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Characterization of the microbial community in ripened Pecorino Toscano cheese affected by pink discoloration

Matteo Daghio,[1,*] Francesco Pini,[1] Anna Espinoza-Tofalos,[2] Giuseppe Conte,[3] Eleonora Mari,[1] Fabiola Giannerini,[4] Luciana Giovannetti,[1] Arianna Buccioni,[1] Andrea Franzetti,[2] Lisa Granchi,[1] Marcello Mele,[3] Giulia Rampazzo,[5] Teresa Gazzotti,[5,6] Elisa Zironi,[5] Carlo Viti [1]

[1]*Dipartimento di Scienze e Tecnologie Agrarie Alimentari e Forestali, University of Florence, Piazzale delle Cascine 18, 50144, Firenze, Italy*

[2]*Dipartimento di Scienze dell'Ambiente e della Terra, University of Milano-Bicocca, Piazza della Scienza 1, 20126, Milano, Italy*

[3]*Dipartimento di Scienze Agrarie, Alimentari e Agro-ambientali, University of Pisa, Via del Borghetto 80, 56124, Pisa, Italy*

[4]*Caseificio Sociale Manciano, Loc. Piano di Cirignano, 58014, Manciano (GR), Italy*

[5]*Department of Veterinary Medical Sciences - University of Bologna, via Tolara di Sopra 50, 40064, Ozzano Emilia, Italy*

[6]*Health Sciences and Technologies-Interdepartmental Centre for Industrial Research (CIRI-SDV), University of Bologna, via Tolara di Sopra 41/E, 40064 Ozzano Emilia, Italy*

[*] Corresponding author: matteo.daghio@unifi.it

Abstract

Pink discoloration defect can cause economic losses for cheese producers due to the impossibility to sell the defected cheese, but few knowledge is currently available on the causes of this defect. To gain more insight on the causes that lead to the formation of pink discoloration in Pecorino Toscano cheese with the Protected Designation of Origin (PDO) status, the bacterial community in defected and not defected cheese was characterized by high-throughput sequencing of bacterial 16S rRNA gene. The bacterial community in the defected cheese significantly differed compared to the control. The relative abundance of the genera *Acidipropionibacterium*, *Enterococcus*, *Escherichia/Shigella*, *Lactobacillus*, *Lentilactobacillus* and *Propionibacterium* was higher in the cheese with pink discoloration defect. The concentration of short chain fatty acids and of lactic acid in cheese was measured and a shift towards the production of propionate in the cheese with pink discoloration defect was observed. Furthermore, the possible involvement of microbially produced vitamin B_{12} in the formation of pink discoloration was not supported by the data, since a tendency to a lower concentration of vitamin B_{12} was measured in the defected cheese compared to the control.

Keywords: microbiota; pink discoloration; propionic bacteria; sheep cheese; vitamin B_{12}

1. Introduction

Sheep cheese includes several varieties of dairy products widely produced in Italy. Several of them, as Pecorino Toscano, have the Protected Designation of Origin (PDO, EC regulation 306/2010) status (Buccioni et al., 2012). Pecorino Toscano PDO cheese is ripened from a minimum of 20 days (for fresh cheese) to over 4 months (for ripened cheese). The ageing is carried out in conditioned chambers in which temperature and humidity favorite the proliferation of the bacteria responsible for aromas. Sometime, undesirable fermentation, due to a microbial contamination, may occur. Pink

discoloration (PD) of the loaf rind or of the cheese inner part is a very common defect. Pink discoloration leads to economic losses for the producers due to the impossibility to sell the defected cheese (Daly et al., 2012). Despite the negative consequences potentially linked to this defect, few knowledge is available on the causes.

In cheese with added colorants (e.g., Cheddar) the PD defect has been often associated to the degradation of the colorant itself (Daly et al., 2012). In cheese without colorant the defect has been associated to Maillard browning or to the microbial activity, such as the activity of some lactobacilli or propionic acid bacteria used as starter cultures (Daly et al., 2012).

