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Unravelling the effect of clostridia spores and lysozyme on microbiota dynamics in Grana Padano cheese: A metaproteomics approach

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Abstract

Grana Padano is a typical Italian Protected Designation of Origin (PDO) hard cheese largely consumed all over the world. The major problem during its production is represented by late blowing. Clostridia are gasogen bacteria responsible of the swelling during ripening, and they are partially counteracted by the use of egg white lysozyme as additive. In this work was applied, for the first time in cheese, a metaproteomic approach that identified the functional dynamics of microbial consortia in relation to the number of clostridial spores and lysozyme treatment using experimental samples of Grana Padano cheese. We used a combined custom BLAST+/MEGAN/STAMP approach to obtain a global taxonomic view associated to low and high clostridial spores' cheese without and with lysozyme. Main differences were highlighted in the bacilli class. Functional

analysis with SEED provided a deep view into several metabolic pathways, highlighting the subsystems "amino acid and derivatives" and "clustering-based subsystem" as the targeted subsystems during lysozyme treatment in the high spore group. In these subsystems, acetate kinase from clostridia was one of the main enzymes affected by the lysozyme treatment.

Biological significance: Metaproteomics is a very promising and useful technique in the control of food safety and quality, from fresh products until 'ready to eat' food. Tools able to identify at molecular level the dynamic fingerprinting of food microbiota could be of great help to improve food safety and quality.

1. Introduction

Cattle farming, especially the one facing the production of milk, plays

a key role in the agricultural economy of the north of Italy. The most common type is the intensive production and it is characterized by significant levels of mechanization, high technology of the installations and professionalism of the employees that contributed to a significant increase in efficiency of production. Other constant of this type of breeding is the presence in the farm of a large number of subjects, with con- sequent high density of animals. The Po Valley is a geographic area intensely dedicated to the agricultural and food products. The typical Italian products of this region of particular economic importance and sold throughout the world are hard cheeses such as Grana Padano. GP) PDO. The alteration that, more often, affects the production of hard cheeses is known as "late-blowing" and consists of a disorganization of the pasta cheese that has "eyes", cracks, shredding and openings in the central part of the form and, sometimes, a spongy consistency [1]. Such defects, if marked, can affect the structure of the product and can be accompanied by unpleasant tastes and odours due to production of butyric acid and acetic aldehyde. The butyric fermentation, which occurs in semi-hard and hard cheeses significantly reduces the quality and the commercial value of the product. The late-blowing starts few weeks or months after production of the cheese, during the aging process, i.e. when the physical and chemical conditions of the pasta cheese become optimal for the development of clostridia, the main responsible for this alteration. The clostridia that affect the dairy pro-

duction are attributable to the group of butyric, further divided into two subgroups: the saccarolytic

(Clostridium tyrobutyricum and Clostridium butyricum) with marked ability fermentation of sugars and organic acids and the proteolytic (Clostridium sporogenes and Clostridium bifermentans) that cause the release of amino acids, on which they carry out actions of deamination, decarboxylation, oxidation and reduction. The appearance of late-blowing is linked to the number of spores, in particular of C. tyrobutyricum, initially present

in the milk [2,3]. The presence of this microflora is a consequence of environmental pollution, the quality of silage and practices of milking. The butyric fermentation, particularly marked in the fer- mentation process of the Grana Padano PDO, is currently being chal- lenged by the addition of egg lysozyme [4]. The lysozyme, antibiotic of natural origin, causes an inhibition of growth of clostridia mini- mizing the butyric fermentation [5].

As described previously, even though some mechanisms are al- ready known, there is still a lot of work to be done for the compre- hension of the bacterial dynamics behind the hard pasta cheese spoilage. If metagenomics analysis can provide a resume of the pres- ence of the different bacterial species, metaproteomics, on the other hand, can provide information on the most representative metabolic pathways active during ripening. For this reason, a complementary approach can provide the most complete information for the com- prehension of this phenomenon.

The metaproteome analysis will allow a dynamic vision of pheno- typic changes during the microbial life and can be used to compare the protein expression levels of microorganisms subjected to differ- ent environmental stresses. The main aim of this investigation is the metaproteomic classification of the microbial community in strictly controlled, experimental samples of Grana Padano PDO with low and high number of clostridial spores. This approach could help to determine what are the dynamics of microbial consortia. In this way it is possible to develop procedures and interventions to reduce the butyric fermentation responsible for the late blowing without use of lysozyme and other additives; in compliance with the specifi- cation of the Grana Padano PDO.

