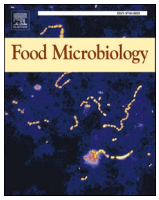
Food Microbiology 106 (2022) 104038

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm



Protein content of the *Oenococcus oeni* extracellular vesicles-enriched fraction

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ARTICLE INFO

Keywords:
Malolactic fermentation
Extracellular vesicles
Proteomics

ABSTRACT

Malolactic fermentation is essential for the quality of red wines and some other wine styles. Spontaneous malolactic fermentation is often driven by *Oenococcus oeni*, and commercial starters for this purpose are also often of this species. The increasing number of microbial species and inoculation strategies in winemaking has prompted a growing interest in microbial interactions during wine fermentation. Among other interaction mechanisms, extracellular vesicles have been hypothesized to play a role in this context. Extracellular vesicles have already been described and analysed for several wine yeast species. In this work, the production of extracellular vesicles by *O. oeni* is reported for the first time. The protein content of these extracellular vesicles is also characterised. It shows differences and similarities with the recently described protein content of *Lactiplantibacillus plantarum*, a bacterial species also capable of performing malolactic fermentation of wine (and used sometimes as an alternative starter). This work further contributes to the development of the field of extracellular vesicles in food biotechnology.

1. Introduction

In addition to yeast-driven alcoholic fermentation, many wines must undergo a second fermentation process before bottling, malolactic fermentation (MLF). This process is almost essential for most red wines (Ribéreau-Gayon et al., 2005), but also for some white wines, such as the base wines of Champagne, which are, in fact, made from red grapes (Ribéreau-Gayon et al., 2005). MLF consist in the bacterially driven decarboxylation of L-malic acid to L-lactic acid, with the concomitant release of carbon dioxide. There are several biochemical and microbiological implications of MLF for wine quality. First, the exchange of a dicarboxylic acid by a monocarboxylic one results in a reduced acidity and a slightly increased pH of the wine. Furthermore, bacterial metabolism during MLF leads to the consumption and release of compounds with sensory impact (Bartowsky and Borneman, 2011). Finally, the depletion of malic acid and other nutrients remaining in the wine after alcoholic fermentation contributes to the microbial stabilisation of wines, thus preventing problems during ageing and storage.

Oenococcus oeni is the main bacterial species responsible for spontaneous MLF, although other lactic acid bacteria (LAB), able to perform MLF, are also often isolated from wines. Spontaneous development of MLF had been a matter of concern for winemakers due to lack of predictability and the possibility of spoilage by other LAB (associated for example to the production of biogenic amines). The use of malolactic starter cultures greatly contributes to solving the issues related to MLF. Nowadays, most commercially available starters for MLF contain some strain of *O. oeni*, followed far behind by *Lactiplantibacillus plantarum* strains.

Currently the choice of microbial starters for winemaking includes *Saccharomyces cerevisiae* starters, commercially developed since the middle of the 20th century; MLF starters; and the more recently developed non-*Saccharomyces* starters. In most cases different kinds of starters are used sequentially; and different interactions, impacting on the fermentation processes and wine attributes, have been described (Mencher et al., 2021; Torres-Guardado et al., 2021). Binary combinations of starters are also relatively common, including those of *S. cerevisiae* and LAB for simultaneous alcoholic and malolactic fermentation (Pardo and Ferrer, 2019; Roudil et al., 2020).

Consequently, there is a growing interest in understanding the interactions between microbial starters during wine fermentation,

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https://doi.org/10.1016/j.fm.2022.104038
Received 13 January 2022; Received in revised form 4 March 2022; Accepted 10 April 2022
Available online 12 April 2022

including both metabolic and other interactions. In this context, it has been recently hypothesized that extracellular vesicles (EVs) might be involved in some of the interactions between wine yeast starters (Morales et al., 2021). It has also been shown that different wine yeast species produce EVs under winemaking-like conditions; and the proteomic content of some of them has also been analysed (Mencher et al., 2020).

EVs are non-replicative particles, delimited by a lipid bilayer, naturally released from cells belonging to all three domains of the tree of life (Théry et al., 2018). They show a broad range of sizes depending on biological species, cell types, and environmental factors. They are involved in intra- and interspecific interactions between living cells in many diverse contexts and are considered as a new paradigm in biological communication (Stahl and Raposo, 2019).

