# Assessment of residual plant DNA in bulk milk for Grana Padano PDO production by a metabarcoding approach

**Plant residues identification in cow milk**

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# Abstract

The aim of this study was to evaluate the ability of DNA metabarcoding, by *rbcl* as barcode marker, to identify and classify the small traces of plant DNA isolated from raw milk used to produce Grana Padano (GP) cheese. GP is one of the most popular Italian PDO (Protected Designation of Origin) produced in Italy in accordance with the GP PDO specification rules that define which forage can be used for feeding cows. A total of 42 GP bulk tank milk samples were collected from 14 dairies located in the Grana Padano production area.For the taxonomic classification, a local database with the *rbcL* sequences available in NCBI on September 2020/March 2021 for the Italian flora was generated. A total of 8,399,591 reads were produced with an average of 204,868 per sample (range 37,002- 408,724) resulting in 16, 31 and 28 dominant OTUs at family, genus and species level, respectively.

The taxonomic analysis of plant species in milk samples identified 7 families, 14 genera and 14 species, the statistical analysis conducted using alpha and beta diversity approaches, did not discriminate the samples by province of origin. However, the milk samples are featured by a high plant variability and the lack of differences at multiple taxonomic levels could be due to the standardisation of the feed rationing, as requested by the GP rules. The results suggest that DNA metabarcoding is a valuable resource to explore plant DNA traces in a complex matrix such as milk.

# Introduction

Current market trends show a growing interest among consumers in the origin of food products [1-3]. In this regard, PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication) products, above others, are able to guarantee authenticity, tradition, taste, typicality, link with the land of origin, safety and traceability [4-5]. In details, the PDO certification ensures that the product is legally guaranteed by the European Union as authentic, or manufactured in a specific geographical area with the expected ingredients [6-7]. An accurate control system is included in the certification itself (public supervision, third-party control and self-control). Designations such as PDO and PGI aim to protect the quality standards of agri-food products, safeguard their production methods, provide consumers with clear information on the characteristics that add value to products. This enormous wealth of information for the consumer is ensured by compliance with production specifications (<https://www.politicheagricole.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/396>) [ ].

Grana Padano (GP) is one of the most popular Italian PDOs, both in Italy and internationally. In 2020, the GP production reached the quota record of 5,255,443 cheese wheels. With a total production value of 4.5 billion Euros, GP represents 59% of the entire Italian PDO-PGI food production, and 52.6% of PDO-PGI food export [8]. GP is typically produced from raw cow’s milk collected from animals bred in 32 provinces of Northern Italy associated with the Grana Padano Protection Consortium which operates in compliance with the rules defined by the product regulations. As such, the quality of the GP cheese is closely related to the typical climatic and environmental conditions, and forage quality[9-10].

In support of certified productions, the EU recognizes the importance of food traceability, *i.e.* the capacity to track food in all stages of the production chain [11]. Food traceability becomes even more important when the area of production influences the quality of the final product. In this context, recent advances in molecular biology make DNA markers potentially valuable tools to track the raw material in the food chain. In fact, DNA has higher stability compared to other biological markers, such as proteins, and can be isolated even in matrices with extremely low contents [12-14]. In this regard different studies have been described for enhancement of GP or other dairy products: Rocchetti et al. (2018) [15] outlined a metabolomic approach to ensure the authenticity of GP, Faustini et al. (2019) [16] reported on a volatilome study on milk destined to the production of both GP and Parmigiano Reggiano cheeses and, metabarcoding of bacterial communities has been used for the preservation and valorisation of similar food products, [17-19]. Several studies have shown that feed-derived plant nuclear and/or chloroplast DNA fragments can also be detected in milk [20]. In fact, small fragments of plant DNA are able to cross the intestinal barrier and enter the bovine bloodstream. However, this DNA is highly fragmented, although it is not possible to establish at what level, as the feed is subjected to the production processes of milling, extrusion, ensiling, grinding, steam heating and pelletizing and, after ingestion, to its elaboration/degradation in the gastrointestinal tract [21-22]. In these cases, where the DNA is subjected to severe treatments and is present in small quantities, its isolation is particularly difficult and, in the absence of commercial kits optimised for this purpose, it is necessary to implement appropriate molecular approaches based on the analysis of very short DNA fragments. Although some studies were conducted for plant DNA detection in milk using classical approaches [19-23], to the best of our knowledge there are no published data about the detection of residual plant DNA in cow’s milk by High-Throughput DNA Sequencing (HTS) metabarcoding.