2. Materials and methods

2.1. Samples

Investigations were made on experimental caseification models of

Grana Padano cheese. During production of Grana Padano lysozyme was added to vat milk up to 50 mg/kg milk to avoid growth of butyric clostridia that are responsible for the late-blowing defect [5]. Anaerobic spore content was obtained throughout the most probable number (MPN) method as described [6]. Eight samples (four experimental sam- ples and two biological replicates for each experimental sample) with a known number of clostridial spores: high (3.14 log10 MPN L− 1), low
(2.33 log10 MPN L−1) and lysozyme (with or without) were processed.
Overall, the whole experimental dataset was obtained from samples categorized as: low spores without lysozyme (LS − L), high spores without lysozyme (HS − L), low spores plus lysozyme (LS + L) and high spores plus lysozyme (HS + L). All samples were obtained from “Consorzio per la tutela del Formaggio Grana Padano” (Desenzano del Garda, Italy).

2.2. Sample preparation and casein depletion.

Samples was grated and 400 mg for each sample (n = 2 for each
condition) was resuspended in 1.6 ml of milliQ water. Grated cheese was sonicated for 120 s at 55% power with an ultrasonic homogenizer (Sonopuls UW2070, Bandelin, Germany) and stirred with a Thermo- mixer comfort (Eppendorf, Germany) at 1400 rpm for 1 h at 40 °C. Sus- pensions were centrifuged at 10,000 × g at 25 °C (Hettich Mikro 200R, Germany) for 10 min to precipitate caseins from samples. Supernatants were removed and stored at − 70 °C until further analysis. Raw samples and depleted samples were solubilized with Laemmli buffer [7], quanti- tated with Bio\\Rad Protein Assay (Bio\\Rad, GmbH) and ten micro- grams separated on a 14% T polyacrylamide gel to check the protein profile after caseins depletion.

2.3. Sample preparation and 2D-LC-MS/MS analysis

The cheese aqueous phases were concentrated about 10 times on a
speed-vac apparatus (Thermo), mixed with cold acetone (1:6 v/v) and precipitated at − 20 °C overnight. Protein pellets were re-suspended in denaturing buffer (8 M urea in 100 mM ammonium bicarbonate). Proteins were quantitated with Bio\\Rad Protein Assay (Bio\\Rad, GmbH). Fifty micrograms of proteins solubilized with denaturing buffer (8 M urea in 100 mM ammonium bicarbonate) were reduced with DTT (10 mM DTT in 50 mM ammonium bicarbonate) for 30 min at room temperature. Then, proteins were alkylated with iodoacetamide (55 mM IAA in 50 mM ammonium bicarbonate) for 20 min at room temperature. Sample

was diluted 4 times in 50 mM ammonium bicar- bonate and digested with trypsin (1 µg enzyme – 50 µg protein) for 16 h at 37 °C. Enzymatic digestion was stopped with formic acid, until reaching pH 1. Peptides were subjected to 2D LC-MS/MS analysis using a bidimensional chromatography approach (SCX\\C18) coupled to an Amazon Speed ETD Ion-trap mass spectrometer (Bruker Daltonics). 6 µL of tryptic peptides solution (3 µg) were loaded on an SCX column (2 cm, 100 µm i.d., IDEX) directly connected with a RP precolumn C18 (2 cm, 100 µm i.d., IDEX) and a C18-Acclaim PepMap column (25 cm, 75 µm i.d., 5 µm p.s., Thermo Fisher Scientific). After 3 min of preconcentration with 100% H2O, 0.1% F.A. flow (3 µL/min) a first gradient was run to elute peptides unbounded to SCX resin (from 3 to 30% ACN in 120 min). Three salt bumps were injected to progres- sively elute charged peptides (18 µL, 10, 100, and 500 mM respectively) with the same gradient as previous. The analytical column was connect- ed with the nano-spray source of a Bruker Amazon ETD Ion Trap work- ing in Auto MSn mode (DDA) recording 10 MS/MS spectra for each survey scan. Raw data were processed with Compass Data Analysis 1.3 (Bruker Daltonics) while protein identifications and 2D fractions data combining was performed with Compass Proteinscape 2.1 (Bruker Daltonics) using Mascot (version 2.4.1) as search engine. Peptide se- quence matching was performed against the NCBInr database, choosing bacteria (eubacteria) as taxonomic restriction (11,349,194 sequences). Cysteine carbamidomethylation and methionine oxidation were set as fixed and variable modifications respectively, and a single miscleavage by trypsin was allowed. Mass tolerance was set to 0.4 Da and 0.5 Da for precursors and fragments respectively. The Protein Extractor option was used to perform the protein list compilation, combining the unfil- tered results of the four gradient runs on the RP column. The final pro- tein list was obtained adjusting FDR to 1%, setting the scoring cutoff above 40 for proteins and above 10 for peptides, and requiring a minimum of two peptide IDs for each protein. Non-redundant peptide list were parsed using a custom pipeline coupled to MEGAN(MEtaGenome ANalyzer) for taxonomic and functional analysis and STAMP (Statistical Analysis of Metagenomic Profiles) for statistical analysis.

