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ABSTRACT

Iron is a vital micronutrient for nearly all microorganisms, serving as a co-factor in critical metabolic pathways. However, cheese is an iron-restricted environment. Furthermore, it has been demonstrated that iron represents a growth-limiting factor for many microorganisms involved in cheese ripening and that this element is central to many microbial interactions occurring in this ecosystem. This study explores the impact of iron fortification on the growth and activity of a reduced microbial community composed of nine strains representative of the microbial community of surface-ripened cheeses. Three different iron compounds (ferrous sulfate, ferric chloride, ferric citrate) were used at three different concentrations, i.e., 18, 36, and 72 μM, to fortify cheese curd after inoculation with the consortium. This treatment significantly enhanced the growth of certain cheese-ripening bacteria in curd, resulting in substantial changes in the volatilome and metabolome profiles. These observations were dose-dependent, with more pronounced effects detected with higher iron concentrations. No statistically significant difference was observed in the microbial composition based on the iron compounds used for fortification, but this factor had an impact on the volatilome and amino acids profile. These findings highlight the importance of iron availability for the behavior of cheese microbial communities. They also open novel perspectives on cheesemakers' use of iron fortification to control microbial growth and improve cheese quality.

1. Introduction

Cheese is one of the most common dairy-fermented products. Several distinct types of cheese are produced worldwide and are unique in their microbial composition and manufacturing technology (Almena-Aliste and Mietton, 2014). Surface-ripened cheeses are characterized by the development of a dense microbial biofilm on their surface during ripening, which is responsible for their typical flavor (Mounier et al., 2005). These cheeses include both mold-ripened varieties, such as Camembert and Brie, and smear-ripened varieties, such as Munster, Livarot, Maroilles, Tilsit, Raclette, and Limburger. Each of these cheeses originates from different countries, including France, Belgium, and Switzerland (Brennan et al., 2004). The surface microbiota of these types of cheeses is generally composed of bacteria from the *Actinomycetota*, *Pseudomonadota*, and *Bacillota* phyla, as well as fungi, including yeasts and filamentous fungi (Mounier et al., 2005; Irlinger et al., 2015; Dugat-Bony et al., 2016).

The colonization ability of these microorganisms on the cheese surface depends on different factors, such as their capacity i) to use the main energy sources present in cheese (e.g., lactose, galactose, lactate, amino acids, proteins, lipids), ii) to tolerate specific abiotic conditions (e.g., presence of oxygen, low pH, cold temperature, high relative humidity, high salt content), iii) to acquire micronutrients such as iron (Monnet et al., 2015). Indeed, iron is essential for the growth and survival of many microorganisms, especially aerobic ones (Golonka et al., 2019). It serves as a co-factor in various cellular processes such as respiration, oxygen transfer, DNA synthesis, redox stress management, and metabolism (Andrews et al., 2003; Cornelis and Andrews, 2010; Andrews and Schmidt, 2007). Previous studies revealed that iron is a frequent growth limiting factor for various cheese microorganisms (Monnet et al., 2012; Albar et al., 2014). Indeed, iron concentration is low in the cheese environment since the natural concentration of iron in bovine milk is weak (0.09–0.90 $mg.kg^{-1}$) (Gaucheron, 2003). Moreover, iron bioavailability is limited due to a combination of factors: the presence of iron-chelating proteins such as lactoferrin (Jenssen and Hancock, 2009), a low iron diffusion index in the solid cheese matrix

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due to compact casein structure, and low iron availability at the cheese surface due to the presence of oxygen (Monnet et al., 2012).

Cheese microorganisms have adopted different strategies to cope with these iron-restricted conditions (Monnet et al., 2015). Many cheese isolates, including some strains belonging to *Glutamicibacter*, *Brevibacterium*, *Corynebacterium, Staphylococcus*, and *Penicillium* genera, produce high-affinity iron-chelating compounds, known as siderophores (Lankford and Byers, 1973), that help to scavenge iron from the cheese environment (Monnet et al., 2010; Schröder et al., 2011; Irlinger et al., 2012; Pham et al., 2017). Others can internalize siderophore-iron complexes produced by neighboring microorganisms through specific transporters such as ABC (Wilkens, 2015). Comparative genomic studies evidenced that the genome of some cheese microorganisms was enriched in genes involved in iron acquisition (siderophore production and transport) compared to their environmental relatives (Monnet et al., 2010; Pham et al., 2017; Bonham et al., 2017; Morin et al., 2018; Lebreton et al., 2020; Ye et al., 2024). Furthermore, recent research revealed the pivotal role of iron in several microbial interactions in cheese rinds (Kastman et al., 2016; Pham et al., 2019; Pierce et al., 2021). For example, co-culture experiments in a cheese model system revealed a mutualistic interaction between *Hafnia alvei* and *Brevibacterium aurantiacum* involving the exchange of siderophores (Pham et al., 2019). Kastman et al. (2016) also observed a bacterial-fungal interaction in cheese where *Scopulariopsis* fungi stimulated the growth of a weak competitor and slow colonizer, *Staphylococcus equorum*, by providing the staphyloferrin B siderophore and free amino acids. Another research study investigated 16 different bacterial-fungal interactions between eight different fungal species from five genera commonly found in cheese environment (*Penicillium*, *Debaryomyces*, *Scopulariopsis*, *Candida*, *Fusarium*) and two bacteria (*Escherichia coli*, *Pseudomonas psychrophila*). The results showed that all fungi modulated iron availability to bacterial species by siderophores production (Pierce et al., 2021). Considering these research findings, modifying iron concentration in cheese could directly impact the microbiology and biochemistry of the product. Until now, modification of iron levels in cheese has only been studied in the context of food fortification, which consists of the deliberate addition of nutrients (mainly vitamins, iron, zinc, and iodine) to food products to enhance their nutritional value and reduce the risk of malnutrition or related health problems in a target population (Allen, 2006). Because of their widespread consumption, milk and dairy products are considered good vehicles for iron fortification programs. Iron salts, particularly ferrous sulfate, are the most used iron fortificants in the dairy industry due to their easy solubility, low cost, and high iron bioavailability (Hurrell, 2021). However, iron salts are known as frequent iron ion donors that interact freely with milk components, leading to modifications in the structure of casein and potentially altering the sensory properties of the final product (Gaucheron, 2000).

The available research literature mainly explored the effect of iron fortification on cheese quality. The research results conflict with iron fortification towards physio-chemical, organoleptic properties, and iron bioavailability (Picciotti et al., 2022). Some studies show that iron fortification in Gouda, Cheddar, and Mozzarella cheese did not compromise the organoleptic properties of these cheeses (Zhang and Mahoney, 1990; Rice and McMahon, 1998; Gaucheron, 2005; Indumathi et al., 2013). On the contrary, negative impacts of iron fortification were observed on Cheddar and Feta cheese's composition, biochemical properties, and sensory profile (Jalili et al., 2017; Arce and Ustunol, 2018; Siddique and Park, 2019). The recommended optimal iron concentration for cheese iron fortification is 144 μM with minimal technological impacts (Jalili et al., 2017).

To the best of our knowledge, the effect of iron addition on the development of the cheese microbial community has not been evaluated. We hypothesized that iron addition disturbs the cheese microbiota in a dose-dependent manner, and different iron compounds result in different responses. To experimentally test this hypothesis, we fortified cheese curd and performed lab-scale fermentation using a reduced-microbial community representing the principal microorganisms of the cheese surface. Three iron compounds and three levels of iron concentrations were used, and we further evaluated the impact of these treatments on the microbial composition and the production of metabolites.

2. Materials & methods

2.1. Study design

Cheese curd was produced on a lab scale under aseptic conditions and inoculated with a reduced microbial community of nine strains mimicking the community of soft surface-ripened cheese and already used in previous research (Dugat-Bony et al., 2015; Dugat-Bony et al., 2016) (Fig. S1). The inoculated curd was fortified with three different compounds, i.e., ferrous sulfate, ferric chloride, and ferric citrate, at 18, 36, and 72 μM concentrations and compared to non-fortified curd (control). Previous work on a similar experimental setup showed that fortification with 1 mg/kg of $FeCl_3$, which corresponds to 18 μM of iron, impacted the growth of some ripening bacteria grown in co-culture with the yeast *Debaryomyces hansenii* (Monnet et al., 2012). We decided to use this concentration as the minimal concentration in our experiment and tried to increase it by 2 (36 μM) and 4 (72 μM) folds. Cheese curd was conditioned into small disposable containers (mini-model cheese system) and ripened for 28 days. Samples were then processed for microbial enumeration, pH measurements, volatile compounds analysis, free amino acids quantification and water-soluble metabolite profiling, to assess the overall impact of iron fortification.