Thermus thermophilus has been identified as a possible responsible of PD defect in Continental-type cheese (Quigley et al., 2016). *T. thermophilus* is a Gram-negative, extremely thermophilic, aerobic, nonpathogenic microorganism, able to produce carotenoids (Tian and Hua, 2010), whose occurrence can lead to the presence of a PD in cheese (Quigley et al., 2016). In a recent work, the formation of PD close the rind of Pecorino Toscano cheese, has been attributed to the presence of *Serratia liquefaciens*, a psychrotrophic and motile organism (Martelli et al., 2020). The authors have hypothesized that the presence of *S. liquefaciens* on Pecorino Toscano cheese with PD defect was due to an environmental contamination (Martelli et al., 2020). Despite the papers reported above, which attempted to understand the mechanisms that lead to the PD defect in cheese, several aspects of this phenomenon remain unclear. Further understanding of the factors involved in the development of PD in cheese can give the opportunity to develop strategies to avoid the formation of this defect, with potential economic benefits for the producers.

The aim of this study was to describe the microbial community associated to PD defect in Pecorino Toscano PDO cheese and to provide new hypotheses on the possible role of the bacterial communities on the development of PD defect.

2. Materials and methods

2.1. Samples

Cheese making was realized in an industrial dairy processing plant located in Tuscany (Caseificio Sociale Manciano, Manciano, Grosseto, Italy) and it was carried out in accordance with the Protected Designation of Origin (PDO) disciplinary of Pecorino Toscano cheese: raw milk was pasteurized and inoculated with milk cultures *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*. Veal rennet was added to the milk and the coagulation occurred at a temperature of 35 °C within 30 minutes. The curds were broken at the size of a corn kernel. After, the curd was placed into 2 kg molds for the whey drain by manual pressing, located in a thermostatic chamber at 35 °C for 2 h and then plunged in a salt solution (NaCl, 19% w/v) at 12 °C for 24 h. Cheese was ripened for 270 days at 8-10 °C.

Cheese samples were collected from two lots of Pecorino cheese: the first lot was composed of non-defected cheese (4 samples were collected from 2 cheese units) and was used as control; the second lot was composed of non-defected cheeses (4 samples were collected from 2 cheese units), used as control, and defected cheese (8 samples were collected from 2 cheese units). Since the defected cheeses showed a PD shading from one flat side to the other, the slices were cut in half, the two samples were processed independently and considered in the defected group (Figure S1). The 8 samples collected from the non-defected cheese were considered as the control group (4 cheese units, 2 samples for each cheese units) while the 8 samples collected from the cheese with PD (2 cheese units, 4 samples each) were considered as the defected group.

2.2. DNA extraction and 16S rRNA gene sequencing

Around 25 g of each cheese sample were homogenized in a mixer and fat was removed. DNA was extracted from 200 mg of each homogenized sample with the DNeasy *mericon* Food Kit (Qiagen) according to the manufacturer's instructions.

The V5-V6 hypervariable regions of the 16S rRNA gene were PCR-amplified using the 783F and 1046R primers (Huber et al., 2007; Wang and Qian, 2009) as previously described (Daghio et al.,

2018). Briefly, the PCR was performed in 2 × 50 µL reactions with GoTaq®Green Master Mix (Promega Corporation, Madison, WI, USA) and 1 µM of each primer. Amplification conditions were: 94°C for 5 min, 29 cycles with 94°C for50 s, 47°C for 30 s, 72°C for 30 s and a final elongation step of 72◦C for 5 min. The amplicons were then purified with the Wizard®SV Gel and PCR Clean-up System (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions and quantified using Qubit® (Life Technologies, Carlsbad, CA, USA). All DNA samples were tested for amplification inhibition by sample dilution. The libraries were sequenced by MiSeq Illumina (Illumina, Inc., SanDiego, CA, USA) using a 300 bp × 2 paired-end protocol. The sequencing produced a total of 1,656,884 reads with an average of 103,555 ± 10,525 reads per sample (average ± standard error).