2.4. Metaproteome bioinformatic analysis

The peptide list from each sample was processed using USEARCH (version 8.1) [8] to obtain only all the non-redundant peptide sequence information and converted in .fasta file. The sequence similarity searching was done using the BLAST + program (version 2.2.31) [9]. Briefly, the

blastp-short application optimized for short query se- quences was used to check the sequences against a custom non- redundant protein sequences database (ftp://ftp.ncbi.nih.gov/blast/db nr.*tar.gz 13.12.2015) limited to the bacteria taxa (taxid:2) using the – gilist option. We set a lower E-value (b 10− 5) to achieve a good strin- gency to obtain very close matches to the database used and to reduce the number of false positives. Output XML files from BLAST + were imported on the MEGAN software (version 5.10.7) [10] to obtain the comparative taxonomic analysis and the functional analysis using the SEED classification function. The LCA (lowest common ancestor) algo- rithm parameters of MEGAN were set as following to keep a high quality matches: (min support 5, min score 50, max expected 10−5, top percent 10). The graphical representation and the statistical analysis was per- formed on the STAMP software (version 2.1.3) [11].

3. Results and discussion

3.1. Casein depletion

In order to better investigate the cheese microbiota, all the samples were depleted from caseins as described in methods. All samples were separated by SDS-PAGE (data not shown). A representative SDS-PAGE of the GP protein profile before and after the depletion is shown in Fig. 1. As it is well known caseins are the most abundant proteins into the extracted cheese samples (Fig. 1 lane 1, black arrow). After the de- pletion at medium gravity, cheese aqueous extracts showed a SDS pro- tein profile free of caseins (Fig. 1, lane 2, black rectangle). Each depleted aqueous extract was processed for the successive MS analysis. This ap- proach is relevant for the workflow of this experiments because allows to exclude the most abundant protein in cheese (e.g. caseins) thus expanding the dynamic range of bacterial proteins that could be identi- fied by our metaproteomic pipeline.

3.2. Metaproteomic pipeline

To best of our knowledge, we applied for the first time for cheese analysis a hybrid bioinformatic approach based on BLAST+/MEGAN and STAMP to investigate the microbiota of Grana Padano in several ex- perimental group. In Fig. 2 is showed the experimental pipeline adopted to obtain the taxonomic and the functional classification. This adopted approach started from a high resolution proteomics analysis via 2D-LC MS/MS of the depleted samples as previously described. Overall 3000 peptides were obtained at FDR 1% that were mapped using the BLAST+ application against a custom bacterial database. The choice of a dedicated custom

database was adopted to reduce the size and im- prove peptide matching, that is a well known problem in metaproteomic studies [12]. The output of the blast search was imported into MEGAN 5.10.7 and roughly 1150 peptides were assigned to taxonomic and functional classes according to the lowest common ancestor approach. It is important to note that we applied the min sup- port parameter of 5, this means that at least 5 peptides is the minimum number of peptides (or reads) that a taxon must obtain for the classifi- cation. With this conservative approach, we exclude one-peptide hits and reduce the number of misassignments. At the end of the pipeline we exported metadata to STAMP to evaluate the statistical significance of taxonomic and functional profiles for each experimental comparison.

