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Abstract

The recent advances in molecular biology, such as the advent of next-generation sequencing (NGS) platforms, have paved the way to new exciting tools which rapidly transform food microbiology. Nowadays, NGS methods such as 16S rDNA/rRNA metagenomics or amplicon sequencing are used for the taxonomic profiling of the food microbial communities. Although 16S rDNA/rRNA NGS-based microbial data are not suited for the investigation of the functional potential of the identified operational taxonomic units as compared to shotgun metagenomics, advances in the bioinformatics discipline allow now the performance of such studies. In this paper, a bioinformatics workflow is described integrating predictive metagenomics profiling with specific application to food microbiology data. Bioinformatics tools pertinent to each sub-module of the pipeline are suggested as well. The published 16S rDNA/rRNA amplicon data originated from an Italian Grana-type cheese, using an NGS platform, was employed to demonstrate the predictive metagenomics profiling approach. The pipeline identified the microbial community and the changes that occurred in the microbial profile during manufacture of the food product studied (taxonomic profiling). The workflow also indicated significant changes in the functional profiling of the community. The tool may help to investigate the functional potential, alterations, and interactions of a microbial community. The proposed workflow may also find an application in the investigation of the ecology of foodborne pathogens encountered in various food products.

1. Introduction

The objective of this work was to suggest and describe a bioinformatics workflow for the analysis of metagenomic data based on the 16S rDNA/rRNA amplicon sequencing originated from the application of next-generation sequencing (NGS) platforms. The pipeline integrates functional metagenomics, which is an emerging technique with potential industrial interest (Coughlan et al., 2015). Usually, papers dealing with the investigation of microbial ecology in food products using NGS methods end up with the taxonomic profiling of the microbial community after the preprocessing of the obtained 16S rDNA/rRNA data (Alessandria et al., 2016; Delcenserie et al., 2014; Ercolini et al., 2012; Liu et al., 2015; Parlapani and Boziaris, 2016; Parlapani et al., 2013; Parlapani et al., 2015; Połka et al., 2015; Sattin et al., 2016). However, data derived from 16S rDNA/rRNA amplicon sequencing can be exploited to investigate the functional potential of the identified operational taxonomic units (OTUs). Only recently, a few studies have performed functional profiling (Ferrocino et al., 2016; Pothakos et al., 2015; Stellato et al., 2016), but in general, this is not a common practice. The 16S rDNA/rRNA amplicon sequencing is a form of metagenomics and not metatranscriptomics, and therefore, the analysis is known as predictive functional profiling (Langille et al., 2013) or predictive metagenomics profiling (Wood, 2016). Other authors have suggested the integration of functional metagenomics into 16S rDNA/rRNA studies (Coughlan et al., 2015; Keller et al., 2014), but a key difference between those studies and the currently proposed bioinformatics pipeline is the inclusion of an additional step for the prediction of metabolic interactions between the microbial species found in a community (Mendes-Soares et al., 2016), an analysis not previously suggested or performed in food

metagenomes. In addition, the proposed food-focused pipeline involves a selection of tools and their specific sequential use along with the statistical tests, describing a step-wise use of each program and statistical test in each submodule. This will provide a quick and easy reference for the user who would like to use the programs in correct order. The 16S rRNA amplicon data originated from a Grana-type Italian cheese using an NGS platform (Alessandria et al., 2016) were used to demonstrate the predictive metagenomics profiling approach.

2. Bioinformatics workflow

The workflow integrates two main stages: the preprocessing (quality control of the sequences) and quantification (identification of the operational taxonomic units – OTUs, their potential interactions, and functional potential). The latter includes two sub-modules: the taxonomic profiling and the predictive metagenomics profiling (PMP) (Fig. 1). To accomplish the objectives of each step of the pipeline there are available various open-source programs which are free for academic use. The available software for the preprocessing and taxonomic profiling of the amplicon sequencing data are numerous. Table 1 presents the use of a specific program and statistical test in each stage and submodule of the pipeline. Alternative software that can be employed is also proposed, to enhance the step-wise description of the analysis workflow. Therefore, this list is not exhaustive but there are several relevant programs which the interested readers can seek in other excellent reviews regarding the existing software tools for bioinformatics analysis of metagenomic data (De Filippo et al., 2012; Dudhagara et al., 2015; Escobar-Zepeda et al., 2015; Ladoukakis et al., 2014; Oulas et al., 2015; Roumpeka et al., 2017;

Scholz et al., 2012; Sharpton, 2014). On the contrary, the number of available tools for PMP of 16S rDNA/rRNA amplicon data is limited (Aßhauer et al., 2015; Iwai et al., 2016; Langille et al., 2013).

3. Case study: taxonomic and functional profiling of the microbial community of a hard, slow-ripened cheese

The data used were from the study of Alessandria et al. (2016). The Sequence Read Archive (SRA) website of the National Center for Biotechnology Information (NCBI) was accessed to download all the deposited sequences in FASTA format (https://trace.ncbi.nlm.nih.gov/Traces/sra/). Three different batches (D, E, and F) of a Grana-type Italian cheese were used to get food metagenomics data by pyrosequencing (Roche 454 GS Junior platform) of the amplified V1 to V3 region of the 16S rRNA marker gene. The authors collected thirty-nine samples in total (n = 39; 13 samples per batch) during manufacture and ripening of the cheese (Whey Starter, WS; Raw Milk, RM; Raw Milk and Whey Starter, MS; Curd after Cutting, CAC; Curd after Heating, CAH; Curd after Pressing, CAP; Curd after Storage Room, CASR; Cheese after Salting, CHAS; Second Ripening Month, CH2RM; Fourth Ripening Month, CH4RM; Sixth Ripening Month, CH6RM; Eighth Ripening Month, CH8RM; Tenth Ripening Month, CH10RM) for pyrosequencing purposes. Preprocessing (stage 1 of the proposed bioinformatics workflow of Fig. 1) of the downloaded sequences had already being performed by Alessandria et al. (2016) with QIIME v1.9.0 (Caporaso et al., 2010), and therefore in this case study only the quantification step (submodule 1 and 2 of the proposed bioinformatics pipeline of Fig. 1) was carried out.

3.1. Submodule 1: Taxonomic profiling

Taxonomic profiling was performed using the SILVAngs 1.3 pipeline (Quast et al., 2013). Each downloaded sequence (264826 sequences in total) was aligned using the SILVA Incremental Aligner (SINA v1.2.10 for ARB SVN, revision 21008) (Pruesse et al., 2012) against the SILVA SSU rRNA SEED and quality controlled (Quast et al., 2013). Quality control of the submitted sequences, using the standard settings of the pipeline, rejected 89660 sequences (number of classified sequences equal to 173225 and number of "No Relative" equal to 1941). Afterward, identical reads were identified (dereplication), unique reads were clustered (OTUs), on a per sample basis, and reference read of each OTU was classified. Dereplication and clustering were made using cd-hit-est (version 3.1.2) (Li and Godzik, 2006) running in "accurate mode", ignoring overhangs, and applying identity criteria of 1.00 and 0.98, respectively. The classification was done by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 128) using blastn (version 2.2.30+) with standard settings (Camacho et al., 2009). Reads without any BLAST hits or reads with weak BLAST hits (Similarity $\leq$ 93%) remained unclassified ("No Relative"). The output of the pipeline, among others, was an OTU table containing the OTU abundances per sample at the genus and species level. The taxonomy at the species level was not possible for all the OTUs. The matrix was filtered further by applying the same filtering criteria with Alessandria et al. (2016), i.e. including only those OTUs with abundance $\geq$ 0.5% in at least two samples. The filtered table was the final output kept for all the subsequent steps.