Therefore, the aim of this work was to investigate whether chloroplast DNA metabarcoding and dedicated data analysis could be a suitable tool to identify and classify the small traces of plant DNA isolated from bulk raw milk, in order to contribute to development of new traceability systems and to support existing one.

# Materials and methods

## Samples collection

A number of 42 GP bulk tank milk samples of 50 ml volumes were collected from cows fed according to PDO Specification rules (9) in 14 dairies located in the GP cheese production area in the provinces of Verona, Piacenza, Trento, Vicenza, Brescia, Cremona and Cuneo, and stored at -20°C until analysis.

Three samples with known composition were also collected for permitting us to monitor all steps of the analysis from DNA extraction and amplification through sequence data filtering and provided a very useful way to evaluate the performance and effectiveness of our experimental procedure. One sample was represented by soy flour (*Glycine max*) and two forages, forage\_1 and forage\_2 allowed by GP PDO production specification rules compounded with variables portion of maize, wheat, soy, sunflower, sugarbeet with different treatments and their by products.

## DNA isolation

Milk samples were thawed at 4°C for 16 hours and total DNA was extracted from 20 ml in phenol/chloroform according to Ponzoni et al. (2009)[20]. As a minor modification of the original method, the first incubation step was conducted at 65°C for 6 hours under constant agitation.

Plant DNA was extracted from foragesusing the DNeasy Plant mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Purity and quantity were determined with Nanodrop Spectrophotometer 2000 (Thermofisher Scientific, Wilmington, DE, USA).

## RuBisCO gene amplicon molecular identification

To confirm the presence of plant nucleic acid in the total DNA extracted from milk, a 351bp RuBisCO gene fragment was amplified with the following primers: RUB F2 TTGGCAGCATTCCGAGTAAC and RUB R2 GTGAGGCGGACCTTGGAAAG [24] by using the GeneAmp PCR System 9700 (Applied Biosystems FosterCity, CA). The PCR condition included a denaturation step at 95 °C for 15’, followed by 35 cycles of 94 °C for 30”, 57 °C for 90’ and 72 °C for 90’ followed by a final elongation at 72 °C for 10’. Fragments were visualised on the Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and only samples with positive amplification were submitted to DNA metabarcoding analysis.

## Library preparation and sequencing

DNA samples were amplified in two steps, as follows: an initial PCR amplification of the variable *rbcL* region of the RuBisCO gene was conducted using locus specific primers with the following Illumina adapter overhangs:

**rbcL F** 5’- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATGTCACCACAAACAGAGACTAAAGC-3’ and **rbcL R** 5’- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTAAAATCAAGTCCACCRCG -3’

and a subsequent amplification that integrates relevant flow-cell binding domains and unique indices (NexteraXT Index Kit, FC‐131‐1001/FC‐131‐1002). Libraries were sequenced on MiSeq instrument (Illumina, San Diego, CA) using the 300 bp paired-end mode.

## Assembly of a local database and metabarcoding analysis

For the taxonomic classification, a local-database of the potential dietary species was created by using 1426 *rbcl* sequences downloaded from NCBI Nucleotide section. Species admitted by the GP PDO Production Specification Rules (clause 4) were also included.