3.3. Taxonomical analysis of experimental Grana Padano bacterial communities

The taxonomic classification associated with the four experimental group investigated: low spores no lysozyme (LS − L), high spores no ly- sozyme (HS − L), low spores plus lysozyme (LS + L) and highs spore plus lysozyme (HS + L), is reported in Fig. 3A. In the heatmap plot thir- teen bacterial classes represent the core microbiome of these samples and are depicted from the most abundant to the less abundant. All the classes account for roughly 95% of the total bacterial population in each group analysed. In particular, Bacilli is the most abundant class (45 to 29%) followed by Gammaproteobacteria (22 to 12%), Alphaproteobacteria (17 to 9%), Clostridia (11 to 7%), Actinobacteria (8.9 to 4.8%), Betaproteobacteria (7.5 to 3.8%) Deltaproteobacteria (4.8 to 2.3%) and Bacteroidia (7.3 to 2.3%). The last five classes contribute in minor part to the bacterial community with a mean abundance rang- ing from 4.3 to 2.3%. A detailed analysis on relative abundances of gen- era was then carried out on the eight most abundant genera present in all groups analysed, which are reported in Fig. 3B. Data reported in Fig. 3B are in agreement with other studies made on wild type Grana Padano using next generation sequencing as 16s rRNA NGS [13,14] and in partial agreement with culture dependent and low throughput dependent methods [15]. This is probably due, in this case, to the limita- tion of the applied methods to the study of complex bacterial communi- ties. The Hahella genus presence was not detected in other studies in the hard cooked cheese matrix but is a common genus present in raw milk as reported by several authors [16].

3.4. Effect of lysozyme on the dynamics of the bacterial community

The effects of lysozyme on the dynamics of microbiota at low level of

clostridial spores (LS − L and LS + L groups) is reported in Fig. 4A. Ly- sozyme caused a decrease in the mean abundance of the genus Lactoba- cillus (p value: 0.018) and no statistically significant changes for the others genera investigated, as reported also by other authors [13]. The dynamics of microbiota in all the groups with a higher level of clostridial spores (HS − L and HS + L) exhibit a trend to the decrease of the genus Lactobacillus but without a significant p value (Fig. 4B).

3.5. Lysozyme-driven changes in the functional profile of bacterial community.

Using the SEED classification function built into MEGAN it was pos-
sible to map peptide reads onto functional roles present in one or more subsystems. In Fig. 5A are reported, for the low spore groups, the mean abundances of 22 functional subsystems in the eight most abundant genera. The subsystems into the heatmap plot are ordered ac- cording to the mean abundance, ranging from the most abundant “Pro- tein metabolism” (25–20%) to the less abundant “Iron acquisition and metabolism” with 0.75%. Of these 22, two were significantly affected by lysozyme and in particular the increased representation of the two subsystems “Membrane transport and RNA metabolism” (Fig. 5B). The effect of lysozyme for the high spore group is related to the significant modulation of two subsystems as reported in Fig. 5C. In particular “Amino acids and derivatives and Clustering-based subsystems” are under-modulated following lysozyme treatment. A deeper analysis of the “Amino acids and derivatives” subsystem highlighted the decrease of several enzymes after the lysozyme treatment. In particular acetate kinase, that is a key enzyme needed in Cl. tyrobutyricum and other clos- tridia to convert acetate and lactate into butyric acid, carbon dioxide and molecular hydrogen [17–19] leading to the late blowing, was undetect- able after the lysozyme treatment (Fig. 6A). To confirm this finding the inspector module of MEGAN was used to retrieve the taxonomic infor- mation associated to this enzyme. It was possible to map the reads to the Clostridium genus thus confirming the close relationship with late blowing phenomena. Conversely, for the enzyme butyryl-CoA dehydro- genase, which is also involved in butyric fermentation, we detected a non-significant increase after lysozyme treatment. A careful mapping of the reads revealed the Thermoanaerobacter genus as the closer to the sequence analysed and not directly involved in the late blowing phenomena.

4. Conclusions

Despite the lack of a complementary NGS investigation and a very high number of identifications, we obtained, using an original metaproteomic approach, taxonomic, functional data and related SEED subsystems. Moreover, our investigation has succeeded to follow sever- al enzymes directly modulated by lysozyme treatment and associated to the different experimental conditions. These information will be useful for developing new biological strategies to control the bacteria for undesirable bacterial activities in hard-cooked cheese. We expect that our metaproteomic investigation of Grana Padano cheese will help the con- sortium and other producers of hard and semi hard cheeses to solve the problem of late blowing and further increase the quality of this product. Anyway, our strategy can be applied also to the food microbiome anal- ysis to detect potential microbiome imbalancements and improve the food quality and safety. Future efforts will be made on selected GP PDO samples from different geographic origin to confirm our findings. The use of a sample-specific metagenomic database and extended LC runs will enhance our understanding of cheese ripening and the rela- tionship between metabolism of starter and bacteria naturally present in the environment.

