01 \pm 0.00) \times 10^{7ab}$	$(7.21 \pm 0.50) \times 10^{7a}$	$(5.29 \pm 1.26) \times 10^{7b}$	$(6.00 \pm 3.74) \times 10^{6c}$
12	1-pentanol	71–41-0	24.33	781	98	42, 55, 41, 70	0.002	$(1.66 \pm 0.08) \times 10^{7b}$	$(1.78 \pm 0.10) \times 10^{7b}$	$(2.14 \pm 0.21) \times 10^{7b}$	$(1.66 \pm 0.25) \times 10^{7b}$	$(2.95 \pm 0.43) \times 10^{7a}$
13	2,3-butanediol	513–85-9	35.94	806	94	45, 57, 75	<0.0001	$(1.01 \pm 0.10) \times 10^{7b}$	$(7.29 \pm 0.78) \times 10^{6b}$	$(1.44 \pm 0.10) \times 10^{7b}$	$(1.46 \pm 0.17) \times 10^{7b}$	$(2.09 \pm 0.14) \times 10^{7a}$
14	o-xylene	95–47-6	38.42	850	96	91, 106, 105, 77	0.004	$(1.34 \pm 0.07) \times 10^{6b}$	$(1.02 \pm 0.21) \times 10^{6b}$	$(1.57 \pm 0.27) \times 10^{6ab}$	$(2.22 \pm 0.18) \times 10^{6a}$	$(2.33 \pm 0.20) \times 10^{6a}$
15	Furfural	98–01-1	38.76	859	93	96, 95, 39, 67, 97	<0.001	$(4.40 \pm 0.65) \times 10^{6a}$	$(4.35 \pm 0.47) \times 10^{6ab}$	$(3.46 \pm 0.19) \times 10^{6ab}$	$(2.54 \pm 0.29) \times 10^{6b}$	$(4.18 \pm 2.13) \times 10^{5c}$
16	1-hexanol	111–27-3	41.16	874	97	56, 55, 43, 69, 84	<0.0001	$(3.04 \pm 0.19) \times 10^{7b}$	$(2.85 \pm 0.20) \times 10^{7b}$	$(4.66 \pm 1.04) \times 10^{7b}$	$(4.69 \pm 1.35) \times 10^{7b}$	$(1.39 \pm 0.04) \times 10^{8a}$
17	N,N-diethylformamide	617–84–5	56.86	912	94	58, 101, 86, 44, 72	0.001	$(1.11 \pm 0.10) \times 10^{7a}$	$(9.27 \pm 0.32) \times 10^{6ab}$	$(6.48 \pm 0.52) \times 10^{6bc}$	$(5.39 \pm 0.49) \times 10^{6c}$	$(5.96 \pm 1.37) \times 10^{6bc}$
18	β-pinene	127–91-3	58.36	947	95	93, 77, 41, 136, 121, 105	<0.0001	$(1.01 \pm 0.10) \times 10^{7bc}$	$(7.29 \pm 0.78) \times 10^{6c}$	$(1.44 \pm 0.10) \times 10^{7b}$	$(1.46 \pm 0.17) \times 10^{7b}$	$(2.09 \pm 0.14) \times 10^{7a}$
19	2-heptenal	2463–63-0	59.08	957	92	83, 55, 41, 70, 112	<0.0001	$(6.18 \pm 0.79) \times 10^{6a}$	$(2.95 \pm 0.05) \times 10^{6b}$	$(2.49 \pm 0.06) \times 10^{6b}$	$(1.69 \pm 0.37) \times 10^{6b}$	$(1.55 \pm 0.28) \times 10^{6b}$
20	Dimethyl trisulfide	3658-80-8	60.16	959	88	126, 45, 79, 64, 111, 128	<0.0001	$(4.11 \pm 0.33) \times 10^{5a}$	$(3.58 \pm 0.12) \times 10^{5ab}$	$(2.68 \pm 0.17) \times 10^{5b}$	$(1.39 \pm 0.19) \times 10^{5c}$	$(8.85 \pm 0.15) \times 10^{4c}$
21	Benzaldehyde	100–52-7	61.01	960	98	106, 105, 77, 51	0.001	$(4.75 \pm 0.32) \times 10^{6ab}$	$(5.34 \pm 0.11) \times 10^{6a}$	$(5.02 \pm 0.21) \times 10^{6ab}$	$(3.68 \pm 0.50) \times 10^{6bc}$	$(2.12 \pm 0.61) \times 10^{6c}$
22	2,2,4,6,6-pentamethyl-3-heptene	123–48-8	61.87	–	92	57, 97, 69, 41, 112, 168, 153	<0.001	$(1.39 \pm 0.16) \times 10^{6c}$	$(9.71 \pm 1.20) \times 10^{5c}$	$(2.49 \pm 0.28) \times 10^{6ab}$	$(1.95 \pm 0.29) \times 10^{6bc}$	$(2.97 \pm 0.26) \times 10^{6a}$
23	Sulcatone	110–93-0	62.17	988	94	43, 108, 55, 69, 93, 126	0.058 /0.005	$(7.06 \pm 0.44) \times 10^{6a}$	$(8.30 \pm 0.34) \times 10^{6a}$	$(1.23 \pm 0.33) \times 10^{7a}$	$(1.07 \pm 0.19) \times 10^{7a}$	$(1.57 \pm 0.47) \times 10^{7a}$
24	α-terpinene	99–86-5	63.69	1012	94	121, 93, 136, 105, 77, 91	<0.0001	$(2.11 \pm 0.25) \times 10^{5c}$	$(1.23 \pm 0.13) \times 10^{5c}$	$(5.57 \pm 0.90) \times 10^{5bc}$	$(8.46 \pm 2.59) \times 10^{5b}$	$(1.97 \pm 0.02) \times 10^{6a}$
25	n-hexyl acetate	143–13-5	64.23	1014	87	97, 43, 55, 56, 69	0.060 /0.021	$(3.05 \pm 0.21) \times 10^{7ab}$	$(2.28 \pm 0.25) \times 10^{7b}$	$(3.46 \pm 1.51) \times 10^{7ab}$	$(3.74 \pm 0.49) \times 10^{7ab}$	$(5.70 \pm 1.05) \times 10^{7a}$
26	γ-terpinene	99–85-4	67.12	1074	92	93, 136, 121, 91, 77, 105, 43	0.005	$(6.14 \pm 0.58) \times 10^{5c}$	$(3.68 \pm 0.19) \times 10^{5c}$	$(2.05 \pm 0.90) \times 10^{6bc}$	$(1.54 \pm 0.23) \times 10^{6b}$	$(3.19 \pm 0.48) \times 10^{6a}$
27	Eicosane	112–95-8	87.92	2000	87	57, 43, 71, 85, 99	0.006	$(1.07 \pm 0.22) \times 10^{6b}$	$(1.42 \pm 0.04) \times 10^{6ab}$	$(1.64 \pm 0.01) \times 10^{6ab}$	$(1.14 \pm 0.26) \times 10^{6b}$	$(2.08 \pm 0.22) \times 10^{6a}$

Different superscript letters within the same row indicate significant differences in the abundance of each VOC among storage days, according to one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$).
* Significance refers to the probability of the ANOVA study. When two significance values are reported, the second value corresponds to the probability of the correlation with time.

significantly over time (except pentane, sulcatone, dimethyl sulfide, and n-hexyl acetate) in the one-way ANOVA analysis. However, subsequent correlation analysis demonstrated that the abundance of the above four exceptional compounds was significantly correlated with storage time, thereby justifying their inclusion in the further analysis.

The PCA analysis was carried out using the average peak areas of the selected VOCs (active variables) obtained from refrigerated meatball samples stored for 12 days with regular sampling points (active observations) as shown in Fig. 3. The plot is highly explanatory, since it retained 89.97% of the total variance of the system, with the first component (F1) accounting for 79.06% and the second component (F2) explaining 10.90% variance, indicating that the biplot provided a reliable representation of the dataset. A clear separation of the meatball samples according to the storage time can be observed along F1. The fresh sample (on day 0) and the sample stored for 3 days are clustered on the left side of the plot (green zone), which indicates similar characteristics of volatile profiles. In contrast, the sample stored for 12 days appears on the right side of the plot, representing the spoilage stage (red zone). Samples stored from days 6 to 9 are positioned between these two extremes (red, but close to the yellow zone). Overall, the PCA biplot reveals a clear opposition between fresh samples (negative F1 values) and spoiled samples (positive F1 values).

The active variables are also clearly separated into two groups according to their correlation with F1. Eight VOCs are strongly negatively correlated with F1, indicating that they decreased during storage. These include six aldehydes (hexanal, 2-methylbutanal, 3-methylbutanal, benzaldehyde, furfural, and 2-heptenal), dimethyl trisulfide (Brewer, 2009; Chen et al., 2019; Flores, 2018, 2023; Sun et al., 2021; Zang et al., 2020) and N,N-diethylformamide. These compounds are mainly associated with the volatile profile of food samples in the satisfactory stage (green zone), where microbial load is below 4 log CFU/g. Conversely, nineteen VOCs are strongly positively correlated with F1, indicating that their intensities increase during storage. These include four alcohols (1-pentanol, 1-hexanol, isoamyl alcohol, and 2,3-butanediol), four aliphatic hydrocarbons (pentane, 1-undecene, eicosane, and 2,2,4,6,6-pentamethyl-3-heptene), three terpene hydrocarbons (β-pinene, α-terpinene, and γ-terpinene), three sulfur compounds (dimethyl sulfide, allyl methyl sulfide and carbon disulfide), two ketones (acetone and sulcatone), one aromatic hydrocarbon (o-xylene), one acetal (1,1-diethoxyethane), and one ester (n-hexyl acetate). The increase in these compounds is associated with an unacceptable stage (red zone) of food samples, where the microbial load exceeds 6 log CFU/g.

Microbiological parameters and pH were also projected onto the PCA plot as supplementary variables to facilitate interpretation of the relationships among VOCs, pH, and microbial growth. The results show that pH is negatively correlated with F1, whereas microbiological variables are strongly positively correlated with F1, suggesting that microbial growth is associated with the formation of those VOCs positively correlated to F1 and maybe with the degradation of those negatively correlated to F1. The different microbial groups are relatively scattered throughout the upper-right quadrant of the PCA plane, highlighting a distinct relationship between specific microorganisms and the VOCs that increased during the storage trial.

The dendrograms generated from the cluster analysis along with a heatmap showing the relative levels of VOCs, microbial, and pH data, at different storage times are shown in Fig. 4. As expected, the eight VOCs that are negatively correlated with F1 in PCA (Fig. 3) are clustered together and decreased during storage, suggesting that they are labile

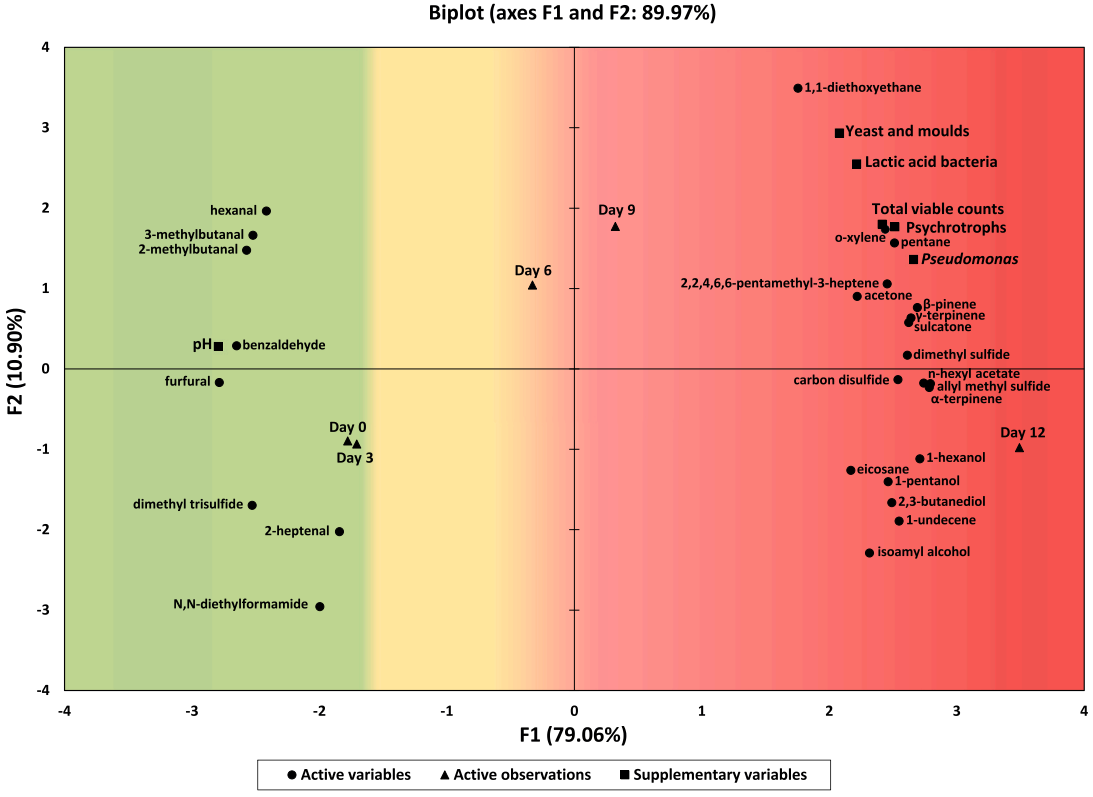

Fig. 3. Biplot corresponding to the first two dimensions of the principal component analysis (PCA), showing VOCs and their relationship with microbial growth over the storage time. Each data point represents the average value of three replicates ($n = 3$). The plot represents the projection of VOCs as active variables (●), five sampling points (days 0, 3, 6, 9, and 12) as active observations (▲), microbial parameters and pH are represented as supplementary variables (■). The background colour gradient denotes the progression of the samples from satisfactory (green) through acceptable (yellow), and to the unacceptable (red) stages of food as defined in the microbiological criteria section 2.4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

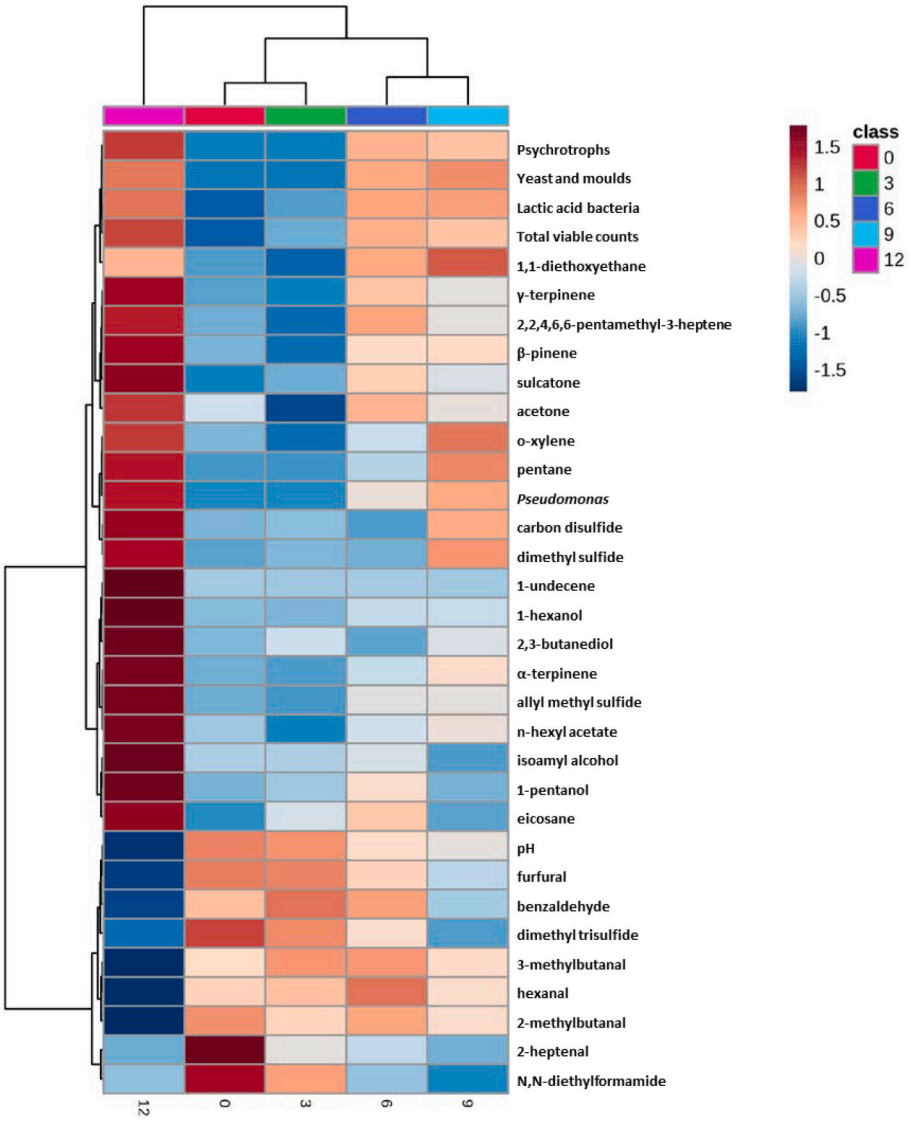

Fig. 4. Heatmap with hierarchical clustering shows the evolution of selected VOCs, microbial population and pH changes during the refrigerated storage of meatballs with almond sauce, where classes with different colours represent storage days, and the colour gradient from blue to red (low to high) indicates the relative abundance of each variable across the storage time. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

VOCs associated with freshly cooked food. Indeed, aldehydes like hexanal and 2-heptenal might arise from the lipid oxidation of fats during cooking, whereas Strecker aldehydes such as 3-methylbutanal and 2-methylbutanal are formed while amino acids react with sugar-derived compounds in the Maillard reaction (Flores, 2018). Other aldehydes, like benzaldehyde, are also detected in thermally processed meats (Chen et al., 2019; Pavlidis et al., 2019; Sun et al., 2021; Zang et al., 2020). Besides, furan compounds such as furfural form during the degradation of sugar molecules via Maillard reaction (Flores, 2023; Mottram, 1998), and dimethyl trisulfide might be generated through the thiamine degradation and sulfur-involving Maillard reaction (Brewer, 2009). Finally, N,N-diethylformamide was detected at very low levels and is likely a contaminant from the packaging materials. Most remarkably, seven out of the eight compounds are aldehydes or have an aldehyde function (N,N-diethylformamide is $(CH_3CH_2)_2$-N-CHO), and the eighth compound is a highly volatile trisulfide, which suggests that the decreasing trend is related to the chemical reactivity of these compounds. The aldehyde group is highly reactive, since it can be oxidised (to acid) or reduced (to alcohol) and has electrophilic properties, so that it can easily react with any chemical species having nucleophilic characteristics, such as glutathione (and any other thiol), lysine, sulphites or many polyphenols. On the other hand, dimethyl trisulfide is also an electrophile which can be easily reduced to methyl disulfide, dimethyl sulfide, methanethiol and hydrogen sulfide. It should be noted that bacterial reductases can carry out both the reduction of aldehydes and trisulfides. Furthermore, the clustering in Fig. 4 reveals that the above mentioned eight compounds followed three different evolution patterns: (i) 2-heptenal and N,N-diethylformamide followed a steady decrease from day 0, reaching a minimum in day 9; (ii) hexanal, 2-methylbutanal and 3-methylbutanal showed a slight increase until day 3, a decrease between days 6 and 9, and a dramatic decrease between days 9 and 12, and (iii) furfural, benzaldehyde and dimethyl trisulfide, together with pH, followed a steady decrease throughout the storage period. It should be observed that the second pattern is particularly consistent with transformations induced by microorganisms.

The nineteen VOCs that are positively correlated with F1 in PCA

(Fig. 3) are grouped into at least five different clusters (Fig. 4), suggesting the existence of five distinct patterns of temporal evolution as follows: (i) 1,1-diethoxyethane is the single VOC clustered with LAB, TVC, psychrotrophs, yeast and moulds and its temporal evolution is characterised by a strong increase between days 3 and 6. This compound has been reported to form through the reaction of ethanol and acetaldehyde, metabolites that can be produced by yeasts and bacteria (Hernández-Macedo et al., 2011; Hernández-Macedo et al., 2012; Papadopoulou et al., 2020; Raj et al., 2014; Stanborough et al., 2017; Thomson et al., 2005). Since this compound formation is favoured under acidic pH conditions, its parallel increase with the number of those microorganisms is consistent. (ii) the temporal evolution profile of γ-terpinene, 2,2,4,6,6-pentamethyl-3-heptene, β-pinene, sulcatone and acetone are characterised by a steady increase from days 3 to 12, with a plateau between days 6 and 9. (iii) o-xylene, pentane, carbon disulfide and dimethyl sulfide, together with *Pseudomonas* showed an overall increasing trend from days 3 to 12; however, carbon disulfide and dimethyl sulfide exhibited a slight decrease on day 6 before increasing thereafter. (iv) 1-undecene, 1-hexanol, 2,3-butanediol, α-terpinene, allyl methyl sulfide and n-hexyl acetate showed a slight increase before day 9, followed by a significant increase between days 9 and 12, and (v) isoamyl alcohol, pentanol and eicosane reached their maximum levels on day 12, while exhibiting a minimum value on day 9.

Following the previous discussion, several significant negative correlations were observed between biochemically related VOCs. In particular, hexanal exhibited a strong negative correlation with 1-hexanol ($r = -0.925$, $p < 0.05$), while 2-methylbutanal and 3-methylbutanal were negatively correlated with isoamyl alcohol ($r = -0.888$, $p < 0.05$ and $r = -0.879$, $p < 0.05$, respectively). Additionally, a significant negative correlation was observed between dimethyl trisulfide and dimethyl sulfide ($r = -0.924$, $p < 0.05$). These correlations support the hypothesis of an active role of bacterial and yeast dehydrogenase in reducing labile VOCs (aldehydes and trisulfides) in the food matrix (Pellissery et al., 2020).

Nevertheless, alcohols such as 1-hexanol, isoamyl alcohol, 1-pentanol and 2,3-butanediol might be formed via microbial carbohydrate metabolism and/or aldehyde-reduction pathways (Montanari et al., 2018; Ramírez & Cava, 2007; Yang et al., 2023). Sulfur compounds also increased with storage time, for example, dimethyl sulfide, allyl methyl sulfide, and carbon disulfide, which may arise from the microbial degradation of sulfur-containing precursors present in both meat and plant ingredients. The specific correlation between carbon disulfide, dimethyl sulfide and *Pseudomonas* is worth mentioning, as previous studies have reported sulfur metabolism and the production of volatile sulfur compounds by this bacterium (Danilo et al., 2009; Edwards et al., 1987; Kang et al., 2026). These sulfur compounds play a key role in the sensory perception of spoilage due to their very low odour thresholds and strong association with sulfury and putrid off-odours (Casaburi et al., 2015). Ketones, such as sulcatone and acetone, have also been reported as microbial volatiles in meat spoilage (Papadopoulou et al., 2020; Zareian et al., 2018). In contrast, hydrocarbons (o-xylene, pentane, 1-undecene, 2,2,4,6,6-pentamethyl-3-heptene, and eicosane) may originate from the food matrix itself (Bleicher et al., 2022) or from environmental or packaging contamination. Similarly, the increase of terpene hydrocarbons (β-pinene, α- and γ-terpinene) is more likely to be associated with ingredient-derived volatiles than with microbial spoilage. These compounds are typically associated with the intrinsic aroma of plant-based ingredients, including spices, vegetables and oils (Bui et al., 2025). Their progressive increase in these compounds during storage may be attributed to enhanced release resulting from the breakdown of the food matrix.

Finally, the results of the PLS-DA model (Fig. 5) helped to identify

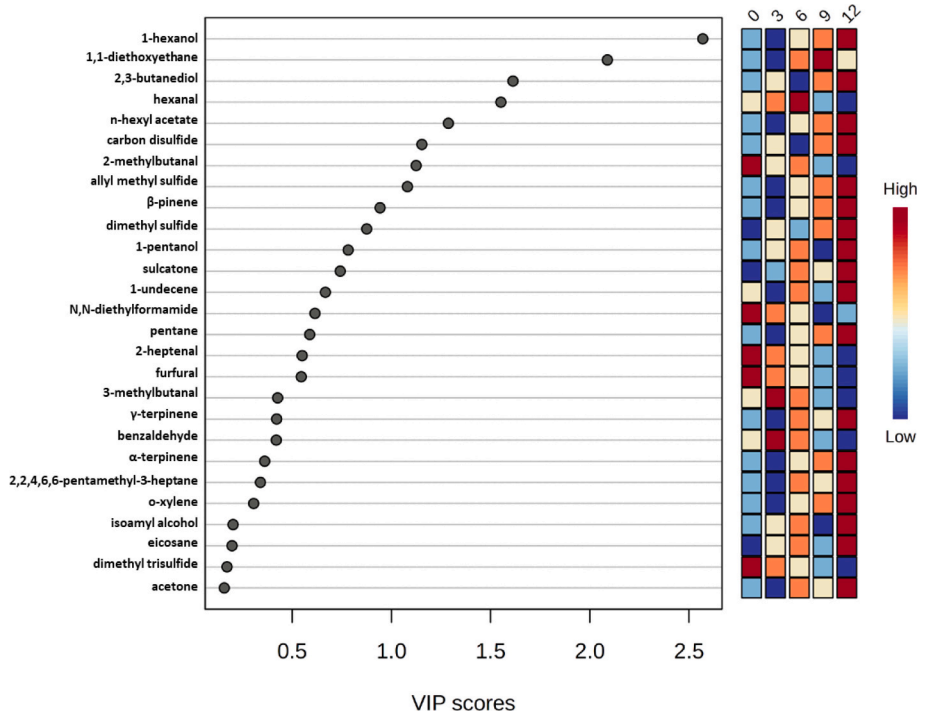

Fig. 5. Variable importance in projection (VIP) scores derived from the partial least squares - discriminant analysis (PLS-DA) model for the selected headspace VOCs in meatballs with almond sauce during the refrigerated storage at 6 °C. Grey circular points in the plot represent the corresponding VIP scores for each VOC, indicating their relative contribution to the sample discrimination. Variables with VIP scores greater than 1 are considered important contributors to the differentiation of samples. The square boxes on the right correspond to the storage days (0, 3, 6, 9, and 12), while the colour gradient from dark blue to dark red indicates a lower-to-higher relative abundance of VOCs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the most influential variables that contributed to the discrimination of VOC profiles. Among these, 1-hexanol, 1,1-diethoxyethane, 2,3-butanediol, n-hexyl acetate, carbon disulfide and allyl methyl sulfide had very high VIP scores in the PLS-DA model. All these compounds increased with storage time. Moreover, all compounds except allyl methyl sulfide have been previously associated with microbial spoilage, suggesting their potential relevance as indicators of microbiological quality deterioration of the food. However, in the case of allyl methyl sulfide, no microbiological metabolic pathway has been reported (Poirier et al., 2023). The compounds such as 1-hexanol and 2,3-butanediol have been reported in vacuum or air-packed meat products and have been associated with microbial metabolic pathways, including proteolytic activity, amino acid catabolism, methyl ketone reduction, and the reduction of aldehydes derived from lipid oxidation (Casaburi et al., 2015). Similarly, carbon disulfide has previously been reported as a spoilage associated VOC in vacuum-packed beef inoculated with *Serratia proteamaculans, Pseudomonas fragi, and Carnobacterium maltaromaticum* (Ercolini et al., 2009) and has also been identified during microbial spoilage of pork stored under low-oxygen packaging conditions (Chen et al., 2023). 1,1-diethoxyethane was likely formed through reactions involving acetaldehyde and ethanol, which might be generated by microbial activity. The accumulation of these precursors is typically associated with carbohydrate fermentation and aldehyde metabolism, while mildly acidic conditions favour the formation of this acetal (Capeletti et al., 2000). Finally, n-hexyl acetate has been identified as a microbial VOC in fermented pork meat, where microbial esterification activity might contribute to the ester formation (Zhao et al., 2023). This compound has also been reported as a product of yeast metabolism resulting from alcohol acetyltransferase-mediated esterification of C6 alcohol precursors. (Dennis et al., 2012). Conversely, hexanal and 2-methylbutanal also exhibited high VIP scores and, unlike the previously discussed compounds, these two compounds showed decreasing trends. These VOCs are primarily formed during cooking and retained during the early days of food storage. As previously discussed, their decrease is most likely attributable to microbiological metabolism. Consequently, these compounds may serve as freshness indicators of the refrigerated foods.

3.3. VOC ratios to assess freshness-to-spoilage transitions

The results presented above demonstrated that microbial growth in refrigerated food had a significant impact on the headspace VOC profiles. One of the most remarkable observations was that compounds associated with cooking gradually decreased and were replaced by microbial VOCs as storage days progressed. In chemical terms, VOC profiles shifted from the initial aldehyde-rich profiles to profiles enriched in alcohols, sulfur compounds and terpene hydrocarbons as storage progressed. In this context, the ratios of compounds that change (increase or decrease) during storage may serve as highly discriminant indicators of food deterioration. Based on this rationale, the results for the selected VOC ratios are presented in Figs. 6 and 7. The aldehydes/terpenes ratio, shown in Fig. 6a, reached a maximum at day 3 and decreased remarkably by day 6. Such a change was highly significant ($Z = -4.24$, $p < 0.001$), indicating a clear switching pattern in VOC profiles that coincided with the defined microbial threshold ($\geq$ 6 log CFU/g). Thus, the aldehydes/terpenes ratio may serve as a potential indicator of imminent spoilage in cooked meatballs. Terpenes, which are derived primarily from plant-based ingredients such as spices and oils and are chemically stable (Gu et al., 2023; Luca et al., 2023; Vichi et al., 2006), hence they might remain relatively stable throughout the storage trial. Nevertheless, several factors may explain the increase in terpenes observed in the present study. One possible explanation is the microbial hydrolysis of terpenic glycosidic precursors through glycosidase activity, whereby microorganisms utilise the sugar moiety as a primary energy source, leading to the release of terpenes (Muradova et al., 2023). Secondly, terpenes may have been retained within the fatty acids and oil during cooking (Falk et al., 1990; Fan et al., 2023), and subsequently, the microbial degradation of these fats may have facilitated their release. A third possible explanation is the gradual degradation of the spice matrix, which may also release terpenes.

The aldehydes/alcohols (Fig. 6b) and aldehydes/sulfur compounds (Fig. 6c) ratios exhibited a high discriminant capacity ($Z = -3.64$, $p < 0.001$ and $Z = -3.39$, $p = 0.001$, respectively) from days 9 to 12, when the food had already reached the spoilage stage. Consequently, these ratios seem to be more useful for confirming food spoilage rather than for predicting imminent spoilage.

In addition, some ratios between aldehydes and specific VOCs showed highly significant changes, as shown in Fig. 7. The aldehydes/1,1-diethoxyethane and aldehydes/2,3-butanediol ratios (**Figs. 7a** and **b,** respectively) showed pronounced changes from days 3 to 6 ($Z = -22.43$, $p < 0.001$ and $Z = 6.52$, $p < 0.001$, respectively), corresponding to the freshness-to-spoilage transition, with the food reaching the unacceptable microbiological threshold ($\geq$ 6 log CFU/g) on day 6. Therefore, these ratios may also serve as primary indicators for assessing imminent spoilage. Additionally, the hexanal/1-hexanol (Fig. 7c) and aldehydes/n-hexyl acetate (Fig. 7d) ratios showed highly significant changes between days 9 and 12 ($Z = 52.72$, $p < 0.001$ and $Z = -4.79$, $p < 0.001$, respectively), suggesting that they may also serve as potential indicators of spoilage progression from days 9 to 12.

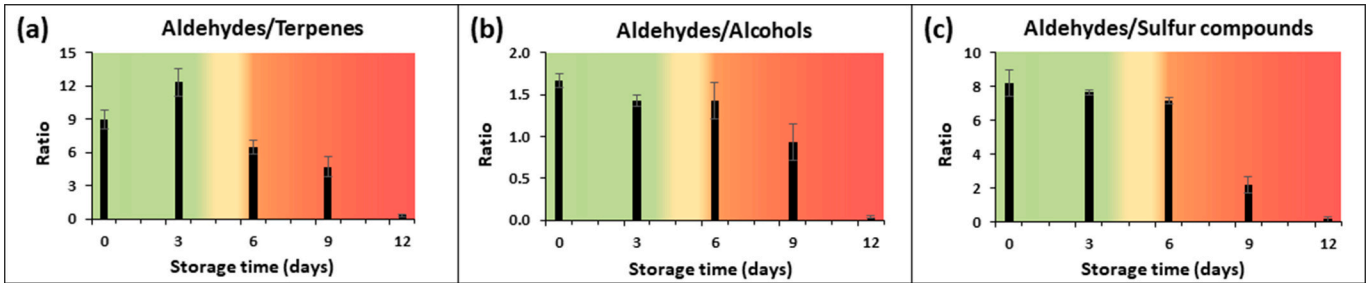

Fig. 6. Temporal evolution of ratios between the selected functional groups of VOCs in cooked meatballs with almond sauce during the refrigerated storage. Ratios of (a) aldehydes/terpenes, (b) aldehydes/alcohols, and (c) aldehydes/sulfurs are shown. The X-axis represents storage time in days, and the Y-axis shows the ratio of compounds. Each bar represents the mean value of three replicates with standard error. The background colour gradient denotes the progression of the samples from satisfactory (green) through acceptable (yellow), and to the unacceptable (red) stages of food as defined in the microbiological criteria section 2.4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

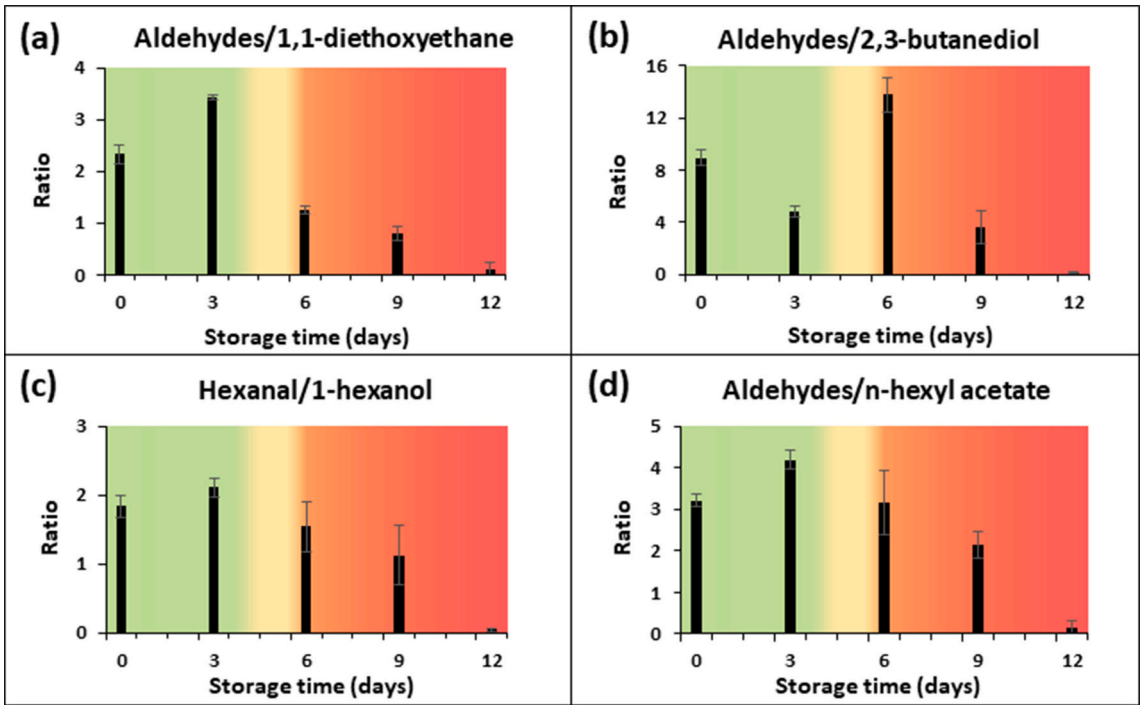

Fig. 7. Temporal evolution of selected VOC ratios during the refrigerated storage of cooked meatballs with almond sauce. Ratios of **(a)** aldehydes/1,1-diethoxyethane (b) aldehydes/2,3-butanediol **(c)** hexanal/1-hexanol and **(d)** aldehydes/n-hexyl acetate are shown. The X-axis represents storage time in days, and the Y-axis shows the ratio of compounds. Each bar represents the mean value of three replicates with standard error. The background colour gradient denotes the progression of the samples from satisfactory (green) through acceptable (yellow), and to the unacceptable (red) stages of food as defined in the microbiological criteria section 2.4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

This study highlights the potential of VOCs to indicate changes in the microbiological quality of cooked meatballs stored under realistic household refrigeration temperature. A clear shift in VOC profiles was observed during storage, with aldehydes predominating during the early stages as a result of the cooking process, followed by the accumulation of alcohols, sulfides, esters, acetal, and other metabolites associated with microbial growth at later stages of storage. Among these, 1-hexanol, 1,1-diethoxyethane, 2,3-butanediol, n-hexyl acetate, and carbon disulfide were identified as key discriminant VOCs. The aldehydes/1,1-diethoxyethane and aldehydes/2,3-butanediol ratios shifted significantly just before microbial development that reached spoilage levels ($\geq$ 6 log CFU/g), suggesting that these ratios may serve as potential indicators of imminent spoilage. These preliminary findings are based on a single production batch and therefore require further validation using additional production batches, different meatball formulations, and different storage conditions. Such validation will support the integration of VOC ratios into smart sensing systems for food freshness monitoring.

CRediT authorship contribution statement

Revathy Gurusamy: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Natalia Merino:** Writing – review & editing, Resources. **Ignacio Ontañón:** Writing – review & editing, Resources, Methodology, Formal analysis. **Jorge Alamán:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Rafael Pagán:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Vicente Ferreira:** Writing – review & editing, Supervision, Resources, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2026.120029.

Data availability

Data will be made available on request.

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