2.3. Bioinformatic elaboration

Bioinformatic elaborations were performed in R 4.0.3 (R Core Team, 2020) using DADA2 package (Callahan et al., 2016), version 1.16.0. According to the quality profiles, forward reads were truncated at 200 bases, and reverse reads were truncated at 180 bases. The first 20 bases were removed from both the forward and the reverse reads. Low quality reads (i.e., reads with expected errors higher than 2 and with Ns) were discarded. Specific error rates were estimated for the forward reads and for the reverse reads. Filtered reads were dereplicated, the estimated error rates were used to infer the Amplicon Sequence Variants (ASVs) (Callahan et al., 2017) and the reads pairs were merged with default parameters. Chimeric sequences were removed and taxonomic assignment (confidence 80%) for each ASV was performed against the RDP database (Cole et al., 2014) using the assign Taxonomy function. Species assignment was performed by add Species function (100% identity). Only the ASVs with a relative abundance of 0.01% (or higher) in at least one sample were considered for further processing. After filtering, merging, removal of chimeric sequences and removal of low abundance ASVs a total of 1,190,658 high-quality sequences were obtained with an average of 74,416 ± 7,958 sequences per sample (average ± standard error).

2.4. Chemical analyses

Short chain volatile fatty acids (SCVFAs) (C2:0, acetic; C3:0, propionic; C4:0, butyric; iso C4:0, isobutyric; C5:0, valeric; iso C5:0, isovaleric) and lactic acid were extracted as follows: 10 g of each sample were added to 50 mL of 0.1 N H_2SO_4 aqueous solution and homogenized for 2 min by UltraTurrax (IKA®-Werke GmbH & Co. KG, Staufen, Germany). After the extraction 15 mL of supernatant were centrifuged at 2500 rpm for 15 min. Five mL of the supernatant were microfiltered (0.22-µm) to remove solid particles. The resulting sample was directly injected in the HPLC apparatus using an Aminex 85 HPX-87 H ion exclusion column (300 mm × 7.8 mm; 9-µm particle size; Bio-Rad, Milan, Italy); the detection wavelength was 220 nm. The analyses were carried out applying an isocratic elution (flux 0.6 mL/min) with a 0.008 N H_2SO_4 solution as mobile phase; the injection loop was 20 µL. Individual SCVFAs and lactic acid were identified and quantified by means of an external calibration curve using a standard solution of 4.50 mg/mL of lactic acid, 5.40 mg/mL of acetic acid, 5.76 mg/mL of propionic acid, 7.02 mg/mL of butyric acid and isobutyric acid, 8.28 mg/mL of valeric acid and isovaleric acid in 0.1 N H_2SO_4 (69775, 338826, 402907, B103500, 58360, 75054, 129542, respectively; Sigma- Aldrich, Milano Italy). The molar concentration of each SCVFA and of lactic acid were estimated and the ratios lactic acid/acetic acid, lactic acid/propionic acid and propionic acid/acetic acid were calculated.

The samples of pecorino cheese were analyzed for the determination of the concentration of vitamin B_{12}. The main natural forms of cobalamin present in food are hydroxocobalamin, 5'-deoxyadenosylcobalamin, methylcobalamin and cyanocobalamin (vitamin B_{12}). All the different forms of cobalamin were converted to cyanocobalamin before analysis, because it is more stable than the others. Ultra-performance liquid chromatography coupled to triple-quadrupole mass spectrometry (UPLC-MS/MS) was used for the quantification of cyanocobalamin in Pecorino cheese. Methotrexate was used as internal standard (IS). The equipment employed consisted of a Waters Acquity UPLC® binary pump, coupled with a Waters Xevo TQ-S Micro triple quadrupole

mass spectrometer equipped with an ESCi™ Multi-Mode Ionization Source (Waters Corporation, Milford MA, USA). Mass spectrometer operated in positive electrospray ionization (ESI+) mode and analysis were performed in MRM (multiple reaction monitoring) mode, following two specific transitions for the target analytes: 678.43 > 147.15, 678.43 > 359.15 for vitamin B_{12} and 455.25 > 308.17, 455.25 > 134.17 for methotrexate (IS). The chromatographic separation was achieved on a Waters Acquity BEH C18 UPLC® column (Waters Corporation, Milford MA, USA). The chromatographic conditions were set as follow: constant flow of 0.350 mL/min; the mobile phase was 5 mM ammonium formate in water acidified with 0.05% of formic acid (A) and acetonitrile with 0.3% of formic acid (B). The extraction procedure was performed on 1 g of Pecorino cheese in accordance with the protocol described by Zironi et al., 2013, with some modifications due to the high lipid component that characterizes the matrix.

