

Moderate wine consumption and inflammatory bowel diseases. Impact in the gut and oral microbiome

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Abstract. This study investigates the effects of moderate red wine consumption on the clinical status and symptomatology of patients with Ulcerative Colitis (UC), including the study of the oral and intestinal microbiome. A case control intervention study in UC patients was designed. Intervention patients ($n = 5$) consumed red wine (250 mL/day) for four weeks whereas control patients ($n = 5$) did not. Moderate wine consumption significantly ($p < 0.05$) improved the parameters related to serum iron, and particularly, faecal calprotectin, considered one of the most used parameters in the diagnosis and remission of IBD. Similarly, the intervention with wine alleviated intestinal symptoms evaluated by the IBDQ-32 questionnaire and, consequently, increased the patient's subjective quality of life appreciation. The metagenomic analysis of the microbial populations present in saliva and faeces indicated a lower bacterial diversity in UC patients compared to healthy individuals. Moderate consumption of red wine seemed to balance the proportions of microbial communities and could promote a microbial profile more similar to that observed in healthy individuals. Finally, analysis of faecal metabolites (i.e., phenolic acids and SCFAs) indicated a non-significant increase ($p > 0.05$) for the UC patients that consumed wine.

1 Introduction

Inflammatory Bowel Disease (IBD) is a heterogeneous group of diseases that manifests itself especially in the inflammation of the intestinal mucosa. One of the most influential factors in the appearance and course of IBD is diet, especially since dietary imbalances can exacerbate the disease. Diet also conditions the composition of intestinal microbiota, which is increasingly associated with the development and evolution of IBD. Due to its content and diversity in phenolic compounds, the relationship between wine and human health is a subject of active research. However, if we refer to Ulcerative Colitis (UC), one of the main IBDs, to our knowledge there is only one intervention study with wine [1], possibly due to the difficulty of studies with this type of patients. These authors suggest that patients with inactive IBD who drink red wine daily may be at an increased long-term risk for disease relapse [1]. Moreover, this study did not address the possible relationship with intestinal microbiota, which nowadays is considered one of the most relevant aetiopathogenic factors of IBD. Moreover, and in spite of the few studies covering this matter, a certain

dysbiosis in the oral microbiota has been reported in UC patients, suggesting also a possible relation between oral microbiome and disease. In order to provide new evidence on this subject, we carried out a comprehensive study of the effects of moderate wine consumption in UC patients in the active phase of the disease. Patients were selected from a previously clinically diagnosed cohort and were divided into a red wine intervention (250 mL/day) group and a control (no wine intake) group. Assessments of clinical parameters, symptomatology and quality of life in both groups before and after the intervention period (four weeks) were carried out. Faecal samples were collected to assess the impact of wine intervention on the gut microbiome of UC patients, and also in comparison with healthy subjects. Gut microbiota metabolic functionality was assessed by means of concentration of short-chain fatty acids (SCFAs) and phenolic metabolites in faeces. Additionally, microbiota from saliva samples were subjected to taxonomic analysis in an attempt to evaluate the effect of moderate wine consumption on oral microbiome.

2 Materials and methods

2.1 Wine

Red wine was produced with the *Cabernet Sauvignon* and *Cabernet Franc* grape varieties (vintage 2006), from the Penedès appellation (Spain). The ethanol content in the wine was 13.5% and the total phenolic content reached 2500 mg of gallic acid equivalents/L as determined by the Folin-Ciocalteu method. The polyphenol profile of the wine was analysed by UPLC-ESI-MS/MS [2].