2.2. Strain cultures and growth conditions

The nine strains used in this study, all originating from cheese, were *Brevibacterium aurantiacum* ATCC 9174, *Geotrichum candidum* ATCC 204307 (from the American Type Culture Collection, Rockville, MD), *Glutamicibacter arilaitensis* Re117 (= CIP 108037 from the culture collection of the Institut Pasteur, Paris, France), *Corynebacterium casei* 2 M01 (= UCMA 3821 from the UCMA culture collection of the University of Caen Normandy, France) *Staphylococcus equorum* Mu2, *Hafnia alvei* GB001, *Kluyveromyces lactis* 3550, *Debaryomyces hansenii* 304, *Lactococcus lactis* subsp. *lactis* S3+ and its protease-negative variant S3- (from the culture collection of INRAE SayFood Research Unit, Palaiseau, France). The lactic acid bacterium *L. lactis* subsp. *lactis* S3+ and its protease-negative variant S3- were cultured in M17 lactose (0.5 %) broth (Biokar Diagnostics, Beauvais, France) under static conditions for 24 h at 30 °C (first pre-culture) and then inoculated at 3 % in reconstituted skim milk (100 g/L, Difco Laboratories, Detroit, MI) and incubated for 16 h at 30 °C (second pre-culture). The pre-culture of the 5 ripening bacteria (*B. aurantiacum* ATCC 9174, *G. arilaitensis* Re117, *H. alvei* GB001, *C. casei* 2 M01 and *S. equorum* Mu2) was accomplished in 50 mL conical flasks with 10 mL of brain heart infusion (BHI) broth (Biokar Diagnostics, Beauvais, France) for 72 h at 25 °C on a rotary shaker at 180 rpm. Meanwhile, 50 mL conical flasks with 10 mL of potato dextrose (PDB) broth (Biokar Diagnostics, Beauvais, France) were used to cultivate the 3 yeast strains (*G. candidum* ATCC 204307, *D. hansenii* 304, *K. lactis* 3550) for 72 h at 25 °C on a rotary shaker at 180 rpm. The cultures were accomplished for bacterial and yeast strains in 250 mL conical flasks with 50 mL of brain heart infusion (BHI) broth (bacterial strains) and 50 mL of potato dextrose (PDB) (yeast strains) for 24 h at 25 °C on a rotary shaker at 180 rpm, after inoculation at a 1:100 (*v*/v) ratio with corresponding pre-cultures.

2.3. Curd production

Cheese curd was produced in a 14.2 L stainless Guerin tank (Sovatech, Cuiseaux, France). Full-fat pasteurized milk was mixed with skim milk to achieve a standardized fat content of 29.1 g/L. The Guerin tank,

placed under aseptic conditions, was filled with milk and pre-heated at 34 °C. After cooling at 28 °C, *L. lactis* subsp. *lactis* S3+ and S3− were added to the milk at a final concentration of 10^6 CFU/mL in addition to 14 mL of filter-sterilized $CaCl_2$ solution (10 % *w/v*). When pH reached a value of 6.3, 4.2 mL of filter-sterilized chymosin solution at 520 mg/L (Coquard, SA) was added to initiate the coagulation process. After 60 min at room temperature, the gel was cut into smaller pieces using a curd cutter with an approximate spacing of 16 × 16 mm. The drained curd (approximately 2 kg) was molded into a cylindrical press mold with dimensions of 200 mm in diameter, 150 mm in height, and 45 mm in thickness. It was kept at 20–22 °C for 24 h to allow maximum whey drainage. The demolded curd was wrapped in sterile plastic bags and stored at −20 °C until use.

2.4. Salting, inoculation, and iron fortification

The curd blocks were thawed at 4 °C for 24 h before use. For salting, 23.4 mL of a NaCl solution at 9 % (90 g/L) was added per 100 g of curd in order to achieve a final NaCl concentration of 1.7 %, and the mixture was homogenized in a sterilized Waring blender (Fisher Scientific, Elancourt, France) at 8000 rpm for 15 s. The cultures of the 5 ripening bacteria and 3 yeasts were centrifuged at 4500 ×*g* for 10 min at 4 °C. The cells were washed using physiological water (NaCl 9 g/L). The curd was inoculated at a final concentration of 10^6 CFU/g for ripening bacteria and 10^4 CFU/g for yeasts, except *G. candidum* ATCC 204307, which was inoculated at 10^3 CFU/g. The inoculated curd was divided into ten different lots prior to iron fortification with ferrous sulfate, ferric chloride, or ferric citrate. Then, each lot was homogenized using a sterile spatula in sterile plastic containers after the addition of 0.1 % (*v/w*) of a specific iron fortification solution (Table S1) or water for controls. The concentrations of iron fortification solutions were calibrated to achieve a final Fe concentration of 18, 36, and 72 μM in the curd (Table S1). The curd was finally transferred onto a circular plastic grid (diameter: 2.7 cm), which rested on a disposable cap (Kim-KapTM, DWK Life Sciences, Rockwood, TN, United States) in a disposable 40 mL sample container (GosselinTM, Dominique Dutscher, Brumath, France). The average cheese weight was approximately 6.5 g per sample. The mini-model cheeses were incubated at 21 °C for 24 h with 95 % relative humidity and then at 14 °C for ripening for 27 additional days. For each condition (iron compound and iron concentration), three mini-model cheeses were produced and used as replicates ($n = 3$).

2.5. Cheese sampling and analyses

Samples were analyzed after 28 days of cheese ripening, providing sufficient time for the principal phenomena of the ripening process to occur. Three mini-model cheeses were analyzed (n = 3) per condition and considered as replicates. Each mini-model cheese was completely recovered from the container and homogenized using a sterile spatula for subsequent analyses. Cheese pH was measured using an InLab® Surface pH electrode (Mettler Toledo, Viroflay, France).

2.5.1. Microbiological analysis

One gram of the sample was dispersed into 9 mL of physiological water (9 g/L NaCl) and homogenized using an Ultra-Turrax Homogenizer (T25Ika Model; Labortechnik, Staufen, Germany) at 24,500 rpm for 1 min. After serial dilutions in physiological water, microorganisms were enumerated by surface plating in duplicate on specific culture media. Three different media were used to enumerate the 9 inoculated strains. Furthermore, these strains are distinct based on their colony color and shape. The five ripening bacteria were enumerated on brain heart infusion agar (BHI, Biokar Diagnostics) supplemented with 50 mg/L Amphotericin B after 3 to 5 days of incubation at 25 °C under aerobic conditions and then transferred for exposure to natural light for colony pigmentation. Bacterial strains were differentiated by their different morphotypes. The three yeasts were enumerated on yeast-glucose-chloramphenicol agar (YEGC, Biokar Diagnostics) supplemented with 0.01 g/L tetrazolium chloride after 2 to 3 days of incubation at 25 °C under aerobic conditions. Yeast strains were differentiated by their different morphotypes. *L. lactis* subsp. *lactis* S3+ and S3- were enumerated on de-Man-Rogosa-Sharpe agar (MRS, Biokar Diagnostics) supplemented with 50 mg/L amphotericin B after 3 days of incubation at 30 °C under anaerobic conditions.

2.5.2. Volatile organic compounds profile

A Dynamic Headspace System (DHS) device combined with a Thermal Desorption Unit (TDU) interfaced with a Gas Chromatography/Mass Spectrometry (GC/MS) system was used to analyze volatile organic compounds. Three grams of each mini-model cheese sample was precisely weighed into a 20 mL glass vial placed in a melting ice container. The glass vial was sealed with a septum-equipped screw cap and stored at −80 °C until use. The frozen samples were thawed at 4 °C overnight before analysis. First, the samples were placed on a DHS plate at an initial temperature of 10 °C. Then, samples were incubated and agitated at 30 °C for 3 min for DHS extraction. The station then purged the sample's headspace by controlling helium gas flow through needles at 120 °C temperature. The helium gas volume was 300 mL, and the flow rate was 30 mL/min. The extracted compounds were trapped and concentrated on a temperature-controlled Tenax polymer at 30 °C. The water in the trap was removed by dry purging with 300 mL helium inert gas at 50 mL/min flow and a temperature of 30 °C.

