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PII: S0958-6946(23)00182-6

DOI: https://doi.org/10.1016/j.idairyj.2023.105763

Reference: INDA 105763

To appear in: *International Dairy Journal*

Received Date: 24 May 2023

Revised Date: 18 July 2023

Accepted Date: 19 July 2023

Please cite this article as: Peralta, G.H., Beret, V., Bürgi, M., Ale, E.C., Martínez, L.J., Albarracín, V.H., Wolf, I.V., Bergamini, C.V., Impact of media culture, freeze-drying and storage conditions on preservation of *Lacticaseibacillus paracasei* 90: viability and metabolic potential as a secondary culture in semi-hard cheese, *International Dairy Journal*, https://doi.org/10.1016/j.idairyj.2023.105763.

Impact of media culture, freeze-drying and storage conditions on preservation of *Lacticaseibacillus paracasei* 90: viability and metabolic potential as a secondary culture in semi-hard cheese

Guillermo H. Peralta [a,b], Victoria Beret[a], Milagros Bürgi [c], Elisa C. Ale [a], Luciano J. Martínez [d], Virginia H. Albarracín [d], I. Verónica Wolf, Carina V. Bergamini [a]

[a] *Instituto de Lactología Industrial, CONCIET, Santiago del Estero 2829. Santa Fe, Argentina.*

[b] *Facultad de Ciencias Agrarias,UNL, Kreder 2805, Esperanza, Argentina.*

[c] *UNL, CONICET, FBCB (School of Biochemistry and Biological Sciences), CBL (Biotechnological Center of Litoral), Ciudad Universitaria, Ruta Nacional 168, Km 472.4, C.C. 242, (S3000ZAA) Santa Fe, Argentina.*

[d] *Centro Integral de Microscopía Electrónica, CONICET, Facultad de Agronomía y Zootecnia, UNT, Finca El Manantial, Camino de Sirga, 4107 Yerba Buena, Tucumán, Argentina.*

*Corresponding author.

E-mail address: gperalta@fiq.unl.edu.ar (G. H. Peralta).

ABSTRACT

Freeze-drying is the main process used to preserve lactic bacterial cultures at an industrial level; however, their viability and metabolic activity can be affected not only by the process per se but also by storage conditions. This study investigated the effect of freeze-drying and long-term storage (14 months) on the viability and metabolic activity of *Lacticaseibacillus paracasei* 90 (L90) grown in three culture media. The freeze-drying process did not influence its culturability and viability, regardless of the culture media used. The storage of the freeze-dried cultures at room temperature had a negative impact on viability, culturability and its ability to grow and acidify milk. However, L90 was able to withstand a long period of storage at low temperatures (14 months, 4 °C) and keep the technological features desirable of a ripening culture. Overall, the results demonstrate the robustness of this strain as a secondary culture in the food industry.

1. Introduction

For the last twenty years, mesophilic lactobacilli have been used by cheese producers to enhance the proteolysis and flavour production, and to avoid the occurrence of defects associated with spoilage microorganisms (Bancalari et al., 2020; Bintsis, Vafopoulou-Mastrojiannaki, Litopoulou-Tzanetaki, & Robinson, 2003; Li et al., 2021; Oberg, McMahon, Culumber, McAuliffe, & Oberg, 2022). The demand for secondary cultures has increased enormously in the last years bringing about the isolation and characterisation of hundreds of strains of several species, such as *Lacticaseibacillus paracasei*, *Lacticaseibacillus casei*, and *Lactiplantibacillus plantarum*, to fulfil this need. Enzymes involved in cheese ripening (proteases, peptidases, and transaminases) have been the most important targets in the selection of potential strains (Jensen & Ardö, 2010; Stefanovic et al., 2018; Thage et al., 2005).

Nowadays, starter and secondary cultures produced in large companies are preserved by freeze-drying, freezing, and spray-drying (Chen & Hang, 2019). Despite the high cost of investment required for the freeze-drying process, it is the most widely used by large plants, as it has advantages in comparison with the other two preservation technologies (Peighambardoust, Golshan Tafti, & Hesari, 2011). Regarding bacteria cultures preserved by freezing technology, it is essential to store them at extremely low temperatures (between −20 °C and –80 °C) during the entire production chain, meaning high costs for culture producers and concomitantly for cheese manufacturers, whilst the storage of freeze-dried cultures does not require extremely low temperatures. Although it is recommendable to store freeze-dried cultures at 4 °C (Taskila, 2017), the viability and activity of many bacteria may not be seriously affected when they are kept at room temperature for several months (Carvalho et al., 2004b; Oddi et al., 2020; Strasser, Neureiter, Geppl, Braun, & Danner, 2009); this is one of the

main reasons why freeze-dried cultures are more convenient than frozen cultures. Regarding spray-drying technology, which involves exposing bacterial suspensions to high temperatures (between 150 and 200 °C), some strains may not be able to withstand this process (Ananta et al., 2004). On the contrary, freeze-drying is a process by which dehydration occurs at very low temperatures (–80 °C) by water sublimation under vacuum conditions, allowing the maintenance of cell viability.

During freeze-drying, the viability and activity of the cells are influenced by many factors, including intrinsic features, growth factors, drying matrix, and drying parameters (Carvalho et al., 2004b; Foerst & Santivarangkna, 2015). In this direction, it has been reported that the storage conditions, such as temperature and time, are critical aspects to preserve the viability and activity of the cells (Carvalho et al., 2004b; Montel Mendoza, Pasteris, Otero, & Nader-Macías, 2014). Furthermore, it is well known that the resistance of bacteria to both freeze-drying process and storage is strain-dependent (Oddi et al., 2020).

Developing starter and secondary cultures that do not require very low temperatures to maintain their functionality is essential to reduce energy costs associated with the long-term storages at frozen temperatures. The use of disaccharides (i.e., lactose, sucrose, and trehalose), polysaccharides (i.e., maltodextrin and soy polysaccharide), polyols (i.e., glycerol, sorbitol, and mannitol), and complex matrices (i.e., skim milk and soybean flour), has been extensively investigated to improve the survival of bacteria during freeze-drying and storage (Ananta et al., 2004; Fonseca, Cenard, & Passot, 2015; Mahidsanan, Gasaluck, & Eumkeb, 2017; Oddi et al., 2020).

It is a common practice to use expensive commercial culture media that contain a lot of nutrients to characterise potential secondary cultures. On the other hand, more economical media formulated with food industry waste or by-products could be applied at industrial scale

(Hayek, Gyawali, Aljaloud, Krastanov, & Ibrahim, 2019). The metabolic activity observed for a bacterial strain grown under optimal conditions at laboratory scale does not always reflect its performance in other industrial media, as it is widely known that the biosynthesis of proteases, peptidases, and transaminases can be modified by the growth medium (Jensen & Ardö, 2010). For instance, Jensen and Ardö (2010) reported that the aminotransferase activities against Asn, Asp, Lys, Gln, His and Pro were detected in four *Lactobacillus helveticus* strains when they were grown in MRS media, but not in skim milk.

In a previous work, we optimised the biomass production of *Lacticaseibacillus paracasei* 90 (L90) using a culture medium composed of an effluent derived from soy protein concentrate production (Beret et al., 2021). The performance of this strain as a secondary culture in cheese has been previously assessed after its growth in MRS (Milesi, Wolf, Bergamini, & Hynes, 2010; Peralta et al., 2017); however, there is no information about its activity in cheese after growing in the culture medium made with the aforementioned effluent. Therefore, the main objectives of this work were to: (i) evaluate the influence of the growth media on the survival rate of L90 after freeze-drying; (ii) assess the impact of the temperature and time of storage on the viability of freeze-dried cultures; and (iii) study the metabolic activity of the freeze-dried cells in cheese after 14 months of storage.

2. Materials and methods

2.1. Culture media

Two residue-based culture media from soybean industrialisation were prepared according to Beret et al. (2021). One of them (RB; residue-based culture medium) only

111 contained the carbohydrates from the soy flour aqueous extract (stachyose, raffinose, and
112 sucrose). Meanwhile, in the second medium (RBG; glucose-supplemented residue-based
113 culture medium), glucose was added as an energy source enhancer. In addition, both media
114 were supplemented with different levels of yeast extract, $MnSO_4$, and $MgSO_4$. The level of
115 glucose, yeast extract, $MnSO_4$, and $MgSO_4$ added to both media are shown in Supplementary
116 material Table S1. MRS (Biokar, Beauvais, France) medium was prepared according to the
117 manufacturer's instructions.
118
119 *2.2. Strain, growth conditions, freeze-drying, and experimental design*
120
121 L90 was routinely stored at −80 °C in MRS broth supplemented with 15% (v/v) glycerol
122 and activated by two successive incubations in MRS at 37 °C for 20 h. Then, L90 was
123 inoculated at 2% (v/v) into 1 L of each medium (RB, RBG, and MRS) and incubated under
124 aerobic conditions at 37 °C for 20 h. The cells were harvested (8000 × *g*, 10 min, 4 °C) and
125 washed twice with 50 mM potassium phosphate buffer, pH=7, and subsequently resuspended in
126 300 mL of a 10% (w/v) lactose solution. Aliquots (2 mL) of each cell suspension were
127 transferred into 10 mL sterilised vials, frozen at −80 °C for 24 h, and freeze-dried (Martin
128 Christ Alpha 1-4-LD Plus, Germany). The freeze-dried (FD) cultures obtained from RB, RBG,
129 and MRS were named FD RB, FD RBG, and FD MRS, respectively.
130 To evaluate the survival rate of L90, microbial counts were made before and
131 immediately after the freeze-drying process; the powdered cultures were rehydrated to their
132 original volume using sterile distilled water. Additionally, scanning electron microscopy
133 (SEM) was performed as per Peralta, Bergamini, and Hynes (2019) using a Zeiss SUPRA
134 55VP scanning electron microscope. Briefly, FD samples were rehydrated in 50 mM potassium

phosphate buffer and fixed in 3.5% formaldehyde to analyse the cell structure. FD powders with no previous hydration were observed as well. The FD samples were stored for 14 months at LT-low temperature (4 °C) and RT-room temperature. Plate counts (MRS, 37 °C, 48 h, microaerophilic conditions) and flow cytometry analyses were carried out during the storage, and the ability to grow in the milk of the freeze-dried powders was assessed at 14 months. Finally, the freeze-dried culture that maintained the greatest viability at 14 months of storage was used as a secondary culture in cheese. A schematic representation of the experimental design and the industrial relevance is shown in supplementary material (Supplementary material Fig. S1).

