Food & Function

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Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: J. I. Mosele, B. Viadel, S. Yuste, L. Tomás, S. García, M. T. Escribano-Bailon, I. García-Estévez, P. Moretón, F. Rodríguez de Rivera, S. de Domingo and M. J. Motilva, *Food Funct.*, 2024, DOI: 10.1039/D4FO03774J.

View Article Online
DOI: 10.1039/D4FO03774J

Application of dynamic colonic gastrointestinal digestion model to red wines: study of flavanol metabolism by gut microbiota and cardioprotective activity of microbial metabolites

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View Article Online
DOI: 10.1039/D4FO03774J

Abstract

Over the last decade, research has emphasized the role of the microbiome in regulating cardiovascular physiology and disease progression. Understanding the interplay between wine polyphenols, gut microbiota, and cardiovascular health could provide valuable insights for uncovering novel therapeutic strategies aimed at preventing and managing cardiovascular disease. In this study, two commercial red wines were submitted to an *in-vitro* dynamic gastrointestinal digestion (GIS) to monitor the flavanol-microbiota interaction by evaluating the resulting microbial metabolites. Furthermore, the cardiovascular protective activity of wine flavanol microbial metabolites was investigated, integrating their effects on antihypertensive activity, cholesterol metabolism and insulin resistance into human endothelial (HAECs) and hepatic (HepG2) cell lines. A significant production of microbial flavanol metabolites, with a prevalence of phenylpropionic and phenylacetic acids, valerolactones and short chain fatty acids like butyric acid was observed, particularly in the transverse and descending colon sections. Incubating HAECs and HepG2 cells with the colon improved cardioprotective parameters. Specifically, an increase in the vasodilator NO, an improvement in the LDL receptors and the HMGCoA enzyme, with positive effects on cholesterol metabolism, and the reduction of the glycogen levels improving the insulin resistance were observed.

Keywords: cardioprotection, colonic metabolism, dynamic digestion model, flavanols, microbial metabolism, red wine, UHPLC-QqQ-MS/MS.

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1. Introduction

Wine differs from other alcoholic beverages due to its heterogeneous (poly)phenol content, among which flavanols are prominent components[1]. In wine, flavanols are present as monomers (catechin, epicatechin and epigallocatechin) as well as proanthocyanidin oligomers or polymers exhibiting varying degrees of polymerization according to the structural monomeric units[1]. Beyond imparting sensory and preservative attributes[2,3], moderate consumption of wine containing flavanols has been associated with the prevention of cardiovascular disease (CVD)[4].

(Poly)phenol bioactivity hinges on their bioavailability. While monomers of flavanols are partially absorbed in the small intestine during gastrointestinal digestion intact proanthocyanidins do not undergo absorption[5]. After gastrointestinal absorption, subsequent biomodifications of flavanol monomers occur, particularly in the liver, yielding diverse sulphated, glucuronidated, and methylated phase-II conjugated metabolites[5,6]. Some conjugated metabolites re-enter the enterohepatic recirculation, reaching the colon together with the non-absorbed flavanols. Within the gut, microbial action catalyzes transformations as dehydroxylation, demethylation, ring fission and decarboxylation generating low molecular weight compounds, such as phenolic acids, phenyl-valerolactones and phenyl-valeric acids[5,7]. These colonic catabolites can be absorbed by colonocytes, increasing the overall bioavailability of flavanols and diversifying the spectrum of bioactive molecules[5,7,8]. The flavanol catabolites generated by the gut microbiota may exert more biological effect than their parent compounds[9]. Moreover, the interactions between flavanols and gut microbiota are intricate and reciprocal, influencing microbiome richness, diversity, composition and function[10,11]. Understanding the colonic metabolism and flavanol-gut microbiota interactions is pivotal

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for unraveling wine-health relationships and identifying molecules potentially involved in CVD prevention.

Therefore, to explore and determine the mechanisms of action of (poly)phenols and their role in disease prevention, it is crucial to understand the factors that constrain their bioactivity. Dynamic *in-vitro* models simulating human digestion serve as simple and ethical alternatives for assessing the digestibility, stability, structural changes and bio-accessibility of food bioactive compounds. The multi-compartmental Gastro-Intestinal Simulator (GIS) systems comprising (i) gastric (ii) duodenal and (iii) a jejunal chambers, coupled with a system of trichamber colonic fermentation including (iv) ascending (AC), (v) transversal (TC) and (vi) descending colon (DC) inoculated with human feces or gut microbiota. Different studies have applied these dynamic digestion *in-vitro* models mimicking the human gut environment to study the two-way interaction between the gut microbiota and phenolic compounds that is pivotal in determining their beneficial effects in human health[12,13].

Many studies on (poly)phenols to date have focused on the bioactivities of one specific molecule in aglycone form, often at supraphysiological doses, whereas foods contain complex mixtures with multiple additive or interfering activities[14]. In the specific case of wine flavanols, most of the bioactivity studies in cell line models have been carried out with the monomers catechin and epicatechin, omitting the complex mixture of proanthocyanidins that during the gastrointestinal digestion are hydrolysed into monomers which are subsequently strongly metabolized by gut microbiota. Therefore, the main objective of this study was to deepen the understanding of the potential cardioprotective effects of colonic metabolites derived from wine flavanols. For this, two commercial Tempranillo red wines (2020 and 2021 harvest) were selected based on their high flavanol contents. The wines were submitted to an *in-vitro* dynamic GIS to monitor

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DOI: 10.1039/D4FO03774J

the stability and transformation of flavanols during the gastro-intestinal digestion and to determine the main microbial metabolites produced after their colonic fermentation. Furthermore, the cardiovascular protective activity of wine flavanol microbial metabolites was investigated, integrating their effects on antihypertensive activity, cholesterol metabolism, and insulin resistance in human endothelial (HAECs) and hepatic (HepG2) cell lines.