The analysis pipeline was performed as briefly described, as follows: raw reads were trimmed in order to eliminate primer sequences by *cutadapt* (Ref1) with the following parameters: *--anywhere* (on both adapter sequences) *--overlap 5 --times 2 --minimum-length 35 --mask-adapter*. Low-quality bases were removed from 3’ by erne-filter (Ref2) by applying default parameters, excluding reads <60 bp from the further analysis, and reads with an error rate >1% were removed. Chimeric sequences were then removed with an uchime\_denovo (Ref3) implemented by usearch (https://www.drive5.com/usearch/), using default parameters.

Reads were clustered to a minimum identity of 97% generating representative sequences (cluster\_fast implemented in usearch) and blasted against the local-database with e-value 0.01. At this point, blast hits were analysed and only the lowest unambiguous taxonomy was reported, *i.e.* if there are best hits with the same score indicating different lineage, last common ancestoris indicated. The information was then compacted in several Operational Taxonomic Unit (OTU) tables, depending on the considered taxonomic level, either family, genus or species.

## Data filtering

A further filter was applied to each resulting OTU table to remove entries having less than 5 reads and binning them to unassigned (UN). Out of the assigned reads for each sample, entries representing less than 1% of the reads of a sample were labelled as “subdominant” and were considered as less important in the analysis [17] on metabarcoding of GP cheese.

## Statistical analysis

Species richness was investigated through the analysis of rarefaction curves to verify that the reading depth was adequate. The function *rarecurve* from R package *vegan* [25] with parameter *step = 100* was used. Alignment results were filtered for *percent\_identity* > 95% and *e-value* < 1e-5. In case of multiple alignments, the one with the smallest *e-value* was retained. A portion of obtained sequences did not align on the *rbcL* database of interest. Clusters not aligning on the *rbcL* database were aligned via blast on the *Bos Taurus* 3.1.1 genome via the NCBI online nucleotide Blast tool (<https://blast.ncbi.nlm.nih.gov/>).

In this study each sample was considered as an isolated ecosystem, thus allowing the use of statistical tools developed in the ecological field such as alpha (Shannon and Simpson) and beta diversity analysis [26,27,28,29]. These two methodological approaches aim to provide objective and comparable measures of biological diversity present in each community. All the considered diversity statistics were computed using the R package *vegan* [25].

# Results

## DNA metabarcoding analysis

Regarding soy flour, forage\_1 and forage\_2, a total of 700,346 paired-end-mode reads (range 217,739-261,868) were obtained with 99% of reads assigned to plant Kingdom. Rarefaction curves showed that samples reached stability in the species richness given the amount of available reads (S1 Fig.).

Results relatively to milk samples are shown in Table 1. Overall, a total of 8,399,591 reads were produced with an average of 204,868 per sample (range 37,002- 408,724). Only one sample showed the extremely low number of reads equal to 31 and was, therefore, discarded from subsequent analyses. More specifically, for milk samples 1-36.6% of reads were assigned to plant Kingdom.

Reads not assigned to plant Kingdom were aligned to the *Bos taurus* 3.1.1 genome. On average, 63% of them were mapped, confirming the bovine origin.

**Table 1. Total number of reads and assigned reads and relative percentages produced in forages and milk samples.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sample Type** | **N\_sample** | **Tot\_Reads** | **Tot\_Reads\_ASS** | **%Eukaryota** | **%UN\_Eukaryota** |
| Forages | 3 | 700,346 | 696,264 | 99.2 | 0.73 |
| Milk samples | 42 | 8,399,591 | 260,784 | 7.21 | 92.78 |

The total sequences obtained were clustered around 4811 centroids and subsequently aligned on the local-database of 1426 *rbcl* sequences. Samples resulted in 16, 31 and 28 dominant OTUs at family, genus and species level, respectively (Table 2).