For the desorption (TDU)-injection (CIS), the molecules were thermally desorbed from the trap in the TDU under inert gas scavenging coupled with the programmable thermal vaporization (PTV) type cool injection system (CIS) to control the concentration and vaporization of the compounds extracted from the samples. A transfer interface set at 300 °C was used between the TDU and the CIS for the duration of the desorption, which involves temperature programming of the TDU from 30 °C to 290 °C, followed by 7 min at 290 °C. Molecule transfer from the TDU to the CIS occurred continuously throughout this temperature program. During desorption, the molecules were cryofocused in the CIS, which was maintained at −100 °C. Following the 7 min desorption period at 290 °C, the CIS underwent a rapid temperature increase from −100 °C to 270 °C at a rate of 12 °C/s. This temperature ramp allowed the molecules to be sent and focused at the top of the capillary column.

For the gas chromatography (GC), the reconcentrated compounds were injected onto a polar capillary column (HP-Innowax, 60 m × 0.32 mm, 0.25 μm film thickness, PEG; Agilent Technologies, USA) swept by Helium at a constant flow rate (1.6 mL/min). The compound injection was done in splitless mode (leak closed for 1 min). These compounds were separated by implementing a chromatograph oven temperature program involving a series of temperature gradients. Initially, the program began at 40 °C for 5 min, followed by a gradual increase of 4 °C/min until reaching 155 °C. Subsequently, there was a shift to a gradient of 20 °C/min from 155 °C to 250 °C, maintained at 250 °C for 5 min. The mass spectrometer (MS) used was a single quadrupole analyzer. Ionization was performed in electron impact mode at 70 eV. ProteoWizard MSConvert software (Version 3.03.9393) converted raw data to mzXML files with the Peak Picking filter option. These files were submitted to the MetaboLights database under the accession number MTBLS10249. Then, data processing was performed using the W4M platform version 23.1.5. dev0 (Giacomoni et al., 2015) following the workflow available at: https://workflow4metabolomics.usegalaxy.fr/u/mahtab66/h/gc-ms esr10exp1.

Chromatographic peak annotation was performed by comparison with the NIST (Version 2.3) Mass Spectral Library (Gaithersburg, MD) based on retention time and mass spectra. Based on the annotations, manual curation was performed in order to remove compounds originating from the capillary column (impurities) and merge features with identical annotations. The chemical category of each compound was determined by expertise.

2.5.3. Quantification of amino acids and analysis of water-soluble metabolites

Ultra-high pressure liquid chromatography coupled with mass spectrometry (UHPLC/MS) was used to produce water-soluble metabolite profiles and quantify free amino-acids using untargeted and targeted approaches respectively. Two grams of cheese were sampled and stored at −20 °C until processing. After defrosting, samples were diluted with 20 mL of LC-MS quality water (Thermo Fischer Scientific, France) and homogenized using an Ultra-Turrax Homogenizer (T25Ika Model; Labortechnik, Staufen, Germany) at 24,000 rpm for 3 min. The homogenate was centrifuged for 30 min at 8000 ×*g* and 4 °C. The supernatant was loaded on a centrifugal filter unit with a molecular cutoff of 10 kDa (Vivaspin 20, Sartorius, Palaiseau, France) and centrifuged for 30 min at 8000 x*g* and 4 °C. The filtrate was then diluted 10 and 100 folds in 1 % formic acid and (Thermo Fischer Scientific, France) analyzed by ultra-high-performance liquid chromatography coupled with a high-resolution mass spectrometer (UHPLC Ultimate 3000 and HR-MS-Q EXACTIVE, Thermo Fischer Scientific, France). UHPLC conditions and data acquisition were the same as Pham et al. (2019) described, except the UHPLC Hypersil GOLD phenyl column length was 150 mm (Thermo Fisher Scientific, France). TraceFinder software version 3.3 (Thermo Fischer Scientific, Waltham, MA, USA) was used to detect and quantify amino acids according to the calibration solution. The full scan was also exploited in an untargeted way to compare the profiles of water-soluble metabolites between samples. Raw spectra were converted to mzXML files using ProteoWizard MSConvert software (Version 3.03.9393) and submitted to the MetaboLights database under the accession number MTBLS10372. Then, data pre-processing and processing were performed using the W4M platform version 23.1.5. dev0, following the workflows available at https://workflow4metabolomics.usegalaxy.fr/u/mahtab66/w/workflow-constructed-from-history-lc-msesr10exp1preprocessing and https://workflow4metabolomics.usegalaxy.fr/u/mahtab66/w/workflow-constructed-from-history-lc-msesr10exp1dataprocessing.

2.6. Statistical analyses

Standard statistical analyses were conducted using R software (R version 4.2.2). Data manipulation and visualization were done with a tidyverse collection of R packages (Wickham et al., 2019). The microbial viable count data were log-transformed, and a two-way analysis of variance (ANOVA) was performed to test the effect of iron fortification. Comparisons between iron compounds and iron concentrations were further analyzed using post hoc tests with Bonferroni correction as implemented in emmeans package version 1.10.2 (Lenth, 2024). Correlations between pH and microbial viable count were assessed with Pearson's product moment correlation coefficient. Principal component analysis, as implemented in the ade4 package version 1.7–22 (Chessel et al., 2004; Dray et al., 2007), was used to explore VOCs, amino acids, and water-soluble metabolites datasets. The Monte-Carlo test with 999 simulations was used to test the effect of iron fortification, and ANOVA simultaneous component analysis (ASCA) with 100 bootstraps, as implemented in the limpca package version 0.99.7 (Thiel et al., 2023), was used to assess further the contribution of both iron compounds and iron concentrations. Kruskal-Wallis and Wilcoxon tests, as implemented in the FSA package version 0.9.5 (Ogle et al., 2023), were finally used to identify statistical differences between sample groups at the level of individual features. Venn diagram was performed with the ggvenn package version 0.1.10 (Yan, 2023), while the heatmap was generated with the pheatmap package version 1.0.12 using Euclidean distances and the “ward.D" clustering method (Kolde, 2019).

3. Results

3.1. Effect of iron fortification on the growth of microorganisms in the model cheese

The growth of the different microorganisms was evaluated by viable plate count after 28 days of ripening, and the surface pH was measured as well (Fig. 1). Regarding ripening bacteria, iron addition significantly increased the viable count of both *Glutamicibacter arilaitensis and Brevibacterium aurantiacum* as compared to the control condition ($p < 0.05$; ANOVA test; Fig. 1A; Fig. 1B), regardless of the iron compounds. Moreover, this increase was dose-dependent ($p < 0.05$; post-hoc test with Bonferroni correction), reaching almost 5 Log CFU/g for *G. arilaitensis* and more than one Log CFU/g for *B. aurantiacum,* with the highest iron concentration. Higher iron concentrations from FeCitrate, $FeCl_3$, and $FeSO_4$ significantly increased the growth of *Corynebacterium casei* compared to the control ($p < 0.05$; ANOVA test; Fig. 1C). The most pronounced growth increases were observed at the highest concentration of 72 μM for all three iron compounds. This effect was again dose-dependent ($p < 0.05$; post-hoc test with Bonferroni correction).

Regarding the lactic acid bacterium *Lactococcus lactis,* we observed a slight but significant increase in the viable count with all iron compounds ($p < 0.05$; ANOVA test; Fig. 1D). This increase was more pronounced with $FeCl_3$ than with other iron compounds and was dose-dependent ($p < 0.05$; post-hoc test with Bonferroni correction). Finally, iron addition did not significantly affect the final count of *Staphylococcus equorum* and *Hafnia alvei* ($p > 0.05$; ANOVA; Fig. 1E and F). The growth of filamentous yeast included in the reduced microbial community, i.e., *Geotrichum candidum,* remained unaffected by iron fortification in the tested conditions, regardless of the iron compounds and concentrations ($p > 0.05$; ANOVA test; Fig. 1G). Meanwhile, the growth of *Kluyveromyces lactis* significantly increased with the highest iron concentrations from FeCitrate and $FeSO_4$ but not from $FeCl_3$ ($p < 0.05$; ANOVA test; Fig. 1H). In contrast, the count of *Debaryomyces hansenii* was below the detection limit after 28 days of cheese ripening.

Overall, all these results revealed that iron fortification profoundly impacts the behavior of the reduced microbial community used in our model cheese system, especially by stimulating the growth of *Lactococcus lactis* and, more importantly, of all ripening bacteria belonging to the *Actinomycetota* phylum (*B. aurantiacum*, *G. arilaitensis,* and *C. casei*). This difference in the growth of certain members of the tested microbial community was accompanied by an increase in the final pH values measured on the model-cheese surface (Fig. 1I). In fact, for these four strains we found a positive and significant correlation between bacterial growth and pH ($r > 0.65$, $p < 0.005$).