2.3. Survival of freeze-dried L90 during storage: plate counts and flow cytometry

Microbiological counts at 1, 3, 6, and 14 months were performed using MRS, as described in section 2.2. The physiological profile of cells at 3 and 14 months of storage was studied by flow cytometry according to Peralta et al. (2023) with slight modifications in the control cells. An overnight culture of L90 grown in MRS broth was used to prepare the controls of dead, permeabilised and live cells. The cells were harvested by centrifugation at 10000 × *g* (4 °C for 10 min) and washed twice with 50 mM potassium phosphate buffer (pH 7.0). For dead and permeabilised controls, the cells of an aliquot (1mL) of this suspension were recovered and treated with isopropyl alcohol or hexadecyltrimethylammonium bromide (Cicarelli, Santa Fe, Argentina), respectively, as per Yanachkina, McCarthy, Guinee, and Wilkinson (2016). Untreated cells of this suspension were used as viable/intact cells (live control). The cells were diluted in PBS buffer with 1 mmol L^{-1} EDTA and 0.01% (v/v) Tween®, and stained with thiazole orange (420 nmol L^{-1}) and propidium iodide (43 mmol L^{-1})

(BD™ Cell Viability kit, BD Biosciences, CA, USA). Fluorescent labelled cells were analysed using a Guava® EasyCyteTH cytometer (Guava Technology, USA) equipped with green (FL1 525/30) and red (FL3 661/15) channels. Samples were analysed in FSC versus SCC plus FL1 versus FL3 dot plots. Guava CytoSoftTM 3.6.1 and FlowJo 10 programs were used for the acquisition and analyses of the data, respectively.

2.4. Growth of freeze-dried cultures in milk

The ability of the cells to grow in milk was studied at the end of the storage. Cells were inoculated in sterile milk and incubated at 37 °C for 24 h. After incubation, microbiological counts (MRS, 37 °C, 48 h) and pH determinations were performed in the fermented milk. The fermented milks (FM) inoculated with the freeze-dried cultures FD RB, FD RBG, and FD MRS were named FM RB, FM RBG, and FM MRS, respectively. In addition, the codes LT and RT indicate low and room temperature of storage of freeze-dried cultures, respectively.

2.5. Cheese making

In this experiment, the freeze-dried culture FD RB stored at LT during 14 months (FD RB LT) was used as a secondary culture in semi-hard cheeses. For that, two types of cheeses (without secondary culture or with secondary culture) were manufactured in triplicate according to Peralta et al. (2023): control cheese (Cheese C) containing *Streptococcus thermophilus* (~10^6 cfu mL^{-1}) as starter culture and no secondary culture; and experimental cheese (Cheese E) containing *S. thermophilus* (~10^6 cfu mL^{-1}), and FD RB LT (~10^6 cfu mL^{-1}) as starter culture and secondary culture, respectively. Briefly, raw cow milk was pasteurised at

63 °C for 30 min and immediately cooled to 37 °C. Then, calcium at 0.02% (w/v) and the cultures were added to cheese milk. After mixing of the cultures in milk, chymosin (ChyMax, Chr. Hansen, 74 IMCU mL^{-1}) was used as clotting agent. The curd was cut to the size of a corn grain and subsequently cooked to reach to 45 °C. Afterwards the whey was drained, and the curd moulded, pressed, and put into a controlled temperature room at 45 °C to reach pH = 5.3. After that, the cheeses were cooled and salted in brine (20% NaCl, w/v, pH 5.40, 5°C) and stored at 10 °C for 7 days. Finally, the cheeses were coated, vacuum-packed and ripened at 10 °C for 120 days counting from the cheesemaking day.

2.6. Composition, pH, and microbiology of cheese

Gross composition was assessed in cheese samples according to the international standard methods: gravimetrical method for moisture (ISO 5534/IDF 4; ISO, 2004), Gerber-Van Gulik for fat (ISO 3433/IDF 222; ISO, 2008), and Kjeldahl for protein (ISO 8968/IDF 20; ISO, 2019). The pH was measured in a slurry prepared by blending 5 g of grated cheese in 5 mL of distilled water using a pH-meter (Bradley et al., 1993).

For microbiological counts, the samples were homogenised in sterile 2% (w/v) sodium citrate solution and then decimal dilutions in casein peptone 0.1% (w/v) were prepared. Analysis of total lactic acid bacteria (PCA milk, 37 °C, 48 h), mesophilic lactobacilli (MRS agar, 37 °C, 48 h), coliforms (VRBL agar, 32 °C, 24 h), yeast and moulds (YGC agar, 25 °C, 5 days) and enterococci (BEA agar, at 37 °C for 48 h) were carried out as outlined by Peralta et al. (2017).

206 *2.7. Carbohydrates and organic acids of cheese*

207

208 The separation and quantification of organic acids and carbohydrates were carried out
209 using a high-performance liquid chromatography (HPLC) system (Perkin Elmer, 200 Series,
210 USA) equipped with a quaternary pump, solvent degasser, column heater (65 °C), UV–visible
211 detector (210 nm) and refractive index detector (Series Flexar). The column used was a 300 ×
212 7.8 mm Aminex HPX-87H (Bio-Rad Laboratories, USA) equipped with a cation H+
213 microguard cartridge. Regarding mobile phase and separation conditions, sulphuric acid (0.01
214 M) with isocratic elution at 65 °C at a flow rate of 0.6 mL min^{-1} was used. The preparation of
215 the cheese sample was carried out as outlined by Peralta et al. (2017). All the standards and
216 reagents used were of analytical-grade and purchased from Sigma Aldrich.

217

218 *2.8. Peptidase activities in the cheese matrix*

219

220 Cheese extracts were prepared according to Nongonierma, Abrlova, and Kilcawley
221 (2013). Five aminopeptidases and one endopeptidase were evaluated in cheese extracts using
222 the chromogenic substrates (Bachem, Torrance, CA, USA): H-Leu-pNA, H-Val-pNA, H-Met-
223 pNA, H-LyspNA.2HBr, H-Arg-pNA.2HCl and Z-Gly-Pro-pNA according to Ale et al. (2023).

224

225 *2.9. Proteolysis of cheese*

226

227 Peptide profiles were carried out by HPLC as outlined by Bergamini Hynes, and Zalazar
228 (2006). Cheese samples (5 g) were homogenised in 10 mM H_2SO_4 (25 mL) using a
229 homogeniser (Ultra Turrax, model T25, IKA, Staufen, Germany) and placed in a heated bath

(40 °C) for 1 h. Then, the suspension was centrifuged at 3000 × *g* for 30 min, filtered using filter paper and subsequently filtered with 0.45 µm membranes (Millex, Millipore, Sao Paulo, Brazil). Finally, this filtered solution was injected into the HPLC system. The column used was a 220 mm × 4.6 mm Aquapore OD-300 C18, 7 mm – 300Å analytical column (Perkin Elmer).

2.10. Volatile compounds of cheese

The volatile compounds were assessed by solid-phase microextraction coupled to gas chromatography using a GC system (SPME-GC) (Perkin Elmer model 9000, USA) with a HP INNOWax column (Agilent Technologies). Analysis was carried out as outlined by Peralta et al. (2017).

2.11. Statistical analysis

Repeated-measures ANOVA was used to analyse the plate counts obtained from the freeze-dried cultures at 1, 3, 6, and 14 months of storage using IBM SPSS Statistics 25.0. Greenhouse-Geisser or Huynh-Feldt correction was applied when Mauchly's test of sphericity was significant (p <0.05). Flow cytometry data and all the data derived from fermented milk and cheese samples were analysed by Infostat software (Info-Stat Group, UNC, Argentina) using the Student's t-test, with a level of significance determined at p <0.05. Pearson's correlation coefficients between cheese parameters (moisture, fat, protein, pH, organic acids, and microbiological counts) at level of p <0.01 was calculated in R (https://www.r-project.org/) using the corrplot R-package. Peptide and volatile profiles of cheeses were analysed by principal component analysis using the statistical software R. A heat map with Z-score values

was obtained using the R packages ggplot2 to visualise connections among cheese samples and the analysed variables: volatile compounds, peptide profiles, organic acids, and carbohydrates.

3. Results and discussion

3.1. Survival of L90 after freeze-drying: plate counts and SEM.

As expected, the reached level of L90 in the three culture media (RB, RBG and MRS) was high (> 9 log cfu mL^{-1}), as both RB and RBG had been optimised for the growth of this strain (Beret et al., 2021). MRS is a commercial medium suitable for most lactic acid bacteria (LAB), including L90 (Beret et al., 2021; Giménez et al., 2021; Peralta et al., 2022). The levels of L90 before and after freeze-drying were similar (9 log cfu mL^{-1}, approximately) regardless of the growth medium. Thus, the freeze-drying process did not have a negative impact on culturability. In agreement with our results, previous studies showed the positive effect of disaccharides on the protection of *Lactobacillus* strains from freeze-drying process (Oddi et al., 2020; Teng, Kawai, Mikajiri, & Hagura, 2017). Although the positive effect of lactose as cryoprotectant is well-known, studies on survival should be carried out to confirm microbial cell viability throughout drying, given that it depends on many factors such as the growth medium, growth conditions, initial concentration of microorganisms, drying matrix, among others (Carvalho et al., 2004b). Furthermore, the resistance to freeze-drying is strain-dependent (de Melo Carvalho, 2018). For instance, Oddi et al. (2020) reported that 10% lactose solution was an excellent cryoprotectant for some strains, while for other strains the loss of viability was significant.

