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One-pot process for the biotransformation of vegetable oils into natural deca- and dodecalactones

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ABSTRACT

Deca- and dodecalactones are highly desired natural compounds that are essential for creating flavor formulations with fruity, peachy, creamy, and floral notes. Although natural ingredients are preferred by consumers, these lactones cannot be extracted from natural sources. Therefore, the biotechnological processes that produce these compounds in their natural form are crucial for the flavor industry. Here, we report a study on the biotransformation of vegetable oils into natural deca- and dodecalactones. The proposed process is performed one-pot, through the sequential use of three different biotransformation steps, namely the lipase-mediated hydrolysis of the triglycerides, the use of probiotic bacteria for the hydration of the unsaturated fatty acids and the transformation of the obtained hydroxy-fatty acids into lactones derivatives employing *Yarrowia lipolytica*. By using a specific vegetable oil in combination with a selected bacterial strain, it is possible to obtain a preferred lactone derivative such as γ-dodecalactone, dairy lactone, tuberose lactone, or δ-decalactone in a concentration ranging from 0.9 to 1.5 g/L. Overall, our method is suitable for the industrial production of these lactones as it is easily scalable, it can be performed in only one bioreactor and it makes use of generally recognized as safe (GRAS) microorganisms.

1. Introduction

The γ- and δ-lactones possessing the chemical structures of type **1** and **2** (Fig. 1), respectively and having from eight to fourteen carbon atoms in their chemical frameworks are compounds of high industrial interest as they have been extensively used as flavour and fragrance ingredients (Surburg and Panten, 2016). Among them, decanolide, undecanolide and dodecanolide derivatives possess very low aroma threshold values, which decrease with increasing the molecular weight in the homologous series (Belitz et al., 2009). These compounds are recognized as key flavours to impart fruity, peachy, coconut-like, creamy, buttery and floral notes to food formulations (Burdock, 2010). In this context, natural lactones **3**-**9** hold particular relevance. These natural products occur in many foods, beverages, fruits and vegetables, where contribute to their specific aroma. More specifically, γ- and δ-decalactone (compounds **3** and **7**, respectively) and γ- and δ-dodecalactone (compounds **4** and **9**, respectively) have been identify in a very large number of foods but are typically present in ripening peaches, nectarines and other stone fruits (Visai and Vanoli, 1997). Dairy lactone (**5**) plays a relevant role in the flavour of dairy products, especially cheeses (Mariaca et al., 2001) and butter (Schlutt et al., 2007) and has been also detected in mango (Kuroki et al., 2021) and white wines (Siebert et al., 2018). Finally, tuberose lactone (**6**) and jasmine lactone (**8**) have been identified into tuberose absolute (Maurer and Hauser, 1982) and tuberose flowers (Mookherjee et al., 1990), respectively. It is worth noting that further researches have assessed the relevance of compounds **6** and **8** as components of butter (Sarrazin et al., 2011) and tea (Zeng et al., 2018) flavour, respectively. Moreover, recent studies indicated that lactones in foods may not only endow food with aroma but also play a role in modulating food pungency by acting on the transient receptor potential channels TRPV1 and TRPA1 (Ogawa et al., 2022).

All the described lactones can be obtained through three different ways: by chemical synthesis, by biotransformation of fatty acid derivatives and by extraction from natural sources. However, since these compounds occur in nature in very minute amounts, it is not feasible to extract them from natural sources at the industrial level. Therefore, the only affordable way to obtain them in their natural form is through the

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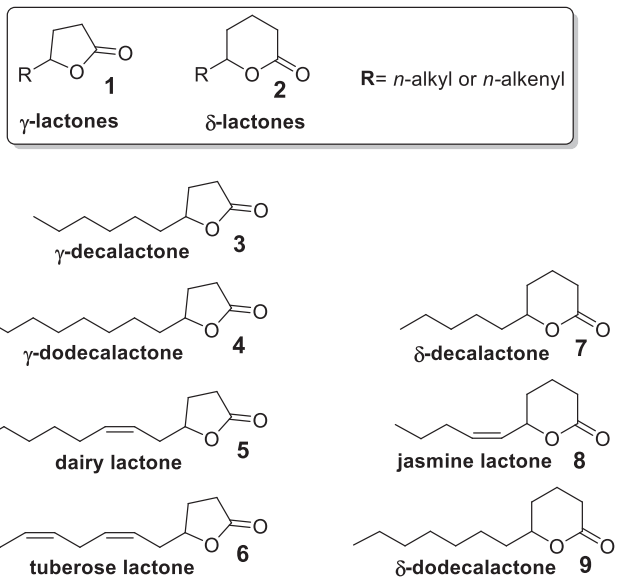

Fig. 1. The general (structures **1** and **2**) and particular (structures **3**-**9**) chemical structures of the lactones described in this work.

biotransformation of natural fatty acid derivatives. Indeed, according to the European (Regulation (EC), 2008) and USA (U.S. FDA, 2018) legislation, the biotransformation of a natural precursor is a 'natural method' of synthesis (Serra et al., 2005). The growing demand from consumers for natural flavor formulations has resulted in different market prices for the same chemical compound. Lactones that hold the 'natural' status are usually much more expensive than their synthetic counterparts, often by hundreds of times. Therefore, any new method that can produce these compounds in their high-value form can be highly profitable.

To date, the biotechnological methods that allow the production of natural lactones are based on the yeasts-mediated degradation of hydroxy-fatty acid (HFA) derivatives (Syed et al., 2022; Romero-Guido et al., 2011). More specifically, oleaginous yeasts are able to use fatty acids (FAs) as the carbon source for their own growth, degrading them through the β-oxidation mechanism (Waché et al., 2003). When the starting lipids contain HFAs, the degradation reaction ends with the formation of γ- or δ-HFAs, which undergo ring closure with lactone production.

This approach has been exploited for the industrial production of γ-decalactone (Braga and Belo, 2016; Singh et al., 2023), in a well-established process based on the biotransformation of castor oil with *Yarrowia lipolytica*. The latter yeast well suited the safety requirements related to food production, as it has been recently included in the list of the GRAS microorganisms (Turck et al., 2019). The main problem related to lactones production through the biotransformation of HFAs concerns the availability of the substrates themselves. Indeed, a very large number of HFAs are present in nature but almost only ricinoleic acid (present as triglyceride in castor oil) can be regarded as a cheap commodity.