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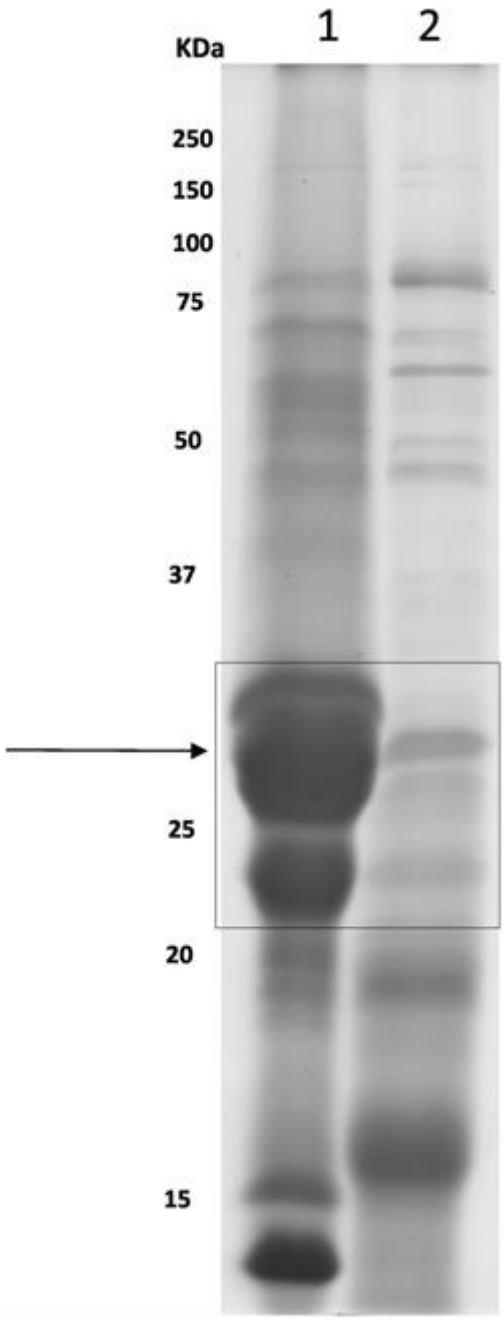

Fig. 1. Representative SDS-PAGE profile of Grana Padano before and after casein depletion. Lane 1: protein profile of Grana Padano cheese aqueous extract, black arrow indicates casein bands. Lane 2: protein profile of cheese extract after pelleting of caseins by centrifugation at 10.000 g. The black rectangle at the same molecular weight of black arrow in lane 1 highlight the absence of caseins from the sample. Molecular weight markers (Precision Plus, Biorad) are shown on the left side of the image.