277 The SEM micrographs of the freeze-dried cultures (before rehydration) showed that
278 most lactobacilli were trapped within the amorphous matrix that lactose produces when
279 dehydrated (Fig. 1a). A similar effect was reported by Pehkonen, Roos, Miao, Ross, and
280 Stanton (2008) and Schoug (2009) for *L. rhamnosus* GG and *L. coryniformis* Si3, respectively.
281 In particular, more cells outside the lactose matrix were observed for FD RBG in comparison
282 with FD RB and FD MRS; this fact probably led to a higher loss of viability during the storage
283 of powders as discussed in the following section (Section 3.2). The cell integrity after
284 rehydration did not show signs of damage (Fig. 1b), in line with our results showing high
285 culturability when analysed immediately after freeze-drying.
286
287 *3.2. Survival of L90 freeze-dried cultures during storage: plate counts and flow cytometry.*
288
289 The results of colony counts and flow cytometry (physiological profile) for the cells that
290 were stored at low and room temperatures for 14 months are shown in Fig. 2 and Fig. 3,
291 respectively. The level of culturability of the three freeze-dried cultures was significantly
292 affected (p <0.05) by the storage temperature, showing lower levels (log cfu mL^{-1}) of L90
293 when stored at room temperature. This difference was more evident when the cells were stored
294 for longer periods. In addition, the negative effect of storage temperature was greater for FD
295 RBG (Fig. 2b) and FD MRS (Fig. 2c) in comparison with FD RB (Fig. 2a), to such an extent
296 that they were not cultivable (<3 log cfu mL^{-1}) at the end of storage at RT. Similarly, several
297 studies have reported loss of viability due to the storage of freeze-dried cultures at temperatures
298 between 22 °C and 37 °C (Carvalho et al., 2004b; Oddi et al., 2020; Strasser et al., 2009). In
299 general, storage at low temperatures allowed a better maintenance of cell culturability.

Fig. 3 shows the results of flow cytometry analyses for the cells at 3 and 14 months of storage, which reflect their physiological profile. At 3 months of storage, most cells (average 96 %) of the three FD cultures were live, while a low percentage were permeabilised (average 4 %). After 14 months of storage, the percentages of live and permeabilised cells decreased and increased, respectively, in relation to the levels at 3 months; this change was significant (p <0.05) for FD RB (Fig. 3a) and FD RBG (Fig. 3b), being very marked in the latter, reaching a similar level of live and permeabilised cells in this freeze-dried culture at 14 months of storage. In addition, dead cells of FD RBG slightly increased during the storage at both temperatures.

This lower survival ratio could be associated with the higher number of cells that remained outside the lactose matrix as shown in the SEM micrographs (Fig. 1a). The changes observed for FD MRS (Fig. 3c) during storage were not significant (p >0.05).

Regarding the storage temperature, the percentages of live and permeabilised cells were significantly (p <0.05) affected in the FD RBG (Fig. 3c).

The different survival rates found for the FD cultures in different storage conditions (temperature and time) could be associated with the carbon source in each culture medium: RB just contained the soluble carbohydrates from soy (stachyose, raffinose, and sucrose), whilst RBG had glucose added. Several studies have demonstrated that the survival ability of a microorganism throughout freeze-drying and storage depends on the carbohydrates present in both the growth medium and drying matrix (Carvalho et al., 2004a,b). The cells grown in RB and stored for 14 months at LT were used as secondary culture in cheesemaking because they showed the highest level of survival at the end of storage at both temperature conditions.

3.3. Microbiological counts and pH of fermented milk

Fig. 4 shows the microbiological counts and pH values of fermented milks prepared with cells stored 14 months at both temperatures. In line with the viability results of the freeze-dried cultures, the ability to grow in milk and to produce acid was significantly altered (p <0.05) when the cells were stored at room temperature. Furthermore, the acidification of the three freeze-dried cultures was lower in comparison with the levels previously reported by Beret et al. (2021). In this study, the L90 cells grown in the same three culture media were inoculated in milk as fresh cultures (without being subjected to lyophilisation and storage) and the pH values were around 5.0. These results indicate that the freeze-drying process followed by 14 months of storage regardless of temperature affects partially the acidification ability of L90. In the same way, Coulibaly et al. (2010) reported a loss of acidification activity for two freeze-dried lactobacilli strains.

3.4. Gross composition, pH, and microbial counts of cheese

Gross composition, pH, and microbiological counts of cheeses were assessed at the end of the ripening time (120 days) and are shown in the Table 1. Moisture, moisture in non-fat substance (MNFS), and protein showed significant difference (p <0.05) between cheese C (without secondary culture) and cheese E (with secondary culture). Meanwhile, fat and fat in dry matter (FDM) showed no significant differences. The pH values in cheese E were lower (p <0.05) than in cheese C.

Significant differences (p <0.05) were found for total lactic acid bacteria and mesophilic *Lactobacillus*. The level of *Lactobacillus* in cheese E was higher than in cheese C. On the contrary, the total LAB levels were higher in cheese C. In addition, the *Enteroccoccus* counts was <3 log cfu mL^{-1} in cheese E, while it was 4.9 log cfu mL^{-1} in the cheese C. The levels of

348 moulds and yeasts were low in both control and experimental cheeses, and no significant
349 differences were found. Coliforms were not present in the cheeses, as expected.
350 The composition and pH of cheeses were similar to those obtained in a previous study,
351 for which L90 was not subjected to freeze-drying and was grown in MRS (Peralta et al., 2023).
352 The lower pH value in cheese E could be associated with the higher level of *Lactobacillus*. The
353 colonies of this microbial group showed the same morphology as the colonies that came from a
354 pure culture of L90, suggesting that this culture might have prevailed among the lactobacilli
355 present in the cheese microbiota. The ability of L90 to reduce the pH values and the
356 *Enterococcus* levels was previously reported by Peralta et al. (2023). The post-acidification
357 verified in cheese E probably caused the diminution of the moisture levels in these cheeses
358 (McSweeney, 2007). Considering that the culture media used in this study are not completely
359 selective (Hayek et al., 2019), future metagenomic studies should be carried out to confirm the
360 effect of L90 on cheese microbiota.
361
362 *3.5. Carbohydrates and organic acids of cheeses*
363
364 Fig. 5a shows the concentration of two carbohydrates and five organic acids quantified
365 in cheeses ripened for 120 days. Galactose, orotic acid, and hippuric acid levels in cheese E
366 were significantly lower (p <0.05) than in cheese C. These compounds can be used as an
367 energy source by LAB. The capacity to metabolise galactose has previously been reported for
368 L90 in different dairy matrices, including, a cheese model incubated for 14 days at 37 °C
369 (Peralta et al., 2016), milk fermented for 24h at 37 °C (Peralta et al., 2022), and Cremoso
370 cheese ripening for 30 days at 4 °C (Giménez et al., 2021; Peralta et al., 2020). The ability to
371 metabolise orotic acid and hippuric acid was also reported for L90 (Giménez et al., 2021,

372 Giménez et al., 2021). Therefore, these abilities were not modified by long-term storage. In
373 particular, using bacteria culture with the ability to metabolise galactose in cheese is a strategy
374 to control the adventitious microbiota and the defects that can emerge from their growth as
375 unwanted production of CO_2 and undesirable volatile compounds. It is interesting to highlight
376 that L90 has prevented the occurrence of these defects in Cremoso cheese (Giménez et al.,
377 2021; Peralta et al., 2020).

378 The lower level of citric acid in cheese C could be related to the higher levels of
379 *Enterococcus*, or to some NSLAB which could use citrate as energy source (Díaz-Muñiz &
380 Steele, 2006; Sarantinopoulos, Kalantzopoulos, & Tsakalidou, 2001). On the other hand, the
381 higher levels of citric acid in cheese E could be associated with the action of citrate synthase
382 (Upreti, McKay, & Metzger, 2006). However, it has been reported that the chromatographic
383 method used in our study may not separate citrate from isocitrate (Upreti et al., 2006), therefore
384 these compounds could co-elute.

385 The higher concentration of lactic acid observed in cheese E could be associated with
386 the higher levels of *Lactobacillus,* and it may explain the lower pH values of these cheeses. In
387 fact, these variables were statistically (p <0.01) correlated. As it was expected, lactobacilli
388 counts were positively correlated with the lactic acid levels, and negatively correlated with the
389 levels of galactose, hippuric acid, orotic acid, moisture, and pH values (Fig. 5b).

390

391 *3.6. Peptidases and peptide profiles of cheese*

392

393 Significant differences were found for both peptidases and peptide profiles in the soluble
394 fractions of cheeses (Fig. 6). In fact, the peptidase activity against the substrates H-Leu-pNA,
395 and H-Val-pNA in cheese E were higher than in cheese C. On the contrary, the peptidase

activity against H-Lys-pNA.2HBr was higher in cheese C. In addition, Fig. 6b clearly shows differences in the peptide profiles of both treatments.

Fig. 6c shows the scores and loading biplot of the principal component analysis that was performed with the area of the 40 peaks of these profiles. Two principal components (PC1: 60.6% and PC2: 22%) were extracted to explain the variance between the cheese samples. The three replicates of cheeses C were located on the left of the PC1 (cluster 1), while cheeses E were placed on the right of the PC1 (cluster 2). Cheeses E were mainly characterised by peaks that eluted early in the chromatograms, which correspond to more hydrophilic peptides, while the contrary occurred in cheeses C. Cheeses C were characterised by peptides that elute later in the chromatograms, which are generally more hydrophobic and associated with bitter taste (Molina, Ramos, Alonso, & López-Fandiño, 1999). It is well-known that *L. paracasei* contributes to proteolysis due to its high peptidolytic activity (Bintsis et al., 2003; Stefanovic et al., 2018). High levels of peptidase activities in cell-free extracts of L90 were recently reported by Peralta et al. (2023). This strain is autochthonous and has been well characterised on its contribution to proteolysis in cheese (Peralta, Wolf, Bergamini, Perotti, & Hynes, 2014; Peralta et al., 2020, 2023). In this sense, it was demonstrated that L90 has the ability to increase the production of free amino acids in a cheese model (Peralta et al., 2014), and can accelerate the proteolysis in Cremoso cheese when used as a secondary culture (Peralta et al., 2020). However, it is interesting to highlight that these studies were performed with the cells grown in the commercial medium MRS, without any preservation process or storage.