EVs have also been described for LAB, mostly in the context of probiotic and functional activities, including those of *L. plantarum* (Bajic et al., 2020). But so far, nothing is known about *O. oeni* EVs. The aim of this work was to characterize the protein content of *O. oeni* EVs, as a first step to understand this feature of the biology of the species, that might have eventual biotechnological implications.

2. Material and methods

2.1. Strain and growth conditions

For this study, strain PSU-1 of *O. oeni* was used. Before inoculation, the culture broth (MRSm) was treated to reduce the background noise caused by particles not produced by the inoculated bacterial strain. MRSm contained, per litre, 52 g of MRS (Merck KGaA, Darmstadt, Germany), 6 g of fructose, 5 g of DL-malic acid, 0.5 g of cysteine, 100 ml of tomato extract. The broth was filtered first through 0.22 μm, and then by ultrafiltration using a 100 kDa tangential filter Vivaflow® 200 (Sartorius, Göttingen, Germany), recovering the flowthrough until the dead volume was reached. Once the filtration was completed, the broth was filter sterilized through 0.22 μm and kept at 4 °C until use.

The precultures were grown in MRSm for 120 h at 28 °C in static tubes under anaerobic conditions. For experimental cultures, three 250 ml bottles filled with 250 ml of MRSm were inoculated to an initial OD_{600} of 0.2 and incubated at 28 °C for 6 days under anaerobiosis. This timing corresponds to the end of exponential growth phase. A representative growth curve under these experimental conditions is shown in supplementary file S1.

2.2. Recovery and concentration of EVs

Vesicles were obtained from three independent cultures. Cells and debris were removed by centrifugation at 5000*g* 10 min at 4 °C. The cell-free supernatant was collected and treated with one tablet per litre of protease inhibitor (complete mini, EDTA-free, Roche, Basel, Switzerland) and filtered through 0.22 μm. Sample concentration was carried out with a 100 kDa tangential filter as described above at 4 °C. When the filter reached dead volume, the sample was dialyzed for three times by adding 50 ml of PBS (phosphate buffered saline) containing: NaCl, 137 mM; KCl 2.7 mM; Na_2HP0_4 10 mM; KH_2P0_4 2 mM, pH 7.4; and running the ultrafiltration until reaching the dead volume again. Another concentration step was performed using Amicon® Ultra-15 100K Centrifugal Filter Devices (100,000 NMWL) (Merck KGaA, Darmstadt, Germany) according to the manufacturer's recommendations. The concentrated culture was centrifuged again at 10000 *g* for 10 min at 4 °C to remove debris. The EV fraction was subjected to ultracentrifugation in 6.0 ml PC Thick-Walled Tubes (16 × 59 mm; Thermo Fisher Scientific, Dreieich, Germany) at 100000 *g* for 70 min at 4 °C in ultracentrifuge Sorvall™ MTX150 with S80-AT3 fixed angle rotor (Thermo Fisher Scientific, Dreieich, Germany). Pellets from ultracentrifugation were washed with 0.5 M triethylammonium bicarbonate (TEAB), ultracentrifuged under the same conditions, and resuspended in 200 μl of TEAB 0.5 M. The final concentration factor was 1250-fold. 20 μl of each sample were pooled in one tube and stored at −80 °C to perform a nanoparticle tracking analysis (NTA). Uninoculated medium was submitted to the same process and samples used as control for NTA. Prior to the proteomic characterization, the sample was evaporated using a centrifugal concentrator miVac (Geneval LTD, Ipswich, England) and stored at −80 °C.

2.3. Nanoparticle tracking analysis

Vesicle size, and concentration were obtained in the ICTS "NANBIOSIS" on a NanoSight NS300 (Malvern Instruments Ltd., Worcestershire, United Kingdom) using NTA 3.4 Build 3.4.4 Nanoparticle Tracking and Analysis software. The samples were diluted 1:50 in PBS with camera shutter and gain optimized for data collection. 10 s videos were taken, and frame sequences were analysed under auto-particle detection and tracking parameters: detection threshold, pixel blur, and minimum track length. All samples were run at room temperature.

2.4. Proteomic analysis

Proteomic analysis was performed in the Proteomics Unit of Complutense University of Madrid. In short, samples were analysed by RP-LC–ESI–MS/MS in an EASY-nLC 1000 System coupled to the Q-Exactive HF mass spectrometer through the Nano-Easy spray source. Database search was performed against UniProt database (SwissProt) with taxonomic restriction to *O. oeni* PSU-1 (1682 sequences; date June 04, 2021). Detailed information of these procedures is described in the supplementary File S2.