Some new biocatalytic methods based on the hydration of the double bond of the unsaturated fatty acids have been studied to find reliable access to further HFAs. This biochemical transformation is catalysed by the enzymes oleate hydratases (OLH, EC 4.2.1.53) that selectively add a hydroxy group to the position 10 (10-hydratases) or to the position 13 (13-hydratases) respectively, of the unsaturated fatty acid chain (Cao and Zhang, 2013; Engleder and Pichler, 2018; Löwe and Gröger, 2020; Prem et al., 2022). Oleic (**10**), linoleic (**11**) and linolenic (**12**) acids (Fig. 2), which are the main components (as triglycerides) of the vegetable oils used for human consumption, are suitable substrates for this kind of reaction.

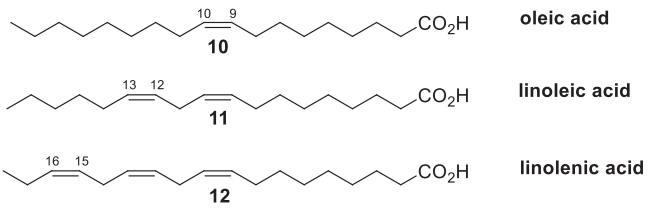

Fig. 2. The most common C_{18} unsaturated fatty acids present in vegetable oils: oleic (**10**), linoleic (**11**) and linolenic acid (**12**).

Different microorganisms are able to hydrate oleic and linoleic acid and the obtained 10-HFAs can be transformed into gamma dodecalactone, dairy lactone and tuberose lactone (Farbood et al., 1994). Unfortunately, most of the microorganisms used to perform the hydration step are potential pathogens and thus are not suitable for flavour industry. This problem has been partially solved by techniques of genetic engineering, through the expression of oleate hydratases in safer microorganisms (Löwe and Gröger, 2020; Jo et al., 2014; Kang et al., 2016). Despite these improvements, the concerns connected to the use of genetically modified microorganisms and the difficulties related to the use of isolated hydratases have hampered the extensive use of these biotechnological approaches.

We have already studied the hydratase activity of different probiotic strains (Serra and De Simeis, 2018; Serra et al., 2020, 2021; Castagna et al., 2020). These bacteria are regarded as beneficial for human health and their use does not involve any safety concerns (George Kerry et al., 2018), thus complying with the strict industrial processes requirements for food flavours production.

In this context, we demonstrated the versatility of some probiotic bacteria strains. In particular, *Lactobacillus rhamnosus* ATCC 53103 and *Lactobacillus plantarum* 299 V, can transform acids **10**–**12** into the corresponding 10-hydroxy derivatives, namely (*R*)−10-hydroxystearic acid (**13a**), (*S*)-(12*Z*)−10-hydroxy-octadecenoic acid (**13b**), and (*S*)-(12*Z*,15*Z*)−10-hydroxy-octadecadienoic acid (**13c**), respectively, in very high enantiomeric purity (ee > 95%) and in good yields (Fig. 3). Differently, *Bifidobacterium animalis* subsp. *lactis* (DSM 15954) hydrates selectively oleic acid and is inactive when linoleic and linolenic acid are used as substrates. Several other probiotics, belonging to the *Lactobacillus* and *Bifidobacterium* genus, possess similar biocatalytic properties with high selectivity toward the hydration of position 10 of the fatty acid chain. Nevertheless, a relevant exception to this general trend concerns *Lactobacillus acidophilus* ATCC SD5212. This strain possesses both 10- and 13-hydratase activity, most likely due to the simultaneous activity of two different enzymes. Therefore, the whole-cell biotransformation of

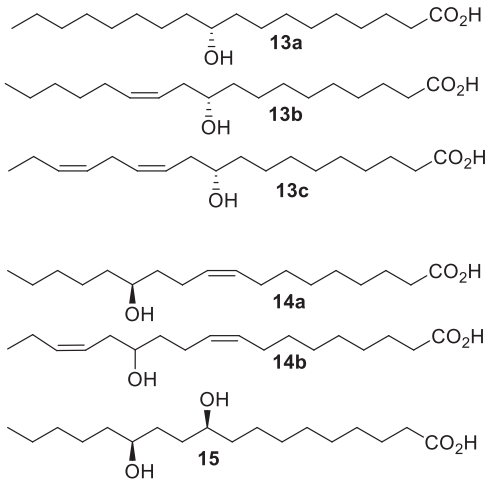

Fig. 3. The most relevant HFAs available by probiotics-mediated hydration of oleic, linoleic and linolenic acid.

linoleic and linolenic acid, using the latter microorganism, can afford a mixture of (*S*)-(12*Z*)−10-hydroxy-octadecenoic acid (**13b**), (*S*)-(9*Z*)−13-hydroxy-octadecenoic acid (**14a**), (9*Z*,15*Z*)−13-hydroxy-octadecadienoic acid (**14b**) and (10*S*,13*S*)-10,13-dihydroxy-octadecenoic acid (**15**).

Overall, we deem that the use of the above-described microorganisms can overcome different drawbacks related to the known biotechnological syntheses of natural lactones. Besides complying with the safety requirements connected to the flavours production, the use of probiotic strains can help to obtain preferentially a given HFA, even using a mixture of fatty acids as a starting material. More specifically, we envisage that the exploitation of the strain chemoselectivity, combined with the selection of the vegetable oil having a suitable fatty acid composition, can allow the direct conversion of this raw material into an HFAs mixture, enriched into the wanted HFA. Finally, it is worth noting that the transformation of vegetable oils into lactones needs at least of three steps, namely the hydrolysis of the triglycerides, the hydration of the obtained unsaturated FAs and the conversion of the HFAs into lactones. In this work, we demonstrated that all the above-described steps can be performed one-pot by a process that exploits the sequential use of probiotic strains and oleaginous yeasts.

2. Materials and methods

2.1. Materials and general methods

All air and moisture-sensitive reactions were carried out using dry solvents and under a static atmosphere of nitrogen.

Reference standard samples of (*Z*)−6-dodecen-4-olide (dairy lactone) and (*Z*,*Z*)−6,9-dodecadien-4-olide (tuberosa lactone) were synthesized as described previously (Maurer and Hauser, 1982).