Even though most mesophilic lactobacilli contribute to the proteolysis of cheese, this effect should be confirmed for each individual strain in cheese-making experiences. For example, a recent study did not notice the effect of *L. paracasei* DPC7150, used as a secondary culture, on the secondary proteolysis of cheese (Leeuwendaal, Hayes, Stanton, O'Toole, &

Beresford, 2022). Stefanovic and McAuliffe (2018) demonstrated significant differences in the genomic features of *L. paracasei* strains isolated from the same ecological niche. Fortunately, neither the culture medium nor the conservation process of the L90 strain negatively affected its proteolytic and peptidolytic activities compared with previous studies with MRS grown fresh culture.

3.7. Volatile compounds of cheese and multivariate analysis

Twenty-one volatile compounds were identified in the headspace of the cheese samples: two aldehydes, four alcohols, eight ketones, two esters, and five acids (Supplementary material Table S2). These compounds have previously been reported in cheese, and their origin is associated with the main metabolic pathways during ripening: metabolism of lactose and citric acid, lipolysis and metabolism of fatty acids, and proteolysis and metabolism of amino acids (Ardö, 2021; Fox & Guinee, 2022; Wilkinson & Kilcawley, 2007). Acetaldehyde can be generated directly from lactose metabolism as a result of pyruvate decarboxylation. Another pathway that can generate acetaldehyde is the metabolism of some amino acids such as threonine and glycine. On the other hand, the reduction of acetaldehyde by an alcohol dehydrogenase activity produces ethanol (Chaves et al., 2002). Both acetaldehyde and ethanol are present in fermented dairy products when *Streptococcus thermophilus* is used as a starter culture. Benzaldehyde can be produced from phenylalanine by both enzymatic and chemical reactions (Nierop Groot & de Bont 1998). Methyl ketones, secondary alcohols, and esters are all odor active and contribute to cheese flavour, and they come from the metabolism of free fatty acids. The presence of water-soluble short-chain fatty acids such as butyric acid, hexanoic acid, and octanoic acid, are associated with the hydrolysis of dairy fat, and they are essential in

444 many cheese varieties because they are relatively easily hydrolysed due to their position on the
445 glyceride backbone (Kilcawley & O'Sullivan, 2018). In particular, the metabolic pathways that
446 lead to diacetyl (2,3-butanedione) and acetoin (3-hydroxybutanone) production are discussed
447 more below in this section.

448 The level of all compounds belonging to each chemical family was analysed (Fig. 7a) in
449 both cheeses. The levels of total ketones and acids of cheese E were significantly (p <0.05)
450 higher than in the control cheese, while no differences were found for the levels of total
451 aldehydes, alcohols and esters. Furthermore, a principal component analysis using the
452 individual peaks area of volatile compounds as variables was performed. The biplot shows a
453 clear separation between cheese C and cheese E (Fig. 7b). The total variance of the two
454 principal compounds was 86.4% (PC1 71% and PC2 15.4%). On the one hand, the control
455 cheeses were located on the left of the PC1 (cluster 1), and they were associated with the
456 variables heptanol, propanone, hexanone, ethanol and diacetyl. The experimental cheeses were
457 located on the right of the PC1, and they were related to some ketons (acetoin, pentanone,
458 heptanone), several acids (octanoic, acetic, butyric, and decanoic acids) and two alcohols
459 (pentanol and hexanol).

460 Cheese ripening is a complex set of biochemical events that involves three groups of
461 enzymes: indigenous milk enzymes (i.e., plasmin, lipoprotein lipase, acid phosphatases, etc.),
462 exogenous enzymes (milk coagulants, enzymes of starter and secondary cultures, enzymes of
463 ripening microorganisms, etc.), and endogenous enzymes (i.e., heat-resistant enzymes of
464 psychrotrophic bacteria or enzymes produced during the cheese ripening by heat-resistant raw
465 milk microbiota) (Ardö, 2021; Fox & Guinee, 2022). In the present study, the level and the
466 type of the indigenous and endogenous milk enzymes were not modified because the cheese
467 making of both control and experimental cheeses was carried out at the same time with the

same raw milk. For this reason, the addition of FD RB LT might explain the main differences observed in the enzymatic profiles of C and E cheeses.

L90 is a flavour-producing strain characterised by having high levels of key enzymes, and its profile of amino transaminases and peptidases was previously studied (Peralta et al., 2016, 2023). In this study an increase of peptidases in the cheese matrix by the incorporation of L90 was verified. In addition, L90 has the ability to produce diacetyl and acetoin (Milesi et al., 2010; Peralta et al., 2017), compounds that are essential in the flavour of several cheese varieties, and, as expected, this property was verified in the present work. Both diacetyl and acetoin can be produced by different metabolic pathways; the main possible pathways for the diacetyl and acetoin production in fermented dairy matrices for L90 are shown in Fig. 8. Furthermore, it is well known that *Enterococcus* can metabolise citrate to diacetyl and acetoin. Considering the higher levels of *Enterococcus* found in cheese C, this pathway could be responsible for the production of these volatile compounds in cheese C. However, it is difficult to elucidate the specific enzymes involved in the cheese volatilome, bearing in mind that the cheese microbiome is complex and dynamic during ripening. Nevertheless, it is highly possible that the changes produced by L90, such as the increase of the lactic acid levels and the depletion of the energy sources, have modified the cheese microbiota and concomitantly the enzymatic activities of the cheese matrix.

In addition, it is interesting to highlight that this is the first study that focused on the ability of L90 to produce flavour compounds after freeze-drying and long-term storage. Freeze-drying is the main technology used worldwide for the preservation of commercial cultures at industrial scale. Thus, all the potential strains to be applied as starter or secondary cultures in the food industry should be tested in terms of their resistance to the freeze-drying process, not only focusing on viability but also on metabolic activity. For instance, Kandil and El Soda

(2015), who studied the impact of freeze-drying on the intracellular enzymatic activities of different lactic acid bacterial species, reported that the intracellular enzymatic activity was significantly reduced by the preservation method.

A cluster analysis was performed on the data of volatile compounds, peptide profiles, carbohydrates and organic acids to classify and discriminate the cheese samples (Fig. 7c). The cluster lines on the left side of the graph correspond to cheese sample clusters, which were packed in two well-defined groups (cluster 1: cheese C and cluster 2: cheese E). The cluster lines on the top of the graph correspond to variable clusters, which were packed also in well-defined groups. For instance, the cluster on the right grouped together hydrophilic peptides (1, 2, 3, 6, 8, 9, and 10) and most acids. Thereby, the results of the heat map indicated that L90 after freeze-drying and storage at low temperatures for 14 months was still viable and able to contribute to the cheese ripening process.

Furthermore, the metabolites produced by L90 such as lactic acid and acetic acid could impact on the cheese microbiota as well, influencing the enzymatic profiles of the cheese matrix. In this direction, the differences between treatments in terms of the metabolites produced might be associated not only with the enzymes of L90 but also with the differences in the composition of the cheese microbiota. However, given that only some microbiological groups were analysed by selective culture media, it would be interesting to perform future metagenomic studies to fully understand the effect of L90 on cheese microbiota.

4. Conclusions

This study shows the metabolic potential of L90 as a secondary culture in cheese after being grown in an industrial waste-based culture medium, submitted to a freeze-drying process,

and stored for 14 months. L90 was successfully preserved for 14 months at low temperature after freeze-drying using lactose 10% (w/v) as cryoprotectant, in terms of viability and metabolic activity. The strain survived the freeze-drying process regardless of the culture media used for its growth. The storage of the freeze-dried cultures at room temperature had a negative effect on the viability, culturability, and ability to grow and acidify in milk. On the other hand, the previously reported technological features of L90, such as its ability to enhance proteolysis and produce flavour compounds, were not affected after using freeze-drying as a preservation strategy, followed by long-term storage at low temperature, even though the strain was grown in a residue-based culture medium (without addition of an extra energy source). In conclusion, this study confirms that the use of freeze-drying and storage at low temperatures are suitable strategies to preserve L90, having no negative effects on its overall performance. The insight into how freeze-drying and a long period of storage affect the viability and activity of a potential secondary culture is key for the design of cultures on the industrial scale.

Acknowledgements

The current study was funded by the Agencia Nacional de Promoción Científica y Tecnológica (PICT-2018-01334; PICT 2019-01633), Agencia Santafesina de Ciencia, Tecnología e Innovación de la Provincia de Santa Fe (IO-2017-00036), and UNL (CAI+D 50620190100105LI). We want to thank Milkaut S.A. for providing the raw milk used in the experiments. Electron micrographs used in this paper were taken at the Centre for Electron Microscopy belonging to UNT and CCT, CONICET, Tucumán, Argentina.

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Table 1

Gross composition, pH and microbiological counts in cheeses ripened at 10 °C for 120 days. [a]

Parameter	Cheese C	Cheese E	*p*-value
Moisture (g 100 g^{-1})	35.9±0.2	33.6±0.3	<0.001
Fat (g 100 g^{-1})	34.2±0.4	34.7±0.7	0.426
MNFS (%)	54.6±0.1	51.4±0.8	0.020
FDM (%)	53.6±0.4	52.2±1.1	0.143
Protein (g 100 g^{-1})	24.9±0.2	26.8±0.8	0.016
pH	5.2±0.1	4.9±0.1	<0.001
Total lactic acid bacteria (log cfu g^{-1})	9.4±0.1	9.2±0.1	<0.001
Lactobacillus (log cfu g^{-1})	6.7±0.5	8.4±0.1	0.004
Enterococcus (log cfu g^{-1})	4.9±0.1	< 3	
Moulds and yeasts (log cfu g^{-1})	1.3±0.6	1.0±0.1	0.423
Coliforms (log cfu g^{-1})	< 1	< 1	

[a] Abbreviations are: Cheese C, without secondary culture; Cheese E, with FD RB LT as secondary culture; cfu, colony forming units; MNFS, moisture in non-fat substance; FDM, fat in dry matter. Values are the mean ± SD (*p*-values by Student's t-test; all treatments were performed in triplicate).