3.2. Effect of iron fortification on the VOCs profile

DHS-TDU-GC/MS was applied to analyze VOCs in control and iron-fortified experimental cheeses after 28 days of ripening. The GC–MS data set with 62 VOCs (Table S2) was classified into eight chemical categories: sulfur compounds, nitrogen compounds, ketones, hydrocarbons, esters, ethers, aldehydes, and alcohols. As illustrated in Fig. 2A, we observed an augmentation in the total peak area of VOCs in the iron-fortified samples relative to the controls. This finding implies a substantive increase in the global concentration of VOCs following iron fortification treatment. More specifically, sulfur, alcohols, and aldehyde categories were significantly higher in iron-fortified cheeses compared to the controls ($p < 0.05$; Wilcoxon test).

We next applied principal component analysis (PCA) to explore the difference in the VOC profiles between control and iron-fortified samples. The corresponding score plot revealed the separation between iron-fortified cheeses and controls according to the first two principal components, which exhibited a cumulative explained variance of nearly 47 % (Fig. S2). The Monte-Carlo test computed from the VOCs profiles statistically confirmed the difference between the two groups ($p < 0.05$,

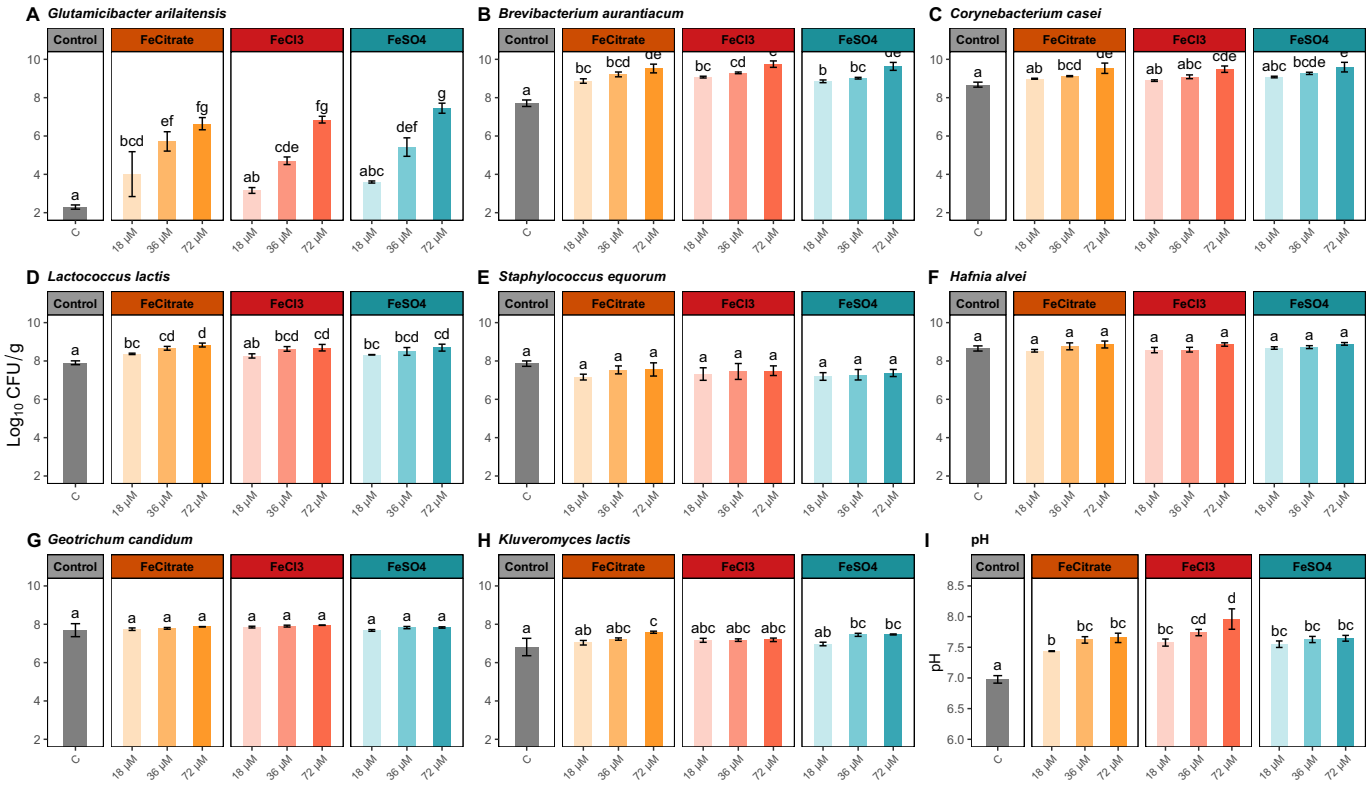

Fig. 1. Effect of iron fortification on microbial viable counts and pH in cheese after 28 days of ripening. (A) *Glutamicibacter arilaitensis.* (B) *Brevibacterium aurantiacum.* (C) *Corynebacterium casei.* (D) *Lactococcus lactis.* (E) *Staphylococcus equorum.* (F) *Hafnia alvei.* (G) *Geotrichum candidum.* (H) *Kluyveromyces lactis.* (I) pH values. Grey: control cheese without iron addition. Orange: cheese samples fortified with ferric citrate. Red: cheese samples fortified with ferric chloride. Metallic green: cheese samples fortified with ferrous sulfate. The color intensity increases with iron concentration. The values correspond to the average of three replicates (expressed in Log_{10} CFU/g for microbial counts), and the error bars represent the standard deviation. Different letters above the bars indicate a significant difference between the mean values according to the post-hoc test with Bonferroni correction ($p < 0.05$).

$n = 999$ simulations).

Forty-three VOCs, representing 69.35 % of the total compounds, were detected in both control and iron-fortified samples. Among these, twenty-nine VOCs (nearly 47 % of the forty-three commonly detected VOCs) were present in all sample conditions, i.e., fortified with all three iron compounds ($FeSO_4$, $FeCl_3$, FeCitrate) and in control as well (Fig. 2B). In contrast, nineteen VOCs (i.e., 31.15 % of the total compounds) were absent in the controls (Table S2), indicating that iron fortification increased the diversity of volatile compounds produced by the microbial community. These nineteen VOC compounds include three nitrogen, six esters, five sulfur, two alcohol, one ketone, and two hydrocarbon volatile compounds (Fig. 2C).

The flavor profiles of the main compounds positively influenced by iron fortification was quite broad, ranging from fruity to cheesy/dairy notes (Table S3). Iron fortification increased the diversity of VOCs belonging to sulfur and esters chemical categories, contributing to the organoleptic properties of cheese. For example, dimethyl trisulfide (DMTS) and dimethyl disulfide (DMDS) are two volatile sulfur compounds (VSCs) found in surface-ripened cheeses that have cheesy, cabbage and garlic-like notes (Landaud et al., 2008). Iron fortification significantly increased the peak intensity of these two compounds when compared to the controls ($p < 0.05$, Wilcoxon test) (Fig. 3A). The production of other VSCs – thioesters - associated with cheesy notes (Berger et al., 1999) was also promoted by iron fortification, especially *S*-methyl thioacetate (MTA) (cabbage, cheese, garlic, sulfur notes), *S*-methyl thiopropane thioate (MTP) (cheese, meat notes) and *S*-methyl 3-methylbutanethioate (dairy note) (Table S3). The production of several esters, which are generally associated with fruity notes, were increased by iron fortification, the major ones being ethyl acetate – associated with aromatic, brandy, and grape notes – together with propyl-butanoate – associated with apricot, fruit, pineapple, solvent notes (Fig. 3B). The production of several alcohols, namely ethanol, 1-propanol (alcohol, candy, pungent), and 3-methyl-1-butanol (= isoamyl alcohol) (burnt, cocoa, floral, malt notes), were also significantly influenced by iron fortification (Fig. 3C).