Oleic acid (94%, lot. MKBZ2615V), linoleic acid (99%, lot. SLBT2627), linseed oil (Lot. MKBT9642V), lipase from *Candida rugosa* (Type VII, 1070 unit/mg), casein peptone, peptone from soybean, yeast extract, meat extract, malt extract, ammonium citrate dibasic, citric acid, Tween 80, sodium thioglycolate, sodium formaldehyde sulfoxylate, resazurin sodium salt, L-cysteine, urea and glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Riboflavin was purchased from Health Leads UK Ltd., (Horeb, UK).

Linolenic acid (85% purity, lot. 81003) was purchased from Nissan—Nippon Oil and Fats Co. (Tokyo, Japan), LTD.

A reference standard samples of (*R*)−10-hydroxystearic acid (**13a**), (*S*)-(12*Z*)−10-hydroxy-octadecenoic acid (**13b**) and (*S*)-(12*Z*,15*Z*)−10-hydroxy-octadecadienoic acid (**13c**) were prepared by *Lactobacillus rhamnosus* mediated hydration of oleic, linoleic and linolenic acids, respectively (Serra and De Simeis, 2018). (*S*)-(9*Z*)−13-hydroxy-octadecenoic acid (**14a**) and (10*S*,13*S*)-dihydroxystearic acid (**15**) were prepared by *Lactobacillus acidophilus* mediated hydration of linoleic acid (Serra et al., 2020).

The reference standards of δ-decalactone and γ-dodecalactone as well as chemical reagents and solvents were purchased from Merck (Merck Life Science S.r.l., Milan, Italy) and used without further purification.

Olive oil was purchased from Fiorentini Firenze S.p.A. (Colle Val D'Elsa, Italy). Sunflower oil and high oleic sunflower oil were purchased from Oleificio Zucchi (Cremona, Italy).

2.2. Analytical methods and characterization of the products deriving from the biotransformation experiments

TLC (Thin layer chromatography): Merck silica gel *60* F_{254} plates (Merck Millipore, Milan, Italy). The TLC plates were eluted with a 7:3 hexane/ethyl acetate mixture and the spots were detected with the cerium-molybdate stain reagent. Using these analytic conditions, we measured the following retention factors (R_f) values:

R_f triglycerides = 0.68; R_f free fatty acids = 0.44; R_f **13a** = R_f **13b** = R_f **13c** = 0.19; R_f **14a** = 0.22; R_f **4** = R_f **5** = R_f **6** = 0.26; R_f **7** = 0.30. The TLC analysis of reaction mixtures containing compound **15** was performed eluting with a 1:1 hexane/ethyl acetate mixture. In these conditions, we measured an R_f **15** = 0.20.

Column chromatography: silica gel.

Mass spectra were recorded on a Bruker ESQUIRE 3000 PLUS spectrometer (ESI detector) (Billerica, MA, USA) or by gas chromatography-mass spectrometry analyses (GC-MS analyses).

GC-MS analyses: A HP-6890 gas chromatograph equipped with a 5973 mass detector, using an HP-5MS column (30 m × 0.25 mm, 0.25 μm film thickness; Hewlett Packard, Palo Alto, CA, USA).

The biotransformations of oleic acid (**10**), linoleic acid (**11**), and linolenic acid (**12**) to give 10-hydroxystearic acid (**13a**), (12*Z*)−10-hydroxy-octadecenoic acid (**13b**), (9*Z*)−13-hydroxy-octadecenoic acid (**14a**), 10,13-dihydroxy-octadecenoic acid (**15**), and (12*Z*,15*Z*)−10-hydroxy-octadecadienoic acid (**13c**) were monitored by means of GC-MS analysis. To this end, a sample of the biotransformation mixture (10 mL) was acidified at pH 4 and filtered on celite. The biomass was washed with ethyl acetate (20 mL) and the aqueous phase was extracted with the same solvent (2 ×10 mL). The combined organic layers were washed with brine, dried (Na_2SO_4) and the solvent was removed under reduced pressure. The residue was treated at 0 °C with an excess of an ethereal solution of freshly prepared diazomethane. As soon as the evolution of nitrogen ceased, the solvent was eliminated and the residue was treated at RT with a 1:1 mixture of pyridine/acetic anhydride (4 mL for about 100 mg of residue) and 4-(dimethylamino)pyridine (DMAP, 10 mg). After five hours, the excess of reagents were removed *in vacuo* and the crude mixture was analysed by GC-MS using the following temperature program: 120° (3 min)—12°/min—195° (10 min)—12°/min—300° (10 min); carrier gas: He; constant flow 1 mL/min; split ratio: 1/30; retention times (t_R) given in minutes.

Oleic acid methyl ester: t_R 18.95.

GC-MS (EI): *m/z* (%) = 296 [M^+] (7), 264 (49), 235 (6), 222 (30), 180 (19), 166 (10), 152 (12), 137 (17), 123 (26), 110 (32), 97 (62), 83 (68), 69 (79), 55 (100).

Linoleic acid methyl ester: t_R 18.52.

GC-MS (EI): *m/z* (%) = 294 [M^+] (18), 263 (15), 234 (1), 220 (4), 178 (6), 164 (10), 150 (16), 135 (15), 123 (18), 109 (36), 95 (70), 81 (93), 67 (100), 55 (56).

Linolenic acid methyl ester: t_R 18.79.

GC-MS (EI): *m/z* (%) = 292 [M^+] (7), 261 (4), 249 (2), 236 (5), 191 (3), 173 (5), 149 (13), 135 (15), 121 (20), 108 (34), 95 (56), 79 (100), 67 (66), 55 (43).

Methyl 10-acetoxystearate: t_R 24.47.

GC-MS (EI): *m/z* (%) = 313 [M+-MeCO] (6), 296 [M+-AcOH] (3), 281 (17), 264 (31), 243 (11), 222 (9), 201 (100), 169 (64), 157 (16), 125 (21), 97 (18), 83 (19), 69 (21), 55 (27).

Methyl (12Z)−10-acetoxy-octadecenoate: t_R 24.28.

GC-MS (EI): *m/z* (%) 311 [M+-MeCO] (<1), 294 [M+-AcOH] (39), 279 (1), 263 (24), 220 (7), 201 (46), 169 (100), 150 (13), 136 (9), 123 (15), 109 (21), 95 (37), 81 (53), 67 (46), 55 (32).

Methyl (9Z)−13-acetoxy-octadecenoate: t_R 24.05.