After removal of the sample identified as outliers, no significant differences were observed between the three D, E and F batches regarding the microbial community profile using the ANOSIM (Analysis of Similarity) statistical test (P = 0.352; $P_{D\text{-}E}$ = 0.311; $P_{D\text{-}F}$ = 370; $P_{E\text{-}F}$ = 0.376) (Fig. 2) of the Past v3.15 software (Hammer et al., 2001). Fig. 3 displays an overview of the microbial community profile at the genus level during manufacture of the Grana-type cheese using the Community-Analyzer program (Kuntal et al., 2013). The arrows show the presence of an OTU in a particular metagenomic sample. Raw milk, for example, was characterized by the presence of microbial taxa with industrial interest and contaminants indicative of the quality of milk used for the manufacture of the product. Taxonomic groups located at the same horizontal level indicates symbiotic relationships amongst them. On the contrary, OTUs placed at a different location across the vertical axis indicate mutually inhibitory relationships, e.g. *Lactobacillus* vs. other contaminants. Grouping of the samples is made based on the similarities in the abundance profile of the OTUs and the relative location of these taxonomic groups. Therefore, the taxonomic abundance profile of the metagenomic sample raw milk, located far away from the other samples, was different in comparison with the rest. The two metagenomic samples "whey starter" and "raw milk plus whey starter" were grouped displaying similar taxonomic abundance patterns. Finally, a third distinct group containing only the samples originated from curd and ripening was formed.

For investigating in more detail the identified taxa within and between the samples, the data of the OTU table obtained with SILVAngs pipeline were introduced to GraphPad Prism v6.07 (GraphPad Software, Inc., San Diego, CA, USA) to construct a stacked bars chart (Fig. 4). The figure presents the main microorganisms found in the

Grana-type cheese samples. *Lactobacillus* species dominated all metagenomic samples. *Lb. helveticus* was in high abundance followed by *Lb. delbrueckii*. In the cured and early ripening samples, *Lb. helveticus* and *Lb. delbrueckii* dominated the manufacturing process. On the contrary, in the middle and late ripening metagenomic samples these two *Lactobacillus* species displayed a decrease in their abundance compared to the other samples. At the same time, *Lb. rhamnosus*, *Lb. casei* group and *Lb. fermentum* occurred during ripening. A similar trend, i.e. presence in curd and ripening samples, was also observed for *Propionibacterium* sp. Finally, *Lb. gallinarum* although in a relatively small amount was detected in all metagenomic specimens. The latter together with *Lb. helveticus*, *Lb. delbrueckii*, *Lactobacillus* sp. and *Streptococcus* sp. comprised the core microbiota. *Lb. brevis* and *Lb. plantarum* as well as *Lactococcus lactis*, recovered from whey starter, curd or ripening samples, were incorporating into *Lactobacillus* sp. and *Lactococcus* sp., respectively, because they were not visible alone in Fig. 4. *Streptococcus thermophilus* also was detected in most of the samples. Statistical comparison of the metagenomic samples with the web-based program METAGENassist (Arndt et al., 2012) revealed the significance of the species *Lb. helveticus*, *Lb. delbrueckii*, *Lb. rhamnosus*, *Lb. casei* group, *Lb. fermentum*, *Streptococcus* sp. and *Str. thermophilus*. These observations highlight the specific role of the *Lactobacillus* species as well as the role of the non-starter lactic acid bacteria (NSLAB) and other species during the Grana-type cheese production (Lazzi et al., 2004; Parente and Cogan, 2004; Rossetti et al., 2008; Rossi et al., 2012). The heat map in Fig. 5 shows the symbiotic (between species with industrial interest) and antagonistic (between contaminants and species with industrial interest) interactions that occurred. The web-based program

METAGENassist for comparative metagenomics was used to construct the heat map. Substantial differences between the two taxonomic profiles, the current (with SILVA as reference database) and the one from Alessandria et al. (2016) (with Greengenes as reference database), were not observed, yet some discrepancies do exist. In the present study, *Lb. gallinarum* was found to belong to the core microbiota; and *Lb. rhamnosus* along with *Propionibacterium* species (other than the contaminant *Propionibacterium acnes* present in the study of Alessandria et al., 2016) were recovered from samples during ripening. Such differences were expected since different databases (SILVA vs. Greengenes) were used to perform the taxonomic profiling (Yilmaz et al., 2014; Balvočiūtė and Huson, 2017).

In the raw milk samples, several contaminants were detected such as *Acidovorax* sp., *Acinetobacter* sp., *Acinetobacter baumannii/calcoaceticus* group, *Anoxybacillus* sp., *Clostridium* sp., *Sphingomonas* sp. and *Staphylococcus* sp. The category "other" of Fig. 4 included other contaminants such as *Pseudomonas* sp., *Enterobacter* sp., *Escherichia-Shigella*, *Rubrobacter* sp., *Bacillus* sp or *Listeria monocytogenes*. The recovery of such microorganisms from raw milk using NGS platforms has been reported elsewhere as well (Quigley et al., 2013). Despite the occurrence of several contaminants, these were decreased gradually due to the antagonistic activity experienced by the rest of microbiota, especially the one originated from the *Lactobacillus* species (Fig. 5) supporting the observation made in Fig. 3.

3.2. Submodule 2: Predictive Metagenomics Profiling

3.2.1. Statistical analysis

The OTU abundance table, obtained from the 16S rRNA data, was used as input for the submodule 2 to presume for metabolic functions. Currently, there are three tools available for PMP: PICRUSt (Langille et al., 2013), Tax4Fun (Aßhauer et al., 2015) and Piphillin (Iwai et al., 2016). In the present study, the Tax4Fun program performed the PMP, which works with the SILVA database. The PICRUSt requires the Greengenes database whereas the Piphillin tool is not obliged to any unique data pre-processing protocol supporting KEGG and BioCyc as a reference database. The output of the Tax4Fun is a table with a similar layout to the OTU abundance containing the functional predictions of KEGG Orthology (KO) or Pathways (ko). Statistical analysis (Kruskal-Wallis H-test with Tukey-Kramer), using the STAMP v2.1.3 software (Parks and Beiko, 2010; Parks et al., 2014) showed that 1629 KO and 121 ko displayed substantial changes. A *P*-value lower than 0.05, corrected for multiple tests according to the Benjamini-Hochberg FDR (False Discovery Rate) procedure, indicated significant differences. PCA (Principal Component Analysis) plots, made with Past v3.15 software, display the orientation of the metagenomic samples and the most abundant KEGG Pathways (ko) (Fig. 6).