The raw data set was filtered to retain only proteins identified in at least two of the three samples with more than two peptides in at least one of them. NSAF (normalized spectral abundance factor) values were calculated according to Zybailov et al. (2007). To perform the subsequent analysis and comparisons, only the proteins present within the NSAF 90% most abundant proteins were used.

The original dataset from Bajic et al. (2020) was filtered according to the same criteria to be able to establish comparisons between *O. oeni* and *L. plantarum*. NSAF values were calculated following the same procedure. To determine the homologous proteins from *L. plantarum* in the *O. oeni* proteome, accession numbers from the dataset were searched against the *O. oeni* PSU-1 (tax id: 203123) proteome annotated in the NCBI database 'nr' using the tool BLAST (Altschul et al., 1990). Gene ontology categories were recovered using the Uniprot database (Bateman et al., 2021), KEGG category analysis was performed using the geneSCF algorithm (Subhash and Kanduri, 2016). Venn diagrams were drawn using Venny 2.1 (Oliveros, 2015) and figures were prepared with R and RStudio software (R Core Team, 2021; RStudio Team, 2021).

3. Results and discussion

3.1. Characterization of O. oeni extracellular vesicles

The sampling time was chosen to focus on EVs produced by living cells, and to minimize the contribution of dead cells. According to preliminary experiments this timing was set to 6 days when the maximum biomass concentration was reached. By this sampling time, the OD_{600} of the cultures ranged from 14.3 to 18.1 and viable cells ranged between 1.55×10^8 and 4.4×10^8 CFU/ml. EVs were recovered from the supernatant of *O. oeni* cultures (three biological replicates). Enrichment protocol consisted in ultrafiltration and ultracentrifugation steps, as described in the experimental section. The concentration factor for EVs in these preparations was 1250x. Size and abundance of EVs were estimated by nanoparticle tracking analysis. The concentration of EVs preparations was $9{,}39 \times 10^9 \pm 6{,}94 \times 10^8$ particles/ml. Particle concentration from the uninoculated medium was $1{,}60 \times 10^9 \pm 1{,}37 \times 10^8$ particles/ml. The mean and mode of particle size for the *O. oeni*

preparation were, respectively, 125,5 ± 1,7 nm and 116,1 ± 5,9 nm. This size is in accordance with previous studies of EVs from Gram-positive (20–400 nm), and Gram-negative bacteria (50–250 nm) (Bajic et al., 2020; Beveridge, 1999; Briaud and Carroll, 2020; Dean et al., 2019).

3.2. Proteome composition of O. oeni EV-enriched fraction

The proteome analysis of the EV-enriched fraction from *O. oeni* identified 129 proteins after applying the filtration criteria described in section 2.5 (supplementary File S3). The 63 most abundant proteins represent 90% of total protein abundance, according to NSAF calculations (Table S1). Further analysis focused on those 63 proteins in order to get a picture of the biological processes represented in the samples, while avoiding an excess weight of proteins present in very low amounts. They were classified according to the cellular component GO terms from the Uniprot database, into six categories (Fig. 1): 'integral component of membrane' (GO:0016021; GO:0005886), 'ribosome' (GO:0005840; GO:0015934; GO:0015935), 'cytoplasm' (GO: 0005737), 'extracellular region' (GO: 0005576) 'other' (including different categories not related to a cell location (Table S2)), and 'unknown'. The most abundant category is 'integral component of membrane' with 24 proteins, representing 29,3% of the NSAF covered by the 63 selected proteins; followed by the category 'ribosome' with 10 proteins and 20% of the NSAF. Within the category 'other', two relevant GO terms can be found, the ATP-binding cassette (ABC) transporter complex [GO: 0043190] and hydrolase activity [GO: 0016787] with 2 and 3 proteins that represent respectively 9,5% and 3,6% of NSAF. Analysis of the same list of 63 proteins with geneSCF showed 5 relevant KEGG categories (p-value < 0,05): ribosome [ooe03010], beta-lactam resistance [ooe01501], protein export [ooe03060], quorum sensing [ooe02024] and cationic antimicrobial peptide (CAMP) resistance [ooe01503] (Fig. 2).