2.5. Statistical analysis

Data from 16S rRNA gene amplicons sequencing were further processed using the vegan package, version 2.5.7 (Oksanen et al., 2020) in R 4.0.3 (R Core Team, 2020). To estimate the alpha-diversity within each samples group a randomly rarefied dataset (28,000 sequences) was generated, then, the Chao1 index and the Shannon diversity index were calculated. A non-metric multidimensional scaling (NMDS) and a permutational multivariate analysis of variance (PERMANOVA) based on Hellinger transformed ASV abundance data were performed using the metaMDS and the adonis2 functions, respectively. Both the NMDS and the PERMANOVA were performed on the Bray-Curtis dissimilarity index. A Kruskal-Wallis test (function kruskal.test) was performed to identify the ASVs and the genera with a different relative abundance between the defected cheese and the control cheese.

A Kruskal-Wallis test was performed in R 4.0.3 (R Core Team, 2020) to identify the significant differences in the content of SCVFAs and of lactic acid, and in the content of vitamin B_{12}.

3. Results and discussion

The taxonomic composition of the microbial community of Pecorino Toscano PDO cheese was investigated by high-throughput sequencing of 16S rRNA gene amplicons to identify bacteria potentially associated to PD defect. The rarefaction analysis, performed on the detected ASVs, indicated that the sequencing depth was enough to describe the biodiversity within the samples (Figure S2). The presence of 74 ASVs was observed within the whole dataset: 18 ASVs were detected only in the control cheese, 19 ASVs were detected only in the defected cheese and 37 ASVs were shared between the two groups (Figure S3). No differences in Chao1 index and in the Shannon diversity index were observed between the defected cheese and the control cheese (Figure 1).

The NMDS plot clearly showed a separation between the bacterial community enriched in the defected cheese and the bacterial community enriched in the control cheese (Figure 2). The difference between the bacterial communities enriched in the two conditions was further confirmed by the PERMANOVA ($R^2 = 0.37$, $p = 0.002$).

The starter culture was composed by microorganisms belonging to the genera *Streptococcus*, *Lactobacillus* and *Lactococcus* which, in total, accounted for ~91 % of the sequences in the control cheese and for ~88% of the sequences in the defected cheeses (Table 1).

To date the main genera that have been clearly associated with the cheese PD defect were *Serratia* in Pecorino Toscano cheese (Martelli et al., 2020) and *Thermus* in continental cheese (Quigley et al., 2016). Sequences classified within the genera *Serratia* and *Thermus* were not detected in the whole dataset (Table S1). Furthermore, the presence of *Serratia* was linked to PD close to the rind of cheese (Martelli et al., 2020), while in this study the discoloration defect was observed in the inner part of the cheese (Figure S1). It is therefore possible to exclude the involvement of these genera in the formation of PD defect in the present study. Quigley and colleagues also observed that the pink color in presence of *T. thermophilus* was more intense raising the levels of *Lactobacillus helveticus* in the starter culture and maintaining *Streptococcus thermophilus* at the same level, but

the biological reason of this difference was not determined (Quigley et al., 2016). A similar pattern in the relative abundance of these two genera was observed also in this work with a higher ($p < 0.05$) ratio *Lactobacillus/Streptococcus* in the cheese with PD (0.024 ± 0.002) compared to the control cheese (0.017 ± 0.002). Furthermore, Martelli and colleagues isolated different strains of *Enterobacter* spp. together with *S. liquefaciens* from the pink spots on the rind of Pecorino Toscano (Martelli et al., 2020) and despite the PD was not attributed to the genus *Enterobacter* it is interesting to observe that, in our study, the relative abundance of this genus was higher ($p < 0.01$) in the samples collected by the defected cheese (Table 1).