The curd and early ripening metagenomic samples were dominated by pathways associated with carbohydrate metabolism (Fig. 6a). Cheese making (curd) and early ripening samples were mainly located in the right part of the graph (Fig. 6b). The ko 02060 (phosphotransferase system – PTS; membrane transport), 00564 (glycerophospholipid metabolism; lipid metabolism) and 00260 (glycine, serine, threonine metabolism; amino acid metabolism) also appeared on the right of the vertical axis (Fig. 6a). The PTS is a mechanism of the bacteria with which they uptake

carbohydrates (Kotrba et al., 2001). Lactobacilli consume sugars such as galactose and lactose, and the glucose can be converted to pyruvate through glycolysis (Hemme et al., 1981; Premi et al., 1972). Pyruvate is an important precursor of many metabolites such as lactic acid, formic acid, acetic acid, acetaldehyde, ethanol, acetoin, diacetyl, and butane-2,3-diol (Hickey et al., 1983). Moreover, thermophilic lactobacilli such as *Lb. helveticus* and *Lb. delbrueckii* can produce peptides, amino acids and other metabolites that stimulate the growth of *Str. thermophilus* (Courtin and Rul, 2004; Hemme et al., 1981) and propionibacteria (Baer, 1995; Kerjean et al., 2000; Piveteau et al., 1995). Metatranscriptomics revealed that genes associated with carbohydrate metabolism (pentose phosphate pathway and glycolysis) were enriched during the cheese making process of the traditional Italian cheese Caciocavallo Silano PDO (Protected Designation of Origin) (De Filippis et al., 2016).

On the top left corner of Fig. 6b, the metagenomic samples of the middle and late ripening formed a separate group, compared to the other samples. Accordingly, the KEGG Pathways located in the top left area of the PCA graph (Fig. 6a) were related to amino acid (ko00280, valine, leucine, isoleucine degradation; ko00360, phenylalanine metabolism) and lipid (ko00061, fatty acid biosynthesis; ko00071, fatty acid metabolism) metabolism. Also, pathways referred to carbohydrate metabolism (ko00020, TCA cycle; ko00640, propanoate metabolism; ko00630 glyoxylate and dicarboxylate metabolism) were also observed, which may participate in the production of aroma compounds. Flavor formation in cheeses is a complex process involving proteolytic and lipolytic activities in which key players are NSLAB and other non lactic acid bacteria (Smit et al., 2005). Interestingly, samples taken during middle and late ripening of the cheese were

characterized by the gradual increase of *Lb. rhamnosus*, *Lb. casei*, *Lb. fermentum*, *Str. thermophilus* and *Propionibacterium* sp. (Fig. 4). These microorganisms are known for their proteolytic and/or lipolytic activity as well as for their ability to produce aroma compounds (González-Olivares et al., 2014; Hong-Xin et al., 2015; Smit et al., 2005; Thierry et al., 2011). The above results showed good correlation with the observations made during ripening of the traditional Italian cheese Caciocavallo Silano PDO using metatranscriptomics (De Filippis et al., 2016).

3.2.2. Metabolic interactions

Usually, thermophilic lactic starters, propionibacteria, and NSLAB follow one another during ripening of Swiss-type cheeses (Gagnaire et al., 2001). A similar trend was observed in the present study for an Italian Grana-type cheese. Propionibacteria growth is dependent on the availability of lactate which is produced by *Lb. helveticus*, *Lb. delbrueckii* and *St. thermophilus* (Kurtz et al., 1959). Propionibacteria preferably utilize lactate as the energy source (Brendehaug and Langsrud, 1985; Fröhlich-Wyder et al., 2002). Despite the fundamental role of NSLAB in cheese flavor, propionibacteria should be present as well, but not in excess, to allow Grana-type cheeses such as Grana Padano and Parmigiano Reggiano develop their typical organoleptic characteristics (Carcano et al., 1995). The uncontrolled growth of propionibacteria may lead to an undesirable situation known as “late blowing” or “late fermentation” (Carcano et al., 1995; Fröhlich-Wyder et al., 2002).

So, both micro-flora NSLAB and propionibacteria have a role to play during the development of the organoleptic characteristics of the Grana-type cheeses. But how do

propionibacteria interact with NSLAB? Facultatively heterofermentative lactobacilli (FHL) such as *Lb. casei* and *Lb. rhamnosus* may compromise propionibacteria growth (Fröhlich-Wyder et al., 2002; Jimeno et al., 1995), especially when FHL are added as supplemental cultures. Fröhlich-Wyder et al. (2002) have showed that the addition of NSLAB in Swiss-type cheeses inhibited lactate fermentation by the propionibacteria. If, however, NSLAB are naturally occurring during cheese ripening, do they have the same effect on propionibacteria growth or not? Most NSLAB do not affect propionibacteria levels in cheese. The influence of *Lactobacillus* spp. on propionibacteria growth is likely to be less important than the impact of technological parameters such as pH and salt in cheeses (Carcano et al., 1995; Noël, 1999).

Consequently, the question above was explored using the microbial metabolic interactions (MMinte) tool (Mendes-Soares et al., 2016) for investigating the interplay between the naturally occurring flora of NSLAB and propionibacteria. Within the microbial community, the MMinte indicates the nature of that interplay (positive, negative or no interaction) based on the comparison of growth rates between the pairs of the microorganisms by constructing predictive genome-scale metabolic models. The sequence data and the subset of correlations between the considered OTUs, as estimated by the METAGENassist program (Fig. 5), were introduced to the MMinte tool and were run through the six widgets available: widget 1, only the representative 16S rDNA/rRNA sequence data were kept for further analysis based on the provided subset of the microorganisms pairs; widget 2, a genome ID is assigned to each OTU using BLAST (the 16S rDNA/rRNA sequences are compared with reference sequences available in NCBI) (Altschul et al., 1990); widget 3, a predictive metabolic model is constructed using

ModelSEED (Henry et al., 2010) for each genome ID; widget 4, a predictive two-species community metabolic model is created by the mean of COBRApy (Ebrahim et al., 2013; Klitgord and Segrè, 2010) for each pair of microbes provided in the first widget; widget 5, predictions on the growth rates are made using flux balance analysis (Heinken and Thiele, 2015; Varma and Palsson, 1994) for each two-species community; widget 6, the metabolic network of the microbial community is drawn and the nature of interactions is indicated using the D3.js visualization tool (Bostock et al., 2011).