2.2 Intervention study in UC patients

The participants in this study were selected from the patient cohort of the Digestive System Department of the “Infanta Sofía Hospital” (San Sebastián de los Reyes, Madrid, Spain). As exclusion criteria, participants should not have received antibiotics for at least six months before the study, or suffer from type I diabetes, have severe cardiac, endocrine or other disorders, have a previous history of alcohol or drug abuse, or follow exclusive diets (vegan or vegetarian). A case control intervention study was designed comprising two consecutive periods: (1) an initial washout period of two weeks during which all patients did not consume wine or any other alcoholic beverages and followed a diet low in polyphenols (excluding excessive intakes of vegetables and fruits); and (2) a period of four weeks during which all patients maintained a low-polyphenol diet, but patients from the intervention group also consumed a daily intake of red wine (250 mL/two doses). The study was approved by the Ethics Committee of the Hospital Infanta Sofía and Hospital La Paz (Madrid, Spain) (reference HULP-PI2908), and incorporated into the ISRCTN register (reference ISRCTN39987). It was conducted according to the guidelines laid down in the Declaration of Helsinki. The minimum sample size was calculated according to previously described [3], taking faecal calprotectin as the calculation biomarker. Initially, a total of 20 patients in the active phase of UC, categorized as mild or moderate (according to the partial Mayo index), agreed to participate. But unfortunately, 10 of them dropped out for different reasons. Therefore, the study finally involved 10 patients of both sexes and aged between 18 and 42 years. All participants signed an informed consent form. Then, participants were randomly allocated to either the control ($n = 5$) or intervention ($n = 5$) group. Both groups showed comparable disease state, and received identical medical prescriptions during the study. After the washing (Initial) and intervention periods (Final), patients from both groups were questioned about their disease symptoms and quality of life (QoL) using the IBDQ-32 questionnaire. Blood, faecal and saliva samples were collected at both times (Initial and Final). Serum biochemical parameters were measured in plasma using an automated biochemical auto-analyser. The blood tests included the measure of glucose levels, lipids, hepatic enzymes, immunological

cell profile, vitamins, micronutrients and haematology. Faecal calprotectin was determined by quantitative enzyme-linked immunosorbent assay (ELISA) immediately after collection. All determinations were carried out at least in duplicate.

Faecal solutions were prepared with 1 gram of fresh samples diluted in 10 mL of PBS. After a vigorous homogenization, samples were centrifuged at 10000 rpm for 10 minutes at 4 °C. The pellet was stored at -80 °C until 16S rRNA gene sequencing, and the supernatant was filtered with a 0.22 µm filter and stored at -80 °C until metabolite analysis. Saliva samples were frozen and kept at -80 °C until 16S rRNA gene sequencing.

2.3 Samples from healthy subjects

Healthy subjects ($n = 8$) from the same area and social environment as the UC patients were recruited from a Primary Care Centre (Madrid, Spain), taking into consideration the same exclusion criteria as the UC patients. They were asked to follow the same washout period of two weeks. After this time, faecal samples were collected, and subjected to the same sample preparation as that indicated above for the UC patients.

2.4 DNA extraction and sequencing

Saliva samples were subjected to DNA extraction using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, USA) following the manufacturer’s instructions with a prior lysis with lysostaphin from *Staphylococcus staphylolyticus* (5000 U/mL), mutanolysin from *Streptomyces globisporus* ATCC 21553 (2500 U/mL) and lysozyme from chicken egg white (50000 U/mL) (Sigma-Aldrich, San Louis, USA). The V3–V4 region of the 16S ribosomal RNA gene was amplified using the primers from Klindword et al., 2013 [4], which produce a PCR product of 460 bp. Faecal pellets were also subjected to DNA extraction using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions. The V3–V4 region of the 16S ribosomal RNA gene was amplified using the following primers: forward 5'-CCTACGGGNBGCASCAG-3' and reverse 5'-GACTACNVGGGTATCTAATCC-3'. The two-step Illumina® PCR protocol was followed to prepare the libraries and both kinds of samples were submitted to 2 x 500 bp paired-end sequencing by means of an Illumina® MiSeq instrument (Illumina®, San Diego, CA, USA). Raw files are available at the National Center for Biotechnology (NCBI) repository under the project code PRJNA749914 for faecal samples and PRJNA749643 for saliva samples. To process the files with raw reads from the Illumina® instrument, RStudio v.4.03 software was used. The FastQC files were filtered for reads of low quality and with the presence of alien DNA using DADA2. The DADA2 algorithm was also employed to denoise, join paired-end reads and filter out chimeras in the raw data [5, 6]. This