Another difference possibly linked to PD defect is related to *Propionibacteriaceae* family. Three ASVs were classified within this family, one ASV (ASV_21) was close to *Propionibacterium freudenreichii* (Table S2), and the other two ASVs (ASV_27 and ASV_64) were close to *Acidipropionibacterium olivae* and *Acidipropionibacterium damnosum* (Table S2), previously classified as *Propionibacterium olivae* and *Propionibacterium damnosum* (Turgay et al., 2020), both isolated from spoiled packaged green olives (Lucena-Padrós et al., 2014). However, ASV_27 and ASV_64 show a high similarity also to *Acidipropionibacterium jensenii* and to *Acidipropionibacterium thoenii* (Table S2), two propionic bacteria associated to dairy products (Turgay et al., 2020).

In our study, *P. freudenreichii* was more abundant in the defected cheese (Table 1, $p < 0.01$). *P. freudenreichii* subsp. *shermanii* was previously described as a possible agent of PD in Swiss cheese, but no mechanism was suggested (Daly et al., 2012; Park et al., 1967). Regarding the genus *Acidipropionibacterium*, the other member of the family *Propionibacteriaceae*, detected in the dataset (Table S1), its presence was observed only in the defected cheese (Table 1, $p < 0.01$). Members of the genus *Acidipropionibacterium* can produce red pigments that can cause discoloration defect in cheese (Turgay et al., 2020), therefore it is possible to hypothesize that members of the family *Propionibacteriaceae* were the main actors involved in the formation of PD defect in the Pecorino Toscano PDO cheese. The higher relative presence of propionic bacteria in

the defected cheese has been evaluated by sequencing of the 16S rRNA gene, therefore there are no clues on their metabolic activity within the cheese. Propionic bacteria are characterized by propionic fermentation, which leads to the production of acetate and propionate using lactate as the substrate (Turgay et al., 2020). Therefore, to obtain more information on their activity the SCVFAs and the lactic acid molar concentrations (Figure S4) and percentages were measured (Figure 3A). Furthermore, the lactic acid/acetic acid, the lactic acid/propionic acid and the propionic acid/acetic acid ratios were calculated (Figure 3B). The percentage of lactic acid was higher in the control cheese compared to the defected cheese ($p < 0.01$), while the percentage of propionic acid ($p < 0.01$) and butyric acid ($p < 0.05$) was higher in the cheese with PD defect (Figure 3A). The ratios lactic acid/acetic acid and lactic acid/propionic acid were considerably higher ($p < 0.01$) in the control cheese compared to the defected cheese. These data suggest a higher consumption of lactate for the propionic fermentation in the cheese in the presence of PD (Figure 3B). This observation is in accordance with the highest abundance of propionic bacteria in the samples collected from cheese with PD. Furthermore, the higher ratio propionic acid/acetic acid in the defected cheese ($p < 0.01$) suggested the presence of different pathways of propionic fermentation in the two groups of cheese. Indeed, the fermentation seemed to shift towards the production of propionate in the cheese with PD defect. Therefore, a change in the activity of propionic bacteria could be involved in the production of PD defect in this study.

A possible metabolite responsible for PD could be vitamin B_{12}: vitamin B_{12} is pink and it could be produced by *P. freudenreichii* when it is growing in food-like conditions (Deptula et al., 2017). Furthermore, microorganisms within the genera *Escherichia/Shigella* and *Lactobacillus*, which are more abundant in the defected cheese (Table 1), have been shown to possess the genes encoding for the enzymes required in vitamin B_{12} biosynthetic pathway (Balabanova et al., 2021). There are four forms of vitamin B_{12}: cyanocobalamin, hydroxocobalamin, methylcobalamin and adenosylcobalamin (Prentice et al., 2013). Hydroxycobalamin, adenosylcobalamin, and methylcobalamin are the major forms of vitamin B_{12} in bovine milk and hard cheese (Gille and