The network in Fig. 7 depicts the predicted interplays between the propionibacteria and NSLAB. When the availability of the metabolites is high ("Complete_100"), propionibacteria is predicted to grow thus take a benefit from the presence of the NSLAB. NSLAB are heterofermentative microorganisms producing lactate among others, which can be used by propionibacteria. *Lb. casei* converts glucose, especially when glucose is limited, to lactate (predominantly), acetate, formate, and ethanol (Liu, 2003). Interestingly, when the availability of the metabolites was reduced by ten times ("Complete_10") there was an increase in the number of negative interactions predicted to occur between propionibacteria and NSLAB, without a positive interplay between them, meaning that NSLAB impairs the growth of propionibacteria when there is higher competition for nutrients. Consequently, the nature of the interaction is altered based on the metabolites availability. This probably explains partially the observation that NSLAB inhibit the propionibacteria growth when added as supplement cultures. This generates a much greater competition by the NSLAB for nutrients, resulting in the accumulation of elevated quantities (excess) of metabolic products such

as acetate, formate, and diacetyl that suspend the increase of propionibacteria (Jimeno et al., 1995).

4. Conclusion

The bioinformatics pipeline described in the present study may also find an application to foodborne pathogens occurring in foodstuffs. As predictive microbiology enters a new era of the integration of meta- and multi- omics in predictive modeling and quantitative risk assessment in foods (Brul et al., 2012; Cocolin et al., 2017; Rantsiou et al., 2011), the workflow proposed here may constitute a useful tool. For instance, it can respond to questions that concern risk assessors, food microbiologists and others dealing with microbiological risk assessment studies: How the foodborne pathogens found in food interact with the rest of microbiota? Why strains of the same species behave differently? How environmental conditions influence important features of the foodborne pathogens such as virulence?

As any novel method, PMP also has constraints. In order, the method to make reliable and accurate predictions about the gene content of an OTU, the genome of reference or at least closely related microorganisms should be sequenced and available. Despite this limitation, PMP is a cost-effective and straightforward method to start with when 16S rDNA/rRNA data are available (Wood, 2016). The tool may help to investigate the functional potential, alterations, and interactions of a microbial community. Thus, it will provide evidence for further exploration of the community and guide future experiments based on the genes or gene groups predicted to change (Wood, 2016).

Finally, PMP is in line with the multi-omics approach in food (safety) microbiology (Ferrocino and Cocolin, 2017).

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Figure legends

Fig. 1. Proposed bioinformatics pipeline for analysis of NGS-based 16S rDNA/rRNA sequencing data derived from food metagenomics integrating both taxonomic and functional profiling. Solid lines show the workflow of the analysis pipeline. Dashed lines indicate the two steps interfering with the analysis workflow. The quantification step includes two submodules.

Fig. 2. Box-plots of the ANOSIM statistical test for the microbial communities of the batch D (Group 1), E (Group 2) and F (Group 3).

Fig. 3. Overview of the microbial community at the genus level (green boxes) found in the Grana-type cheese samples (blue boxes). WS, whey starter; RM, raw milk; MS, raw milk and whey starter; CAC, curd after cutting; CAH, curd after heating; CAP, curd after pressing; CASR, curd after storage room; CHAS, cheese after salting; CH2RM, cheese after two months of ripening; CH4RM, cheese after four months of ripening; CH6RM, cheese after six months of ripening; CH8RM, cheese after eight months of ripening; and CH10RM, cheese after ten months of ripening.

Fig. 4. Stacked bars chart depicting the percentage of taxa in each metagenomic sample and the changes occurred between samples. WS, whey starter; RM, raw milk; MS, raw milk and whey starter; Curd, all curd samples (curd after cutting, curd after heating, curd after pressing and curd after storage room); ER, early ripening samples (cheese after salting and cheese after two months of ripening); MR, middle ripening samples (cheese after four and six months of ripening); and LR, late ripening samples (cheese after eight and ten months of ripening).

Fig. 5. Heat map of the correlation matrix between the taxa.

Fig. 6. The orientation of the a) most abundant KEGG Pathways (ko) and b) metagenomic samples using Principal Component Analysis (PCA). Two principal components (1 and 2) were extracted based on the total variance explained. The percentage shows the variance explained by each particular linear component. Upper-right quadrant has higher readings than points in the lower-left quadrant. Colors indicate KEGG Pathways (ko) related with specific metabolism or function such as carbohydrate metabolism (green), lipid metabolism (red), amino acid metabolism (blue), metabolism of cofactors and vitamins (gray), xenobiotics biodegradation and metabolism (salmon), and membrane transport (orange). Phosphotransferase system – PTS (ko02060), Glycolysis/Gluconeogenesis (ko00010), Galactose metabolism (ko00052), Starch and sucrose metabolism (ko00500), Pentose phosphate pathway (ko00030), Glycerophospholipid metabolism (ko00564), Glycine, serine, threonine metabolism (ko00260), Pyruvate metabolism (ko00620), Valine, leucine, isoleucine degradation (ko00280), Phenylalanine metabolism (ko00360), Fatty acid biosynthesis (ko00061), Fatty acid metabolism (ko00071), Benzoate metabolism (ko00362), Aminobenzoate metabolism (ko00627), Folate biosynthesis (ko00790), Ascorbate metabolism (ko00053), Glyoxylate and dicarboxylate metabolism (ko00630), TCA (citrate) cycle (ko00020) and Propanoate metabolism (ko00640). WS (whey starter – khaki), RM (raw milk – gold), MW (raw milk plus whey starter – salmon), Curd (cheese making – green), ER (early ripening – blue), MR (middle ripening – orange), LR (late ripening – red) are the group names of the metagenomic samples.

Fig. 7. Metabolic interaction network between propionibacteria and NSLAB; "Complete_100" and "Complete_10" indicate the availability of the metabolites to the

microbial community; “Complete_100”, over 400 metabolites are available, with a flux for the import reactions of 100 mmol/gDW/h (high availability); “Complete_10”, the same metabolites but with reaction fluxes of 10 mmol/gDW/h (ten-times lower availability); The percentage next to the microorganisms indicates the similarity between the OTU sequence provided and the reference sequence (genome ID assigned); The thickness and the length of the links inside the network imitate the correlation values provided. When the correlation value increases, the line is becoming thicker and shorter.

Table 1. Software and statistical tests used in each stage of the pipeline. Alternative software is also proposed.