**Table 2. Number of raw OTUs, OTUs with reads > 5 and dominant OTUs at family, genus and species level in forages and milk samples.**

|  | **Forages (n=3)** | | | **milk samples (n=41)** | | |
| --- | --- | --- | --- | --- | --- | --- |
| **Taxonomical**  **level** | **Raw OTUs** | **Otus reads >5** | **Dominant OTU** | **Raw OTUs** | **Otus reads >5** | **Dominant**  **OTU** |
| Phylum | 1 | 1 | 1 | 1 | 1 | 1 |
| Family | 11 | 11 | 4 | 10 | 8 | 7 |
| Genus | 30 | 27 | 9 | 25 | 16 | 14 |
| Species | 44 | 34 | 9 | 36 | 16 | 14 |

## Taxonomic analysis

The taxonomic analysis for the three forage samples showed that the most abundant families in forage\_1 and forage\_2 were Poaceae (69.3% and 24.4%, respectively) and Fabaceae (74.8% and 7.3%, respectively), while for the soy flour the most represented family was confirmed to be Fabaceae (99.5%). At genera level, in forage\_1 the most represented were Glycine (16.28%) followed by Pisum (5.32%), Ceratonia (2.57%), Gossypium (2.18%), Helianthus (1.64%) and Zea (1.54%) (ricontrollare le % con anna). In forage\_2, the percentage of Glycine was equal to 14.38% followed by Helianthus (2.39%), Gossypium (2.36%), Ceratonia (1.68%), Pisum (1.02%) and Zea (0.87%). Regarding soy flour sample, Glycine was equal to 99.39% with minimal percentages of other genera. At species level, for forage\_1 and forage\_2 the most abundant specie was Glycine max (16.02% and 14.26%, respectively) followed by Pisum sativum (5.3% and 1.02%, respectively), Ceratonia siliqua (2.5% and 1.68%, respecrtively), Helianthus annuus (1.64% and 2.39%, respectevely) and Zea mays (1.54% and 0.88%, respectively). Graphical representations of taxonomic analysis were reported in figures 1, 2 and 3.

Fig 1. Taxonomic contents of the forage\_1.

Fig 2. Taxonomic contents of the forage\_2.

Fig 3. Taxonomic contents of the soy flour.

In the milk samples, the metabarcoding analysis associated all the assigned reads to the Streptophyta Phylum. Analysis of deeper taxa identified 7 families (*Fabaceae, Convolvulaceae, Poaceae, Rubiaceae, Malvaceae, Asteraceae and Ranunculaceae*), 14genera (*Ceratonia, Cuscuta, Glycine, Triticum, Gallium, Gossypium, Medicago, Panicum, Lolium, Helianthus, Pisum, Zea, Delphinium and Vicia*) and 14 plant species (*Ceratonia siliqua, Triticum aestivum, Medicago sativa, Gossypium mustelinum, Glycine max, Panicum pygmaeum, Panicum wiehei, Lolium perenne, Pisum sativum, Helianthus annuus, Vicia faba, Zea mays, Delphinium grandiflorum and Secale cereale*). Among the sevenfamilies identified, the most abundant were: Fabaceae (55.9%), Convolvulaceae (26.6%) and Poaceae (9.4%), followed by Rubiaceae (4.3%), Malvaceae (3.4%), Asteraceae (0.2%) and Ranunculaceae (0.1%). At a lower taxonomic level, the most representative genera were Ceratonia (33.9%), Cuscuta (26.9%), Glycine (21.2%) and Triticum (5.8%)**.**

In Figs. 4, 5 and 6, with regard to the abundance, are shown the heatmaps for dominant (>1%) centroids at different taxonomic levels: family, genus and species, , respectively.

Fig 4. Abundance detected in milk sample at family taxonomic level

Fig 5. Abundance detected in milk sample at genus taxonomic level

Fig 6. Abundance detected in milk sample at species taxonomic level

## Statistical analysis

Ma la alpha viene elimiata? Riscrivere alpha diversity per i campioni suddivisi per provincia come figura 5

Alpha diversity analysis was performed using the “Shannon-Wiener” and “Simpson” diversity indexes; in Table 3 are reported the p-values from the Wilcoxon rank sum test.