Schmid, 2015). The involvement of vitamin B_{12} in the PD defect in this study was excluded since the content of vitamin B_{12} did not differ in the defected cheese (24 ± 1 ng/g) compared to the control cheese (29 ± 2 ng/g), though a tendency ($p < 0.1$) to a lower concentration in the defected cheese was observed (Figure S5). These values are in accordance to the values reported in literature for other dairy products (Gille and Schmid, 2015; Souci et al., 2008) since a vitamin B_{12} concentration of 3.8 ng/g was reported for curd, but a concentration ranging from 10 ng/g to 31 ng/g was reported for other cheeses (Gille and Schmid, 2015). The work of Prentice et al. (2013), on formation of pink color in therapeutic proteins, suggests a threshold between 300 and 500 ng/g hydroxycobalamin for visible pink. This threshold, even if referred to a different matrix, is well above the average values found in this work.

4. Conclusions

Pink discoloration is an important issue in cheese manufacturing but its causes are still not completely understood. In this study, bacteria belonging to the genera *Thermus* and *Serratia*, which have been associated to PD defect were excluded as their presence was not observed in the samples analyzed. As well, data do not support the involvement of vitamin B_{12} in the formation of PD. Considering the differences observed in the bacterial community of Pecorino Toscano PDO cheese with PD defect, the involvement of microorganisms belonging to the genera *Propionibacterium* and *Lactobacillus*, has been hypothesized. These genera were more abundant in the defected cheese compared to the control and a different propionic fermentation was observed in the two groups of cheese. Anyway, further studies could be helpful to elucidate the mechanisms that causes PD in Pecorino cheese.

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Tables

Table 1 – Average relative abundance of the microorganisms detected in the defected cheese and in the control cheese. Only the genera with a relative abundance of 0.1%, or higher, in at least one sample are reported. Values are reported average ± standard error. N.D. = not detected (i.e., relative abundance = 0). Significance codes: * $p < 0.05$; ** $p < 0.01$.

	Control (%)		Defect (%)			
Genus	Average	Std. Error	Average	Std. Error	p-value	
Acidipropionibacterium	N.D.		0.06	0.02	0.001	**
Corynebacterium	0.01	0.01	N.D.		0.317	
Enterococcus	0.01	0.01	0.06	0.02	0.008	**
Escherichia/Shigella	0.07	0.02	0.19	0.04	0.046	*
Lacticaseibacillus	1.78	0.64	0.75	0.18	0.142	
Lactiplantibacillus	0.41	0.13	0.49	0.08	0.401	
Lactobacillus	1.44	0.18	1.96	0.18	0.046	*
Lactococcus	4.49	0.76	4.07	0.42	0.674	
Lentilactobacillus	1.37	0.38	3.58	0.49	0.006	**
Leuconostoc	0.67	0.15	0.30	0.19	0.027	**
Pediococcus	0.24	0.07	0.04	0.02	0.004	**
Propionibacterium	0.06	0.06	0.09	0.02	0.016	**
Streptococcus	84.94	1.90	81.87	0.92	0.208	
Weissella	0.00	0.00	0.04	0.02	0.371	
Other genera	0.06	0.01	0.07	0.01		
Unclassified	4.44	0.20	6.44	0.45		

Figure captions

Figure 1 – Alpha diversity indexes calculated for ASV abundance. The alpha diversity is not different in the defected cheese compared to the control cheese.

Figure 2 - Non Metric Mutidimensional Scaling (NMDS) based on the Bray-Curtis distance, calculated on the Hellinger transformed ASV relative abundance data. Stress = 0.061. The bacterial community enriched in the defected cheese and the bacterial community in the control cheese were different.

Figure 3 – Relative abundance of the detected SCVFAs and lactate in the control cheese and in the defected cheese (A). The proportion of lactic acid was lower in the defected cheese, and the proportion of propionic acid was lower in the control cheese. Ratios of fatty acids and of lactic acid in the control cheese and in the defected cheese (B). Lactic acid/acetic acid and lactic acid/propionic acid ratios were higher in the control cheese, while propionic acid/acetic acid ratio was higher in the defected cheese. Significance codes: * $p < 0.05$; ** $p < 0.01$.