Pipeline step	Statistical test and Software used	Alternative software
Preprocessing	Performed by Alessandria et al. (2016) using Qiime v.1.9.0	SILVAngs pipeline BMPOS pipeline (Pylro et al., 2016)
Taxonomic profiling (Taxonomic Operational Units – OTUs)	SILVAngs pipeline using the SILVA database	BMPOS pipeline (Greengenes database) EzBioCloud database (Yoon et al., 2017) One Codex pipeline (Minot et al., 2015)
Statistical comparison of the metagenomic samples	Analysis of Similarity (ANOSIM) using the Past software	Stamp MicrobiomeAnalyst (Dhariwal et al., 2017) Explicet (Robertson et al., 2013)
Microbial community overview	Community-Analyzer Stacked bars chart using the GraphPad Prism software	MicrobiomeAnalyst Explicet
Statistical significance of the identified OTUs	METAGENassist	MicrobiomeAnalyst Explicet
Symbiotic and antagonistic relationships within the microbial community	Heatmap constructed based on the Pearson correlation using the METAGENassist software	MicrobiomeAnalyst Explicet
Predictive Metagenomics Profiling (PMP)	Tax4Fun	Picrust Piphillin MicrobiomeAnalyst
Statistical analysis of the PMP results	Kruskal-Wallis H-test with Tuckey-Kramer corrected for multiple tests according to Benjamini-Hockberg False Discovery Rate using the Stamp software	MicrobiomeAnalyst
Orientation of the metagenomic samples of the most abundant KEGG pathways	Principal Component Analysis (PCA) using the Past software	MicrobiomeAnalyst Stamp
Metabolic interactions within the microbial community	MMinte	–

Fig. 1

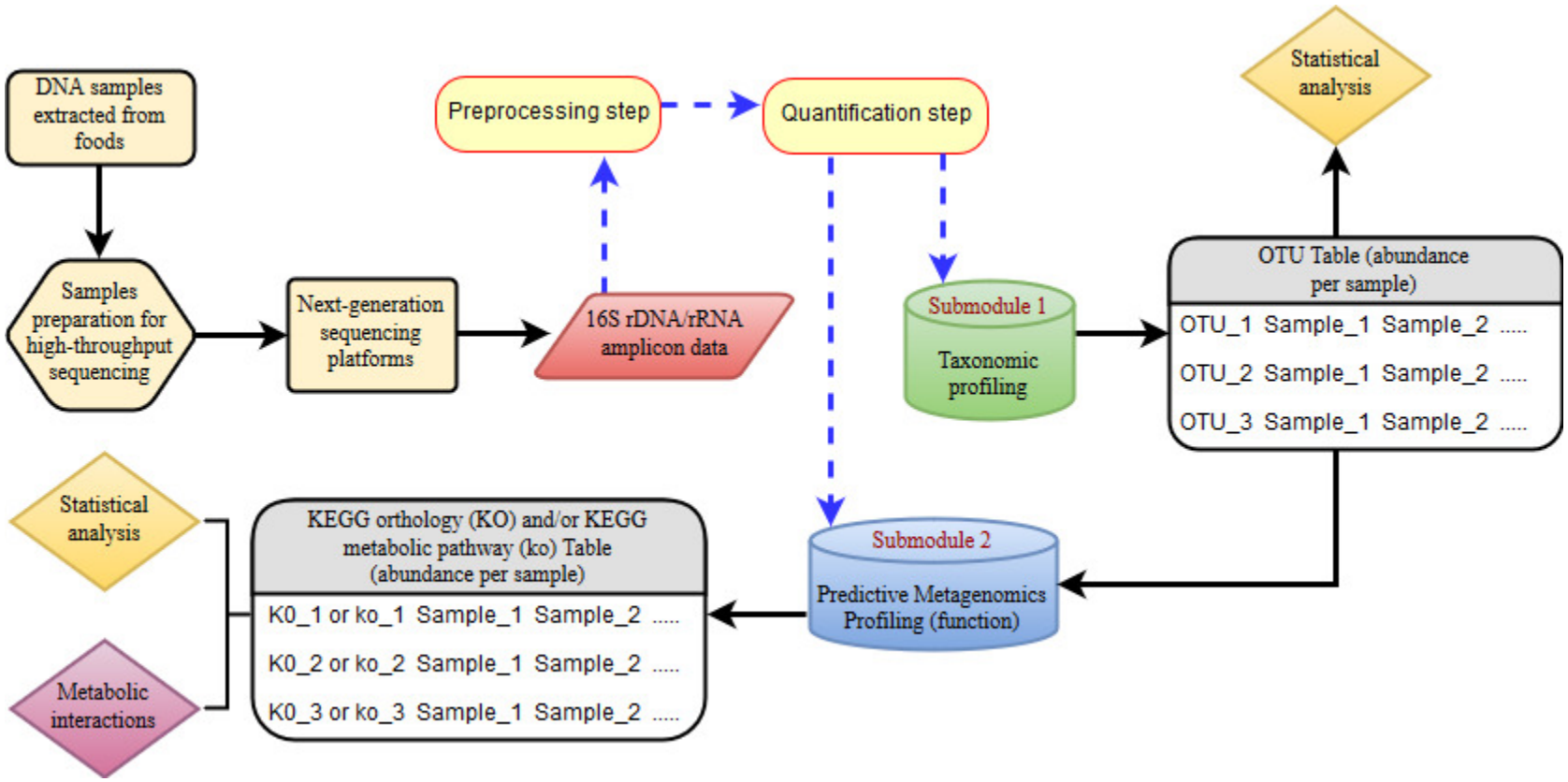
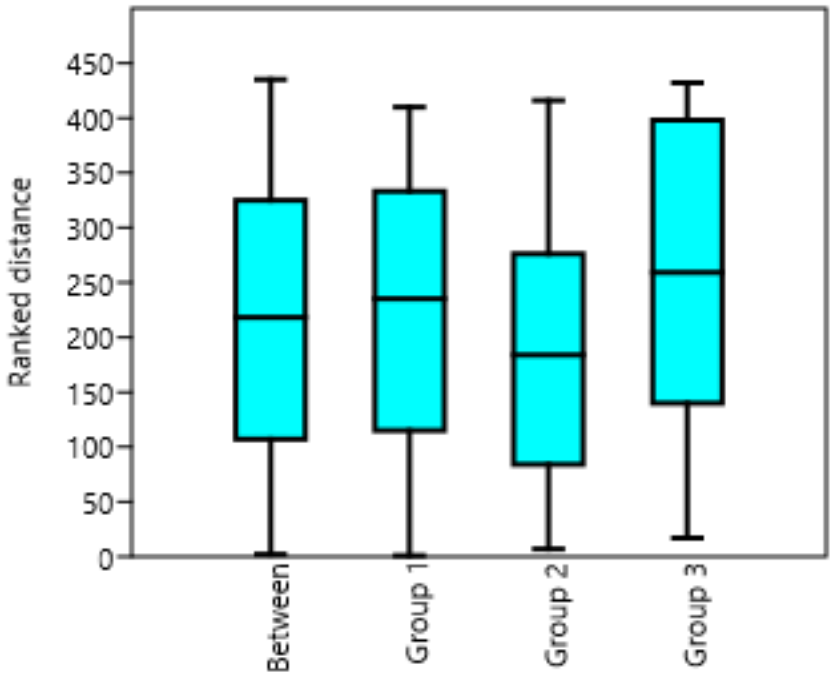

DNA samples extracted from foods
Samples preparation for high-throughput sequencing
Next-generation sequencing platforms
16S rDNA/rRNA amplicon data
Preprocessing step
Quantification step
Statistical analysis
Submodule 1
Taxonomic profiling
OTU Table (abundance per sample)
OTU_1 Sample_1 Sample_2 .....
OTU_2 Sample_1 Sample_2 .....
OTU_3 Sample_1 Sample_2 .....
Submodule 2
Predictive Metagenomics Profiling (function)
KEGG orthology (KO) and/or KEGG metabolic pathway (ko) Table (abundance per sample)
K0_1 or ko_1 Sample_1 Sample_2 .....
K0_2 or ko_2 Sample_1 Sample_2 .....
K0_3 or ko_3 Sample_1 Sample_2 .....
Statistical analysis
Metabolic interactions