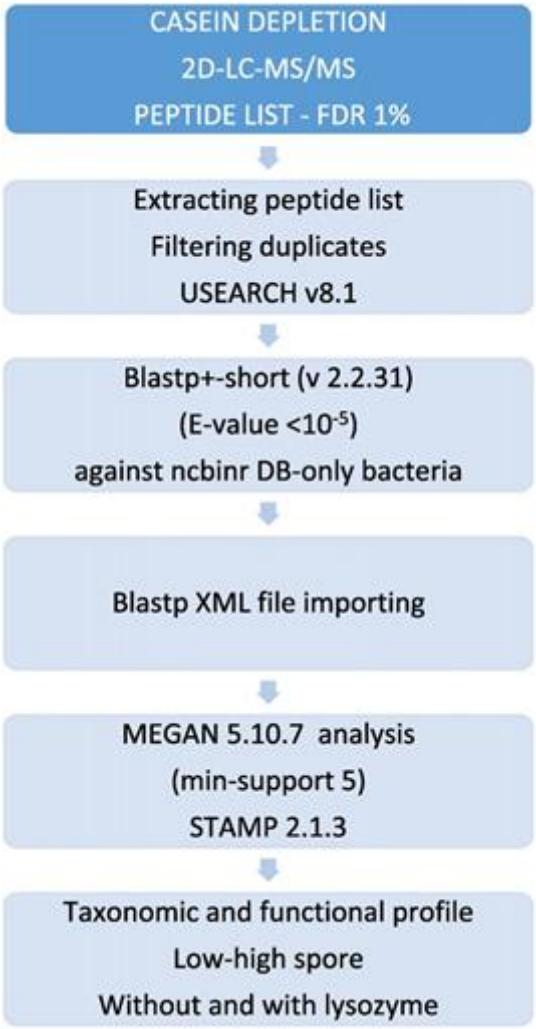

Fig. 2. Experimental pipeline adopted to obtain taxonomic and functional classification. The experimental approach started from a high resolution proteomics analysis via 2D-LC MS/MS of depleted samples. N 3000 peptides were obtained at FDR 1% that were mapped using the BLAST+ application against a custom NCBInr bacterial database. The output of the BLAST+ search was imported into MEGAN 5.10.7 and roughly 1150 peptides were assigned to taxonomic and functional classes according to the lowest common ancestor approach. Obtained metadata were analysed through STAMP to evaluate the statistical significance of taxonomic and functional profiles for each experimental comparison.

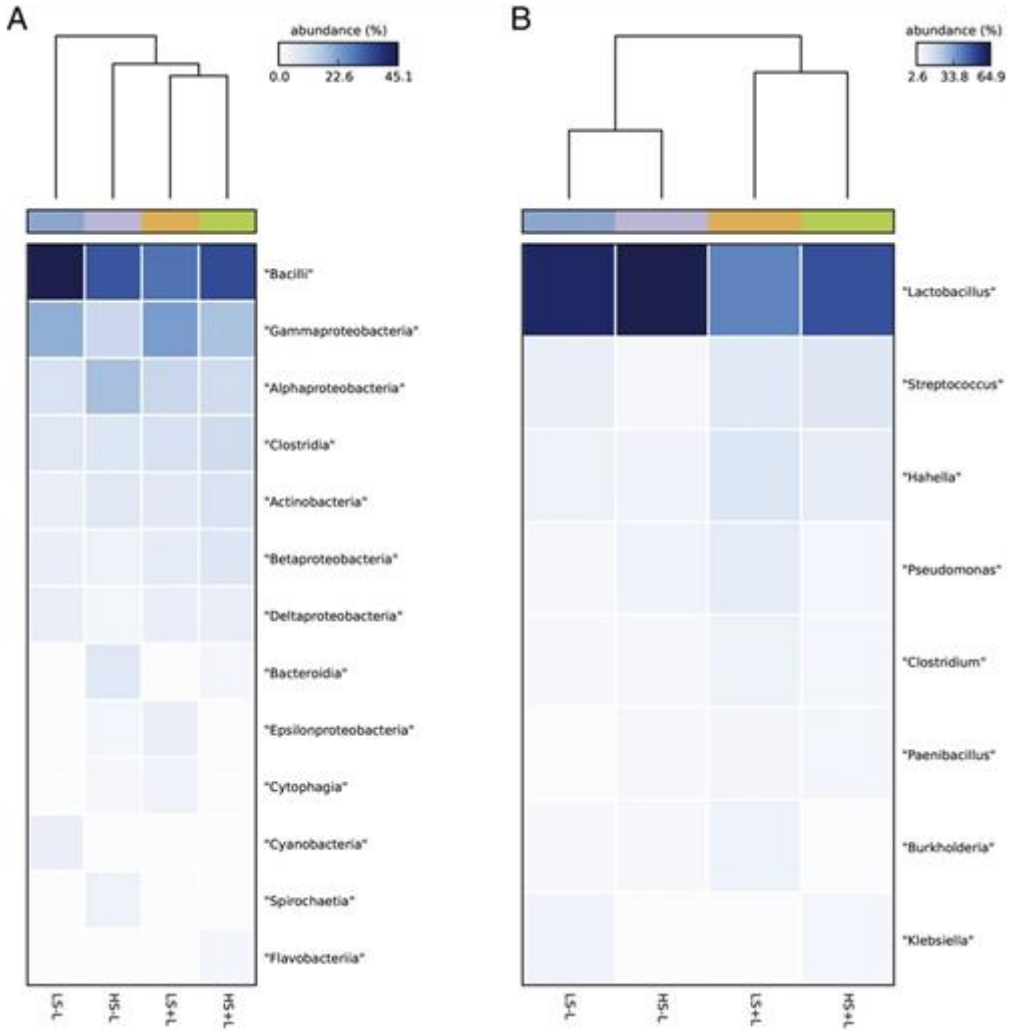

Fig. 3. a. Heatmap plot representing the core microbiome of analysed samples from the most abundant to the less abundant. All the classes account for roughly 95% of the total bacterial population in each group analysed. b. Analysis on relative abundances of the eight most abundant genera present in all experimental groups.