GC-MS (EI): *m/z* (%) 311 [M+-MeCO] (<1), 294 [M+-AcOH] (40), 279 (1), 263 (29), 241 (3), 220 (9), 210 (10), 196 (12), 178 (22), 164 (28), 150 (24), 136 (26), 123 (27), 109 (41), 95 (78), 81 (100), 67 (95), 55 (79).

Methyl (12Z,15Z)−10-acetoxy-octadecadienoate: t_R 24.33.

GC-MS (EI): *m/z* (%) 292 [M+-AcOH] (76), 277 (1), 261 (20), 201 (33), 169 (100), 149 (19), 135 (28), 121 (41), 108 (42), 93 (57), 79 (87), 55 (39).

Methyl (10,13)-diacetoxy-stearate: t_R 26.61.

GC-MS (EI): *m/z* (%) 383 [M+-OMe] (2), 355 (1), 336 (1), 323 (2), 311 (11), 294 (58), 279 (6), 263 (42), 241 (70), 214 (18), 201 (41), 169 (48), 141 (100), 123 (47), 109 (25), 95 (50), 81 (78), 67 (58), 55 (69).

The transformation of the hydroxy-acids into lactones was monitored using GC-MS analysis. To this end, γ-undecalactone (internal standard;

0.5 mg/mL) was added to a sample of the biotransformation mixture (20 mL). The pH was adjusted to 4 adding citric acid and the sample was heated to 100 °C for 15 minutes. After cooling, ethyl acetate (one-fifth of the sample volume) was added and the heterogeneous mixture was shaken vigorously. Hence, the organic phase was separated by centrifugation and analysed using the following GC-MS temperature program: 60° (1 min) − 6°/min − 150° (1 min)− 12°/min − 280° (5 min); carrier gas: He; constant flow 1 mL/min; split ratio: 1/30; t_R given in minutes.

γ-dodecalactone (***4***): t_R 20.72.

GC-MS (EI): *m/z* (%) = 198 [M^+] (<1), 180 (2), 169 (1), 162 (2), 151 (2), 136 (5), 128 (11), 114 (4), 110 (4), 100 (5), 96 (5), 85 (100), 69 (8), 55 (12).

(Z)−6-dodecen-4-olide (dairy lactone, ***5****)*: t_R 20.40.

GC-MS (EI): *m/z* (%) = 196 [M^+] (2), 178 (<1), 167 (<1), 136 (3), 121 (1), 109 (1), 96 (8), 85 (100), 81 (5), 67 (4), 55 (5).

(Z,Z)−6,9-dodecadien-4-olide (tuberosa lactone, ***6****)*: t_R 20.49.

GC-MS (EI): *m/z* (%) = 194 [M^+] (2), 179 (<1), 165 (2), 147 (8), 134 (6), 121 (9), 105 (14), 94 (38), 85 (100), 79 (41), 67 (18), 55 (11).

γ-undecalactone: t_R 19.09.

GC-MS (EI): *m/z* (%) = 183 [M+-1] (<1), 166 (<1), 148 (<1), 137 (<1), 128 (8), 122 (2), 114 (2), 100 (3), 85 (100), 69 (8), 55 (11).

δ-decalactone (***7***): t_R 17.95.

GC-MS (EI): *m/z* (%) = 152 (5), 141 (3), 114 (11), 99 (100), 84 (6), 81 (6), 71 (50), 55 (37).

2.3. Microorganisms and growth media

Yarrowia lipolytica (DSM 8218) was purchased from DSMZ GmbH collection (Braunschweig, Germany).

Lactobacillus rhamnosus (ATCC 53103, trade name Kaleidon 60), was purchased from Malesci Spa (Bagno a Ripoli, Italy).

Lactobacillus acidophilus (La-14 = ATCC SD5212, trade name GSE AcidophiPlus) was purchased from Prodeco Pharma S.r.l (Castelfranco Veneto, Italy).

Lactobacillus plantarum (299 V, trade name Smebiocta LP299V) was purchased from Ipsen Pharma (Boulogne-Billancourt, France).

Bifidobacterium animalis subsp. *lactis* (BB-12 = DSM 15954, trade name Bifido Lactis Infant) was purchased from SOFAR S.p.A. (Trezzano Rosa, Italy).

The growth media used in this work are MRS Medium (MRS), Bifidobacterium Medium (BM), Minimal Medium for Oleaginous Yeasts (MMOY) and Universal Medium for Yeasts (YUM).

MRS composition: casein peptone (10 g/L), meat extract (10 g/L), yeast extract (5 g/L), glucose (20 g/L), Tween 80 (1 mL/L), K_2HPO_4 (2 g/L), NaOAc (5 g/L), ammonium citrate dibasic (2 g/L), $MgSO_4{\cdot}7H_2O$ (0.2 g/L) $MnSO_4{\cdot}H_2O$ (50 mg/L), L-cysteine 0.1% (w/w), sodium thioglycolate (2 g/L).

BM composition: casein peptone (10 g/L), peptone from soybean (5 g/L), yeast extract (5 g/L), meat extract (5 g/L), glucose (10 g/L), NaCl (5 g/L), K_2HPO_4 (2 g/L), Tween 80 (1 mL/L), $MgSO_4{\cdot}7H_2O$ (0.2 g/L) $MnSO_4{\cdot}H_2O$ (50 mg/L), L-cysteine 0.1% (w/w), sodium thioglycolate (2 g/L), sodium formaldehyde sulfoxylate (1 g/L), salt solution [$CaCl_2{\cdot}2H_2O$ (0.25 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), KH_2PO_4 (1 g/L), K_2HPO_4 (1 g/L), $NaHCO_3$ (10 g/L), NaCl (2 g/L)] 40 mL/L.

MMOY composition: urea (5 g/L), $Na_2HPO_4{\cdot}12H_2O$ (1.4 g/L), KH_2PO_4 (0.35 g/L), yeast extract (2 g/L), $MgSO_4{\cdot}7H_2O$ (1 g/L), Tween 80 (0.5 mL/L), riboflavin (10 mg/L), trace elements solution (10 mL/L). Trace elements solution: $FeCl_3$ (50 mM), $CaCl_2$ (20 mM), $MnCl_2$ (10 mM), $ZnSO_4$ (10 mM), $CoCl_2$ (2 mM), $CuCl_2$ (2 mM), $NiCl_2$ (2 mM), Na_2MoO_4 (2 mM), Na_2SeO_3 (2 mM), H_3BO_3 (2 mM).