**Table 3. Average values for Shannon and Simpson richness indexes for all samples, computed at three different taxa levels. Significance test p-values computed via Wilcoxon rank sum test.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | | **Taxa level** | | |
| **Richness index** | **Area** | **Family** | **Genera** | **Species** |
| Shannon | GP | 0.684 | 1.038 | 0.46 |
| outside | 0.326 | 0.867 | 0.311 |
| p-value | 0.108 | 0.128 | 0.322 |
| Simpson | GP | 0.35 | 0.557 | 0.245 |
| outside | 0.16 | 0.494 | 0.155 |
| p-value | 0.083 | 0.267 | 0.285 |

Results revealed no statistically significant differences for both indexes at three taxonomic levels (family, genus and species), with all p-values ≥ 0.05. The Shannon index shows a clearly non-normal distribution (Fig. 4). In fact, it appears to be bimodal, *i.e.* as if it was the union of two distributions, one with lower and one with higher average diversity. This bimodal trend is exacerbated by one single sample of commercial milk, that at species level has a very high diversity (Shannon alpha value ≥ 0.875). On average, we found that in the GP milk samples the richness indexes are higher than the commercial milk samples (Table 3). We then tested the differences among groups for statistical significance. The data distributions did not pass the Shapiro normality test (data not reported), so we used Wilcoxon rank sum test to compute the p-values associated with differences in significance.

An analysis of variance (ANOVA) on both Shannon and Simpson scores was performed, grouping samples by province and considering progressively deeper taxonomic levels (Fig 5). No statistically significant differences in richness were observed between the provinces. The Table 4 reports the resulting p-values.

**Fig 5. Distribution of alpha diversity as computed using Shannon’s and Simpson’s indexes.** Colours indicate different provinces of origin. The three boxes report indexes computed at different taxonomic levels.

**Table 4. p-values from ANOVA.**

|  |  |  |
| --- | --- | --- |
| **Level** | **Shannon\_pvalue** | **Simpson\_pvalue** |
| Family | 0.219 | 0.205 |
| Genus | 0.924 | 0.955 |
| Species | 0.857 | 0.792 |

Beta diversity also considers information about biological richness, but measures the variation between pairs of different communities using the Bray-Curtis index. The results are visualised in Fig 6 after the application of Multi Dimensional Scaling (MDS).

**Fig 6. Diversity analysis - beta diversity.** Beta diversity computed using Bray–Curtis index and then transformed via Multi-Dimensional Scaling for milk samples by province.

# Discussion

In this work, bulk milk samples collected within the GP production area were investigated by a metabarcoding approach in order to identify and classify the residual plant DNA content. As far as we know, there are no published studies on DNA metabarcoding for the detection of residual plant DNA in bulk cow's milk. In this regard, only a few studies have been conducted with the same goal using classic PCR approaches [20], while others have used different biological matrices such as faeces, intestinal contents etc. [30-31]. One of the difficulties in characterising plant DNA in milk compared to other biological matrices derives from the fact that plant components resulting from the diet are strongly degraded and fragmented due to their passage through the digestive system. At the same time, it is important that the chloroplast DNA (cpDNA) target can be extracted with the least possible amount of nuclear and mitochondrial DNA and other contaminants, such as polysaccharides and resins, that can interfere and reduce the efficiency of the subsequent applications. The contamination, in fact, could reduce the efficiency of the subsequent applications. [32].

With particular reference to these aspects, the extraction method adopted in the present work proved to be effective for recovering chloroplast DNA from plants in milk. Furthermore, of considerable importance for the subsequent development of the method, the Consortium for the Barcode Of Life (CBOL) Plant Working group identified plastid coding region *rbcl and matK as* core-barcode for plant identification [33]. In the present work the *rbcl* marker was chosen because it is the most characterised plastid coding region in the GenBank database and for its higher universality compared to other DNA barcode markers described in the literature [34]. Although *matk* is considered a good marker of DNA due to its high-resolution rate, it was excluded since it is difficult to amplify with a single primer pair, especially when compared with *rbcl* which is less problematic not only in amplification [13], but also in sequencing and bioinformatics analysis.