Taken together, these analyses point to a relevant presence of ribosomal and integral membrane proteins in *O. oeni* EVs. This observation is in agreement with previous studies on EVs from *Staphylococcus aureus* (Jeon et al., 2016; Lee et al., 2009; Tartaglia et al., 2018, 2020), *Bacillus anthracis* (Rivera et al., 2010), *Propionibacterium acnes* (Jeon et al., 2017), *Bacillus subtilis* (Brown et al., 2014), *Mycobacterium tuberculosis* (Lee et al., 2015) and *Streptococcus pneumoniae* (Codemo et al., 2018), and is further analysed and discussed in Section 3.3.

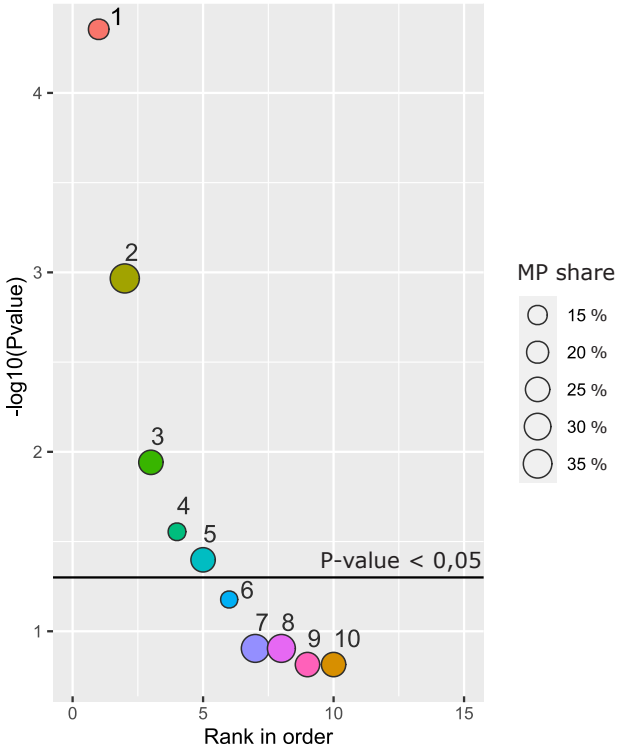

Fig. 2. GeneSCF graph representing significant KEGG categories from the *O. oeni* EV-enriched proteome. "MP share" stands for the percentage of the corresponding metabolic pathway covered by the proteins included in each category. 1: ooe03010-Ribosome; 2: ooe01501-beta-Lactam resistance; 3: ooe03060-Protein export; 4: ooe02024-Quorum sensing; 5: ooe01503-Cationic antimicrobial peptide (CAMP) resistance; 6: ooe00550-Peptidoglycan biosynthesis; 7: ooe00625-Chloroalkane and chloroalkene degradation; 8: ooe00626-Naphthalene degradation; 9: ooe00071-Fatty acid degradation; 10: ooe00350-Tyrosine metabolism.

3.3. Comparison between O. oeni and L. plantarum EV proteome

Until recently, the production and biogenesis of EVs was relatively neglected regarding Gram-positive bacteria in general and lactic acid

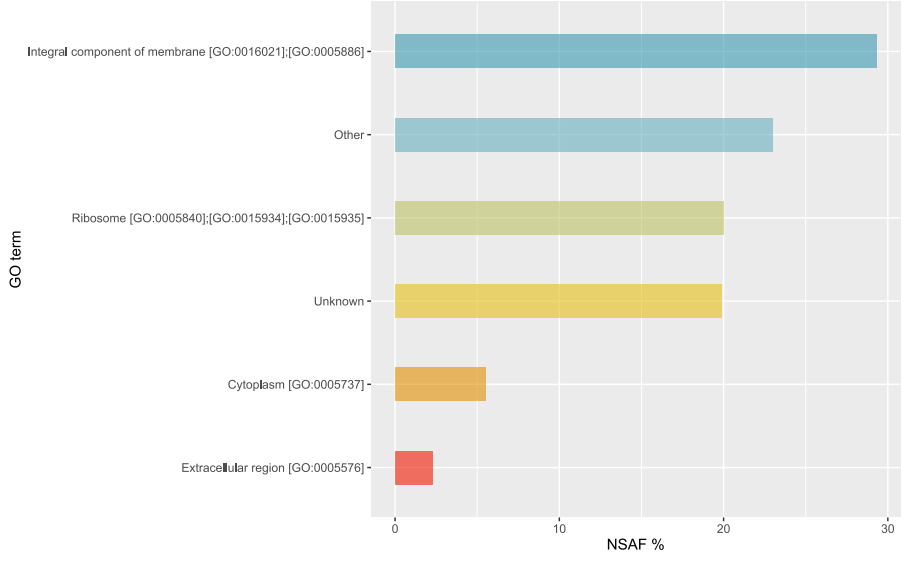

Fig. 1. Fraction of the global NSAF covered by the most abundant proteins in the *O. oeni* EV-enriched fraction sharing different GO terms for cellular component.