Given the notable impact of iron fortification on the VOC profiles of cheese, our next objective was to assess whether this effect varied according to the iron compounds or concentrations. PCA was therefore conducted to investigate the clustering of samples based on iron compounds and concentrations. The PCA score plots revealed some degree of overlap between the different iron compounds and concentrations (Fig. 4A, B). We applied the ANOVA Simultaneous Component Analysis (ASCA) cumulative approach to evaluate the statistical significance of these two factors. The results revealed that both iron concentration and iron compounds contributed significantly (Bootstrap *p*-value <0.05) to the differences observed within the iron-fortified samples (Fig. 4C). Following the identification of the significant impact of iron concentrations and iron compounds, we executed the Kruskal-Wallis test to validate the differences among the three iron concentrations (18 µM, 36 µM, and 72 µM) as well as among three iron compounds (FeCitrate, $FeCl_3$, $FeSO_4$). The Kruskal-Wallis test confirmed a significant difference between iron concentrations ($p < 0.05$), and a similar trend was observed between iron compounds ($p < 0.05$). The peak area of eight compounds was significantly influenced by iron concentration (Kruskal-Wallis, $p < 0.05$), i.e. 3-methyl-1-Butanol, Phenylethyl Alcohol, 2-Heptanone, 3-methyl-Butanal, Ethanol, Benzene ethanethioic acid, *S*-methyl ester, n-Hexane, and Acetone (Fig. S3). Additionally, 19 compounds were significantly different according to the iron compounds used for fortification (Kruskal-Wallis, $p < 0.05$) including 7 ketones, 3 hydrocarbons, 3 esters, 3 nitrogen compounds, 2 sulfur compounds and 1

alcohol (Fig. S4).

3.3. Effect of iron fortification on water-soluble metabolites

Having observed the impact of iron fortification on the VOCs profile in cheese, we wondered if the same applies to other types of metabolites. Therefore, we employed an untargeted metabolomics approach using the UHPLC/MS technique to profile water-soluble metabolites in the same samples. A total of 2669 ions were detected across whole chromatograms. The resulting metabolomic profiles were subjected to PCA to explore the possible impact of iron fortification. The PCA results indicated a partial overlap but a general trend towards separation between control and iron-fortified samples along PC1 and PC2, which collectively accounted for nearly 45 % of the total variance (Fig. 5A). This separation was statistically significant, as confirmed by a Monte-Carlo test ($p < 0.05$, $n = 999$ simulations). Focusing on iron-fortified samples, we next applied the ASCA method, which revealed a non-significant contribution of iron concentration and iron compound ($p > 0.05$) (Fig. 5B).

Using the same data, we also investigated the proteolysis efficiency by performing a targeted analysis with standards for the principal amino acids. In total, we were able to detect and quantify 15 of them in our samples. Iron fortification did not significantly increase the total concentration of amino acids in cheese after 28 days of ripening ($p > 0.05$; Wilcoxon test) (Fig. S5). To compare the amino acid profiles of iron-fortified and control samples, we conducted PCA, which revealed a clear separation between the two groups (Fig. 6A). The first two principal components explained 33 % and 21 % of the total variance. This difference was statistically confirmed by a Monte-Carlo test ($p < 0.05$, $n = 999$ simulations). ASCA was applied to investigate further the statistical contribution of the iron compound and iron concentration to the profile observed in iron-fortified samples. The results showed a slight but significant effect of iron compound only (Bootstrap $p < 0.05$) (Fig. 6B). A Kruskal-Wallis test validated the differences among the three iron compounds ($FeCl_3$, $FeSO_4$, and FeCitrate) ($p < 0.05$) and revealed that the concentration of 9 amino acids was significantly different according to this factor (Fig. S6).

We represented the amino acid profile through a heatmap (Fig. 6C) to observe differences in our samples. We found that four amino acids, namely lysine, L-glutamic acid, methionine, and L-tyrosine, exhibited significant differences between control and iron-fortified samples ($p < 0.05$; Wilcoxon test). Specifically, L-glutamic acid, methionine, and L-tyrosine concentrations increased in iron-fortified samples, while lysine decreased (Fig. 6D).

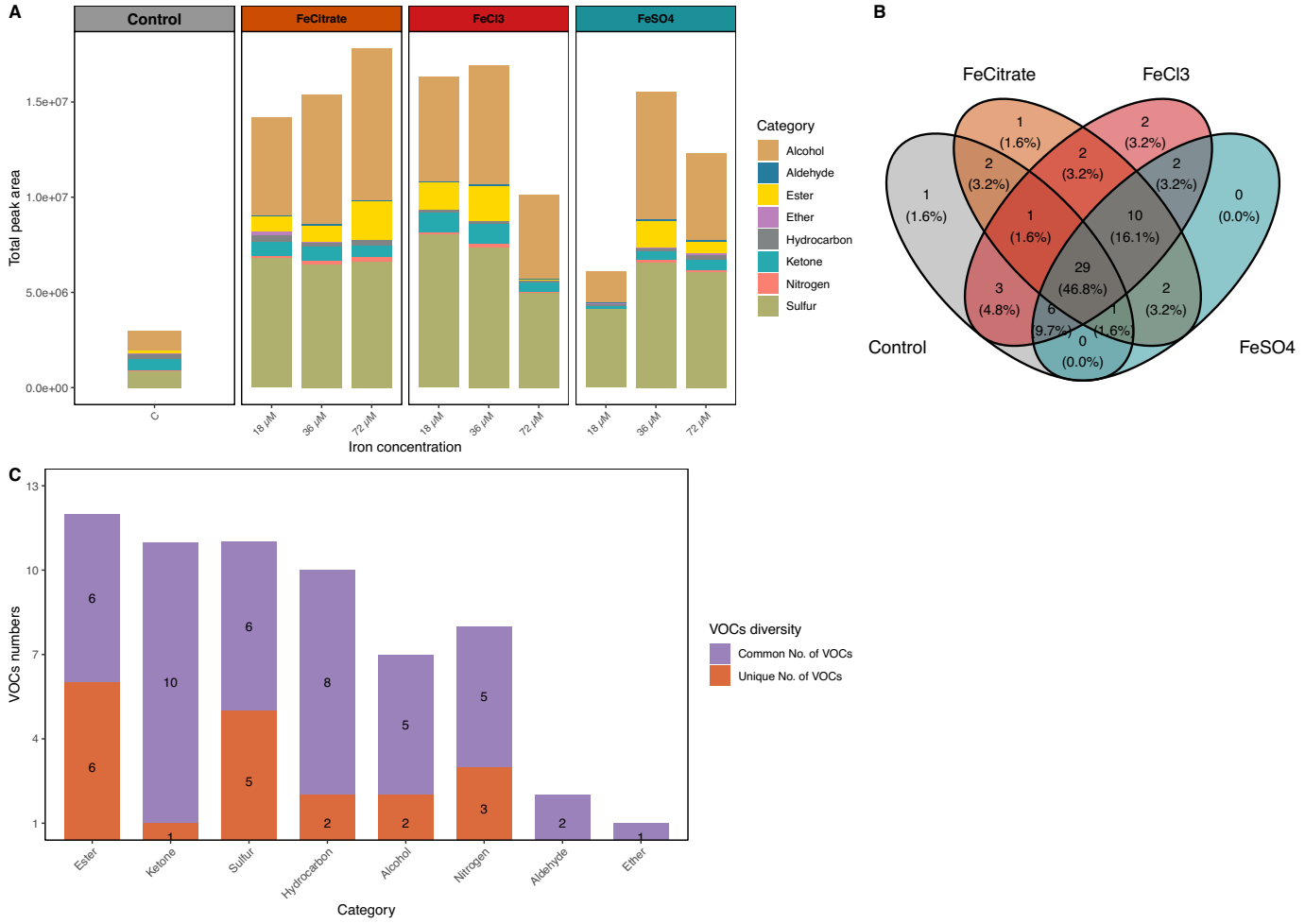

Fig. 2. Effect of iron fortification on volatile organic compounds in cheese after 28 days of ripening. (A) Combine stacked bar plot representing the total peak area of VOC chemical categories in control and iron-fortified samples with three iron compounds (FeCitrate, $FeCl_3$, $FeSO_4$). Each bar represents the average values of three biological replicates. (B) Venn diagram showing the detection of the 62 VOCs in control cheeses and cheeses fortified with the three different iron compounds. Grey: control cheese without iron addition. Orange: cheese samples fortified with ferric citrate. Red: cheese samples fortified with ferric chloride. Metallic green: cheese samples fortified with ferrous sulfate. (C) Stacked column bar graph depicts the number of VOCs per chemical category common between iron-fortified cheeses and controls or unique to iron-fortified cheeses.