YUM composition: yeast extract (3 g/L), malt extract (3 g/L), peptone from soybeans (5 g/L), glucose (10 g/L).

MRS was used for all *lactobacillus* species.

BM was used for *Bifidobacterium animalis* subsp. *lactis*.

YUM and MMOY were used for the pre-growth of *Yarrowia lipolytica*.

All the biotransformations were carried out in triplicate and the presented results are the mean of three experimental data.

All the preparative biotransformation experiments were performed using a 5 L fermenter (Biostat A BB-8822000, Sartorius-Stedim (Göttingen, Germany).

2.4. General procedures for the biotransformation experiments

2.4.1. Chemoselectivity of the microbial-mediated hydration of oleic, linoleic and linolenic acids

The anaerobic flasks were prepared by loading 40 mL of the suitable medium (MRS, BM) in 100 mL conical vacuum flasks followed by the addition of cysteine (40 mg), and resazurine sodium salt (1 mg) or methylene blue (for BM; 1 mg). The flasks were flushed with nitrogen until complete removal of the oxygen content, then were sealed with silicone rubber septa and sterilized (121 °C, 15 min.). Each flask was inoculated via a syringe with the suitable lyophilized bacteria strain (about 2×10^9 colony forming units (CFU), suspended in 2 mL of sterilized skimmed milk). Then, the flasks were incubated at 37 °C and 150 rpm. As soon as the lactic acid bacteria started to grow (4–8 h), a 1:1:1 mixture of oleic/linoleic/linolenic acid (240 mg) in ethanol (300 μL) and 2 mL of a sterilized solution of glucose (300 g/L) in water, were added and the stirring was prolonged for further 48–80 h. Hence, the whole microbial culture was acidified at pH 4 by the addition of diluted HCl (3 % w/v) and then filtered on celite. The biomass was washed with ethyl acetate (40 mL) and the aqueous phase was extracted with the same solvent (2 × 30 mL). The combined organic layers were washed with brine, dried (Na_2SO_4) and the solvent was removed under reduced pressure. The crude biotransformation mixtures were derivatized and analyzed by GC-MS as described above. The results of the experiments are collected in Table 1.

2.4.2. Preparation of the lactic bacteria inoculum

The anaerobic flasks (100 mL volume, 40 mL of medium) were prepared as described above (2.4.1). Each flask was inoculated via a syringe with the suitable lyophilized bacteria strain (about 2×10^9 CFU, suspended in 2 mL of sterilized skimmed milk) and was incubated at 37 °C and at 120 rpm for 72 h. The whole microbial culture was centrifuged at 3220×*g* for 3 min (4 °C), the supernatant was removed and the cells were resuspended in 5 mL of sterilized skimmed milk. The obtained suspension was used to inoculate a sterilized fermenter vessel containing the suitable nitrogen-flushed medium (2 L).

2.4.3. Preparation of the Yarrowia lipolytica inoculum

A 100 mL conical flask was loaded with 40 mL of YUM, sealed with a cellulose plug and sterilized (121 °C, 15 min.). Then, the flask was inoculated with an active culture of *Yarrowia lipolytica* and was

Table 1
Microbial-mediated hydration of an equimolar mixture of oleic/linoleic/linolenic acids.[a]

Entry	Microorganism	Fermentation time[2]	Hydroxy fatty acids ratio[3,4]
1	*Bifidobacterium animalis* sub. *lactis*	48	**13a/13b/13c** = 99:1:0
2	*Lactobacillus rhamnosus*	48	**13a/13b/13c** = 47:20:33
3	*Lactobacillus rhamnosus*	68	**13a/13b/13c** = 59:16:25
4	*Lactobacillus rhamnosus*	80	**13a/13b/13c** = 71:14:15
5	*Lactobacillus plantarum*	48	**13a/13b/13c** = 37:32:31
6	*Lactobacillus acidophilus*	48	**13a/13b/13c/14a/14b/15** = 50:7:0:31:0:12

[a] The biotransformation experiments were performed in a 100 mL anaerobic flask, at 37 C° and using a 1:1:1 mixture of oleic/linoleic/linolenic acids with a starting concentration of 6 g/L (stirring 150 rpm). [2] Number of hours between the inoculum of the fatty acids mixture and the analysis of the fermentation broth. [3] The ratio was measured through GC-MS analysis. [4] We assigned a value equal to zero when a given hydroxy-acid derivative was not detected by GC-MS analysis or its relative amounts was very low (<1 %)

incubated at 28 °C, at 120 rpm for 72 h. The obtained active culture (5 mL) was inoculated into a previously sterilized 500 mL conical flask containing MMOY (100 mL) and suitable vegetable oil (2 g). The flask was sealed with a cellulose plug and was stirred (150 rpm) at 28 °C for 4 days. The whole microbial culture was centrifuged at 4000×*g* for 5 min (4 °C), the supernatant was removed and the cells were resuspended in 5 mL of sterilized saline. The obtained suspension was used to inoculate a fermenter vessel containing the biotransformation mixture (2 L) of the vegetable oil.

2.4.4. General procedure for the direct transformation of vegetable oils into deca- or dodecalactone derivatives

The selected lactic bacteria inoculum was added to a sterilized fermenter vessel containing the suitable anaerobic medium (2 L). The temperature, the stirring speed, and the pH were set to 37 °C, 160 rpm and 6.8, respectively. The pH was controlled by the dropwise addition of sterilized aqueous solutions (10 % *w/w* in water) of either sulfuric acid or ammonia.