Fig. 2


Ranked distance
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400
350
300
250
200
150
100
50
0
Between
Group 1
Group 2
Group 3

Fig. 3

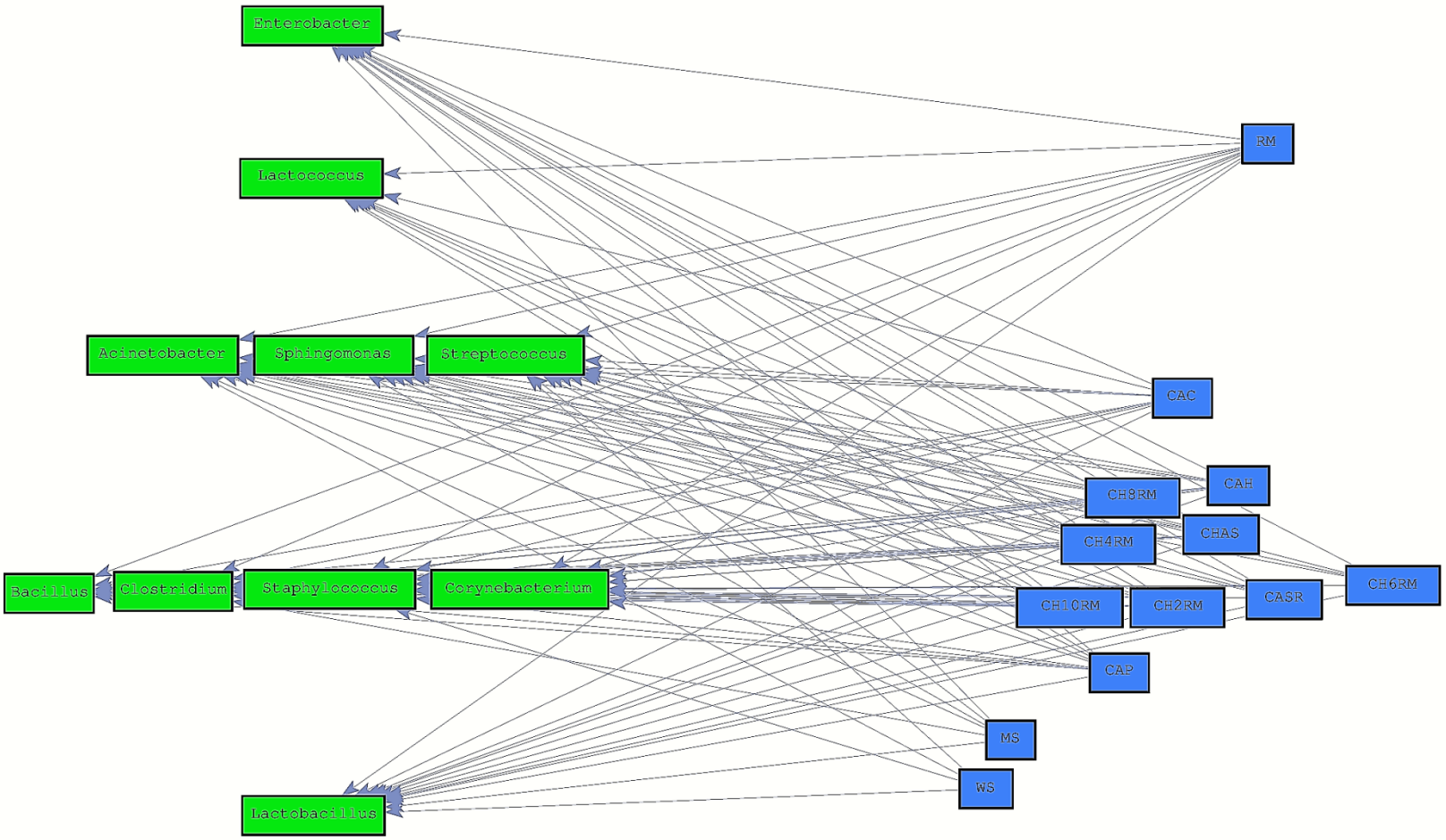

Enterobacter
Lactococcus
Acinetobacter
Sphingomonas
Streptococcus
Bacillus
Clostridium
Staphylococcus
Corynebacterium
Lactobacillus
RM
CAC
CH8RM
CH4RM
CH10RM
CAP
MS
WS
CAH
CHAS
CASR
CH2RM
CH6RM