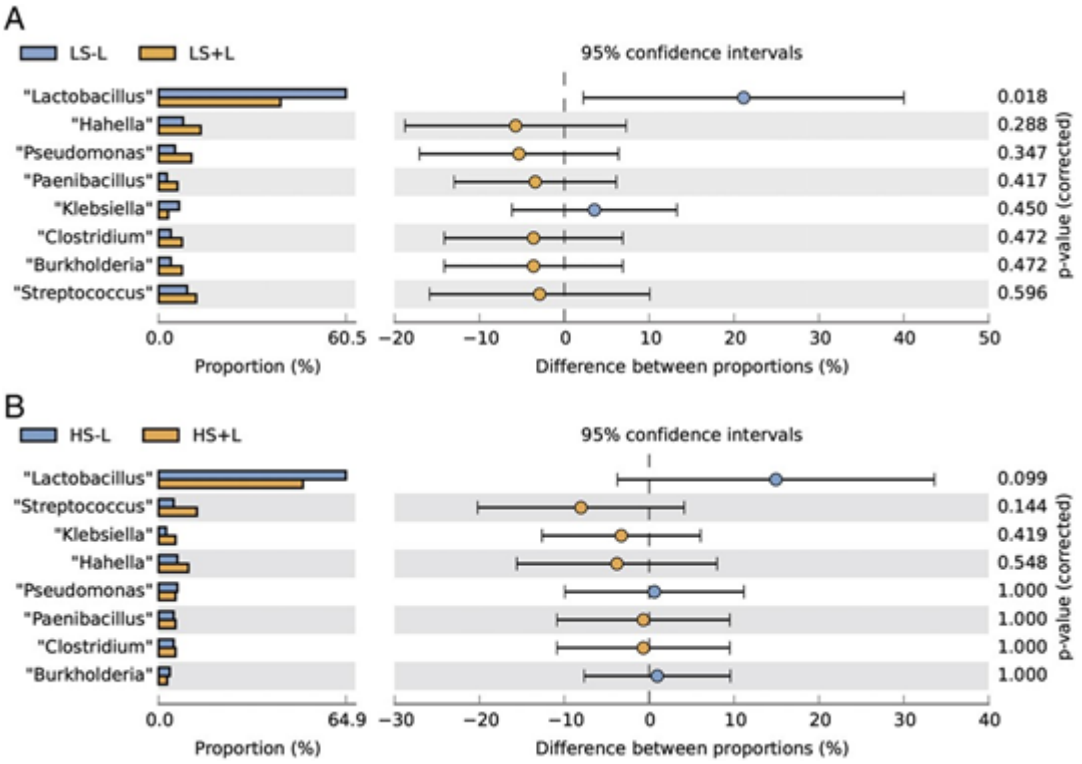

Fig. 4. a. Plot representing the effects of lysozyme on the dynamics of microbiota at low level of clostridial spores (LS − L and LS + L groups). b. Plot representing the effects of lysozyme on the dynamics of microbiota at high level of clostridial spores and lysozyme treatment (HS − L and HS + L groups).

