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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 consequent 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 production 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

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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 fermentation process of the Grana Padano PDO, is currently being challenged by the addition of egg lysozyme [4]. The lysozyme, antibiotic of natural origin, causes an inhibition of growth of clostridia minimizing the butyric fermentation [5].

As described previously, even though some mechanisms are already known, there is still a lot of work to be done for the comprehension of the bacterial dynamics behind the hard pasta cheese spoilage. If metagenomics analysis can provide a resume of the presence 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 comprehension of this phenomenon.

The metaproteome analysis will allow a dynamic vision of phenotypic changes during the microbial life and can be used to compare the protein expression levels of microorganisms subjected to different 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 specification 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 samples and two biological replicates for each experimental sample) with a known number of clostridial spores: high (3.14 $\log_{10}$ MPN L^{-1}), low (2.33 $\log_{10}$ 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 caseins 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 Thermomixer comfort (Eppendorf, Germany) at 1400 rpm for 1 h at 40 °C. Suspensions 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], quantitated with Bio—Rad Protein Assay (Bio—Rad, GmbH) and ten micrograms 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 bicarbonate 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% H_2O, 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 progressively elute charged peptides (18 μL, 10, 100, and 500 mM respectively) with the same gradient as previous. The analytical column was connected with the nano-spray source of a Bruker Amazon ETD Ion Trap working 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 sequence 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 unfiltered results of the four gradient runs on the RP column. The final protein 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 sequences 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 ($<10^{-5}$) to achieve a good stringency 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) algorithm parameters of MEGAN were set as following to keep a high quality

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