Fig. 4

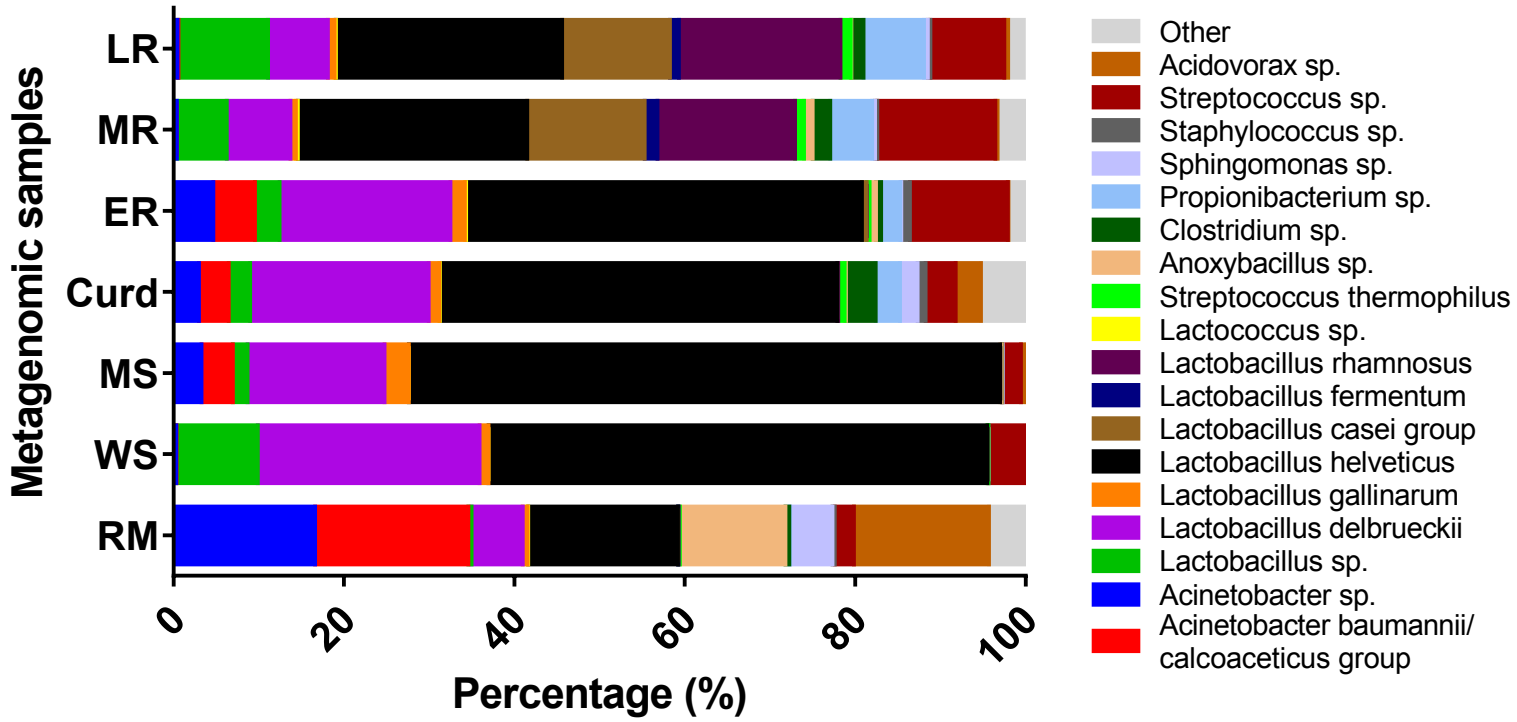
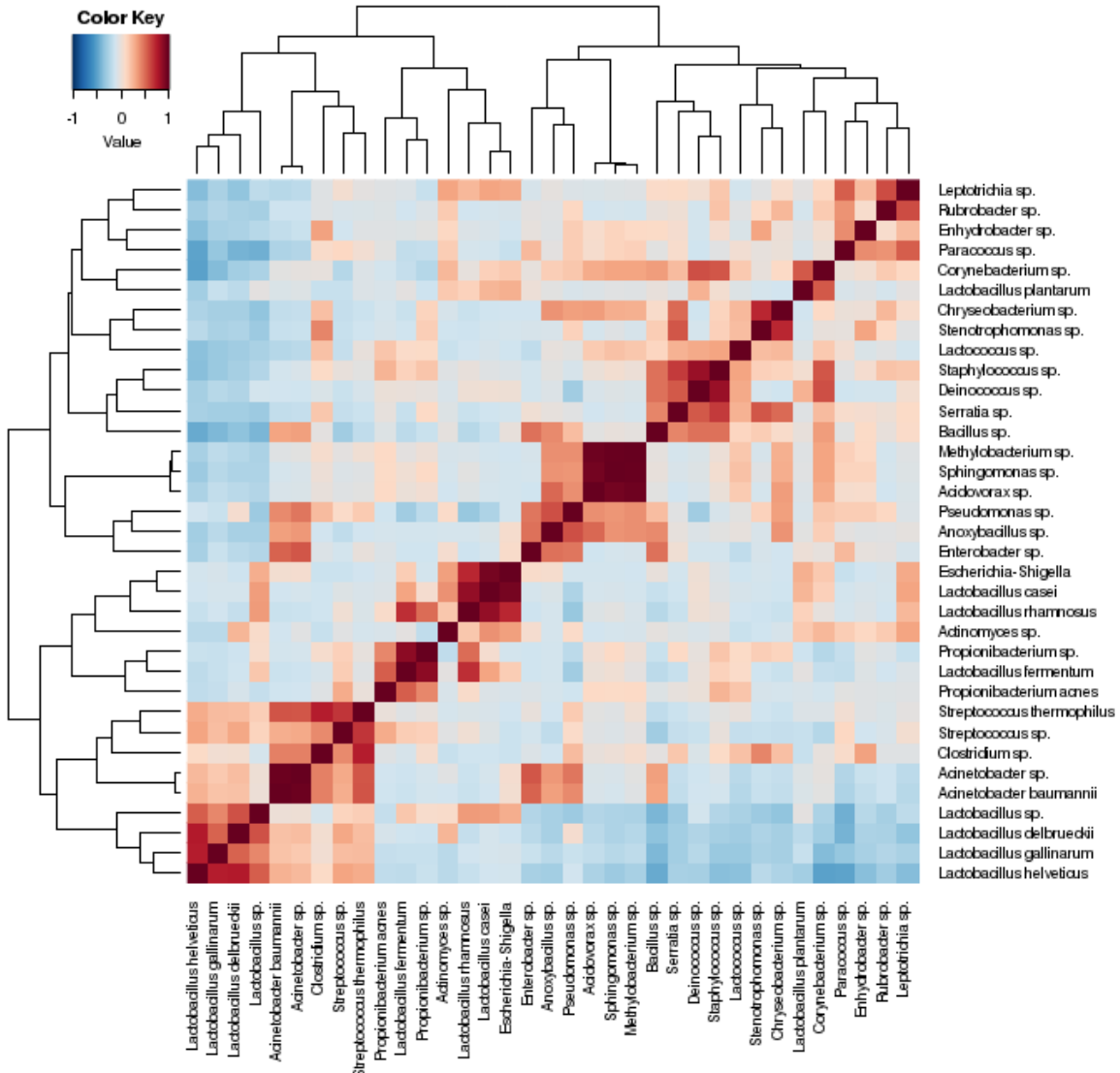

Metagenomic samples
LR
MR
ER
Curd
MS
WS
RM
0
20
40
60
80
100
Percentage (%)
Other
Acidovorax sp.
Streptococcus sp.
Staphylococcus sp.
Sphingomonas sp.
Propionibacterium sp.
Clostridium sp.
Anoxybacillus sp.
Streptococcus thermophilus
Lactococcus sp.
Lactobacillus rhamnosus
Lactobacillus fermentum
Lactobacillus casei group
Lactobacillus helveticus
Lactobacillus gallinarum
Lactobacillus delbrueckii
Lactobacillus sp.
Acinetobacter sp.
Acinetobacter baumannii/ calcoaceticus group