The taxonomic analysis of plant DNA isolated in all samples was performed with a local database created specifically on the basis of the Italian plant taxa. In fact, as demonstrated by several authors [31,33,35,36], for the taxonomic assignment the use of local dedicated databases reduces the possibility of wrong identifications compared to existing databases that contain millions of reference DNA sequences [37]. DNA metabarcoding was found to be effective to characterise the plant component of each sample and then to measure the abundance of sequences at phylum, family and species level. n samples with known composition forage\_1, forage\_2 and soy flour, metabarcoding identified the taxa to which the raw materials used as fodder belong. With regard to milk samples, at the family and genus level the major number of taxa were identified in milk samples of cows belonging to GP consortium.

Two unexpected plants were detected: *Ceratonia siliqua* and *Cuscuta* for which no deeper classification was possible. The causes could be due to several factors as reported from other authors: DNA metabarcoding can produce different proportion of reads which are not related to the real quantities of each species in the sample analysed, furthermore also the sequence abundance can be affected by the density of chloroplasts in different species. Another reason could be the bias of amplification efficiency in PCR towards different species and loci due to the high level of plant DNA fragmentation, that could bring to a sharing of sequences between relative species. In particular with a manual alignment in Clustal, the *Ceratonia Siliqua (*Fabaceae family) showed an important sequence similarity (95%) with the *Glicyne max*.

Although the milk samples belonged or were external to the production area of the GP, the statistical analysis conducted using the two alpha and beta approaches did not discriminate the samples either by type or by area of origin. In particular, the beta analysis highlighted the subdivision of the samples into two distinct clusters showing how, in some cases, the milk of a specific area could actually belong to both clusters [38]. Even though the milk samples are characterised by high plant variability and came from different provinces of the GP production area, the lack of differences at multiple taxonomic levels could be due to the standardisation of feed rationing as required by the GP standard to ensure the product quality.

In addition, more information on the type and composition of the diet may be required as technological processes for feed production can also degrade DNA thereby distorting the final taxonomic analysis.

The results suggest that DNA metabarcoding is a valuable resource to explore plant DNA traces in a complex matrix such as milk.

The results are encouraging and represent a good starting point to develop new molecular approaches, or to improve existing ones, in order to add more information to the food traceability, such as the geographical origin of the milk for the production of GP PDO.

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# Supporting information

**S1 Fig. Rarefaction curves.**

**S2 Fig.** **Species heatmap.** Heatmap showing the abundance of the fourteen dominant species (≥1% total reads on at least one sample) found in milk samples. The colour scale indicates the species’ relative abundance: more intense is the colour, more abundant are the species. The province of origin is indicated by the first two letters of each sample, with the exception of commercial milk samples which are explicitly marked as such.

**S3 Fig.** **Genus heatmap.** Heatmap showing the abundance of the fourteen dominant genera (≥1% total reads on at least one sample) found in milk samples. The colour scale indicates the species’ relative abundance: more intense is the colour, more abundant are the species. The province of origin is indicated by the first two letters of each sample, with the exception of commercial milk samples which are explicitly marked as such.

**S4 Fig.** **Family heatmap.** Heatmap showing the abundance of the seven dominant families (≥1% total reads on at least one sample) found in milk samples. The colour scale indicates the species’ relative abundance: more intense is the colour, more abundant are the species. The province of origin is indicated by the first two letters of each sample, with the exception of commercial milk samples which are explicitly marked as such.

**S1 Table. Family distribution.**

**S2 Table. Genera distribution.**

**S3 Table. Species distribution.**