bacteria in particular (Briaud and Carroll, 2020). Proteomic information on them was even more scarce. A comprehensive proteomic analysis of a dairy isolate of *L. plantarum* was recently published (Bajic et al., 2020). Strains of this species are also often isolated from spontaneous malolactic fermentation of wines, and some strains are commercially used as starter cultures for this purpose. So, this was the closest reference to compare with results obtained with *O. oeni* in this work.

The complete EV-enriched proteome was retrieved from raw data provided by the authors of (Bajic et al., 2020). Then, to facilitate comparisons, accession numbers from the *L. plantarum* proteome were converted to putative orthologs in *O. oeni*. The comparison of the proteomes of the EV-enriched fractions of both species showed above 50% of *O. oeni* vesicular proteome (32 proteins) had a counterpart in the *L. plantarum* dataset (Fig. 3).

The GeneSCF analysis of the 32 shared proteins shows a similar rank of KEGG categories (Fig. S1). The only difference in this analysis between the full *O. oeni* EV proteome and the shared one is the substitution of 'ooe: 00550-Peptidoglycan biosynthesis' by 'ooe: 01503-Cationic antimicrobial peptide (CAMP) resistance'. The coverage of metabolic pathways is lower than for the full *O. oeni* EV-enriched fraction proteome, as expected for lower number of total proteins in the analysis.

In contrast, the analysis of the full *L. plantarum* EV-enriched fraction dataset highlights a different set of protein categories (Fig. 4). The putative orthologs in *O. oeni* were used again for this analysis, since the number of proteins identified in the KEGG database was higher than using the *L. plantarum* accession numbers directly. The results obtained the original accession numbers were rather similar, and are made available in supplementary File S4. This analysis returned 17 relevant KEGG categories with metabolic pathway coverages from 28% to 80%. Besides ribosomal proteins, the categories in this analysis were mainly related to the biosynthesis and metabolism of different metabolites. These proteins are usually associated with the intracellular compartment; while in the *O. oeni* analysis the categories were related to cellular communication and resistance to different antimicrobials, with proteins usually associated with the cell surface (ribosomal proteins aside). These differences might be species-specific but could also be explained by differences in culture conditions and sampling time between both studies. *O. oeni* was grown in MRSm at 28 °C for 6 days while *L. plantarum* was grown in standard MRS at 30 °C for 20 h. Interestingly despite these differences, the most relevant KEGG category was 'ooe03010-Ribosome' in both cases.

The presence of ribosomal proteins in EVs from Gram-positive bacteria has been previously observed in different species, as described in Section 3.2. Ribosomal proteins in EVs are not restricted to Gram-positive bacteria; they were found in EVs from other prokaryotic and eukaryotic organisms. Ribosomal proteins have been described in the EV-enriched fractions from Gram-negative bacteria such as *Francisella tularensis* (Klimentova et al., 2021), *Helicobacter pylori* (Melo et al., 2021), *Acinetobacter baumanii* (Kesavan et al., 2020), *Myxococcus xanthus* (Zwarycz et al., 2020), *Rhizobium etli* (Taboada et al., 2019), *Escherichia coli* (Lee et al., 2007); from yeasts such as *Candida* species (Zamith-Miranda et al., 2021), *Torulaspora delbrueckii*, or *Saccharomyces cerevisiae* (Mencher et al., 2020). Ribosomal proteins are also present in EV fractions from higher organisms such as *Arabidopsis thaliana* (He et al., 2021), *Mus musculus* (Anand et al., 2018) *or Homo sapiens* (Bijnsdorp et al., 2013) among others. More examples can be found in the database vesiclepedia (Kalra et al., 2012). Although ribosomal proteins are present in EVs from all biological kingdoms, their relative abundance is variable and their function in the EVs has not been elucidated.