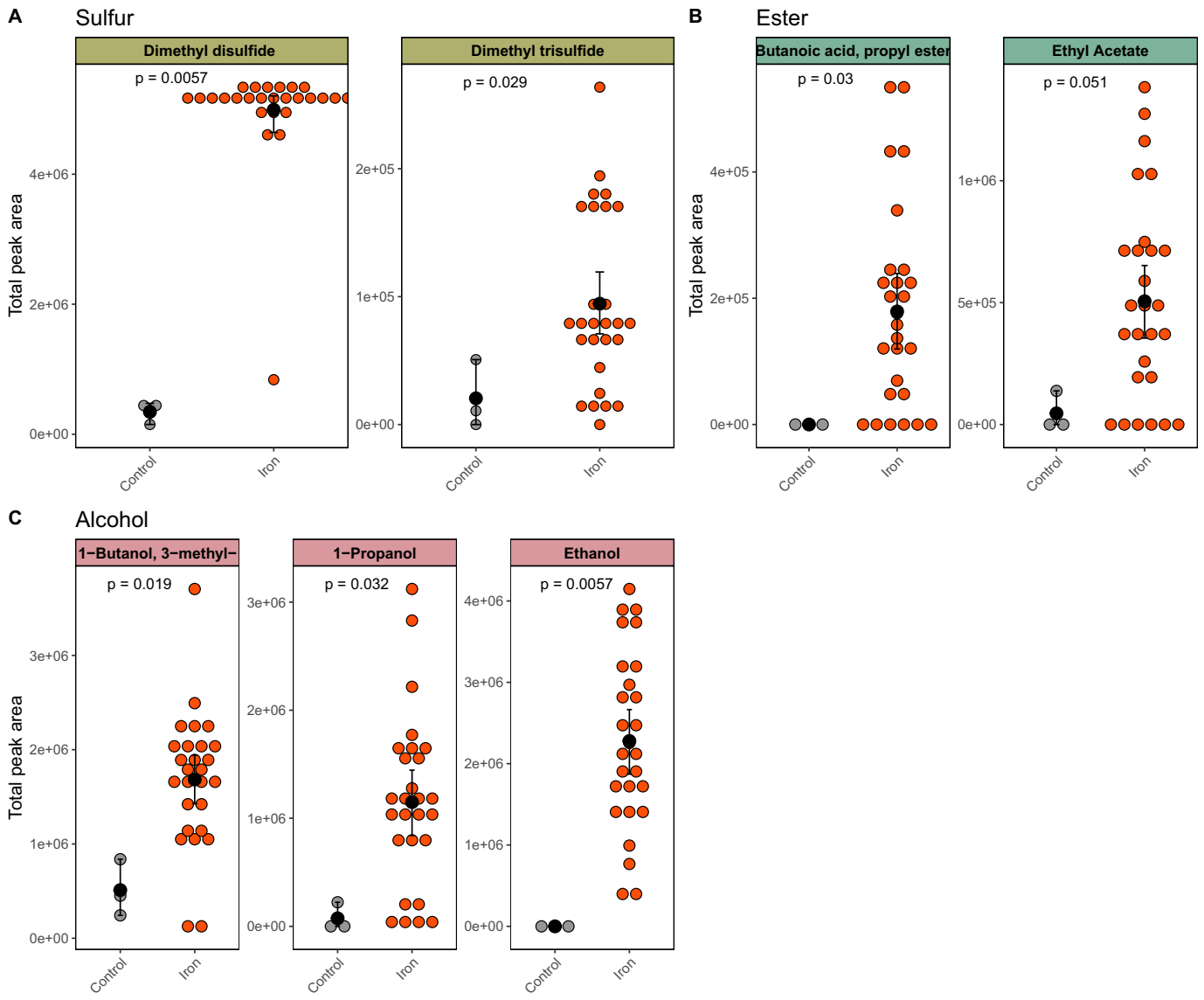

Fig. 3. Effect of iron fortification on key volatile organic compounds of cheese flavor. (A) Dot plots showing the total peak area values of two specific volatile sulfur compounds (DMTS, DMDS) in control versus iron-fortified cheese samples. (B) Dot plots displaying the total peak area values of butanoic acid, ethyl ester, and butanoic acid, propyl ester, in the same sample groups. (C) Dot plots illustrating the total peak area values of 3-methyl-1-butanol, 1-propanol, and ethanol in control versus iron-fortified cheese samples. The *p*-value was obtained by comparing the average values between the two groups using a Wilcoxon test.

4. Discussion

In this study, we observed a strong effect of iron fortification on the behavior of a 9-strain microbial community, mimicking that of a surface-ripened cheese grown in cheese curd. Specifically, iron fortification significantly increased the growth of L. *lactis*, the sole LAB present in the studied system, as well as all the ripening bacteria belonging to the *Actinomycetota* phylum, namely *G. arilaitensis, B. aurantiacum,* and *C. casei.* Comparable dose-dependent effects were obtained with the three tested iron compounds, i.e. $FeCl_3$, $FeSO_4$, and FeCitrate. In a previous study conducted on a simpler co-culture model consisting of one yeast (*D. hansenii*) and one ripening bacteria in the curd, supplementation with $FeCl_3$ or the siderophore deferoxamine B caused the stimulation of the growth of several strains of *Glutamicibacter arilaitensis*, *Brevibacterium aurantiacum,* and *Corynebacterium* spp. (Monnet et al., 2012). This experiment was also performed using *G. arilaitensis* Re117, one of the strains we used in the present study, leading to its growth stimulation (Monnet et al., 2010). Genomic studies have shown that several cheese-ripening *Actinomycetota*, including members of the genera *Glutamicibacter*, *Brevibacterium*, and *Corynebacterium* possess several genes involved in iron acquisition such as siderophore biosynthesis gene clusters, ABC transporters, related to iron uptake, Mn^{2+}/Fe^{2+} transporters, EfeUOB transporters, and IrtA/B-like iron transport components (Monnet et al., 2015; Monnet et al., 2010; Noordman et al., 2006; Schröder et al., 2011; Pham et al., 2017). However, it is well acknowledged that the expression of iron uptake systems, particularly siderophore biosynthesis, is costly for microbial cells and is often tightly regulated by the availability of iron (Hider and Kong, 2010). Previous work on cheese ripening bacteria also evidenced that iron addition reduces the expression of genes involved in siderophore biosynthesis, as well as those encoding components of the iron-siderophore ABC transport system (Monnet et al., 2012). Based on this, we hypothesized that the increased iron availability through iron fortification represses the expression of these systems, which could, in turn, enhance the fitness of such bacteria in our experimental conditions. Iron acquisition genes are also frequently exchanged through horizontal gene transfer (HGT) within cheese-associated *Actinomycetota*, sometimes as part of large genomic regions such as actinoRUSTI, an island of ~47 kbp containing