After some hours (4–8 h, depending on the strain used) the fermentation showed an exponential phase of growth, as indicated by starting of the continuous addition of base, necessary to neutralize the lactic acid produced by the glucose bacterial catabolism. At this point, the suitable vegetable oil (10–30 g), Tween 80 (1 mL) and a suspension of *Candida rugosa* lipase (CRL, 0.6 g) in aqueous glycerol (50 % w/v, 4 mL) were added at once. The stirring rate was increased to 300 rpm. As soon as the lactic acid production decreased (about 24 h since bacterial inoculum), 100 mL of a sterilized solution of glucose (300 g/L) in water was added and the anaerobic biotransformation was prolonged until the TLC analysis indicated that the hydration reaction didn't proceed any further (48–72 h). After this time, the activity of the lactic acid bacteria was stopped switching the biotransformation to the aerobic conditions, by flushing the fermenter with a continuous stream of air (0.6 v/v/min). Hence, the *Yarrowia lipolytica* inoculum, prepared as described above, was added at once and the transformation of the hydroxy-acid derivatives into lactones was monitored by TLC analysis. During the first 18–24 h after the yeast inoculum, the oxygen probe recorded a rapid p-O_2 decrease without any lactone formation. Hence, we observed that p-O_2 reached a minimum value whilst simultaneously the formation of the lactones started. During the second 18–24 h, the TLC analyses showed a slow decrease of the hydroxy-acids concentration and a rapid increase of the lactones concentration. Before partial transformation of the hydroxy-acids, we observed the initial formation of different side products, deriving from the degradation of lactones themselves. As soon as this over-oxidation reaction was detectable by TLC analysis, the biotransformation was stopped. The pH was adjusted to 4 adding citric acid, the fermentation broth was downloaded and heated to 100 °C for 15 minutes. Hence, the isolation of the lactones mixture can be performed through two different procedures:

-Extraction and purification.

The broth was cooled and was mixed with diethyl ether (300 mL). The obtained heterogeneous mixture was filtered through a celite pad and the collected biomass was washed thoroughly with diethyl ether (300 mL). The upper layer was separated and the aqueous phase was extracted again with diethyl ether (300 mL). The combined organic phases were dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by chromatography using *n*-hexane/AcOEt (9:1–6:4) as the eluent. All the fractions containing lactones were pulled together, the solvent was evaporated and the residue was bulb-to-bulb distilled to give the lactones mixture.

-Steam distillation.

The broth was heated at 100 °C, under stirring, while a slow stream of steam was fluxed throughout the heterogeneous biotransformation mixture. The distillation was prolonged until no further lactones were detectable by TLC analysis of the distillate (3 h). The collected aqueous fractions were extracted with CH_2Cl_2 (2 × 150 mL) and the combined organic phases were dried (Na_2SO_4) and concentrated *in vacuo*. Then, the residue was bulb-to-bulb distilled to give the lactones mixture.

The above-described general procedure was applied using the following combination of lactic bacteria and vegetable oils:

- *Bifidobacterium animalis* subsp. *lactis*/olive oil (5 g/L):
- *Bifidobacterium animalis* subsp. *lactis*/high oleic sunflower oil (5 g/L).
- *Bifidobacterium animalis* subsp. *lactis*/high oleic sunflower oil (15 g/L).
- *Lactobacillus rhamnosus*/sunflower oil (5 g/L).
- *Lactobacillus plantarum*/sunflower oil (9 g/L).
- *Lactobacillus acidophilus*/sunflower oil (5 g/L).
- *Lactobacillus rhamnosus*/linseed oil (5 g/L).

Yields and composition of HFAs and lactones are described in Table 3.

3. Results and discussion

As mentioned above, our previous researches (Serra and De Simeis, 2018; Serra et al., 2020) have outlined the potential of different *Lactobacillus* and *Bifidobacterium* strains in performing the hydration of acids **10-12**. According to these findings, we selected *Lactobacillus rhamnosus* ATCC 53103, *Lactobacillus plantarum* 299 V, *Bifidobacterium animalis* subsp. *lactis* (DSM 15954) and *Lactobacillus acidophilus* ATCC SD5212, as suitable strains to be employed in this study. Indeed, the latter microorganisms have shown better hydration performances in terms of efficiency, versatility and stereoselectivity (Serra et al., 2020). These results concern the biocatalytic capabilities of each one of the latter strains toward a specific fatty acid (oleic, linoleic or linolenic acid) without considering a possible chemical selectivity of the hydration reaction. Since we were interested in the preparation of HFAs by biotransformation of vegetable oils, we had to take into account that the latter raw material is usually made up of a complex mixture of fatty acids. Therefore, in order to study the chemical selectivity of the above-described four strains, we planned a new set of experiments. Accordingly, each one of the selected strains was used as a whole-cell biocatalyst to perform the hydration reaction of an equimolar mixture of the three acids **10-12**. We chose to employ a very high substrates concentration (6 g/L) to ensure a minimal variation of their composition during the biotransformation. Hence, we measured the chemioselectivity of the hydration reaction by determining the relative ratio of the formed HFAs. The obtained results (Table 1) suggest a number of considerations.

Firstly, *Bifidobacterium animalis* confirmed to have almost complete selectivity for oleic acid (entry 1). The hydroxy group was introduced in position 10 only and the relative ratio of the HFAs formed (**13a**/**13b**/**13c** = 99:1:0) clearly indicated that this is the strain of choice for converting vegetable oils, rich in oleic acid, into 10-hydroxystearic acid (**13a**).

Similarly, both *Lactobacillus rhamnosus* and *Lactobacillus plantarum* hydrated fatty acids **10-12** in position 10 only, although with a different chemical selectivity. *Lactobacillus rhamnosus* showed a hydration rate in the order oleic>linolenic>linoleic (entry 2). Noteworthy, with longer incubation time (entries 3 and 4), we observed an increase in the 10-hydroxystearic (**13a**) content, formally corresponding to an increasing oleic acid transformation rate. We could explain this odd result by taking into account that acid **13a** has very low solubility in the biotransformation medium and starts to precipitate as soon as it is formed. Therefore, the probiotic strain activity is not inhibited by **13a**, which keeps forming at a constant rate. Otherwise, acids **13b** and **13c** are liquid at the biotransformation temperature and thus their formation rate decreases with longer incubation times.

Lactobacillus plantarum didn't show a defined chemoselectivity (entry 5). The experiment indicated that this strain hydrated the three fatty acids with the same efficiency.

Lactobacillus acidophilus hydrated efficiently oleic and linoleic acids, leaving linolenic acid unaffected (entry 6). The latter observation is

noteworthy as the lack of formation of compounds **13c** and **14b** results in the impossibility of producing tuberose lactone (**6**) and jasmine lactone (**8**) using *L. acidophilus*. This strain showed both 10- and 13-hydratase activity, and linoleic acid was transformed into 13-hydroxy derivate (**14a**), 10-hydroxy derivate (**13b**) and diol **15**, formed tank to the combined action of both hydratase. The relative ratio of the HFAs formed (**13a**/**13b**/**14a**/**15** = 50:7:31:12), clearly indicated that using as a substrate a fatty acid mixture enriched in linoleic acid, the biotransformation process could afford a HFAs mixture enriched in the acid **14a**.