Figure legends

Fig. 1. SEM micrographs of (a) the FD RB, FD RBG and FD MRS cells (arrows) trapped within the amorphous matrix of lactose (left and right panels depict the same sample image at different magnifications on separate areas of observation) and (b) the same freeze-dried cultures after rehydration (ah). No significant morphological change among samples was observed.

Fig. 2. Survival of FD RB (a), FD RBG (b), and FD MRS (c) after 1, 3, 6 and 14 months of storage at low temperature (LT,) and room temperature (RT,). The data represent mean ± SD performed in triplicate. Asterisks (*) indicate significant (p <0.05) differences between the storage conditions (LT and RT). #1 limit of detection: < 3 log cfu mL^{-1}.

Fig. 3. Percentages of sub-populations of cells (dead , permeabilised , and live) in FD RB (a), FD RBG (b), and FD MRS (c) at 3 and 14 months of storage at low temperature (LT) and room temperature (RT). The symbols * and # indicate significant differences (p <0.05) between the storage times (3 and 14 months) and the storage temperatures (LT and RT), respectively.

Fig. 4. Microbiological counts (log cfu mL^{-1}) (a;) and pH (b;) values in fermented milk (FM) inoculated with FD RB, FD RBG, and FD MRS stored 14 months at low temperature (LT) and room temperature (RT). The symbol * indicates significant differences (p <0.05) between the storage temperatures (LT and RT).

Fig. 5. Panel (a): concentration (mg 100 g^{-1}) of organic acids and carbohydrates in cheeses ripened for 120 days. C: cheese made without secondary culture. E: cheese made with the freeze-dried culture of L90 produced in the RB media and stored at low temperature (LT) for 14 months (FD RB LT) as secondary culture. The symbol * indicates significant differences (p <0.05) between treatments (C and E). Panel (b): correlations among the results of several parameters of cheeses: gross composition, pH, microbiological counts (total lactic acid bacteria), lactobacilli, and moulds and yeasts), organic acids and carbohydrates. Correlations marked with × were not significant (p >0.01). Blue squares represent positive correlations, and red ones represent negative correlations. Darker colour tones and larger squares represent higher correlation coefficient magnitudes. Blank squares indicate correlation coefficients close to zero.

Fig. 6. Panel (a): peptidase activity against the chromogenic substrates H-Val-pNA (), Z-Gly-Pro-pNA (), H-LyspNA.2HBr (), H-Arg-pNA.2HCl (), H-Met-pNA (), and H-Leu-pNA () in the cheeses ripened for 120 days. C: cheese made without secondary culture. E: cheese made with the freeze-dried culture of L90 produced in the RB media and stored at low temperature (LT) for 14 months (FD RB LT) as secondary culture. The symbol * indicates significant differences (p <0.05) between treatments (C and E). Panel (b): peptide profiles of the three replicates of each treatment. Panel (c): scores and loading biplot of the principal component analysis of the peptide profile data.

Fig. 7. Panel (a): levels of total acids (), esters (), ketones (), alcohols (), and aldehydes () in the cheeses ripened for 120 days. C: cheese made without secondary culture. E: cheese made with the freeze-dried culture of L90 produced in the RB media and stored at low

temperature (LT) for 14 months (FD RB LT) as secondary culture. Panel (b): principal component analysis (PCA) of the volatile compounds of cheeses. Panel (c): heat map of correlations among carbohydrates, organic acids, peptides, and volatiles compounds of cheeses. The colour scale indicates the abundance of each variable (Z-score). Red and blue indicate high and low abundance, respectively.

Fig. 8. Schematic representation of possible pathways for the diacetyl and acetoin production by *Lactobacillus paracasei* 90: AAt, amino acid transport system; DtpT, di-/tripeptide transport system(s); Opp, oligopeptide transport system. EC and IC correspond to the extracellular medium and the intracellular medium, respectively.

(a)

(b)