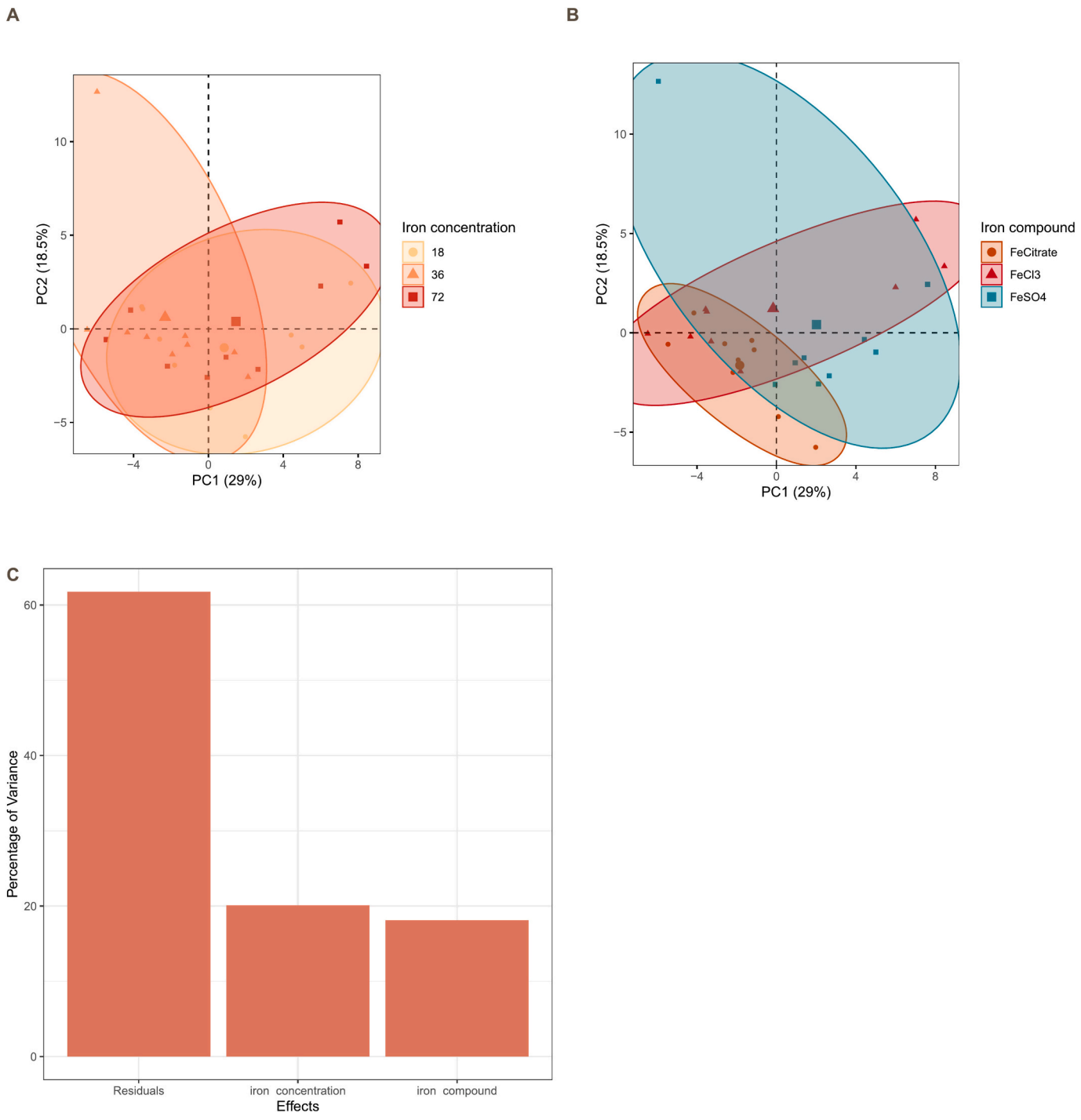

Fig. 4. Effect of iron fortification on volatile organic compounds (VOCs) profile in cheese after 28 days of ripening. (A) PCA score plot showing the effect of iron concentration on the VOCs profile of iron-fortified samples. (B) PCA score plot showing the effect of iron compounds (FeCitrate, $FeCl_3$, $FeSO_4$) on the VOCs profile of iron-fortified samples. (C) Bar graph describing the contribution of iron compounds and concentrations to the variance in iron-fortified samples, based on ANOVA simultaneous component analysis (ASCA).

several genes encoding a siderophore import complex (Bonham et al., 2017). Such HGT events represent a major molecular mechanism involved in bacteria's evolution and adaptation to new ecological niches (Wiedenbeck and Cohan, 2011). Iron acquisition may provide a competitive advantage to *Actinomycetota* for development in iron-restricted environments such as cheese.

The production of volatile organic compounds (VOCs) in cheese is intricately linked to microbial biodegradation activity towards lactose, lactate, citrate, proteins, and lipids (Bertuzzi et al., 2018). We observed that iron fortification significantly increased both the quantity and diversity of VOCs detected in our model-cheese system, suggesting greater microbial activity in iron-fortified samples, possibly due to higher microbial growth of certain ripening bacteria. In particular, the production of sulfur compounds such as dimethyl trisulfide (DMTS) and dimethyl disulfide (DMDS) was enhanced upon iron fortification. These compounds, which are well-known odor-active compounds providing garlic and cabbage notes typically found in surface-ripened cheese, originate from the catabolism of methionine (Landaud et al., 2008).

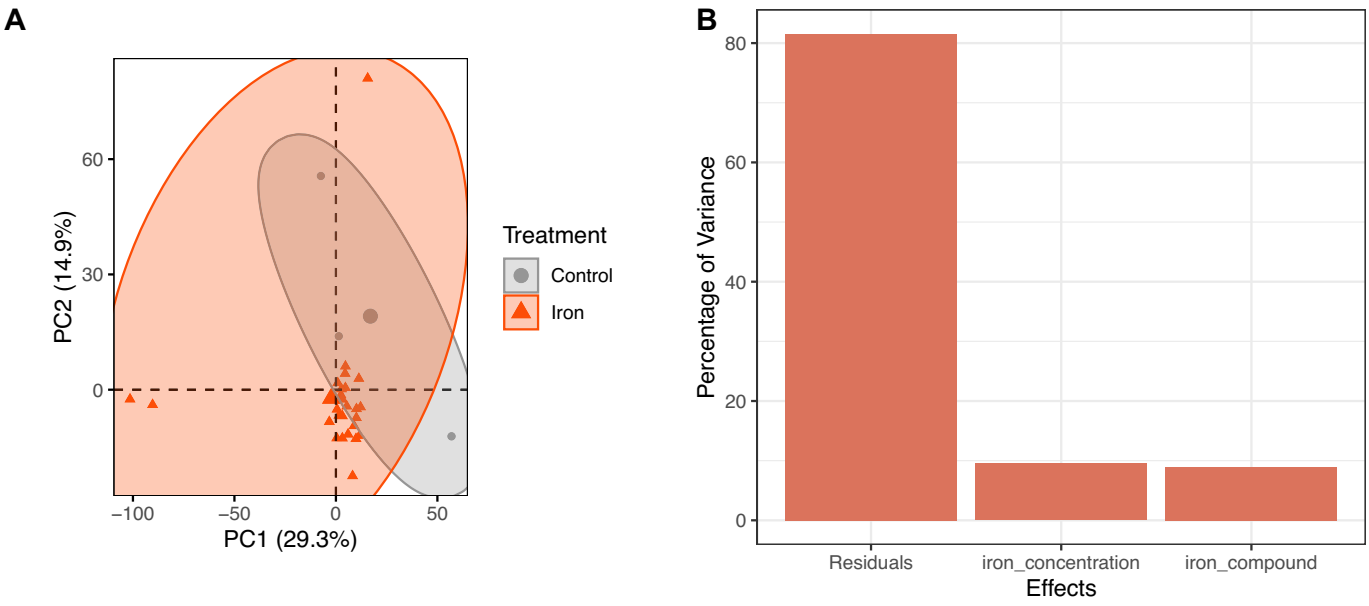

Fig. 5. Effect of iron fortification on untargeted water-soluble metabolites profile detected in cheese after 28 days of ripening. (A) PCA score plot showing the effect of iron fortification on water-soluble metabolite profiles (B). Bar plot illustrating the contribution of iron compounds and concentrations to the water-soluble metabolites data variance in iron-fortified samples, based on ANOVA simultaneous component analysis (ASCA).

Brevibacterium aurantiacum, whose growth was strongly stimulated by iron fortification in our experiment, is a typical orange-red pigmented bacteria that is widely used by cheesemakers as an adjunct culture for surface-ripened cheese production (Irlinger et al., 2017). This species possesses the L-Methionine-γ-lyase (MGL) enzyme catalyzing the conversion of methionine into methanethiol (MTL) (Amarita et al., 2004), a common precursor for DMTS, DMDS, and *S*-methyl thioesters. The production of *S*-methyl thioesters, namely MTA, MTP, and *S*-methyl 3-methylbutanethioate, is also increased by iron addition. The biosynthesis of *S*-methyl thioesters results from the reaction of MTL with acyl-CoA compounds (Hélinck et al., 2000), suggesting that acyl-CoA compounds, produced from the microbial community metabolism, are respectively acetyl-CoA, propionyl-CoA and 3-methylbutyryl-CoA. The production of esters, mainly contributing to fruity notes, was also increased through iron fortification. A major contributor to ester biosynthesis in the microbial community is the yeast *K. lactis* (Arfi et al., 2002). The most influenced esters by iron fortification are ethyl-acetate and propyl-butanoate, the alcohol moiety being ethanol and propanol respectively, whose production is also enhanced by iron fortification. Another alcohol, isoamyl alcohol, is also favorably influenced by iron fortification. This alcohol, along with other alcohols, is produced by yeasts as fuel oils and results from amino acid catabolism through the Ehrlich pathway (Hazelwood et al., 2008). Owing to oxidative conditions, alcohols produced by the microbial community can possibly be oxidized to the corresponding acids, which could serve, in turn, as possible precursors for other VOCs like methyl thioesters (e.g., *S*-methyl 3-methylbutanethioate, MTP).

We observed that both iron concentration and the type of iron compound used for fortification (FeCitrate, $FeSO_4$, or $FeCl_3$) led to distinct profiles of VOCs in cheese. Using different iron compounds also influenced the amino acid composition of the fortified cheese. Iron is involved in various metabolisms including of glucose, lipids, and amino acids degradation (Zhang et al., 2022) that contribute to aroma compound production. Moreover, iron plays a crucial regulatory role in the activity and expression of TCA cycle enzymes, such as mitochondrial aconitase and isocitrate dehydrogenase (Sun et al., 2023). An increase in iron concentration have been shown to enhance enzyme activity in several strains of *Propionibacterium* (Sakharova et al., 2022), an important bacterial genus for the dairy industry. The solubility and bioavailability of iron vary across different iron compounds due to their distinct chemical properties (Hurrell, 2021). These differences likely result in variations in microbial enzymatic activities, thereby influencing the production of metabolites. Casein, the primary substrate for proteolysis, is known to bind iron but the nature of this interaction depends on both the type of casein (αs1-, αs2-, β-, and κ-casein) and the specific iron compound (Gaucheron, 2000). We hypothesize that the variations observed in VOCs and amino acids profiles in our study when using different iron compounds for fortification, could be the result of the distinct biochemical properties of each iron compound. However, a dedicated study would be required to further characterize these effects.