algorithm allows the differentiation of even a single nucleotide, leading to the formation of amplicon sequence variants (ASVs). The taxonomic assignment was performed using the naïve Bayesian classifier implemented in DADA2 using Silva v.138 as the reference database [7], with a bootstrap cut-off of 80%. A total of 3,152,891 complete good-quality reads for faeces and 1,283,321 for saliva were used for the analysis.

2.5 Analysis of phenolic metabolites by UPLC-ESI-MS/MS

Phenolic metabolites were analysed in faecal supernatants in triplicate using an UPLC-ESI-MS/MS following a previously reported method [8]. Data acquisition and processing were realized with MassLynx 4.1 software.

2.6 Analysis of Short-chain Fatty Acids (SCFAs)

Analysis of SCFAs in faeces was carried out by duplicate following the SPME-GCMS method described previously [9]. Quantitative data were obtained using calibration curves of each of their corresponding standards compared to the internal standard (2-methylvaleric acid).

2.7 Statistical analysis

Main changes in blood markers, faecal metabolites, the IBDQ-32 answers, relative abundances of the different taxa, and alpha diversity indices between the initial and the final sampling times were evaluated using the non-parametric Wilcoxon signed-rank test through the “stats” package. The Mann-Whitney U test was chosen to check non-related samples (healthy subjects vs. UC patients). Biodiversity, expressed in terms of alpha diversity, was estimated by calculating the Shannon and Simpson indices through the “phyloseq” package. Differences between samples (beta diversity) were obtained employing a Bray-Curtis dissimilarity matrix represented by non-metric multidimensional scaling (NMDS). Permutational multivariate analysis (PERMANOVA) belonging to the “vegan” package was conducted to find statistically significant differences between experimental groups. All tests were conducted with RStudio v.4.03.

3 Results and Discussion

3.1 Effects of moderate wine consumption on clinical parameters and QoL questionnaire

Differential trends between the control and intervention groups were observed for some serum parameters (Table 1). Total circulating iron was found to increase after moderate wine consumption (from 63.4 ± 29.2 to 102 ± 40 $\mu\text{g/dL}$, as mean \pm DS values), a fact that was

not found for the control group (Table 1). Accordingly, all the patients that consumed wine increased their transferrin level and transferrin saturation index after the intervention period, although changes were only statistically significant ($p < 0.05$) for the transferrin saturation index. In relation to ferritin, the main iron storage protein, its levels significantly decreased in both groups during the intervention time (Table 1).

Table 1. Mean values \pm standard deviations of some serum biochemical parameters and faecal calprotectin for the control and intervention groups before (Initial) and after (Final) the intervention period. Only changes with p -value < 0.01 are showed.