Figure 1

Click here to access/download;Figure;Figure1.tif

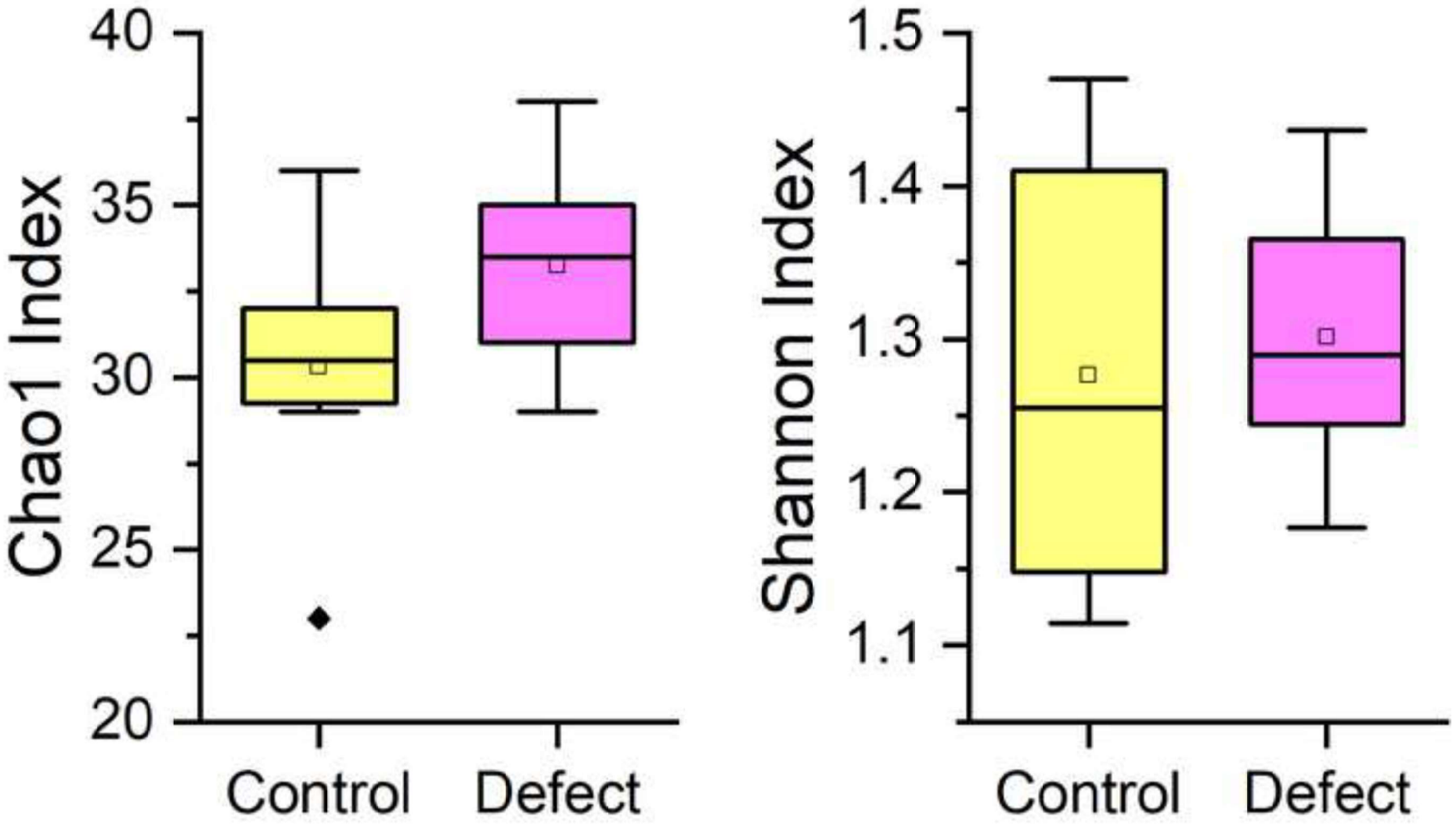

Figure 2