Fig. 5


Color Key
-1
0
1
Value
Leptotrichia sp.
Rubrobacter sp.
Enhydrobacter sp.
Paracoccus sp.
Corynebacterium sp.
Lactobacillus plantarum
Chryseobacterium sp.
Stenotrophomonas sp.
Lactococcus sp.
Staphylococcus sp.
Deinococcus sp.
Serratia sp.
Bacillus sp.
Methylobacterium sp.
Sphingomonas sp.
Acidovorax sp.
Pseudomonas sp.
Anoxybacillus sp.
Enterobacter sp.
Escherichia- Shigella
Lactobacillus casei
Lactobacillus rhamnosus
Actinomyces sp.
Propionibacterium sp.
Lactobacillus fermentum
Propionibacterium acnes
Streptococcus thermophilus
Streptococcus sp.
Clostridium sp.
Acinetobacter sp.
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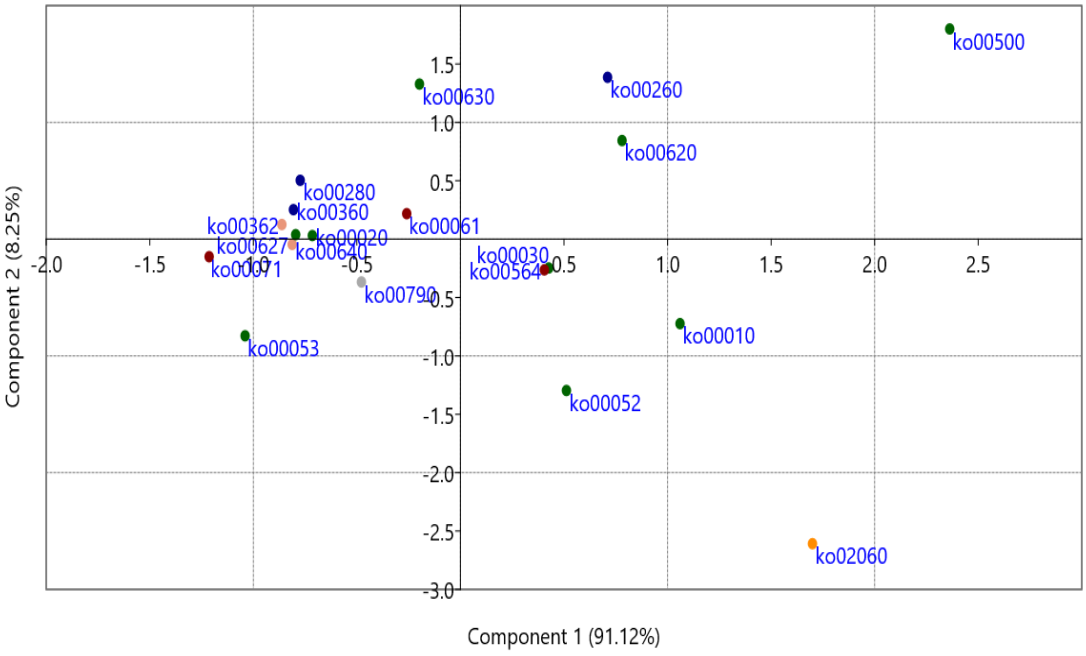
Lactobacillus sp.
Lactobacillus delbrueckii
Lactobacillus gallinarum
Lactobacillus helveticus

Fig. 6

a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ko00020
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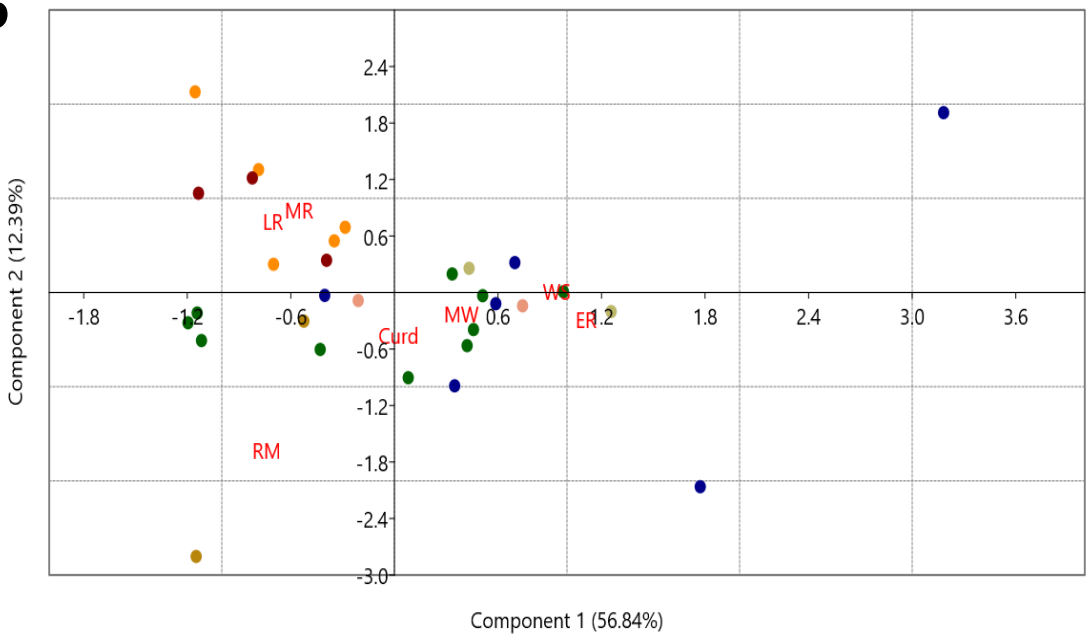
ko00790
ko00053
ko00010
ko00052
ko02060
Component 2 (8.25%)
Component 1 (91.12%)

b


LR
MR
MW
WR
ER
Curd
RM
Component 2 (12.39%)
Component 1 (56.84%)

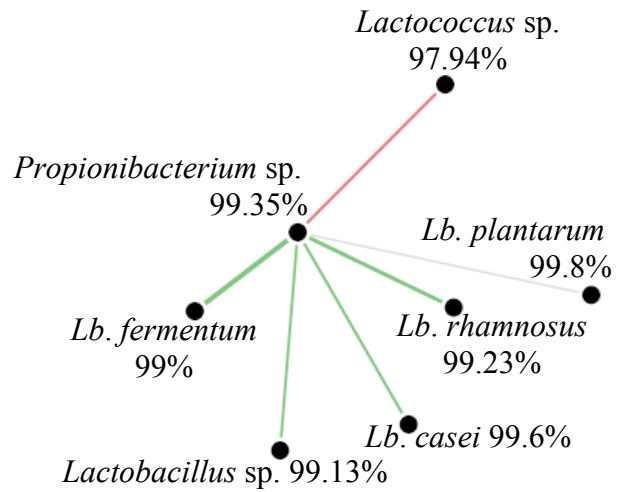
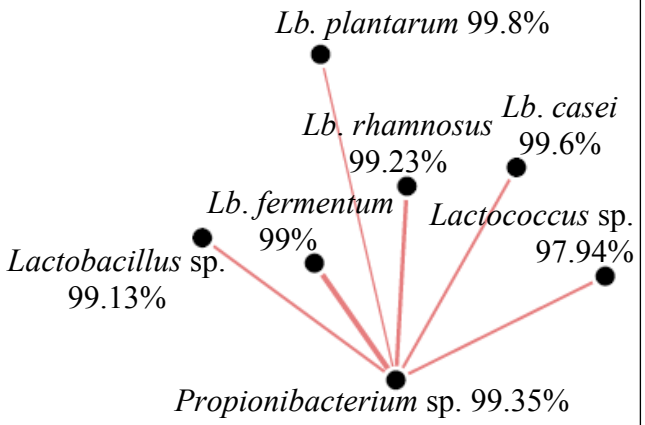
Metabolites availability	Interaction	Number	Network
Complete_100	Positive	4	
	Negative	1	
	No interaction	1	
Complete_10	Positive	0	
	Negative	6	
	No interaction	0	

Fig. 7