	Control			Wine Intervention		
	Initial	Final	p value	Initial	Final	p value
LUC ($10^3/\mu\text{L}$)	0.14 ± 0.05	0.14 ± 0.07	0.892	0.13 ± 0.04	0.18 ± 0.05	0.068
LUC %	1.32 ± 0.58	1.79 ± 0.79	0.080	1.70 ± 0.57	2.30 ± 0.49	0.043*
Haematocrit %	36.2 ± 2.4	39.8 ± 2.9	0.138	41.8 ± 4.5	45.1 ± 2.0	0.042*
Iron ($\mu\text{g/dL}$)	63.6 ± 27.0	58.2 ± 24.5	0.500	63.4 ± 29.2	102 ± 40	0.043*
Total bilirubin (mg/dL)	0.44 ± 0.21	0.48 ± 0.25	0.999	0.54 ± 0.23	0.82 ± 0.19	0.059
B12 vitamin (pg/mL)	493 ± 156	384 ± 306	0.225	391 ± 185	279 ± 70	0.043*
C-reactive protein (mg/L)	3.25 ± 4.53	3.14 ± 2.63	0.715	3.96 ± 1.61	1.34 ± 1.2	0.080
Ferritin (ng/mL)	213 ± 316	34.6 ± 25.5	0.068	216 ± 161	159 ± 147	0.043*
Transferrin (mg/dL)	234 ± 26	258 ± 64	0.500	243 ± 43	276 ± 40	0.068
rin saturation index	19.5 ± 8.5	16.6 ± 8.0	0.225	18.8 ± 8.1	27.5 ± 12.4	0.043*
Faecal calprotectin ($\mu\text{g/g}$)	1400 ± 1510	1808 ± 2754	0.681	1964 ± 2686	91 ± 104	0.122

LUC: Large Unstained Cells.

Calprotectin, the most important marker of ulcerative colitis in faeces, was reduced from 1964 ± 2686 to 91 ± 104 $\mu\text{g/g}$ in patients who consumed moderate amounts of wine whereas a slight increase was observed in the control group (from 1400 ± 1510 to 1808 ± 2754 $\mu\text{g/g}$), although differences were not significant ($p > 0.05$) in any case. In this sense, in general, a similar steady trend was observed for patients from both groups, with the exception of patient C3 in the control group that experimented a notable increase, and patients W2 and W4 from the intervention group that exhibited a drastic decrease after wine consumption.

Among the four categories evaluated in the IBDQ-32 questionnaire (bowel symptoms, systemic symptoms, emotional involvement and social involvement) (Table 2) the score for bowel symptoms registered a significant ($p < 0.05$) improvement (33% increase) in the final sampling for the intervention group, whereas no change was observed for the control group. These trends after the intervention period in the bowel category were reflected in the total QoL score. As a whole, our outcomes revealed an improvement of the bowel and systemic symptoms in patients who had consumed moderate wine (Table 2), being consistent with those of previous works evaluating supplementation with resveratrol, one of the most noticeable polyphenols in wine, in subjects with mild or moderate UC [9].

Table 2. Mean values \pm standard deviations of IBDQ-32 scores for the control and intervention groups before (Initial) and after(Final) the intervention period.

		Variation interval	Initial	Final	<i>p</i> -value
IBDQ Intestinal	10-70	Control	39.8 \pm 9.8	45.0 \pm 10.8	0.225
		Wine Intervention	40.4 \pm 11.5	53.0 \pm 6.9	0.043*
IBDQ Systemic	5-35	Control	20.2 \pm 4.8	19.6 \pm 5.8	0.786
		Wine Intervention	18.0 \pm 7.5	25.4 \pm 3.8	0.080
IBDQ Emotional	12-84	Control	43.4 \pm 15.1	49.0 \pm 20.7	0.225
		Wine Intervention	44.8 \pm 14.2	54.0 \pm 15.8	0.225
IBDQ Social	5-35	Control	20.2 \pm 4.4	20.8 \pm 9.3	0.892
		Wine Intervention	19.2 \pm 9.4	26.6 \pm 7.1	0.066
IBDQ Global	32-224	Control	123 \pm 23	134 \pm 45	0.560
		Wine Intervention	122 \pm 32	159 \pm 40	0.036*

3.2 Effects of wine intervention on the gut ecosystem

3.2.1 Changes in the gut microbiome after moderate wine consumption

A total of 1856 ASVs were sequenced from the faecal samples ($n = 5$ control initial, 5 control final, 5 intervention initial and 5 intervention final). For all of the identified genera, non-statistically significant differences ($p > 0.05$) in their relative abundance (%) after the intervention period for both control and intervention groups were found. However, the results revealed some interesting trends, including a decrease in the relative abundance of *Streptococcus*, *Escherichia/Shigella* and *Granulicatella* and an increase in *Faecalibacterium* and *Dialister* after the intervention period. Comparison of alpha diversity indices did not show significant differences ($p < 0.05$) after the intervention period for either the control or the intervention group (Fig. 1A), although a slight improvement of the Shannon and Simpson indices was observed in the case of wine consumption.