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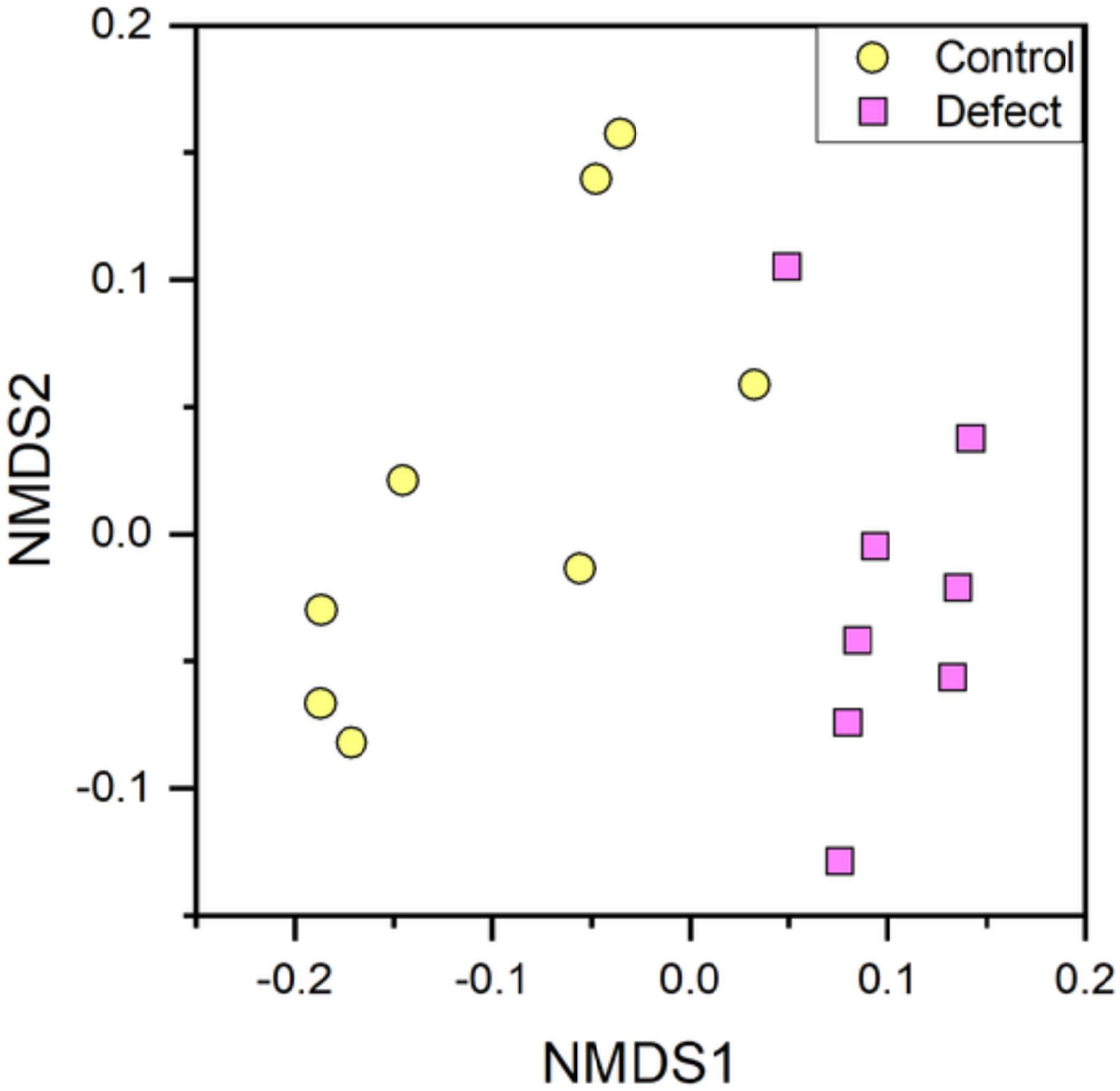

Figure 3

Click here to access/download;Figure;Figure3.tif