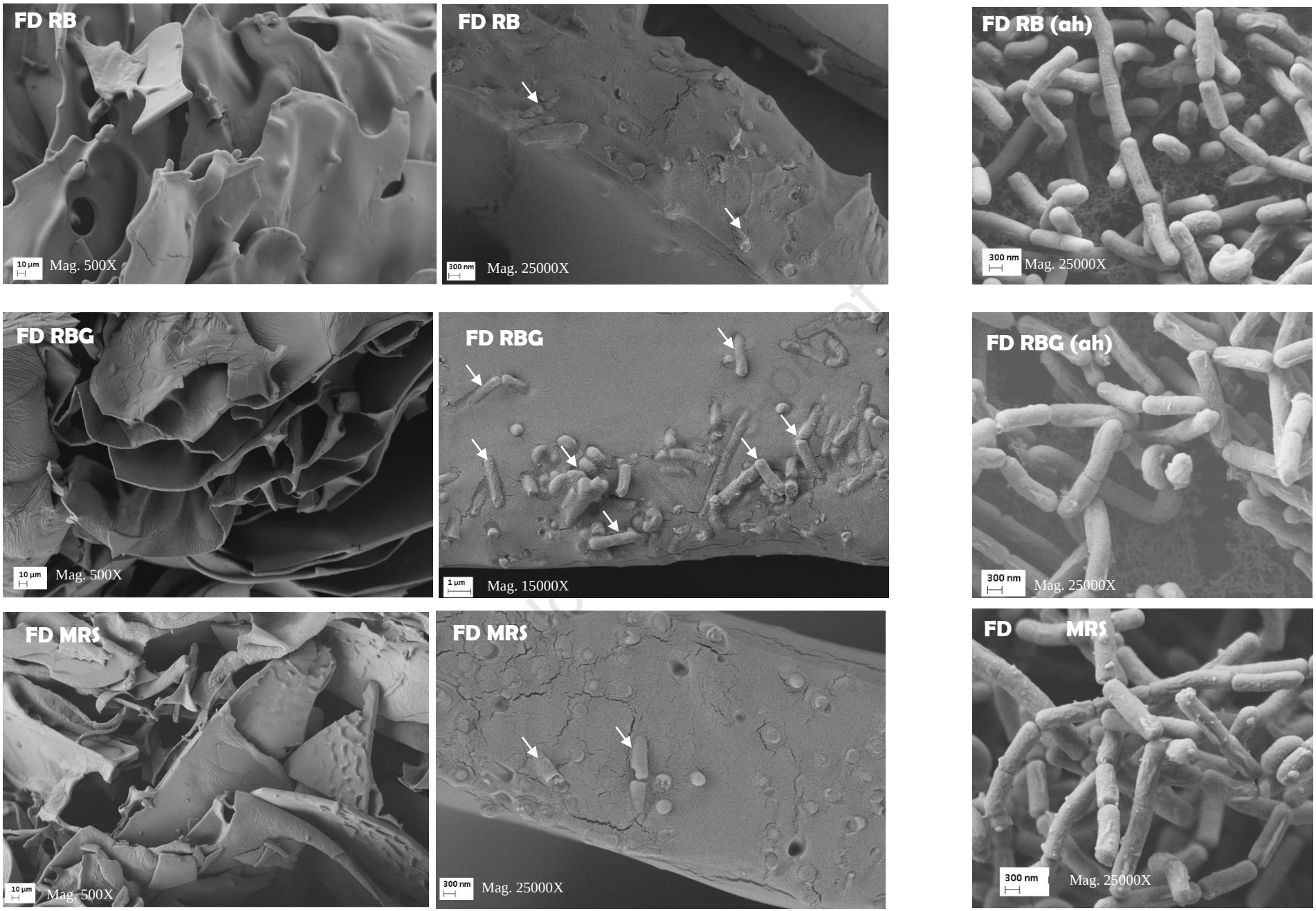


Figure 1

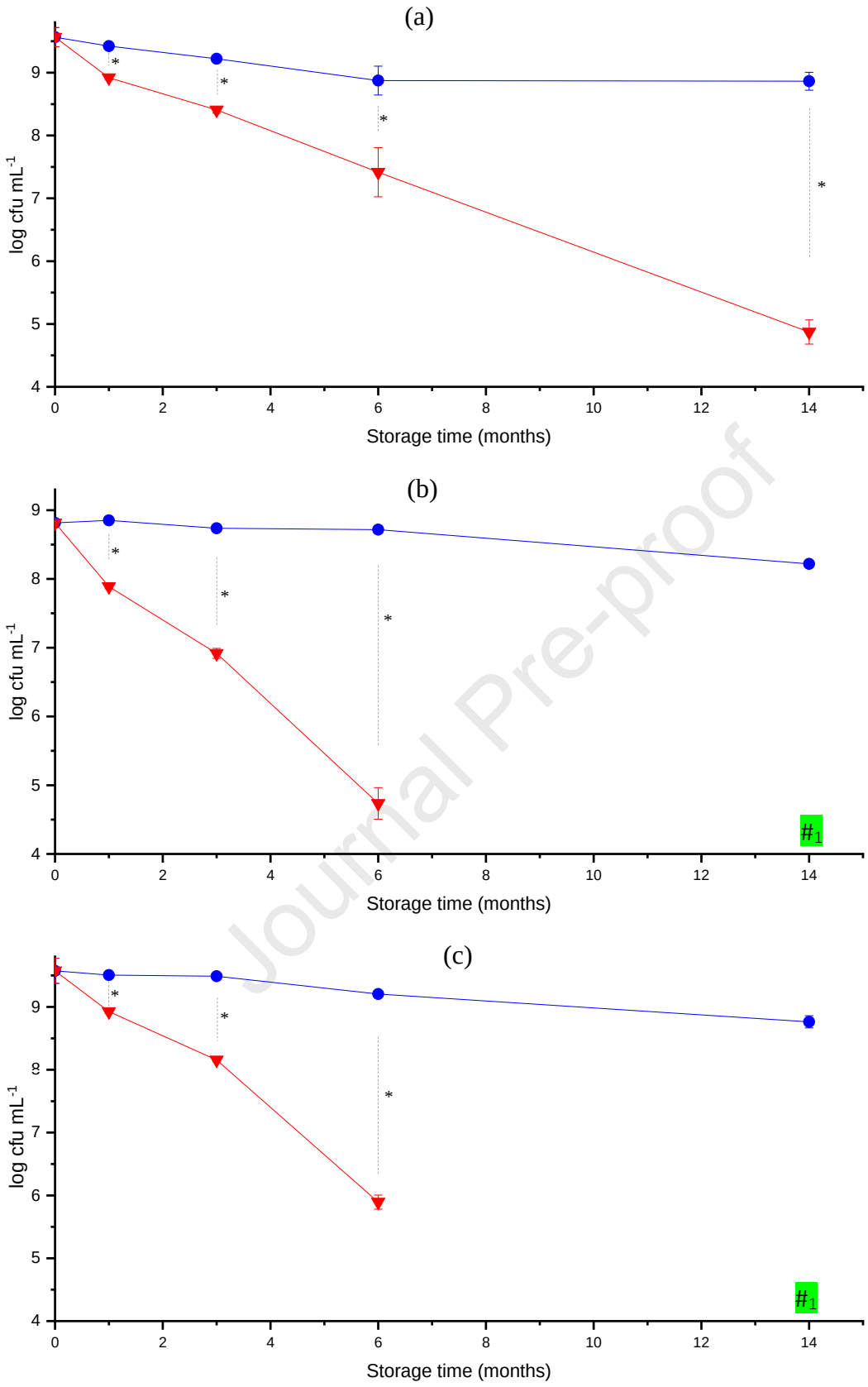


Figure 2

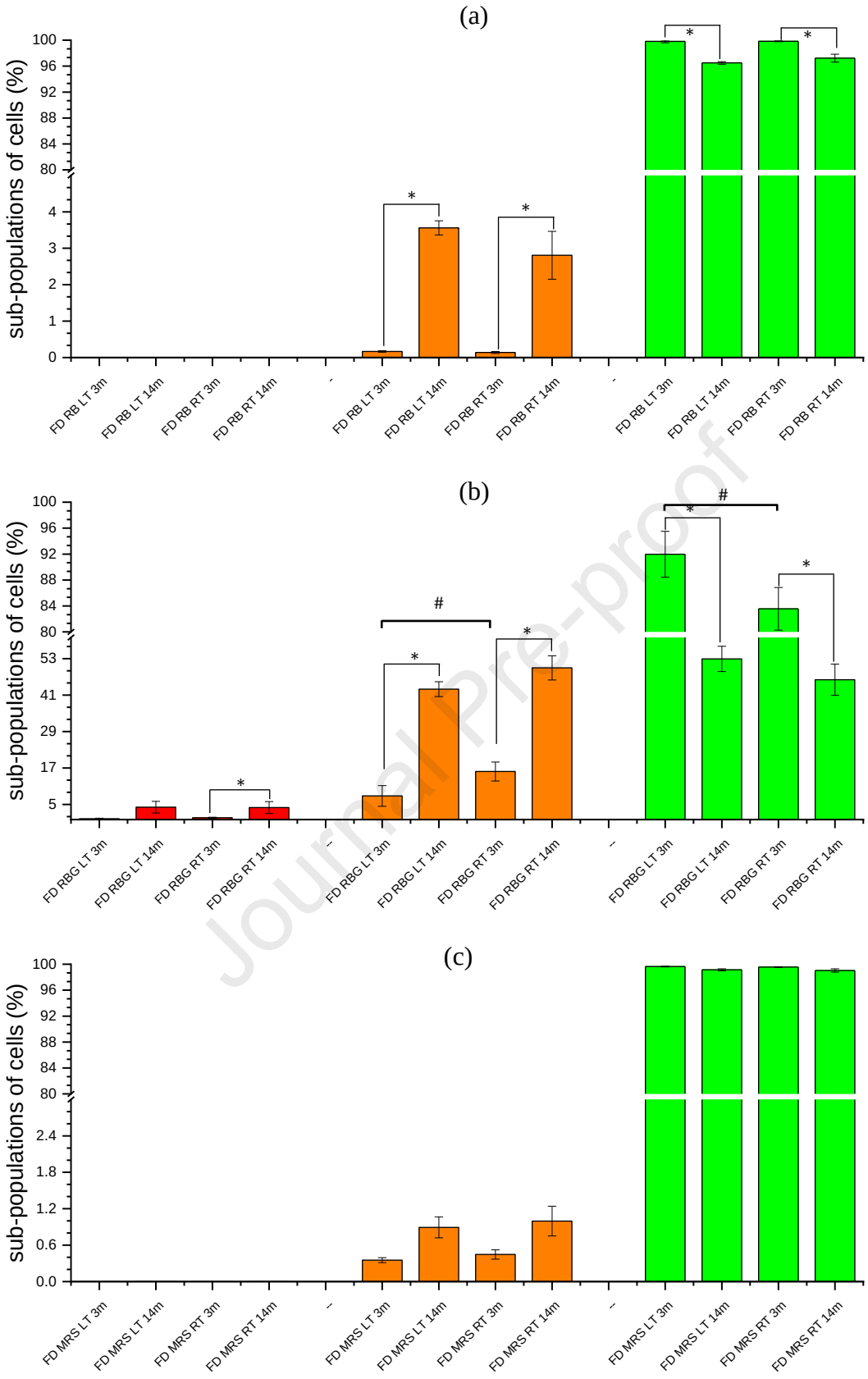

(a)
sub-populations of cells (%)
100
96
92
88
84
80
4
3
2
1
0
*
*
*
*
FD RB LT 3m
FD RB LT 14m
FD RB RT 3m
FD RB RT 14m
FD RB LT 3m
FD RB LT 14m
FD RB RT 3m
FD RB RT 14m
FD RB LT 3m
FD RB LT 14m
FD RB RT 3m
FD RB RT 14m
(b)
sub-populations of cells (%)
100
96
92
88
84
80
53
41
29
17
5
*
*
#
*
*
#
*
FD RBG LT 3m
FD RBG LT 14m
FD RBG RT 3m
FD RBG RT 14m
FD RBG LT 3m
FD RBG LT 14m
FD RBG RT 3m
FD RBG RT 14m
FD RBG LT 3m
FD RBG LT 14m
FD RBG RT 3m
FD RBG RT 14m
(c)
sub-populations of cells (%)
100
96
92
88
84
80
2.4
1.8
1.2
0.6
0.0
FD MRS LT 3m
FD MRS LT 14m
FD MRS RT 3m
FD MRS RT 14m
FD MRS LT 3m
FD MRS LT 14m
FD MRS RT 3m
FD MRS RT 14m
FD MRS LT 3m
FD MRS LT 14m
FD MRS RT 3m
FD MRS RT 14m

Figure 3

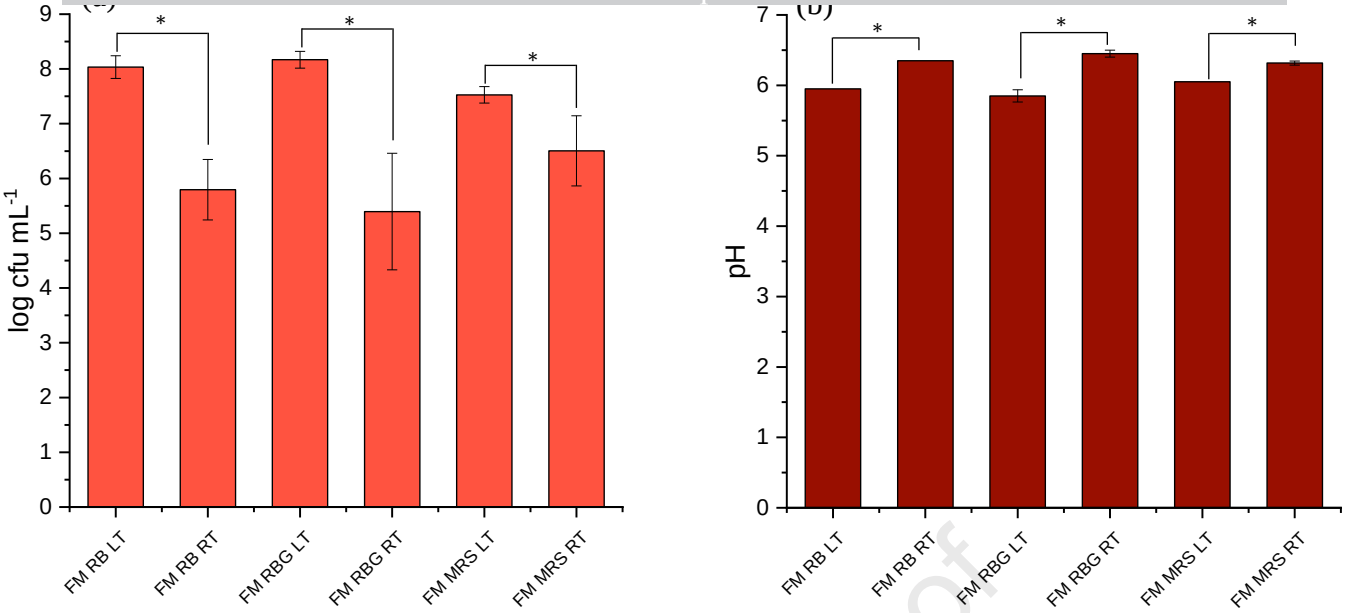
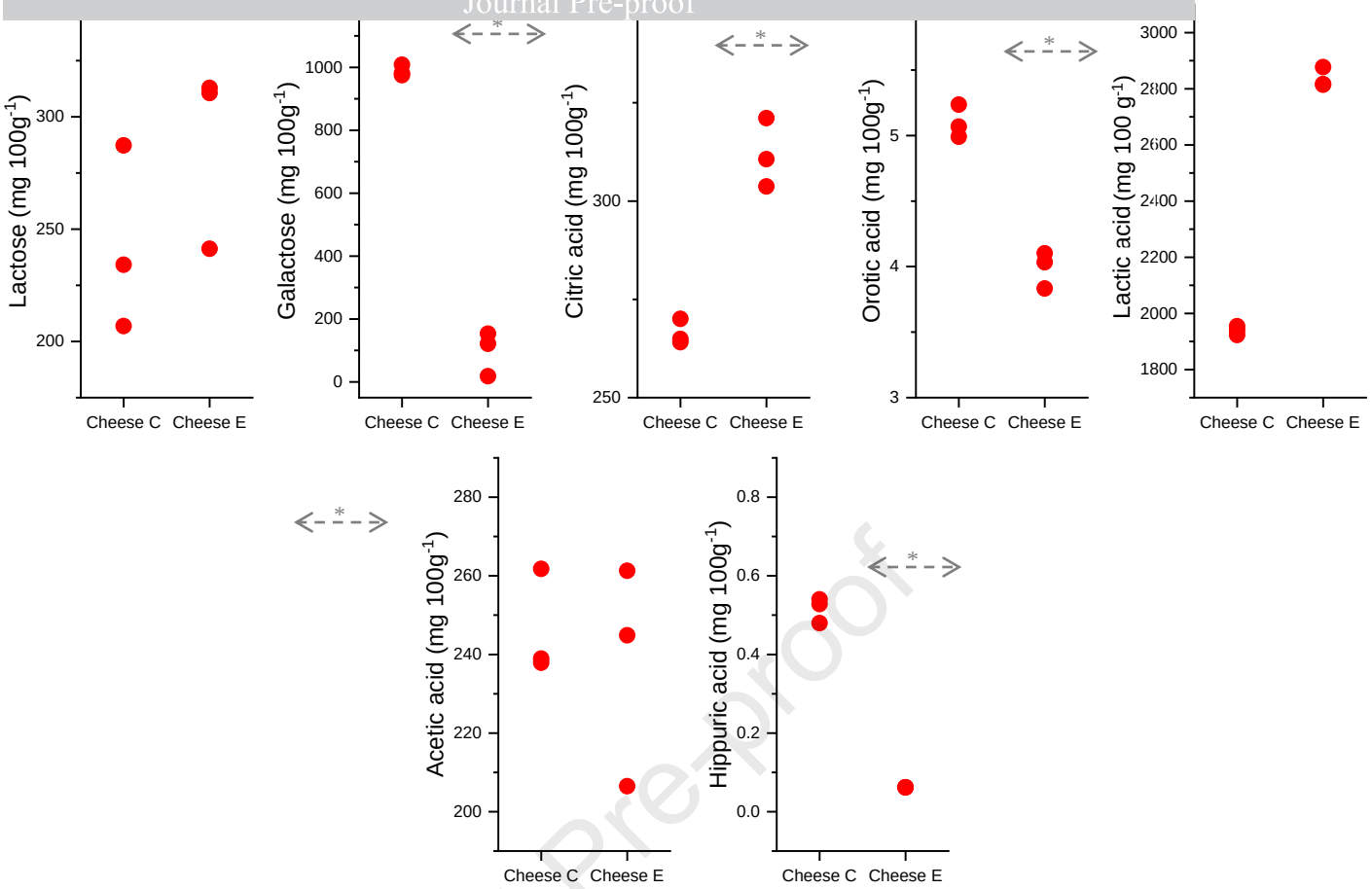