To explore the degree of dysbiosis in the gut microbiota of UC patients, taxonomic data of UC patients ($n = 10$) were compared to those of healthy subjects ($n = 8$) after the washout period of two weeks (Initial sampling). The most remarkable event at family level was a significantly lower relative abundance, almost to undetectable levels, for the UC participants in *Akkermansiaceae* ($p = 0.022$), which exclusively consists of the genus *Akkermansia*. A significant alteration was also detected in the proportions of other healthy-associated families such as *Christensenellaceae*, *Eggerthellaceae* and *Ruminococcaceae*, among others, in UC patients. This was directly related to altered populations of the genera *Christensenellaceae_R-7*, *Ruminococcaceae_NK4A 214*, *Ruminococcaceae_UCG-003*, *Ruminococcaceae_UCG-005* and *Subdoligranulum*, which belong to those families. Further, other notorious genera like *Dialister*, *Fusicatenibacter* and *Parabacteroides* turned out to be damaged by the UC pathology, at the same time as

Streptococcus was significantly most abundant in the faeces of these individuals. In regard to microbial alpha diversity, microbiota from UC patients exhibited lower values for the three indices (Observed, Shannon and Simpson) than those from healthy subjects (Fig. 1B), although the differences were not statistically significant ($p > 0.05$).

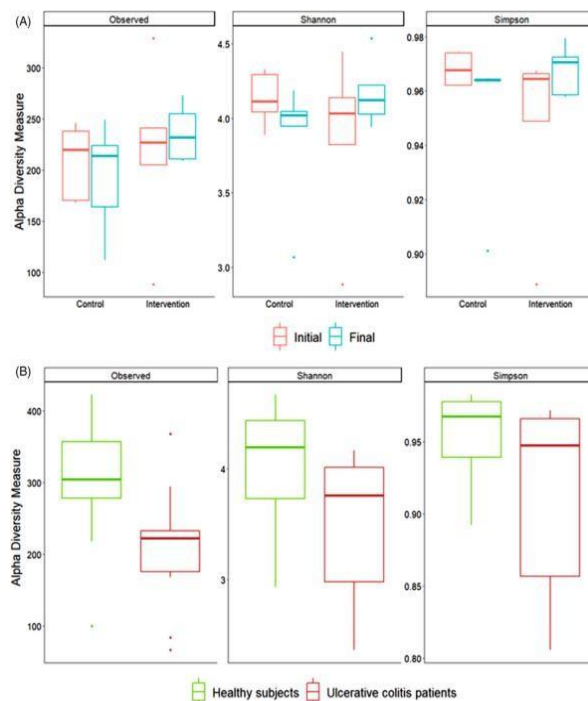


Figure 1. Alpha diversity analysis (Observed, Shannon and Simpson indices) of fecal samples from the control and intervention groups at initial and final sampling A) and from healthy subjects and UC patients before the intervention period B). The Mann–Whitney *U* test confirmed no significant differences between groups.

The dysbiosis status of the two groups of UC patients (control and intervention) in comparison to healthy subjects was also measured as means of beta diversity. Before the intervention period, samples corresponding to healthy subjects were located quite close, whereas samples corresponding to UC patients (both control and intervention groups) were widely distributed, thereby indicating greater inter-individual variability (Fig. 2A). After the intervention period, samples were situated closer and equidistant, especially for those corresponding to the UC patients consuming wine (Fig. 2B). *P*-values confirmed that the greater dispersion exhibited by UC intervention patients at the initial sampling ($p < 0.05$) was corrected after moderate wine consumption, bringing them closer to healthy subjects at the final sampling ($p > 0.05$) (Fig. 2C). One of the most notorious effects of the wine intervention was the restoration of intestinal microbial dysbiosis as means of alpha diversity indices. They seemed to be more stable after wine consumption compared with controls, something described previously in CU-induced rodents subjected to dietary interventions with polyphenols that are also abundant in wine, like quercetin [11,12].