Concerning vegetable oils, we selected four different commercial products (Table 2). Olive oil and high oleic sunflower oil showed a higher oleic acid content (75 % and 78 %, respectively) with a rather low (8–9 %) linoleic acid content. Sunflower oil and linseed oil are the best sources of linoleic acid (60 %) and α-linolenic acid (53 %) respectively, although they contain a significant amount of oleic acid (26 % and 21 %, respectively).

Other cheap and easily available vegetable oils, such as canola, soybean and peanut oils contain high levels of the unsaturated FAs **10** and **11** but there is not a very significant prevalence of one over another.

Taking advantage of all the above-described findings, we devised a new, one-pot process that allows converting the four selected vegetable oils into γ-dodecalactone, dairy lactone, tuberose lactone, δ-decalactone or mixtures thereof. The procedure is schematically described in Fig. 4.

The first step, namely the hydrolysis of the triglycerides, must be performed enzymatically because chemical reactions are not allowed in processes for natural flavour preparation. According to our preliminary experiments, we selected *Candida rugosa* lipase (CRL) as the most efficient lipase to catalyze the latter transformation. Indeed, all the selected oils are efficiently hydrolyzed to free fatty acids in a very short time (< 1 h with 300 mg/L of the commercial enzyme with activity =1070 unit/mg) even at high concentrations (up to 15 g/L).

From a preparative standpoint, we had to face several problems. Firstly, the hydration step proceeds efficiently only if the probiotic bacteria are put in contact with the free fatty acid during the exponential phase of growth. It is worth noting that triglycerides are unaffected and late addition of the free fatty acids (during the stationary phase of growth) leads to very low production of HFAs. A second problem concerns microbial metabolism. *Lactobacillus* and *Bifidobacterium* strains grow properly only in anaerobic conditions whereas oleaginous yeasts transform the HFAs into lactones, through β-oxidation, only with a high oxygen supply.

We fulfill all the described requirements setting sequentially all the biochemical steps, in only one bioreactor and devising a new general protocol for the direct transformation of vegetable oils in lactones. Accordingly, the suitable probiotic strain was grown anaerobically on a glucose-rich medium (MRS broth). As soon as the exponential phase of growth was started, the vegetable oil (from 5 to 15 g/L) and CRL were added to the bioreactor. The hydrolysis of the triglyceride ended in less than one hour and simultaneously was possible to observe the formation of the HFAs. After 2–4 fermentation days, the activity of the probiotic bacteria was inhibited, switching the bioreactor environment from anaerobic to aerobic. The residual nutrients present in the biotransformation broth supported the initial growth of the oleaginous yeast

Table 2
The fatty acid composition[a] of the vegetable oils used in the biotransformation experiments.

Vegetable Oil	Saturated	Unsaturated	Other
Olive	15 %	Oleic (75 %), linoleic (8 %)	2 %
High Oleic Sunflower	10 %	Oleic (78 %), linoleic (9 %)	3 %
Sunflower	11 %	Oleic (26 %), linoleic (61 %)	2 %
Linseed	8 %	Oleic (21 %), linoleic (17 %), α-linolenic (53 %)	1 %

[a] Determined by GC-MS analysis after transformation of the glycerides into fatty acid methyl ester.

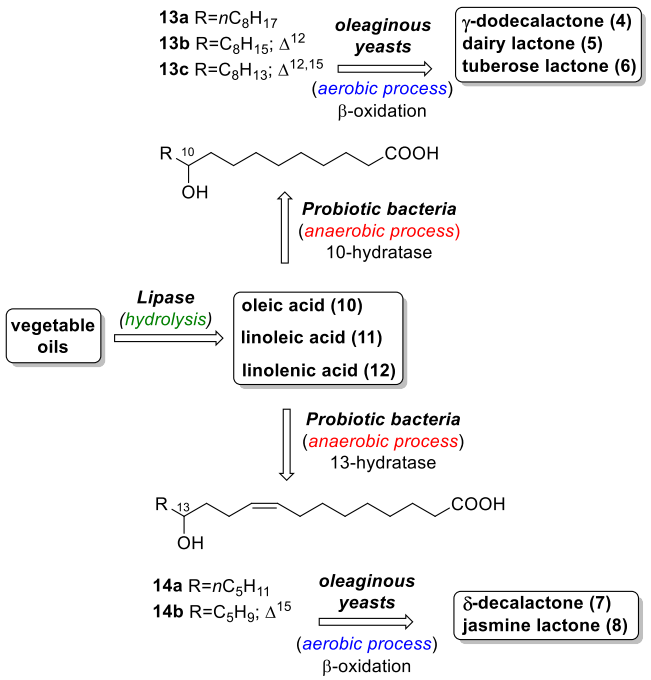

Fig. 4. The proposed one-pot process for the transformation of vegetable oils into high-value natural lactones.

without FAs transformation (about 24 h). The latter microorganism, once its biomass was increased markedly, started to degrade FAs and HFAs. To this end, we selected a specific strain of *Yarrowia lipolytica* (DSM 8218), which possesses very high oxidative activity (Cerniglia and Crow, 1981). After a further 24–48 h, the lactone mixture was isolated by steam distillation or by extraction with solvents followed by chromatographic purification.

We applied the above-described protocol employing a suitable probiotic strain/vegetable oil combination. More specifically, the *Bifidobacterium animalis* subsp. *lactis* was used to transform vegetable oils with a high oleic acid content, such as olive oil or high oleic sunflower oil. *Lactobacillus rhamnosus*, *Lactobacillus plantarum* and *Lactobacillus acidophilus* were employed in the biotransformation of sunflower oil, which is rich in linoleic acid. Finally, *Lactobacillus rhamnosus* was exploited for the hydration of the linolenic acid, contained in linseed oil.

All the biotransformations were performed on a preparative scale (2 L medium, 5 L bioreactor) using an oil concentration ranging from 5 to 15 g/L. The results of the experiments are collected in Table 3 and consent to outline some relevant points. Firstly, we can observe that the procedure allows transforming the investigated vegetable oil into HFAs with good overall yields (from 37 % to 55 %, in two biochemical steps). Lower yield (37 %, entry 3) refers to a biotransformation experiment in which we employed an oil concentration (15 g/L) higher than those used in the remaining trials. These data suggest that the right oil concentrations to be used in our process should range from 5 g/L to 10 g/L. Concerning the HFAs ratio, the study confirms that *Bifidobacterium animalis* subsp. *lactis* gives almost exclusively 10-hydroxystearic acid (**13a**) both using olive oil and high oleic sunflower oil (entry 1–3). Therefore, the following *Yarrowia*-mediated oxidation gave almost pure γ-dodecalactone (**4**).

The biotransformation of sunflower oil, which contains mainly linoleic acid, generated very different results depending on the probiotic strain used. *Lactobacillus rhamnosus* (entry 4) produced a nearly equimolar mixture of the HFAs **13a** and **13b** whereas the HFAs mixture obtained employing *Lactobacillus plantarum* contained **13b** as the main component (entry 5). *Lactobacillus acidophilus* yielded a more complex mixture of HFAs (entry 7) in which the 13-hydroxy derivative (**14a**, 45 %) was the main component followed by compound **13a** (38 %). Finally,

Table 3
One pot[a] biotransformation of vegetable oils into dodeca- and decalactones.

Entry	Microorganism	Vegetable oil	HFAs yield[2](HFAs ratio)[3]	Total Lactones concentration[4](Lactones ratio)[4]
1	*Bifidobacterium animalis* sub. *lactis*	Olive oil (5 g/L)	51 %(**13a/13b** = 99:1)	**1.2 g/L(4/5/nd**[5] = 96:2:2)
2	*Bifidobacterium animalis* sub. *lactis*	High oleic sunflower oil (5 g/L)	55 %(**13a/13b** = 99:1)	**1.4 g/L(4/5/nd**[5] = 97:1:2)
3	*Bifidobacterium animalis* sub. *lactis*	High oleic sunflower oil (15 g/L)	37 %(**13a/13b** = 98:2)	**1.3 g/L(4/5/nd**[5] = 97:1:2)
4	*Lactobacillus rhamnosus*	sunflower oil (5 g/L)	48 %(**13a/13b** = 52:48)	**1.1 g/L(4/5/nd**[5] = 57:39:4)
5	*Lactobacillus plantarum*	sunflower oil (9 g/L)	48 %(**13a/13b** = 31:69)	**1.5 g/L(4/5/nd**[5] = 35:63:2)
6	*Lactobacillus rhamnosus*	linseed oil (5 g/L)	41 %(**13a/13b/13c** = 30:16:54)	**1.0 g/L(4/5/6/nd**[5] = 34:12:48:6)
7	*Lactobacillus acidophilus*	sunflower oil (5 g/L)	53 %(**13a/13b/14a/15** = 38:7:45:10)	**0.9 g/L(4/5/7/nd**[5] = 43:8:46:3)

[a] The biotransformation experiments were performed in a 5 liters bioreactor, using two liters of medium. [2] Overall yield was calculated on the basis of the weight of the isolated hydroxy acids mixture. [3] The ratio was measured through GC-MS analysis. [4] The total lactones concentration and the lactones ratio were measured by GC-MS analysis using γ-undecalactone as internal standard. [5] Lactones of unknown chemical structures are indicated as **nd** (not determined)

the biotransformation of linseed oil with *Lactobacillus rhamnosus* gave a mixture of 10-hydroxy derivatives in which compound **13c** was the main component followed by **13a** and **13b** (entry 6). It is worth noting that the final biotransformation step, namely the transformation of HFAs into lactones by *Yarrowia lipolytica*, proceeded without any chemoselectivity. Consequently, the lactone mixture compositions reflect the compositions of the starting HFAs mixtures.

Overall, our process was used for the preparation of different lactones, in natural form. More specifically, we combined *Bifidobacterium animalis* subsp. *lactis* with high oleic sunflower oil, *Lactobacillus plantarum* with sunflower oil, *Lactobacillus rhamnosus* with linseed oil and *Lactobacillus acidophilus* with sunflower oil. The process, performed with the above-described oil-strain combinations, affords the following lactones mixtures: γ-dodecalactone (**4**) in almost pure form, dairy lactone (**5**) in mixture with γ-dodecalactone (**4**), tuberose lactone (**6**) in mixture with γ-dodecalactone (**4**) and dairy lactone (**5**), δ-decalactone (**7**) in mixture with lactone **4** and **5**, respectively. The proposed one-pot process for the transformation of vegetable oils can provide all the high-value lactones described in Fig. 4 with the single exception of jasmine lactone (**8**), whose HFA precursor **14b** wasn't produced by any of the probiotic strains used.

It is also worth pointing out that we didn't optimize the biotransformation procedure in order to maximize the lactones yields. The careful selection of specific oleaginous yeasts (Jo et al., 2014) as well as the addition of further vegetable oil during the exponential phase of yeast growth (Farbood et al., 1994) are biotechnological tricks that could greatly increase the lactones productivity. These procedures have been already exploited in different industrial processes. Therefore, the optimization of our process, according to the latter findings, doesn't provide any scientific novelty. Thus, we limited our study to the use of only one strain of *Yarrowia lipolytica*, without performing any further screening experiments.

4. Conclusions

We propose a new biotechnological process that allows transforming vegetable oils, namely olive oil, sunflower oil and linseed oil, into natural lactones of great industrial relevance. The starting oils are cheap commodities with a cost of a few euros per kilogram, whereas the obtained lactones, such as γ-dodecalactone, dairy lactone, tuberose lactone and δ-decalactone, are high-value natural flavours, whose cost is superior to 1000 euros per kilogram. The process exploits the biocatalytic activity and selectivity of certain probiotic strains of *Bifidobacterium* and *Lactobacillus*, along with the use of the proper vegetable oil, selected based on its fatty acid composition. Three different biocatalytic steps are involved but the overall transformation is performed one-pot. The microorganisms used are generally recognized as safe (GRAS) and are not genetically modified. Therefore, our methods compare favourably with the previous processes for lactone preparation, which need the isolation of the intermediate HFAs and make use of GMOs (Genetically Modified Organisms) or potential pathogen microorganisms.

CRediT authorship contribution statement

Stefano Serra: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Davide De Simeis:** Methodology, Investigation, Data curation.

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.

Data availability

Data will be made available on request.

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