On the other side, 'glycolysis/gluconeogenesis' and related categories appear highlighted for the *L. plantarum* EV-enriched fraction (Bajic et al., 2020). This trait is common among EV-enriched fractions of other bacterial species (Bäuerl et al., 2020; Domínguez Rubio et al., 2017; Hong et al., 2019; Jeon et al., 2017; Lee et al., 2009; Taboada et al., 2019; Tartaglia et al., 2018) as well as eukaryotes (Bleackley et al., 2019; Mencher et al., 2020). In contrast, the only glycolytic enzyme found among the 63 proteins more abundant *O. oeni* proteins in the EV-enriched fraction was glyceraldehyde-3-phospate dehydrogenase. This enzyme has shown moonlighting attributes (Gancedo and Flores, 2008) and is present in vesicles of other organisms where it plays a role of adhesion, membrane fusion, internalization, and virulence (Bajic et al., 2020; Bleackley et al., 2019; Jeon et al., 2017; Klimentova et al., 2021; Lau et al., 2013; Mencher et al., 2020; Park et al., 2010; Taboada et al., 2019; Tartaglia et al., 2020). Extending the search to all 129 proteins fulfilling the initial filtration criteria shows just a few more enzymes and an ABC transporter related to carbon metabolism in *O. oeni* (Table 1). Interestingly, the malolactic enzyme is among the top 63 most abundant proteins in the EV-enriched fraction of *O. oeni* (supplementary File S3). Whether there is a specific mechanism for the localisation of this enzyme in EVs or whether this is an indirect consequence of the protein abundance in the bacterial cytoplasm remains an interesting question for future research.

In summary, this is a first report on the production of EVs by the biotechnologically relevant species *O. oeni*. They show a size distribution in the range described for other bacteria. The most notable features in terms of protein content of the EV-enriched fraction of *O. oeni* are the abundance of ribosomal and integral membrane proteins. These features are also found, to different extents, in EVs from other bacterial and eukaryotic species. The availability of this information will help future studies on microbial interactions in winemaking, especially those where an impact of *O. oeni* on the physiology or performance of other microorganisms has been revealed (Rossouw et al., 2012). In the winemaking context, knowledge on *O. oeni* (and *L. plantarum*) EVs would be especially relevant to understand bacterial-yeast interactions in procedures involving the simultaneous inoculation of yeasts and lactic acid bacteria. Also, EVs might play a role in the ecological interactions between different bacteria during conventional spontaneous or inoculated MLF. Accordingly, future steps would involve studying the physiological impact of *O. oeni* EVs on wine isolates of other lactic acid bacteria, as well as *S. cerevisiae* and other wine yeast species. Paying attention to intraspecific diversity would also be important for the advancement of this field.

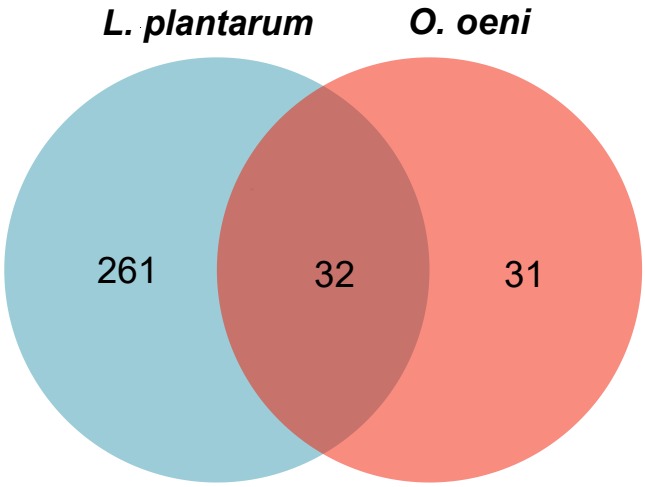

Fig. 3. Venn diagram representing the number of proteins shared by *O. oeni* and *L. plantarum* EV-enriched fractions.

Funding information

This work was funded by the Spanish Government through grants PID2019-105159RB-I00 funded by MCIN/AEI/10.13039/501100011033 and BES-2016-077557 and PRE2020-093420 funded by