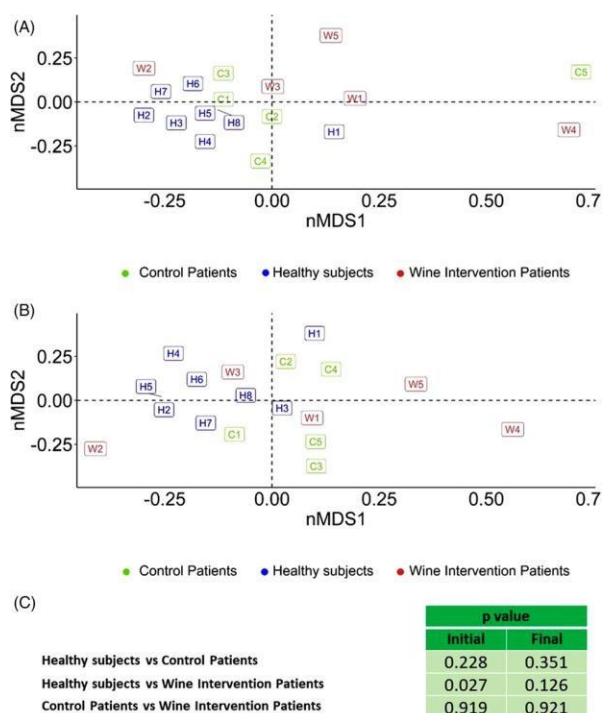


Figure 2. Beta diversity analysis of fecal samples from healthy subjects, control patients, and intervention patients before A) and after B) the intervention period. PERMANOVA was carried out to check statistical differences between three groups both at initial and final sampling and the *p*-values are shown in the table C).

3.2.2 Changes in bacterial metabolites after moderate wine consumption

After intervention with wine, no significant changes were observed in phenolic profiles, probably due to the great variability among patients. However, increased levels of several microbial-derived metabolites such as 3-O-methylgallic acid, 4-hydroxybenzoic, 3,4-dihydroxyphenylacetic and 3-hydroxyphenylacetic were observed. UC participants consuming wine also experienced non-significant ($p > 0.05$) rises in propionic acid, butyric acid and especially acetic acid levels. These results revealed that UC patients showed a poorer profile of phenolic metabolites in comparison to healthy volunteers [8,13], probably due to the gut dysbiosis associated with the disease.

3.3 Effects of wine intervention on the oral ecosystem

A total of 1257 ASVs were sequenced from the saliva samples ($n = 20$). From a taxonomic point of view, the results did not show statistical significance ($p > 0.05$) after the intervention period for any of the taxa evaluated. However, bacterial diversity analysis revealed a slight non-significant increase ($p > 0.05$) in alpha diversity measurements after the wine intervention (Fig. 3), which was more evident in the case of Simpson indices of dominance, contrary to what was observed for the control group, in which a decrease can be seen in the three alpha diversity measurements.

Joint beta diversity analysis of faecal and saliva samples from the control and intervention groups ($n = 20$) at both sampling times (Initial and Final) clearly separated them according to their microbial environment (Fig. 4).

The analysis also showed greater dispersion among patients for the faecal samples than for the saliva samples at both sampling times, indicating a richer and more complex microbial niche for the gut environment. After the intervention period, both oral and gut environments tended to reduce their dispersion in parallel, leading to greater differentiation between them (Fig. 4).