Figure 4

(a)



(b)

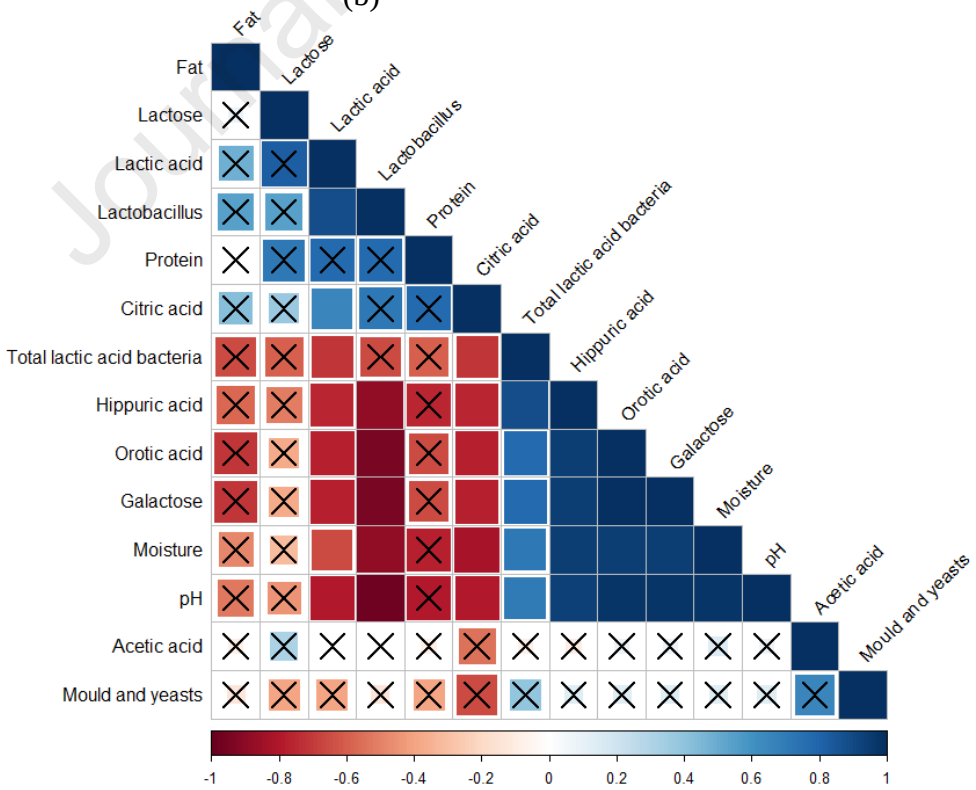


Figure 5

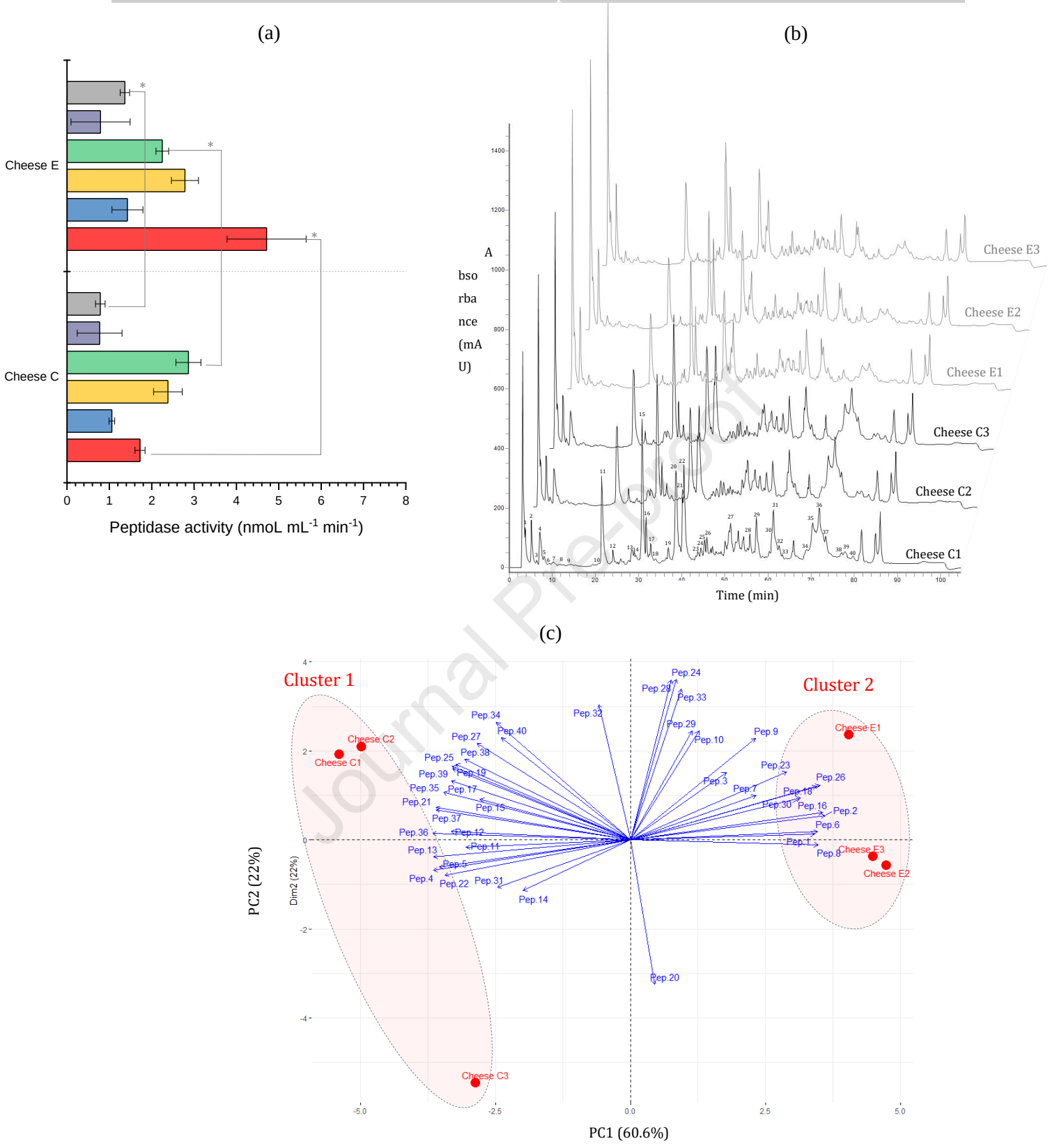
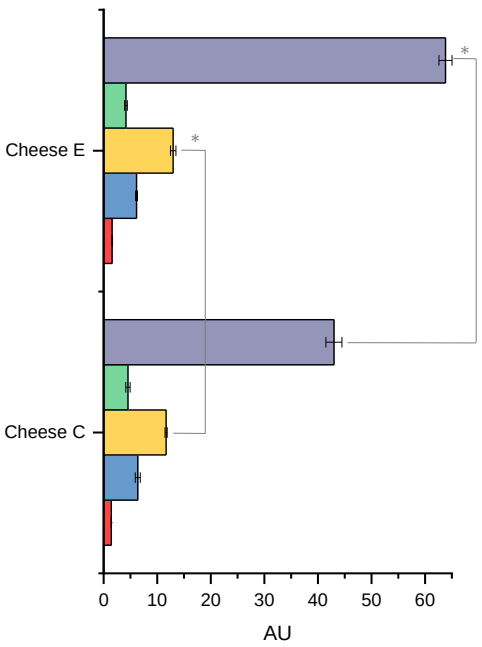
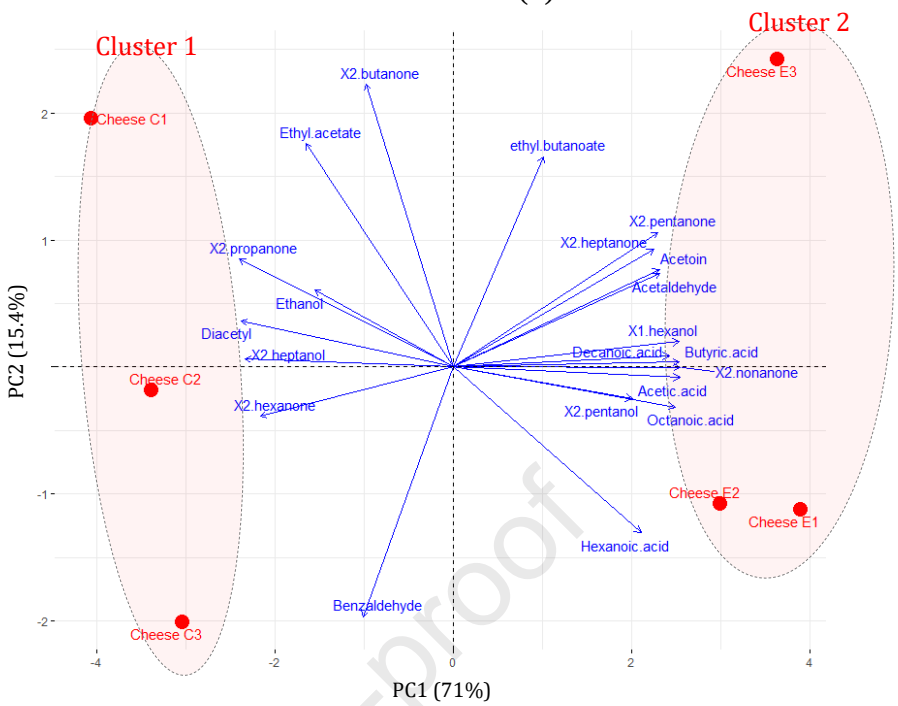