The addition of iron to cheese is currently considered by the dairy industry only for a specific application, namely fortification. However, our results demonstrate that, in the case of surface-ripened cheeses, increasing the amount of available iron significantly impacts the microbial community composition, particularly with dose-dependent and selective stimulation of the growth of certain ripening bacteria and, consequently, the metabolic profiles of the product. These initial observations thus pave the way for a new application of iron in cheesemaking to control the development of the microbial ecosystem better, ultimately providing cheesemakers with a new tool to master the expression of certain functionalities. However, the outcomes of iron fortification on cheese microbial communities may be affected by various biotic and abiotic factors, including cheese specific microbial strains, milk iron levels, cheese type, production methods, and ripening conditions such as relative humidity, temperature, and oxygen availability. Among the potential impact of using iron fortification, we observed an increase in the abundance of key cheese ripening bacteria, resulting in a stronger matrix degradation and a higher diversity and quantity of volatile compounds. This would therefore modify both the taste and aroma of cheese, although the positive or negative impact of this change should be properly assessed through dedicated sensory analyses. Iron could also be used to modify cheese color, as certain species sensitive to iron fortification, such as *Brevibacterium aurantiacum* and *Glutamicibacter arilaitensis*, are known as pigment producers (Galaup et al., 2015; Giuffrida et al., 2016). To achieve this goal, future research should be conducted at a pilot scale or under real production conditions to evaluate the impact of iron addition on surface-ripened cheese organoleptic properties. The threshold iron levels required to maximize these properties while maintaining other essential cheese characteristics and consumer acceptance should also be appropriately determined.

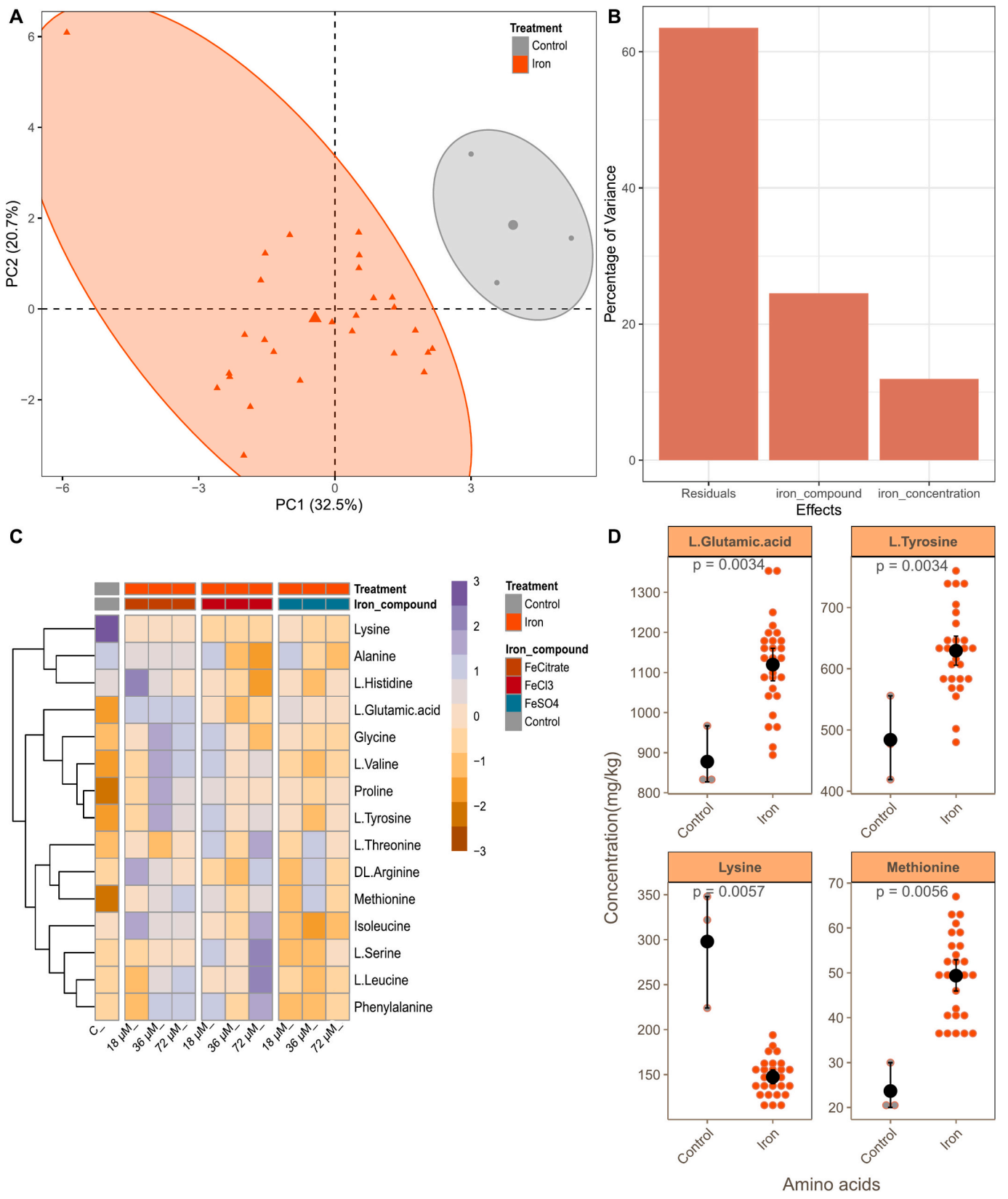

Fig. 6. Effect of iron fortification on free amino acids (FAAs) profile quantified in cheese after 28 days of ripening. (A) PCA score plot of FAAs profile showing the difference between control and iron-fortified samples. (B) Bar graph explaining the contribution of iron compounds and concentrations to the variance in iron-fortified samples, based on ANOVA simultaneous component analysis (ASCA). (C) Heatmap representing the level of the 15 free amino acids (FAAs) in cheese samples according to iron treatment (Control and Iron-fortified), iron compounds (Control, FeCitrate, $FeCl_3$, and $FeSO_4$), and iron concentrations (18 µM, 36 µM, and 72 µM). Each bar represents the average values of three biological replicates. (D) Dot plots showing the comparison of the concentrations of four FAAs between control and iron-fortified samples, i.e., L-Tyrosine, L-Glutamic acid, Lysine, and Methionine ($p < 0.05$, Wilcoxon test).

From a more fundamental perspective and to provide a better understanding of the system, the impact of iron addition on microbial physiology and metabolism during cheese ripening should also be investigated, for example, using multi-omics approaches (Afshari et al., 2020; Yap et al., 2022; Ferrocino and Cocolin, 2017).

5. Conclusions

This study evaluated the impact of iron addition in surface-ripened cheese with different doses and iron compounds. The results confirmed that iron addition increased the growth of key ripening bacteria, namely, *Glutamicibacter arilaitensis*, *Brevibacterium aurantiacum,* and *Corynebacterium casei,* in a dose-dependent manner but independently of the iron compounds used for fortification. Iron addition also altered the cheese volatilome, increasing the diversity and concentration of volatile organic compounds (VOCs) with a significant effect of both dose and iron compounds. Regarding water-soluble metabolites, iron addition significantly impacted both the untargeted and amino acids profiles. We observed a slight but significant effect of iron compounds on the amino acids profile but no significant effect of the dose. Overall, these findings highlight the potential of using iron fortification as a new lever for improving cheese quality through the modulation of microbial growth, thereby offering cheesemakers valuable insights for developing iron-fortified products.

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CRediT authorship contribution statement

Mahtab Shoukat: Writing – review & editing, Writing – original draft, Visualization, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Vincent Hervé:** Writing – review & editing, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Data curation. **Anne-Sophie Sarthou:** Methodology, Investigation, Formal analysis. **Anne-Claire Peron:** Methodology, Investigation, Formal analysis, Data curation. **Alice Danel:** Methodology, Investigation, Formal analysis, Data curation. **Dominique Swennen:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Pascal Bonnarme:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Eric Dugat-Bony:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, 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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Data availability

ESR10_Exp1 (Original data)

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