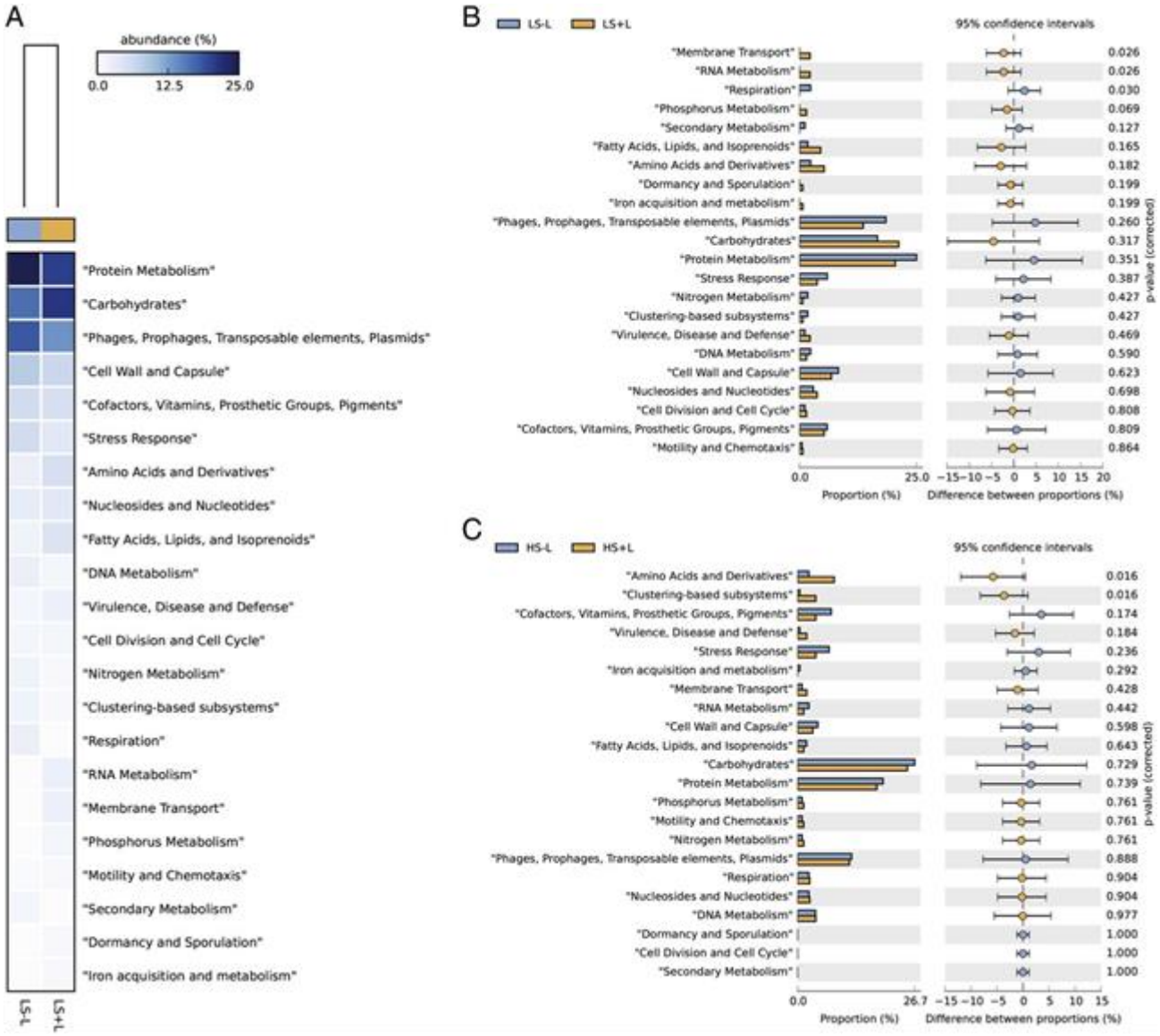

Fig. 5. a. Results obtained using the SEED functional classification tool built into MEGAN. Low spores groups: mean abundances of 22 functional subsystems in the eight most abundant genera. b. Plot resuming the differences in the metabolisms according to the lysozyme treatment in low spores groups. c. Plot resuming the differences in the metabolisms according to the lysozyme treatment in high spores groups.

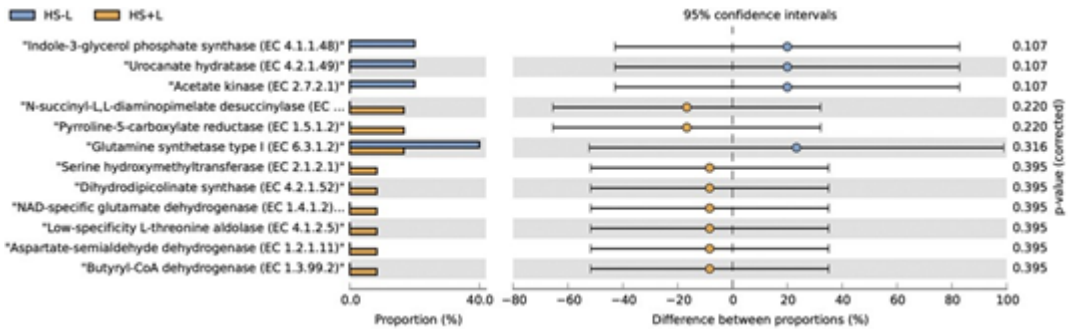

Fig. 6. A. Plot representing the uncollapsed subsystem “Amino acids and derivatives”. The effects of lysozyme at the single enzyme level are highlighted for the high spores samples without (HS − L) and with lysozyme (HS + L).