Figure 6

(a)



(b)



(c)

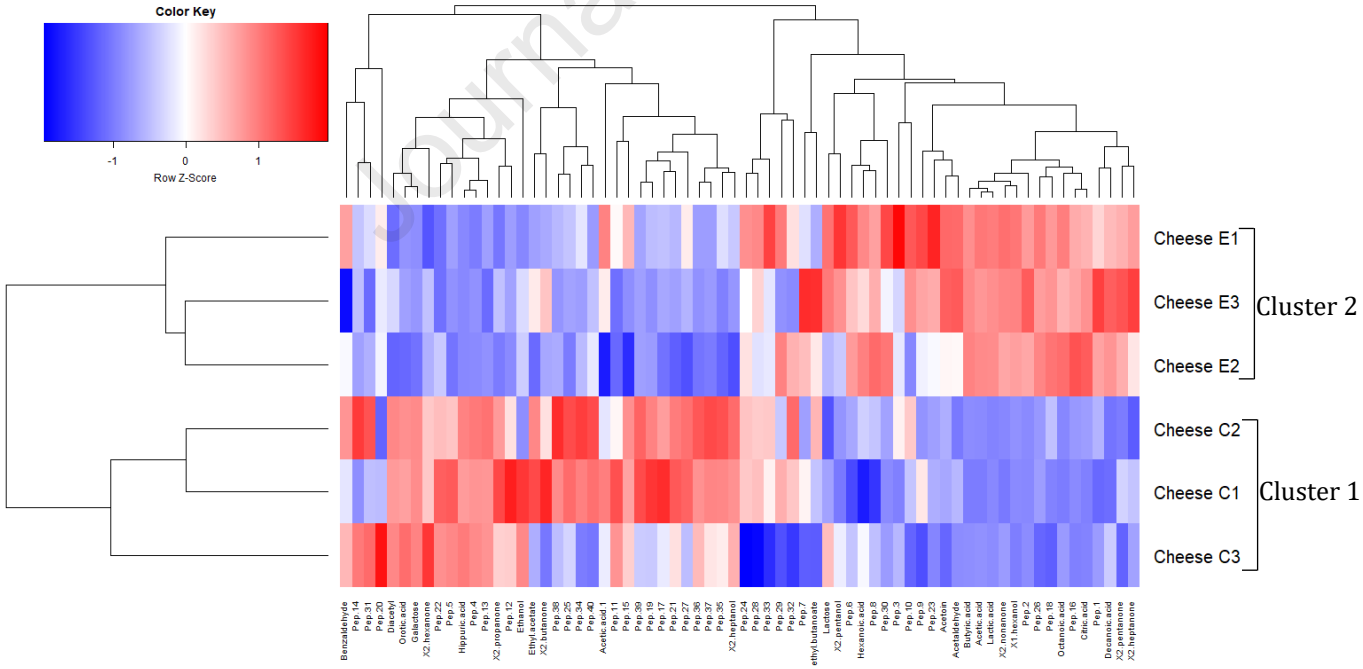


Figure 7

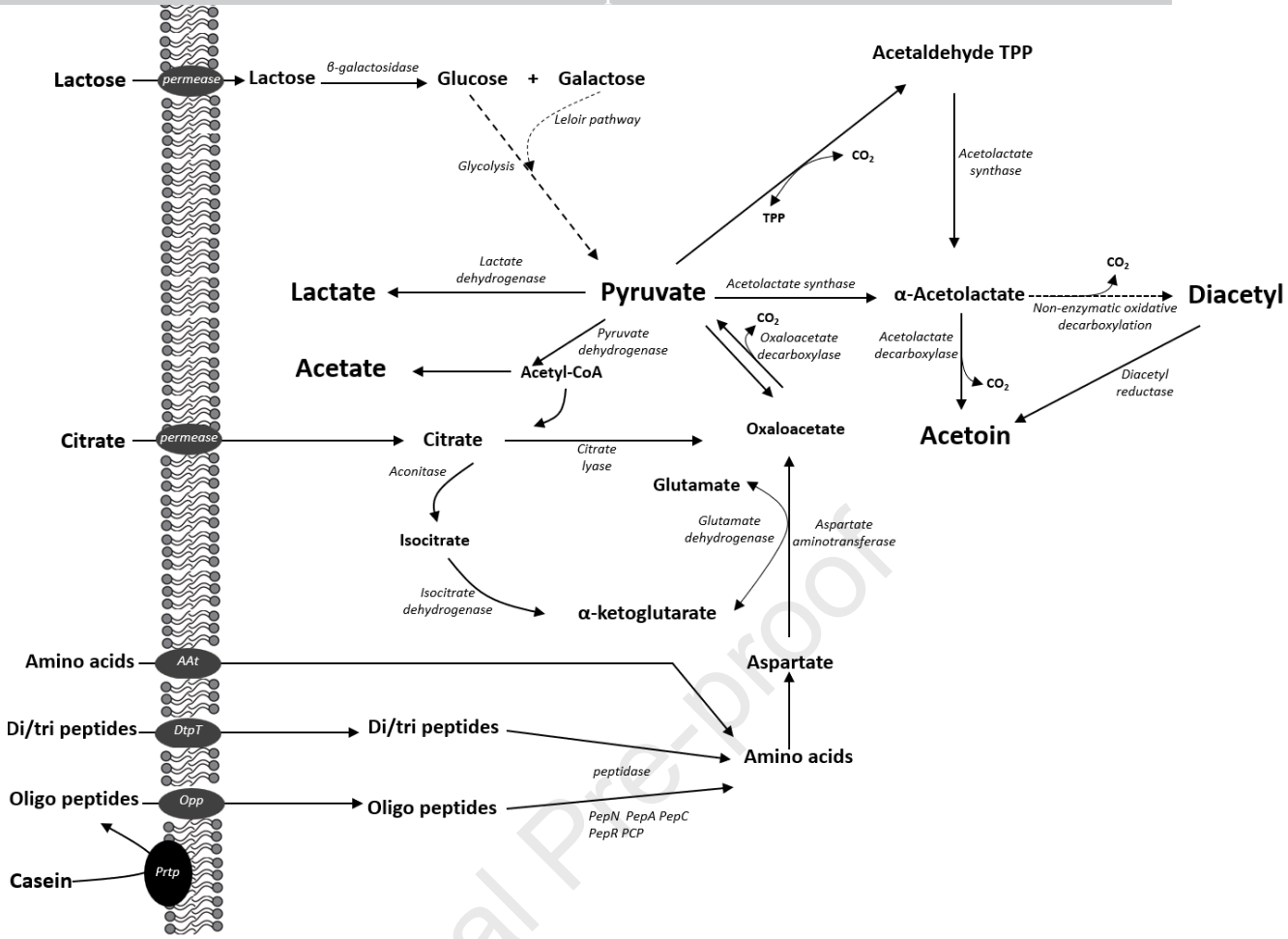


Figure 8

Credit author statement

Peralta GH: Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; writing – original draft; writing. **Beret MV**: investigation. **Bürgi MDM**: Investigation; **Ale EC**: Investigation. **Martínez LJ**: Investigation. **Albarracín VH**: Investigation. **Wolf IV**: Investigation. **Bergamini CV**: funding acquisition; investigation; methodology; project administration.

All authors contributed to writing, review, and editing.

Declaration of interests

X 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: