



Biotechnological formation of dairy flavor inducing δ -lactones from vegetable oil

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ARTICLE INFO

Keywords:

Aroma-active lactones
Vegetable oil
Flavor generation
Fermentation
Heat stress
Lactic acid bacteria

ABSTRACT

Agroscope Culture Collection was screened to identify bacterial strains effective in production of dairy flavor inducing lactones using grapeseed oil as a substrate. *Lentilactobacillus parafarraginis* FAM-1079, *Lactococcus lactis* subsp. *lactis* FAM-17918, and *L. lactis* subsp. *lactis* biovar *diacetylactis* FAM-22003 showed the most efficient formation of targeted δ -lactones. The application of sublethal heat stress significantly increased target lactone production. The most profound improvement was for *L. lactis* subsp. *lactis* biovar *diacetylactis* where δ -octadecalactone generation was improved by factor of 9. The pre-fermentation step as well as growth phase in which bacteria are harvested did not have a significant impact on lactones yield. The lactone production process from vegetable oil developed in this study offers a new way of developing a natural flavor ingredient for incorporation into plant-based products.

1. Introduction

The global market of plant-based food products has grown tremendously over the past decade due to nutritional, environmental, and ethical reasons. Improving the flavor profile of plant-based products in view of increasing consumer acceptability is focus of ongoing research. In addition to attenuation of plant material-based off-flavors, the incorporation of flavor-inducing natural ingredients, which impart dairy-like flavor, offers potential solution for achieving increased consumer acceptability for plant-based dairy alternatives. Lactones with γ (five-membered ring) and δ (six-membered ring) structure have high organoleptic relevance in dairy products and wide application in the flavor industry. In particular δ -lactones have been evidenced to contribute to milky-cream flavor of full fat cream (Schlutt, Moran, Schieberle, & Hofmann, 2007). The desired buttery flavor in milk fat has been attributed to the presence of δ -decalactone (δ -C10), δ -dodecalactone (δ -C12), δ -tetradecalactone (δ -C14), and to a lesser extent also γ -lactones (Boldingh & Taylor, 1962). The δ -C10 and δ -C12 contribute to the characteristic coconut-like flavor found in milk-based products (Tharp & Patton, 1960). The desirable aroma imparted in fried and baked goods by butter was partially attributed to δ -C10 and this

characteristic dairy flavor note was absent when vegetable shortening was used instead (Keeney & Patton, 1956). The longer chain lactones are semi-volatile yet contribute to creamy taste and smooth texture: δ -C14 is responsible for the creamy mouthfeel in full fat cream, δ -hexadecalactone (δ -C16) induces melted butter-like aroma in cream and δ -octadecalactone (δ -C18) influences the texture by having a direct effect on the melting behavior of cream in the oral cavity (Schütt & Schieberle, 2017). γ and δ -lactones have been reported to have similar flavor threshold values in butter oil (Siek, Albin, Sather, & Lindsay, 1971), and in several studies, the content of δ -lactones in milk fat was reported to be more abundant than that of γ -lactones (Jurriens & Oele, 1965; Kinsella, Patton, & Dimick, 1967; Siek & Lindsay, 1970). This depicts the key role of δ -lactones in terms of their contribution to dairy flavor.

The lactones are commonly present in an array of natural products and can be extracted using different isolation techniques (Maga, 1976). However, apart from the low yields, the limited number of natural ingredients rich in lactones makes their extraction of low interest, economically. A more affordable alternative to generate lactones is by chemical synthesis but the acquired flavor ingredient does not qualify for natural flavoring declaration. An increasing number of market

Abbreviations: δ -C10, δ -decalactone; δ -C12, δ -dodecalactone; δ -C14, δ -tetradecalactone; δ -C18, δ -octadecalactone; HFA, Hydroxy Fatty acid; ACC, Agroscope Culture Collection; MEP, Mid-exponential phase; ESP, Early stationary phase; OD, Optical density; QT, Quantifier ion; QF, Qualifier ion; LOQ, Limit of quantitation; UFA, Unsaturated fatty acid; HS, Head space; GC-O, Gas-chromatography-olfactometry; LAB, Lactic acid bacteria; SPE, Solid phase extraction; RI, Retention index.

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<https://doi.org/10.1016/j.fochx.2022.100220>

Received 23 June 2021; Received in revised form 5 December 2021; Accepted 14 January 2022

Available online 19 January 2022

2590-1575/© 2022 The Author(s).

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surveys and studies have revealed that over the past few decades the trend of consuming food items that are free of chemical additives and are labeled natural has increased considerably (Román, Sánchez-Siles, & Siegrist, 2017). It has become crucial to find new ways of natural lactone production in economically viable quantities. Biotechnological formation of natural lactones by means of fermentation is a promising route enabling decent yields as well the use of 'natural' label for the produced flavor ingredient.

The biochemical formation of aroma-active lactones consists of three main steps: hydroxylation, β -oxidation, and lactonization (Fig. 1). The knowledge of the biochemical pathway allows to screen bacteria with the help of genomic analysis tools, which dispose of genes encoding for enzymes that catalyze the biochemical steps in lactone generation.

With the advancement in the field of biotechnology, more and more research has been directed towards exploiting the potential of bacterial strains to optimize the yields of value-added components like lactones from natural and renewable ingredients. An obvious choice for such raw materials is vegetable or fish oil as they are rich in poly-unsaturated fatty acids and can readily undergo hydroxylation reaction to produce hydroxy fatty acid (HFA). The use of vegetable oils is the raw material of choice enabling application in pure plant-based products. To our knowledge, there have been just a few studies where vegetable oil has been used as a precursor material for lactone formation using fungal fermentation only (Bonnarme et al., 1997; Laufenberg, Rosato, & Kunz, 2004).

Little is known about factors that potentially play a role in optimizing lactone production. Hydratase enzyme, which catalyzes hydroxylation, is involved in the first step of the biosynthetic pathway. In a study by Rosberg-Cody et al. (2011) it was shown that *Lactococcus lactis* and *Corynebacterium glutamicum*, when genetically biotransformed with a hydratase gene, exhibited enhanced cell viability under heat stress. This shows that the hydratase enzyme may play a role in countering heat stress in bacteria. It is therefore suggested that heat stress enhances hydratase activity in a hydratase-containing bacterium, which might lead to increased lactone formation. Moreover, it is well established that the metabolism and production of enzymes in microbial cells, particularly bacteria, is greatly dependent on the physicochemical elements present in the surrounding environment and the growth phase in which they are in (Dubos, 1940; Horak, Engelbrecht, van Rensburg, & Claasens, 2019). In the light of this information, it is justified to evaluate the effect of a pre-fermentation step (an additional fermentation step that allows the bacteria to adapt to the new media) and the growth phase in which bacteria is harvested on lactone formation. A study by Boratyński, Szczepańska, De Simeis, Serra, and Brenna (2020), did investigate the effect of the growth phase and found the stationary phase to be favorable for γ -dodecalactone formation by *Micrococcus luteus*. However, due to limited literature available on this matter, it is not established if this is a general trend, is species specific, and/or whether this holds for lactones other than γ -dodecalactone. Furthermore, to best of our knowledge there has not been a study that investigated the effect of heat stress and pre-fermentation step on aroma-active lactone generation by bacteria.

This study aimed to screen the Agroscope Culture Collection (ACC) to identify bacteria that show optimal efficiency and selectivity towards the production of target lactones using vegetable oil. δ -C10, δ -C12, δ -

C14, δ -C16 and δ -C18 were identified as the target lactones due to their imperative role in contributing to dairy flavor for e.g. milky and creamy characters. The best performing strains in terms of target lactone production were further employed in experiments determining if pre-fermentation, growth phase, and heat stress influences target lactone production. Gas chromatography-mass spectrometry (GC-MS) with and without Olfactometry were used to evaluate the aroma profile of grapeseed oil fermented with the three best-performing strains-both in qualitative and semiquantitative manner. As a result of these objectives, a recommendation of a new process for natural lactone ingredient production for plant-based food applications will finally be proposed.

2. Material and method

2.1. Chemicals

The chemicals were purchased from Sigma-Aldrich Chemie GmbH (Switzerland), Givaudan (Switzerland), Honeywell (Germany) and, Chemrise (Germany): dimethyl trisulfide (Aldrich W327506), ethanol (Aldrich 02860), ethyl butanoate (Aldrich W242705), 3-methylbutanal (Aldrich W269204), oct-1-en-3-ol (Aldrich W280518), oct-1-en-3-one (Aldrich W351504), 2-phenylethanol (Aldrich 77861), hexanal (Aldrich 115606), allyl sulfide (Aldrich A35801), 2-phenylethanol (Aldrich 77861), pentane-2,3-dione (Aldrich W284106), butanoic acid (Aldrich W222119), polyethylene glycol 200 (Aldrich P3015), ethyl heptanoate (Aldrich W243701), isoamyl acetate (Aldrich W205508), furfural (Aldrich W248908), acetyl-3-methyl pyrazine (Aldrich W396400), 3-ethyl phenol (Aldrich 04688), acetone (Honeywell 34850), Methanol (Honeywell 34860-1L), δ -octalactone (Aldrich w321405), δ -nonalactone (Aldrich), δ -decalactone (Aldrich W236101K), δ -undecalactone (Aldrich W239401K), δ -dodecalactone (Aldrich W320109K), δ -tetradecalactone (Aldrich W359009), γ -octalactone (Aldrich W279617), γ -decalactone (Aldrich W236012), and γ -dodecalactone (Givaudan 269352). δ -octadecalactone was provided by Nestlé (Konolfingen, Switzerland) in form of a 2 mg/mL solution in ethanol.

2.2. Strain selection

The screening of the strain library was done in two steps. In the first step, information regarding enzymes and their corresponding genes that are potentially involved in lactone biosynthesis via biotransformation in bacteria was gathered from the literature. The gene sequences for the identified enzymes were obtained from the literature or from online public databases like UniProt (Consortium, 2018) or the non-redundant protein database of NCBI. In the case of the absence of the entire gene sequence, the protein families in the enzyme and the corresponding gene sequence were determined using the HMMER database 3.3.1 (<http://hmmer.org>). Genomic analysis was conducted to identify strains that had similar genes using the BLAST tool that was available in the Dialact software (sequence database of Agroscope). The identified strains were grouped into species based on their identity numbers. Based on the number and copies of desired genes present in the genome, strains were selected per species for the second step of the screening. Ideally, one

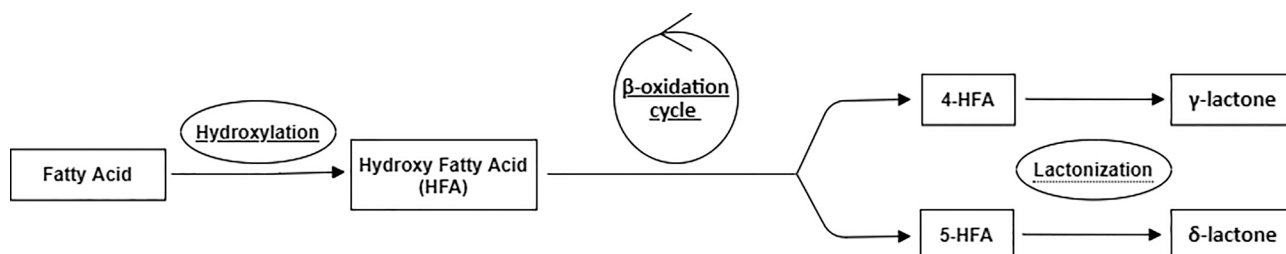


Fig. 1. General scheme for the biotransformation of fatty acid to lactones.

strain was selected as representative per species so to minimize the number of fermentation trials needed for the second phase of screening. In instances where there were more than one strong candidate in a species group, strains were selected based on the intra-species genetic diversity so that the strains chosen were genetically dissimilar to further widen the radar of screening. Intra-species diversity of strains was determined using cluster diagrams constructed with *t*-distributed stochastic neighbor embedding (*t*-SNE) and Principal component analysis (PCA) algorithms. An example of a cluster diagram is shown in Figure S.1 in the Supplementary material. The input for these reduction algorithms was Pfam data mined from assembled whole-genome sequences (El-Gebali et al., 2019). 25 strains belonging to 21 different species were selected for the second step of screening: fermentation trials. After the fermentation trials, the three-best performing strains in terms of higher target lactone production were chosen for further experiments.

2.3. Fermentation

The frozen bacteria conserves were reactivated for 24 h with 1 % (v/v) inoculum in sterilized, airtight 100 mL Schott bottles without shaking using Agroscope's standard growth media (recipe confidential) for the selected species. Strains that were obligatory aerobic, the reactivation was done inside a sterilized 250 mL baffled flask with a perforated cap and kept in a shaker at 160 rpm. In the fermentation step, 2 % (v/v) of the reactivated strain was added to the fresh fermentation medium inside a sterilized 250 mL baffled flask along with 20 % (v/v) of commercially purchased grapeseed oil (Migros, Switzerland). A perforated cap was used to seal the flask for strictly aerobic bacteria otherwise a standard plastic cap was utilized. The flasks were kept in the incubator at a shaking speed of 160 rpm. The temperature of the incubator was kept at the optimum growth temperature of the respective strains during the reactivation step and the fermentation trials (Table S.1 in the Supplementary material). The fermentation time was kept such that the strains were in the early stationary phase at the end of fermentation. After fermentation, the sample was sterilized at 121 °C, 0.5 bar for 60 min. After sterilization, the oil was separated from the fermentation media using separating funnel and stored in sterile 15 mL falcon tubes at -40 °C until further use. Figure S.2 in the Supplementary material illustrates the complete fermentation scheme.

Growth curves were determined using pH curves and absorbance curves so to decide on the fermentation time. A 2 % (v/v) inoculum of reactivated strain was added to fresh growth media. The pH of the mixture was followed to estimate the growth curve since the rate of pH drop corresponds to growth rate. The pH curves were constructed using an automatic pH probe system by iCinac (AMS Alliance, Italy) and the recorded data was processed using iCinac version 1.2.3.1 (AMS Alliance, Italy). For the bacteria that did not acidify the medium, optical density (OD) was monitored instead using Absorbance Microplate Reader ELx808 (BioTek Instruments GmbH, Switzerland) at a wavelength of 600 nm, and recorded data were later processed with Gen5 version V.10.11 (BioTek Instruments GmbH, Switzerland).

2.4. Effect of pre-fermentation step and growth phase on lactone production

Two-step fermentation was conducted for five randomly chosen strains from the pre-selected 25 strains for two different fermentation times: shorter time corresponding to harvesting lactones in mid-exponential phase (MEP) and longer fermentation time corresponding to harvesting lactones in early stationary phase (ESP). In a two-step fermentation scheme, an additional fermentation step (referred to as a pre-fermentation step in this article) is introduced and served as the source of inoculum for the main fermentation step. Each fermentation step is conducted in the same way as described previously under Section 2.3. The scheme for the two-step fermentation is illustrated in Figure S.3

in the Supplementary material. At the end of each fermentation, the sample was sterilized, and the oil was collected and stored as described earlier. The sterile oil sample from pre-fermentation and the main fermentation step was labeled as P1 and P2 respectively for each of the five strains. To determine the effect of the pre-fermentation step the target lactone production in P1 and P2 was compared for each of the five strains with the same fermentation time. The effect of the growth phase was investigated by comparing target lactone production in P1 for the two different fermentation times for each strain.

2.5. Effect of heat stress on lactone production

Two different heat stress conditions, referred to as V1 and V2 in this article, were used to elucidate the effect of heat-stress on the biotransformation capability of strains to produce the target lactones. The heat stress experiments were done only for the three best-performing strains. In the V1 condition, the bacteria were subjected to heat stress of 40 °C for 30 min after reactivation and then used as an inoculum in the proceeding fermentation step. In V2 the heat stress was applied to the bacteria during fermentation when they are in the late exponential phase. To apply heat stress in V2 condition the fermentation flask was taken out from the shaker two hours before the fermentation ends and was kept in an incubator (without shaking) at 40 °C for 30 min. After 30 min, the fermentation flask was placed back in the shaker for another two hours until the fermentation time is completed. The fermentation was conducted in the same way as described earlier in Section 2.3 under both conditions V1 and V2. At the end of fermentation, the sample was sterilized and stored in the same way as described earlier. The number of bacteria (cfu/mL) in fermentation media was followed with the standard plate count method: after reactivation, before and after heat stress application, and at the end of fermentation. The prepared plates were analyzed with automatic plate count reader SphereFlash (IGZ Instruments AG, Switzerland) coupled with software SphereFlash version 1.01.11 (IGZ Instruments AG, Switzerland).

2.6. Sample preparation for quantitative GC-MS analyses

The method for lactone extraction from fermented grape seed oil was adapted from Obi et al. (2018) where methanol was used for selectively extracting the lactones from coconut oil and melted butter against the triglycerides which often interfere with lactone analysis. Furthermore, having lactones in a volatile organic solvent like methanol enables concentrating lactones in smaller sample volume for efficient analysis in later stage of sample preparation. An approximate mass of 500 mg of sterile fermented oil sample was weighed in 15 mL falcon tubes and 1.5 mL of methanol was added for the extraction of lactones from the grape seed oil. The solution was vortexed and later centrifuged at 4 °C, 4000 rpm for 4 min using Centrifuge 5804R (Eppendorf AG, Germany). After centrifugation, 1 mL of methanol that had settled over the oil layer was collected in 1.5 mL Eppendorf tubes using a micropipette. The methanol was completely evaporated using Refrigerated CentriVap Concentrator (LABCONCO, USA). Afterward, 100 µL of methanol was added back to Eppendorf tubes and was vortexed to homogeneously re-dissolve the compounds left inside it. Finally, the 100 µL methanol was transferred to the GC glass vials for analysis.

2.7. Quantitation of target δ -Lactones

The quantitative analysis was conducted using an MPS2 autosampler (Gerstel, Sursee, Switzerland) and an Agilent 7890B gas chromatography (GC) system coupled with an Agilent 5977B mass selective detector (MSD) (Agilent Technology, Santa Clara, CA, USA) equipped with a capillary column HP-5MS UI (5 %-phenyl)-methylpolysiloxane, 30 m \times 0.25 mm i.d., 0.25 µm thickness, (Agilent Technologies, Germany). The oven temperature was programmed to remain at 60 °C for 5 min, increase to 280 °C at 5 °C/min, hold for 1 min at 280 °C. The total GC run

time was 50 min. The split/splitless (SSL) injector temperature was set at 250 °C. The flow rate of the carrier gas (helium) was 1.2 mL/min (27 cm/s). The split ratio was 5:1 (v/v) and the injection volume was 1 µL. The transfer line to the MS was set at 230 °C. The analytes were monitored in selected ion monitoring mode (SIM) with a gain of 10 and a solvent delay of 5 min. The quantifier (QT) and qualifier (QF) ions for target lactones were: 114 *m/z* (QT), 101 and 152 *m/z* (QF) for δ-C10; 99 *m/z* (QT), 114 and 180 *m/z* (QF) for δ-C12; 99 *m/z* (QT), 114 and 164 *m/z* (QF) for δ-C14; 99 *m/z* (QT), 264 and 282 *m/z* (QF) for δ-C18. The SSL injector and autosampler were controlled with Maestro1 software V.1.4.8.14/3.5 (Gerstel). The detector response signals were integrated using Masshunter quantitative analysis software version B.08.00 (Agilent). The NIST/EPA/NIH mass spectral library (NIST14) version 2.2 (NIST, Gaithersburg, MD, USA) was used for peak identification. The calibration curves were made for each lactone for concentrations 0.025, 0.05, 0.1, 0.6, 1.2, 1.6 and 2 mg/L. An additional five concentrations for δ-dodecalactone were prepared as some samples had a higher concentration of this lactone: 2.5, 3.5, 4.5, 5.5, and 6 mg/L. For constructing the calibration curves 40 µL of the parent lactone solution was used to spike 500 mg of unfermented, sterilized grape seed oil in a 15 mL falcon tube. The mixture was vortexed, and the methanol extraction step was repeated. The samples for GC–MS in solution analysis were prepared as described and later analyzed with GC–MS methodology described previously in this section.

2.8. GC-Olfactometry analyses

The aroma profile for the three best-performing strains was determined using Gas-chromatography–olfactometry (GC-O). Volatile organic compounds were extracted from grape seed oil using a newly developed extraction technique of Dynamic Headspace Vacuum Transfer In-Trap Extraction (DHS-VTT) by Fuchsmann et al. (2019) as it enabled the use of shorter extraction times coupled with lower temperatures and improved sensitivity of extraction for volatile compounds compared to HS-ITEX and solid-phase microextraction (HS-SPME) sampling. The ITEX-2 option (Brechtbühler, Schlieren, Switzerland) was used on the MPS2 autosampler controlled with the Cycle Composer V. 1.5.4 (CTC Analytics, Zwingen, Switzerland). The headspace (HS) was extracted using a Tenax® TA/Carbosieve SIII-trap (GB Analytik AG, Boeckten, Switzerland). A sample of 800 mg of sterile fermented oil was weighed in an HS vial and then sealed with septum caps. The HS vials were in the agitator for an incubation time of 15 min, an incubation temperature of 60 °C, and an agitation rate of 250 rpm. The HS was extracted under continuous reduced pressure of 500 Pa using a vacuum pump Buchi V-300 equipped with a pressure control interface I-300 (Büchi, Flawil, Switzerland) for an extraction time of 15 min. The bound volatiles were desorbed from the sorbent material at 250 °C in a CIS4 injector equipped with a glass liner filled with TTA at 10 °C. The injector was then heated at a rate of 12 °C/s to 240 °C. The odor-active compounds in the sample were identified using a two-way odor detection port (ODP2; Gerstel) connected to an Agilent 7890B gas chromatography (GC) system coupled with an Agilent 5977A mass selective detector (MSD) (Agilent Technology, Santa Clara, CA, USA). Volatile compounds were separated on an Optima FFAP plus (100 % PEG with nitroterephthalic acid, bonded and crosslinked, 60 m × 0.32 mm × 1.0 µm film; Macherey-Nagel, Switzerland) with helium as the carrier gas at a constant flow of 2.5 mL/min (30 cm/sec). The oven temperature of the GC was programmed to stay at 40 °C for 5 min at the start, increase to 240 °C at 10 °C/min, and hold there for 10 min. The total GC run time was 35 min. The MS settings were as follows: transfer line at 250 °C, source temperature at 230 °C, and analytes monitored in SCAN mode between 29 and 350 amu with a solvent delay of 3 min. The sample was split through deactivated silica capillaries into the proportions MSD/ODP/ODP 1:2:2, corresponding to flow rates of 0.5 mL/min, 1 mL/min, and 1 mL/min, respectively. The odorant compounds were identified with NIST/EPA/NIH massspectral library (NIST14) version 2.2 (NIST,

Gaithersburg, MD, USA) and AcquiSniff version 6.5.9 (INRA, Clermont-Ferrand, France).

The GC/olfactory analysis was carried out with the help of six trained panelists (4 females, 2 males with ages in between 25 and 55) and with 14 odor families of interest: cheesy/rancid/acids, cooked/potato, dairy, earthy/mushroom/forest, empyreumatic/plastic/chemical, fatty/oxidized, flowery, fruity, green/grassy, grilled/meaty, malty/cereal/aldehyde, spices/fir tree, sulfury/onion/garlic, and animal. The order of panelists in which the sniffing sessions were conducted was randomized such that each of the six panelists got to sniff each sample at least once and no panelist was repeated more than twice per sample. Each sniffing session was 35 min long. A total of eight GC-O analysis results were collected per sample and later used for constructing the corresponding aromagram. The panelists were provided with microphones to record their responses during the session and were also requested to simultaneously press a button while describing the odor and indicate an intensity on a scale of 1 to 5 (1: very weak odor and 5: very strong odor). The recorded responses were processed with AcquiSniff version 6.5.9 (INRA, Clermont-Ferrand, France). The aromagrams were realized with the VIDEO-Sniff method (Berdague, Tournayre, & Cambou, 2007) and the AcquiSniff software.

2.9. Statistical analysis

The data reported in this article were mean of at least two biological and technical replicates per sample. The Spyder 4.1.4 was used for the right-tailed Wilcoxon–Mann–Whitney test, frequency tests, and to prepare other data representation diagrams. The cut-off value of $p < 0.05$ was used to judge the statistical significance. Limit of quantitation (LOQ) was defined as five times the amplitude of noise in the baseline of the chromatogram around the peak of the target lactone. Data below LOQ was recorded as N/A and was not used in statistical analysis.

3. Results

3.1. Identification of the most efficient stains in the formation of δ lactones

Enzymes in bacteria that are involved in different steps of biosynthesis of lactones from fatty acids (Fig. 1) were identified after a comprehensive literature research. The corresponding genetic sequences for the enzymes were obtained from literature or from other public databases. Genomic analysis of strains from Agroscope Culture Collection (ACC) was conducted with the gathered information about the enzymes (data not shown). The strains that tested positive for desired genes were grouped in 21 bacterial species based on their identity. A total of 25 strains were selected with at least one strain per species to proceed on with the second step of screening (the list of strains can be found in Table S.1 in the Supplementary material). Fermentation trials with grapeseed oil (the choice for vegetable oil in this study) were conducted with the pre-selected 25 strains in view of evaluation of their efficiency and specificity in generating the target δ-lactones. The GC–MS methodology developed in this study was used for quantitation of the target δ-lactones. The developed methodology, however, could not successfully quantitate the δ-C16 content in the sample due to a strong co-elution with oleic acid. This lactone was therefore excluded from the analysis in this study for reasons explained in the result Section 4.1.

The unit used to express concentration of target lactones corresponds to mg of lactones per liter of the fermentation medium. LOQ for target lactones were in the range of 0.005 mg/L to 0.05 mg/L. The calibration curves constructed for quantitation of the target lactones all had the Pearson's correlation coefficient >0.99 (Table S.2 in the supplementary material). δ-C10, δ-C12, δ-C14 and δ-C18 were generated in the range of 0.053–0.255 mg/L, 0.046–1.33 mg/L, 0.033–0.74 mg/L, and 0.029–0.16 mg/L (Fig. 2). *Marinilactibacillus psychrotolerans* FAM-24235 was the only strain that did not produce any target lactones. Quantities

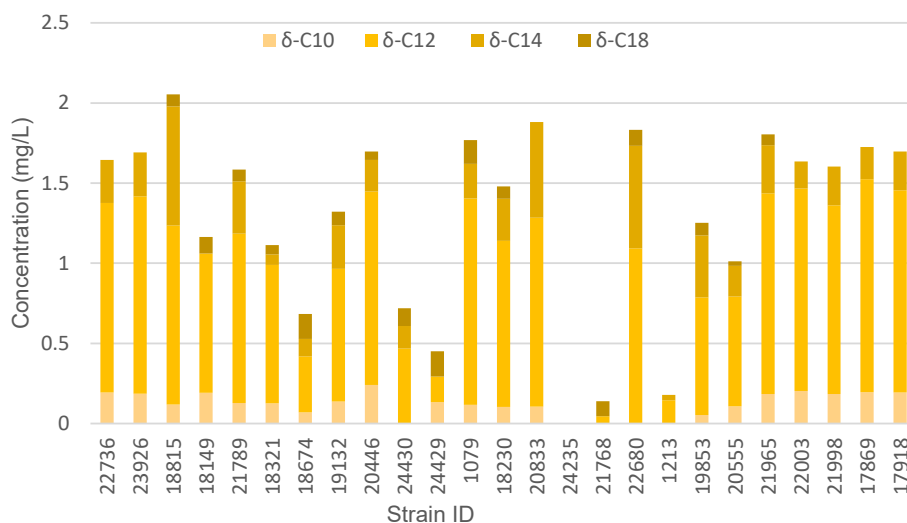


Fig. 2. Average concentration (in mg/L of the fermentation medium) of target lactones for the 25 pre-selected strains subjected to fermentation trials. The identities of the respective strains can be found in [Table S.1](#) in the [Supplementary material](#). δ -decalactone(δ -C10), δ -dodecalactone(δ -C12), δ -tetradecalactone(δ -C14), and δ -octadecalactone(δ -C18).

of organoleptically relevant γ -lactones were also determined to investigate the selectivity of the substrate to produce δ -lactones ([Table S.3](#) in the [supplementary material](#)). Compared to γ -lactones, δ -lactones were produced in higher quantities from grapeseed oil.

The three best-performing strains were identified based on higher yield of target lactones δ -decalactone (δ -C10), δ -dodecalactone (δ -C12), δ -tetradecalactone (δ -C14) and δ -octadecalactone (δ -C18) and minimum

corresponding standard deviation: *Lentilactobacillus parafarraginis* FAM-1079, *Lactococcus lactis* subsp. *lactis* FAM-17918, and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* FAM-22003. FAM-1079 was one of the top producers of both δ -C12 and δ -C18, a specificity not seen in the other strains. FAM-22003 and FAM-17918 were top producers of δ -C10 and δ -C12 and had corresponding standard deviations in the range of 5–15% of the value which is much smaller compared to other comparable

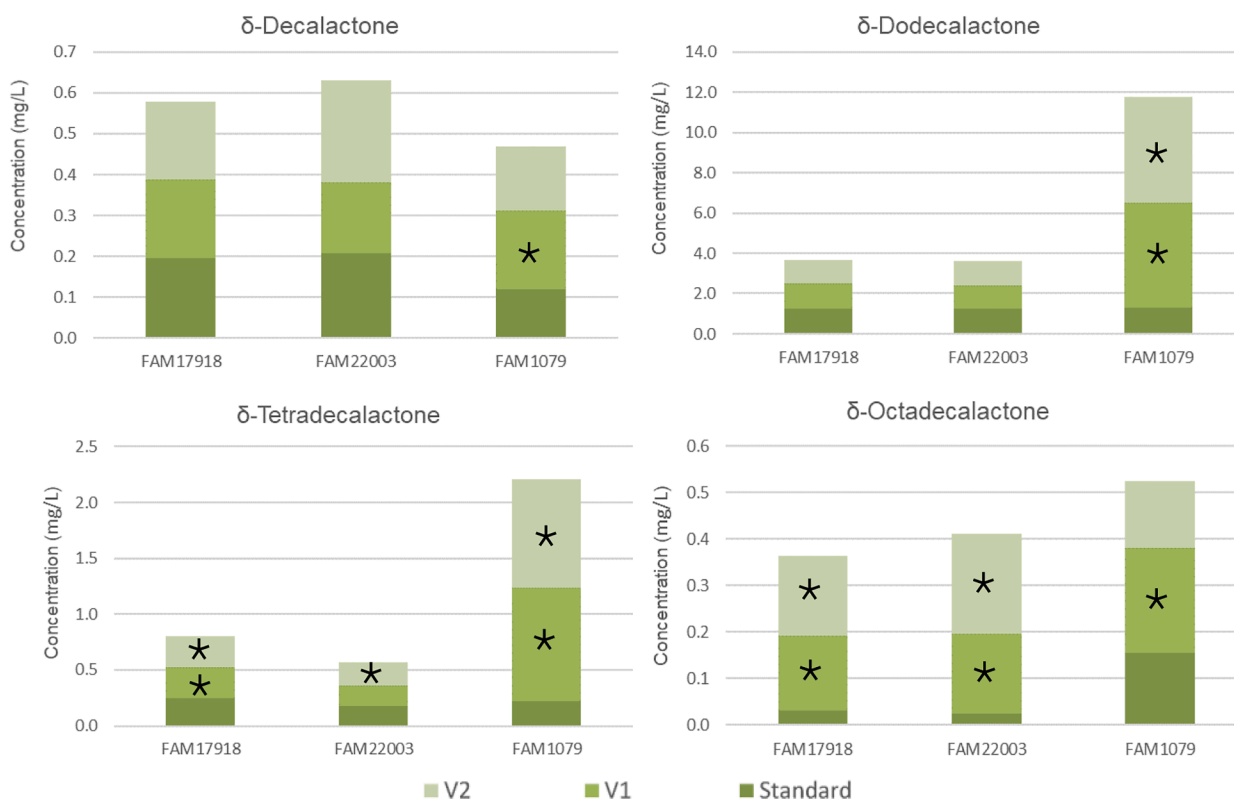


Fig. 3. Average concentration (in mg/L of the fermentation medium) of target lactones by the three best performing strains under three different fermentation conditions: standard, V1 and V2. The star icon represents significant difference in lactone production under heat stress condition in comparison to the standard condition. *Lentilactobacillus parafarraginis* FAM-1079, *Lactococcus lactis* subsp. *lactis* FAM-17918, and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* FAM-22003. Standard condition refers to fermentation without application of heat stress. V1 and V2 are fermentation conducted with heat stresses as described in [Section 2.5](#).

strains. These strains were chosen due to the efficient production of δ -C10 and δ -C12, especially δ -C10 which is an important lactone for inducing buttery flavor. *Lactococcus lactis* subsp. *cremoris* FAM17869 generated δ -C10 and δ -C12 in similar quantities but the associated sample standard deviation was higher (Table S.3 in the supplementary material). *Latilactobacillus sakei* FAM-18674 and *Latilactobacillus curvatus* FAM-24429 did perform better than FAM-1079 in terms of δ -C18 yields, but they produced other target lactones in much smaller quantities and hence were not considered in the final selection. *Pediococcus stilesii* FAM18815, *Staphylococcus xylosum* FAM-20833, and *Lactobacillus delbrueckii* subsp. *bulgaricus* FAM-22680 generated δ -C14 in much higher yields than the chosen strains but produced smaller quantities of other target lactones, especially δ -C10 which, as mentioned earlier, is a lactone of special interest due to its characteristic buttery-milky aroma.

3.2. Optimization of the fermentation process

Two different heat stress conditions, referred to as V1 and V2 in this report, were used to elucidate the effect of heat-stress on target lactone production by the strains. In V1 the bacteria were subjected to heat after reactivation and then used as an inoculum in the proceeding fermentation step. In V2 the heat stress was applied to the bacteria during fermentation when they are in the late exponential phase. Heat stress did play a positive role in improving target lactones production in comparison to the standard fermentation condition where no heat stress was applied (Fig. 3). In the case of FAM-1079 the heat stress significantly improved the production of all target lactones under V1 condition; δ -C10 production improved by a factor of 1.6, δ -C12 by a factor of 4, δ -C14 by a factor of 4.5 and δ -C18 by a factor of 1.5. Apart from *Lentilactobacillus parafarraginis* FAM-1079 under V1 condition, none of the other strains displayed a significant improvement in δ -C10 production under either of the heat stress condition. Similarly, with the exception *Lentilactobacillus parafarraginis* FAM-1079 that showed an improvement by a factor of 4 in δ -C12 production under both heat stress conditions, none of the other strains exhibited a significant improvement in δ -C12 generation under either of the heat stress condition. *Lactococcus lactis* subsp. *lactis* FAM-17918 and *Lactococcus lactis* subsp. *lactis* biovar *diacetyllactis* FAM-22003 showed significant improvement in production of δ -C18 under heat stress. The production of δ -C18 improved by approximately a factor of 5 for FAM-17918 under V1 and V2 condition. Moreover, an improvement by factor of 7 and 9 under V1 and V2 condition, respectively, was found for FAM-22003 in terms of production of δ -C18.

The number of bacteria (cfu/mL) in fermentation media was followed with the standard plate count method: after reactivation, before and after heat stress application, and at the end of fermentation (Table 1). The bacterial count before and after the stress is similar. Furthermore, bacteria count at the end of fermentation in V1, V2, and standard conditions for each strain were also similar.

The effect of having a pre-fermentation step (P1) in the fermentation scheme before the main fermentation step (P2) on lactone yield was also investigated in the study. The lactone yield in P1 and P2 was compared for five randomly chosen strains subjected to a two-step fermentation process as described in Section 2.4. The introduction of a pre-

fermentation step had a positive effect on the yield of target lactones, as their concentration was higher in P2 compared to P1 for the same fermentation time (Fig. 4), however, the difference was not significant. Lastly, the effect of growth phase on lactone yield was also examined in the study. Results in Fig. 4 reveal that harvesting strains in early stationary phase (ESP) vs. mid exponential phase (MEP) did not have a significant effect on the obtained target lactone yields. However, in majority of the cases investigated in the current study the mean target lactone production was higher in ESP than MEP.

3.3. GC-MS/Olfactometry

The aromagrams A, B and C belonging to strains *Lentilactobacillus parafarraginis* FAM-1079, *Lactococcus lactis* subsp. *lactis* FAM-17918, and *Lactococcus lactis* subsp. *lactis* biovar *diacetyllactis* FAM-22003, respectively, were developed to explore the olfactory space of the ferments beyond target lactones (Fig. 5). The target lactones could not be detected in the GC-Olfactometry analysis. In all three aromagrams an olfactory note classified under the odor class malty/cereal/aldehyde was detected which was attributed to 2- and 3-methylbutanal with retention index (RI) of 941 (peak 1 in A, B, and C). Moreover, all three aromagrams showed a dairy note with RI of 999 due to 2,3-butanedione (diacetyl) production (peak 3 in A, B, and C). The sulfury/garlic/onion note with RI of 1460 (peak 13 in A and B, and C) was also detected in all three aromagrams and could be attributed to dimethyl trisulfide. Multiple fruity notes were present in all aromagrams but were more abundant in FAM-1079 followed by FAM-17918. Ethyl 3-methylbutanoate with RI of 1089 (peak 4 in A, B, and C) was the compound common among all three aromagrams contributing to fruity odor. Furthermore, all panelists also had reported to have detected a cooked/potato or grilled/meaty like odor note in all three ferment samples (peak 15 in A, B, and C, RI = 1551) which potentially was derived from 2-ethenyl-6-methylpyrazine. All the panelists reported to have detected strong cheesy/rancid/acid odor notes during the GC-Olfactometry analysis of FAM-1079 sample around RI of 1475 and one of the responsible compounds was identified as acetic acid. The detailed identification of the peaks in the aromagrams can be found in Table S.4 in supplementary material.

4. Discussion

4.1. Identification of the most efficient stains in the formation of δ lactones

The majority of the strains that were identified from culture collection containing enzymes potentially involved in biosynthesis of lactones belonged to the group of lactic acid bacteria (LAB). This does not come as a surprise since LAB are typically known for being able to carry out hydroxylation of fatty acids – an important step in lactone biosynthesis (Yoshinaga et al., 2019). A 20 % (v/v) of a grapeseed oil was added to the fermentation medium. A higher concentration of grapeseed oil in the fermentation medium was avoided since the intrinsic phenolic compounds can induce oxidative damage to the cell membrane of the fermenting microbe (Garavaglia, Markoski, Oliveira, & Marcadenti, 2016).

Table 1

Bacterial count made with the standard plate count method to follow change in biomass in the samples. The standard fermentation is the control condition without heat stress. FAM-1079: *Lentilactobacillus parafarraginis*; FAM-17918: *Lactococcus lactis* subsp. *lactis*; and FAM-22003: *Lactococcus lactis* subsp. *lactis* biovar *diacetyllactis*.

Strain FAM	Plate Count \pm SD ($\times 10^8$ cfu/mL)						End of standard fermentation
	V1			V2			
	Before Stress	After Stress	End of fermentation	Before Stress	After Stress	End of fermentation	
22003	4.42 \pm 0.99	4.44 \pm 0.74	4.67 \pm 0.12	4.22 \pm 0.66	3.84 \pm 0.44	4.52 \pm 0.18	3.20 \pm 0.38
17918	3.66 \pm 0.48	4.16 \pm 0.91	5.39 \pm 0.11	6.49 \pm 0.13	7.11 \pm 0.15	6.50 \pm 0.14	5.93 \pm 0.19
1079	2.26 \pm 0.58	1.73 \pm 0.34	4.42 \pm 0.18	5.31 \pm 0.26	5.02 \pm 0.27	5.10 \pm 0.24	4.44 \pm 0.19

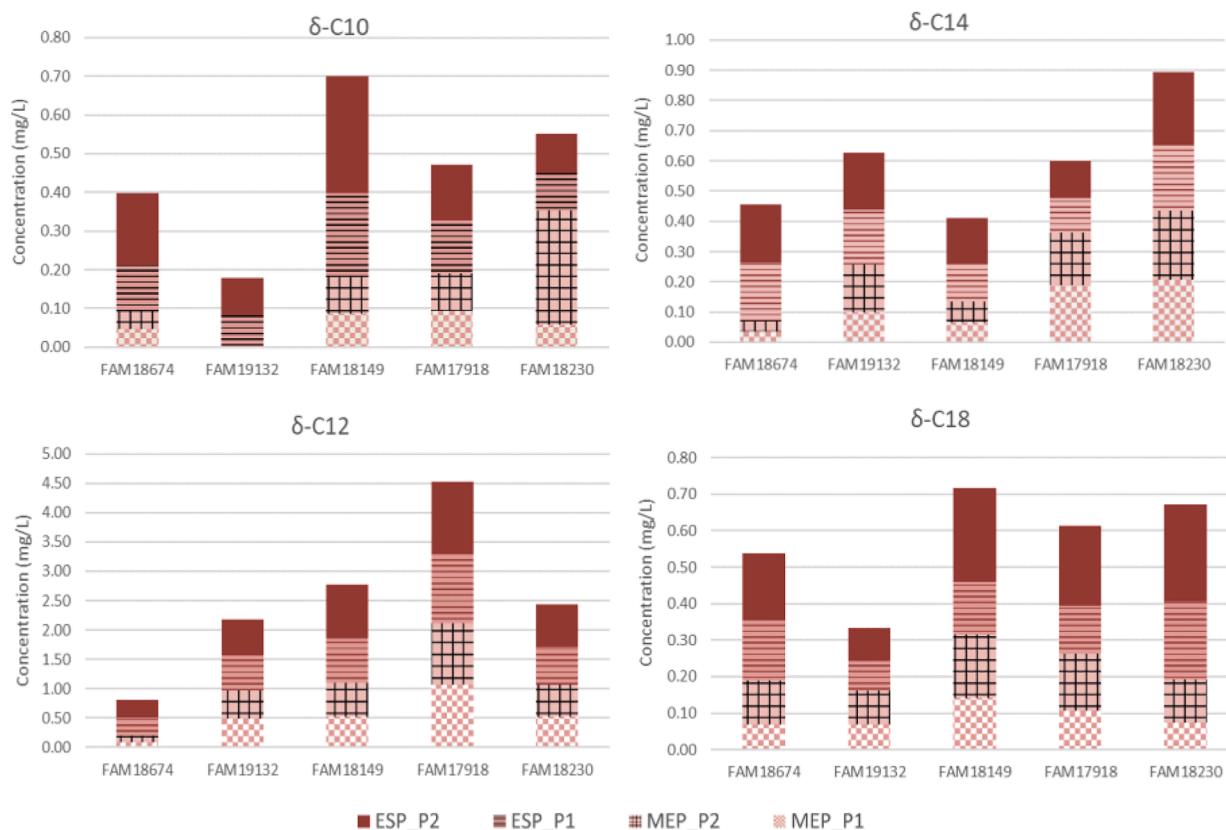


Fig. 4. Average concentration (in mg/L of the fermentation medium) of the target lactones for the five randomly chosen strains for experiments to study the effect of the pre-fermentation step and growth phase on target lactone production. MEP: mid exponential phase, ESP: early stationary phase. *Lentilactobacillus parafarraginis* FAM-1079, *Lactococcus lactis* subsp. *lactis* FAM-17918, and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* FAM-22003.

The idea behind using vegetable oil for fermentation was to find a renewable, natural substrate for lactone production, which enables the application of the resulting natural ingredient in plant-based food products. An additional advantage of using a hydrophobic substance like vegetable oil was also its ability to quench lactones from the fermenting medium, which helps in minimizing their toxicity towards the fermenting microbe (Romero-Guido et al., 2011). The vegetable oil chosen in this study was grapeseed oil due to its high content of unsaturated fatty acid (UFA). 85–90 % of the fatty acids present in grapeseed oil are UFA which includes linoleic acid, oleic acid, and palmitoleic acid (Garavaglia et al., 2016). A comprehensive review of microbial hydroxylation enzymes by Kim et al. (Kim & Oh, 2013), describes how most of the enzymes prefer UFA as a substrate for hydroxylation to produce hydroxy fatty acid – a prime precursor for lactones.

The GC–MS methodology developed in this study successfully determined all target lactone but δ -C16 due to its co-elution with oleic acid – a free fatty acid co-extracted with the lactones from the fermented grape seed oil. In order to remove free fatty acids from the sample a clean-up step after methanol extraction can be applied by using solid-phase extraction (SPE) on aminopropyl bonded to silica as described in study by Brede et al. (2002). Another possible solution could be to try a different column that might offer a better resolution between the two compounds. However, none of the suggestion was utilized in this study due to time constraints in the research project i.e. additional SPE step will significantly prolong the sample preparation time. Additionally, δ -C10 was a lactone of special interest among the lactones investigated in the current study due to its significant contribution to dairy flavor hence time and resources were prioritized for its determination and yield optimization.

There is scarce literature available on dairy lactone production from

plant-based material by means of fermentation, hence it is difficult to benchmark the lactone yields obtained in this study. Emmentaler cheese was found to contain 1.4 mg/Kg of δ -C10 (Preininger, Warmke, & Grosch, 1996). Similarly, Gouda cheese was reported to have 4.7 mg/Kg, 13.4 mg/Kg, and 17.8 mg/Kg of δ -C10, δ -C12, and δ -C14, respectively, after 45 weeks of ripening (Alewijn, Smit, Sliwinski, & Wouters, 2007). The relatively higher quantity of δ -lactones in cheese could be explained by the difference in fermentation time, a synergistic effect due to other microbes in the starter culture, and/or matrix effects of milk as milk proteins can protect bacteria from the detrimental effect of fatty acids (Kim & Liu, 2002). It is important to mention that yields of lactones reported in this study were determined in a semiquantitative manner since recovery rates for the lactones in the methanol extraction step were not studied. However, recovery rates of 90 to 100% have been reported for δ -C10, δ -C12, and δ -C14 from coconut and butter oil with a similar extraction method (Obi et al., 2018) hence the reported yields can still be used for drawing meaningful conclusions and achieve the objectives of this study.

Genes for enzymes potentially involved in biosynthesis of lactones were identified in *Marinilactibacillus psychrotolerans* FAM-24235 but no target lactone were produced during the fermentation trials (Fig. 2). The growth of FAM-24235 was followed under the same fermentation conditions for 24 h by monitoring the absorbance value. This anomalous result can be explained by the fact that the fermentation conditions and the medium used for FAM-24235 were not ideal for the growth of the bacteria since the absorbance value did not vary much over the 24-hour period. Compared to γ -lactones, δ -lactones were produced in higher quantities from grapeseed oil. δ -lactones require the presence of hydroxyl group at odd-numbered carbon in the acyl chain of a hydroxy fatty acid (Joo & Oh, 2012). It has been reported that some microbial

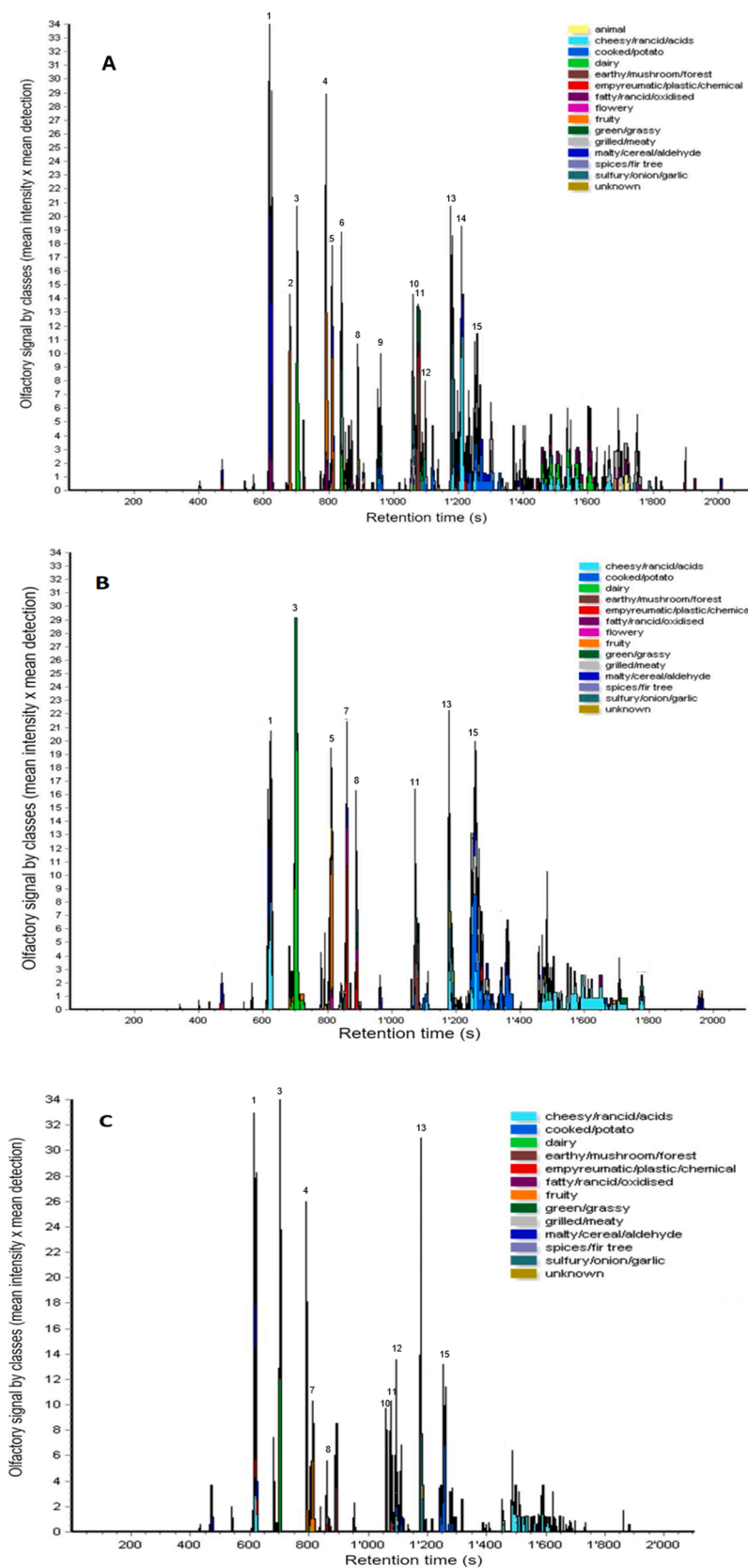


Fig. 5. The figure shows the mean olfactory signal by odor classes of strains: *Lentilactobacillus parafarraginis* FAM-1079 (A), *Lactococcus lactis* subsp. *lactis* FAM-17918 (B), and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* FAM-22003 (C) over eight sniffing results by six trained panelists. The x axis corresponds to the time in seconds and the y axis corresponds to mean intensity x mean detection. The comments of the panelists were distributed in the 14 aroma classes that have been determined. The colors of the aromatic classes are chosen arbitrary. The details of the labelled peaks can be found in Table S.4 in the [Supplementary material](#).

hydratase variants can carry out the hydroxylation of linoleic acid at an odd position to produce 13-hydroxy-cis-9-octadecenoic acid (Oh et al., 2015). Since 77 % of unsaturated fatty acid in grapeseed oil exist as linoleic acid (Garavaglia et al., 2016) there are higher chances for hydroxylation reaction that produces hydroxy fatty acid with hydroxy group at an odd position which can then be converted to δ -lactones. This can explain the higher production yields of δ -lactones as observed in this study.

In this study the recorded GC–MS data were not normalized with an internal standard since the aim of the study was to identify strains that could produce target lactones from grapeseed oil and not absolute yield determination. However, considering the many steps in sample preparations it would be wise to standardize the GC–MS data for better estimate for yields in follow up studies. A reactivation step of 24 h was used for all strains for ease of execution of the experiments in the study. A possible shortcoming of this approach is that due to the difference in growth rate among strains the cells were not in the same physiological states when the reactivation ended, and they were added to the lactone production step. The growth and activity of bacteria are influenced by the physiological state (Serrazanetti, Gottardi, Montanari, & Gianotti, 2013) which can affect their biotransformation capability and could be a potential factor behind the difference in target lactone production by different strains rather than their innate capability. Future studies, designed in a way that assures strains entering the lactone production step are in the same physiological state, will enable precise comparison among the strains.

4.2. Optimization of the fermentation process

The study explored the potential role of sub-lethal heat stresses, growth phase and pre-fermentation step in optimizing lactone yield obtained from fermentation by the three best performing strains identified from the culture collection. The application of sublethal heat stress led to a significant increase in target lactone production. This positive influence could be due to the up-regulation of the Myosin-cross-reactive antigen (MCRA) gene, a protein family found in hydratase enzyme, as it is regulated by a stress-responsive transcription factor (Bischoff et al., 2004; Rosberg-Cody et al., 2011). When bacteria are subjected to high temperatures it struggles to maintain the plasma membrane of the cell in its normal liquid crystalline state (Jenske, Lindstrom, Grobner, & Vetter, 2008). Hydroxy fatty acids (HFA) help in stabilizing the liquid crystalline structure by lowering down the phase transition temperature and therefore, microbial cell under heat stress has a higher hydratase activity that results in higher HFA production (Black, Zannini, Curtis, & Ganzle, 2013). A higher concentration of HFA in the cell as a response to heat stress could also lead to a higher lactone yields as HFA are key precursor molecules in the formation of lactones. Heat itself is suspected to directly play a role in enhancing lactone production i.e. by thermal degradation of triglycerides to produce free fatty acids, which are key precursors for lactone biosynthesis or facilitating chemical reactions involved. However, Schütt and Schieberle (2017) reported that the lactone content in dairy cream was not affected by heating at 70 °C for 20 min, a temperature–time combination that is much stronger than what was used in this study, hence it would be rational to conclude that heat directly does not influence lactone production in the ferment. The strains used in this study demonstrated similar response under both types of the heat stress conditions (V1 and V2) however usually the time taken for stress response to take effect is strain and treatment-dependent (Serrazanetti et al., 2013).

The bacterial count in the medium was followed in experiments conducted to investigate the effect of sub-lethal heat stress on target lactone production. This was done to see if the applied heat stress was indeed sub-lethal in nature and did not affect the cell biomass. The bacterial count before and after the stress is similar which shows that the chosen heat stress condition is not fatal for the bacteria and can potentially trigger a heat stress response (Table 1). Furthermore,

bacteria count at the end of fermentation in V1, V2, and standard conditions for each strain were similar which corroborates the fact that the difference in lactone production is probably due to a change in the biotransformation capability of microbes under stress and not due to the difference in biomass.

Introduction of a pre-fermentation step before the main fermentation step did increase the target lactone yield by strains, however the effect was not found to be significant in this study ($p > 0.05$). Adaptive enzymes are a set of enzymes produced by microbes in response to the presence of a certain substrate in the medium around them. For example, the production of a hydration enzyme found in *Rhodococcus rhodochrous* is inducible by oleic acid (Hou, 1995). Although the effect was not found to be significant in this study, the pre-fermentation step can play a role in optimizing the production of a certain microbial metabolite by allowing enough time for the fermenting microbe to tailor its traits according to the surrounding physical conditions like temperature, available substrate, and pH, i.e. production of adaptive enzymes. Similarly, target lactone yields obtained in the early stationary phase (ESP) were higher compared to that in the mid exponential phase (MEP), however the difference was not significant in this study ($p > 0.05$). Ramin and Allison (2019) describes the preference of bacteria to allocate resources for growth associated functions during the exponential phase rather than enzyme production. As lactone biosynthesis involves several enzymes, it is advisable to harvest lactone in ESP instead of MEP for higher lactone yield as higher enzyme activity will be expected in the former case.

4.3. GC–MS/Olfactometry

The analysis was conducted to explore olfactory space of the fermented grape seed oil sample by the three best performing strains from the culture collection. The target lactones could not be detected in the GC-Olfactometry analysis probably due to their low volatility in grapeseed oil, leading to low quantities of lactones released from the fermented grapeseed oil into the headspace during the extraction step. Desired dairy notes, due to 2,3-butanedione (diacetyl), were present in all the samples. This is expected as diacetyl is typically produced by majority of LAB (Mallikarjuna & Yellamma, 2019). Dimethyl trisulfide, the compound responsible for sulfury/garlic/onion odor note in the fermented grape seed oil samples, has been reported to contribute to sulfury notes in cheddar cheese produced by some LAB like *Lactobacilli*, *Lactococcus lactis* ssp. *lactis*, and *Lactococcus lactis* ssp. *cremoris* present in the starter culture (Seefeldt & Weimer, 2000). The GC–MS/Olfactometry analysis provides insight on what odors other than that of the target lactones exists in the ferments. Knowing the undesirable odor notes and compounds responsible for it in the ferments can lead to future studies that investigate ways of minimizing or selectively removing such compounds from the ferments. Furthermore, this knowledge can be used to assess the applicability of the fermented grape seed oil as a natural flavor ingredient in plant-based products.

5. Conclusion

The current study provided insight into the development and optimization of a novel fermentation process for the biotechnological production of δ -decalactone, δ -dodecalactone, δ -tetradecalactone, and δ -octadecalactone based on grapeseed oil as starting material in combination with lactic acid bacteria strains. After screening strains *Lentilactobacillus parafarginis* FAM-1079, *Lactococcus lactis* subsp. *lactis* FAM-17918, and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* FAM-22003 from Agroscope Culture Collection were identified as best candidates to generate targeted δ -lactones using grapeseed oil as substrate. Grapeseed oil was found to favor the formation of δ -lactones vs. γ -lactones. This study was the first of its kind that investigated the effect of sub-lethal heat stress on aromatic lactone production. Applying appropriate heat stress on the fermenting microbe significantly improved

target lactone production and is a promising optimization strategy. The pre-fermentation step and growth phase had a positive effect on target lactone production, although the effect was not found to be significant in this study. The GC-MS/Olfactometry analysis revealed the presence of strong dairy notes in the ferment due to diacetyl production which is supposed to positively impact the overall aroma profile of natural lactone ferment. The sensory analyses were not part of the current study as the ferments were not produced in food grade environment. However, additional sensory analysis is needed to assess the impact of generated δ -lactones on the creaminess and milky-buttery flavor in dedicated food applications. The lactone production process from vegetable oil developed in this study offers a new way of developing a natural flavor ingredient for integration in vegan/plant-based products. Future studies can be directed towards exploring other factors to optimize lactone production from vegetable oil like exploring synergies in co-fermentation system with yeast or other complimentary bacteria, investigating the effect of other stresses like solvent or nutrient stress. This study offers a new production process for aroma active δ -lactones with the potential of developing an ingredient that induces dairy notes in vegan products.

CRedit authorship contribution statement

H. Zia: Conceptualization, Methodology, Formal analysis, Investigation, Software, Writing – original draft, Visualization, Data curation. **U. Von Ah:** Methodology, Writing – review & editing, Resources. **Y.H. Meng:** Methodology, Writing – review & editing. **R. Schmidt:** Visualization, Formal analysis. **J. Kerler:** Conceptualization, Supervision, Writing – review & editing. **P. Fuchsmann:** Supervision, Writing – review & editing, Project administration, Resources.

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.

Acknowledgments

This project could not have been completed without the help of our wonderful colleagues at Agroscope, Liebefeld. A special thanks to Mir-eille Tena Stern for her continuous support as our laboratory technician. Lastly, we would like to thank the panelists that took part in the olfactometry analysis.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2022.100220>.

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