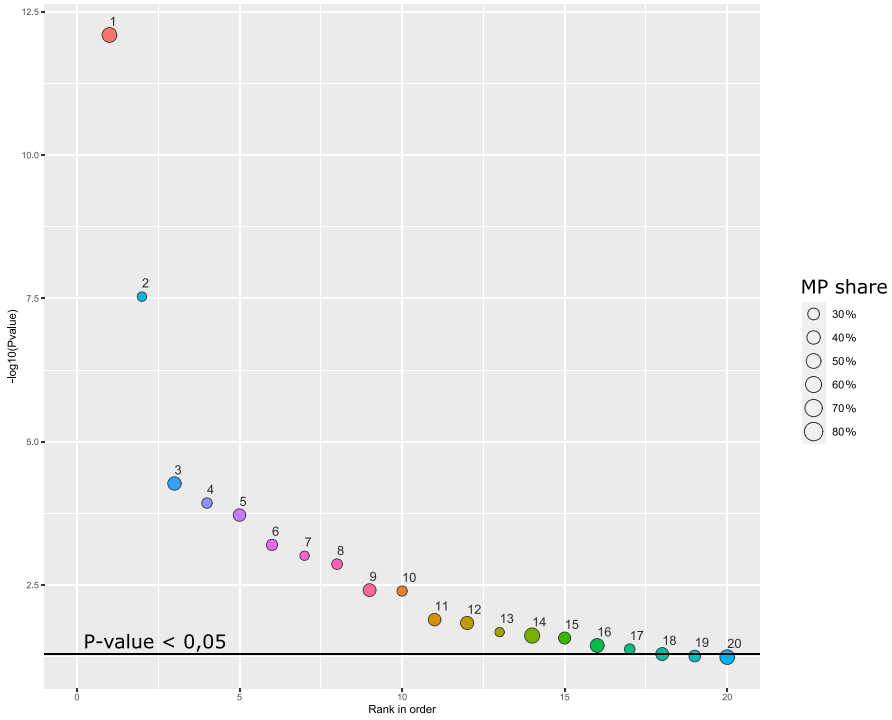

Fig. 4. GeneSCF graph representing significant KEGG categories from the *L. plantarum* EV-enriched proteome (analysed as the putative orthologs in *O. oeni* to ease comparisons). "MP share" stands for the percentage of the corresponding metabolic pathway covered by the proteins included in each category. 1: ooe03010-Ribosome; 2: ooe01100-Metabolic pathways; 3: ooe00520-Amino sugar and nucleotide sugar metabolism; 4: ooe01120-Microbial metabolism in diverse environments; 5: ooe00010-Glycolysis/Gluconeogenesis; 6: ooe01200-Carbon metabolism; 7: ooe01110-Biosynthesis of secondary metabolites; 8: ooe01240-Biosynthesis of cofactors; 9: ooe00052-Galactose metabolism; 10: ooe01230-Biosynthesis of amino acids; 11: ooe00190-Oxidative phosphorylation; 12: ooe00541-*O*-Antigen nucleotide sugar biosynthesis; 13: ooe00970-Aminoacyl-tRNA biosynthesis; 14: ooe03020-RNA polymerase; 15: ooe00250-Alanine, aspartate and glutamate metabolism; 16: ooe00480-Glutathione metabolism; 17: ooe00240-Pyrimidine metabolism; 18: ooe00450-Selenocompound metabolism; 19: ooe00640-Propanoate metabolism; 20: ooe00261-Monobactam biosynthesis.

Table 1
Proteins related to central carbon metabolism present in *O. oeni* EV.

Accession	Protein name	NSAF
Q04GQ8	Glyceraldehyde-3-phosphate dehydrogenase *	0.0168
Q04D23	Phosphoketolase	0.0020
Q04FG3	6-phosphogluconate dehydrogenase, decarboxylating	0.0017
Q04G44	Glucose-6-phosphate isomerase	0.0014
Q04E02	Carbohydrate ABC transporter substrate-binding protein, CUT1 family	0.0002

MCIN/AEI/10.13039/501100011033 and by "ESF Investing in your future" (training contracts for AM and MM). JT was funded by FGCSIC by the COMFUTURO program.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

The authors would like to thank Cristina Juez and Laura López for technical assistance, Patricia Ruas and Abelardo Margolles for sharing the raw datasets of *L. plantarum*. The characterization of the EV samples has been performed by the ICTS "NANBIOSIS", more specifically by the Biomaterial Processing and Nanostructuring Unit of the CIBER in Bioengineering, Biomaterials & Nanomedicine (CIBER-BBN) at the Institut de Ciència de Materials de Barcelona (ICMAB). The proteomic analysis was performed in the Proteomics Unit of Complutense University of Madrid, a member of ProteoRed and is supported by Grant PRB3 (IPT17/0019 - ISCIII-SGEFI/ERDF).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104038.

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