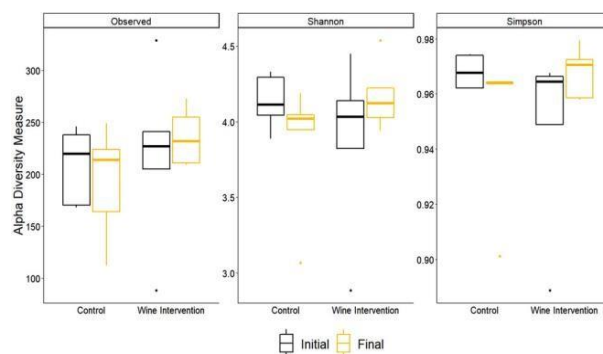


Figure 3. Alpha diversity analysis (Observed, Shannon and Simpson indices) of saliva samples from the control and intervention groups before (initial) and after (final) the intervention period.

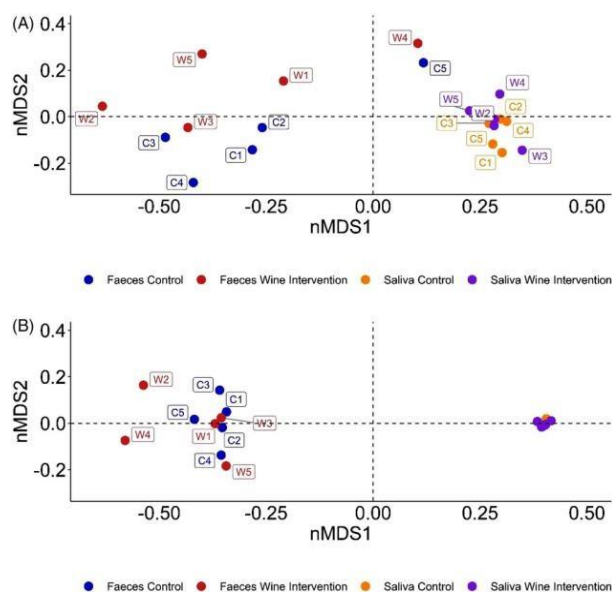


Figure 4. Beta diversity analysis of fecal and saliva samples from the control and intervention groups before A) and after B) the intervention period.

Interestingly, wine intervention increased the three alpha diversity indices after four weeks of consumption. Moreover, the great dispersion in beta diversity at the initial sampling tended to diminish in faeces and saliva in parallel. This was interpreted as the existence of an interrelationship between the two ecosystems in the context of UC, something previously described as the gum-gut axis [14]. In fact, it has been hypothesized that, once established, IBD can be driven by microbial

and inflammatory changes originating specifically from the gingival niche through saliva, thereby worsening IBD outcomes and thus perpetuating a vicious cycle [14].

4 Conclusions

Wine intervention resulted in an improvement of anaemia-related biomarkers, such as iron and transferrin levels. Also, moderate wine consumption seemed to reduce levels of faecal calprotectin, that was related to iron malabsorption and with an exacerbated UC state, indicating a possible anti-inflammatory effect of wine polyphenols in UC patients. Our study confirmed the presence of dysbiosis in UC patients. In spite of the small sample size of the intervention, we reported an effect of wine in the gut and oral microbiomes, including stabilization of biodiversity and increases in some key bacteria and their metabolites. All these outcomes led to an improvement in the quality of life of the participants of this exploratory study as revealed by the IBDQ-32 questionnaire. Although studies with a greater sample size are required, the integrated vision provided in this study has allowed us to establish the most complete scientific evidences to date on the impact of moderate wine consumption in IBD patients, as well as on the oral and intestinal microbiome and its physiological relevance[2,15].

Research in our labs is funded by Grants AGL2015- 64522-C2-R and PID2019-108851RB-C21 (Spanish Ministry of Science and Innovation), and ALIBIRD-CM 2020 P2018/BAA-4343 (Comunidad de Madrid, Spain). The authors would like to thank Bodegas Miguel Torres S.L. for providing the red wine.

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