2. Materials and Methods

2.1. Wine samples and determination of flavanol content

Red wines from both the 2020 and 2021 harvests were elaborated from *Vitis vinifera* L. cv. Tempranillo grapes by Bodegas Pradorey (Burgos, Spain). Alcoholic fermentation was performed in stainless steel tanks and malolactic fermentation and ageing was carried out in French oak barrels for 1 year before bottling. Prior to conducting the HPLC-DAD-ESI-MS analysis of flavanols, wines were fractionated using a cationic exchange cartridge (Oasis MCX, Waters Corp., Milford, MA, USA) as previously reported[1215]. Chlorogenic acid (Sigma-Aldrich, St. Louis, MO, USA) was incorporated into the samples as an internal standard, achieving a final concentration of 0.025 mg/mL. Then, chromatographic separation was achieved using an Agilent 1200 series HPLC system equipped with an Agilent Poroshell 120 EC-18 column (2.7 μm, 4.6 mm × 150 mm) (Agilent Technologies, Waldbronn, Germany), maintained at a temperature of 25° C. The mobile phase was composed by solvent A, 0.1% (*v/v*) formic acid (VWR International, Fontenay-sous Bois, France) aqueous solution, and solvent B, HPLC grade acetonitrile (Merck KGaA, Darmstadt, Germany). Flavanols were quantified through mass spectrometry using a 3200 QTRAP triple quadrupole mass spectrometer (AB Sciex, USA) equipped with an electrospray ionization source (ESI Turbo V™ Source). The detailed conditions for the HPLC and mass spectrometry procedures are provided by

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DOI: 10.1039/D4FO03774J

García-Estévez et al. (2017)[15]. Calibration curves for (+)-catechin, (−)-epicatechin, procyanidin dimers B1 and B2, procyanidin trimer C1, (−)-epicatechin 3-*O*-gallate, (+)-gallocatechin, and (−)-epigallocatechin were utilized for quantification. Monomeric flavanols were purchased from Sigma-Aldrich (St. Louis, MO, USA), whereas procyanidin dimers and trimer were purchased from Extrasynthèse (Genay, France). When the corresponding standard was not available, the flavanol was quantified as equivalents of the most related flavanol on the basis of their structure. Thus, procyanidin dimers B3 and B7 were quantified as procyanidin dimer B1 equivalents; procyanidin dimers B4, B5, B6 and B7 were quantified as procyanidin dimer B2 equivalents, procyanidin trimers, tetramers and pentamers were quantified as procyanidin trimer C1 equivalents and prodelphinidins were quantified as gallocatechin equivalents. The flavanol composition of red wines from the 2020 and 2021 harvests is detailed in Table 1. The wine flavanol concentration was expressed as mean ± standard deviation (SD) of the average of 3 replicates per wine.

Table 1. Flavanol concentration in red wines from the 2020 and 2021 harvests.

Compound (mg/L wine)	Wine 2020	Wine 2021
Catechin	5.0 ± 0.3	4.7 ± 0.2
Epicatechin	6 ± 1	13.4 ± 0.3
Gallocatechin	1.57 ± 0.09	1.62 ± 0.01
Epigallocatechin	1.01 ± 0.02	1.4 ± 0.1
Epigallocatechin gallate	0.003 ± 2E-05	0.002 ± 2E-05
Epicatechin gallate	0.010 ± 0.001	0.11 ± 0.01
Total flavan-3-ols monomers	*13.6 ± 1*	*21.2 ± 0.6*
Procyanidin dimer B1	42.3 ± 0.4	29.9 ± 0.4
Procyanidin dimer B2	19.5 ± 0.6	16.8 ± 0.6
Procyanidin dimer B3	3.3 ± 0.1	4.3 ± 0.1
Total proanthocyanidins dimers	*82 ± 2*	*82 ± 2*
Total proanthocyanidins trimers	*7.6 ± 0.9*	*9.0 ± 0.5*
Total proanthocyanidins tetramers	*3.9 ± 0.2*	*5.6 ± 0.1*
Total proanthocyanidins pentamers	*0.36 ± 0.01*	*0.54 ± 0.01*
Total gallocatechins and prodelphinidins	*6.4 ± 0.3*	*7.3 ± 0.3*
Total catechins and procyanidins	*105 ± 7*	*114 ± 2*

Total flavanols	**111 ± 8**	**122 ± 2**

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DOI: 10.1039/D4FO03774J

Results are expressed as mean ± standard deviation (SD)(n=3)

2.2. Simulated digestion in the Dynamic Colonic Gastrointestinal Digester

The Dynamic-Colonic Gastrointestinal Digester (D-CGD) was developed by AINIA Technology Center (Valencia, Spain)[16]. The system consists of a computer-assisted model of five interconnected double jacket vessels imitating the physiological conditions of the stomach (G: vessel 1), small intestine (I: vessel 2), and the three colonic sections: the AC (vessel 3), the TC (vessel 4), and the DC (vessel 5) (Supplemental Figure S1). All the compartments were connected by peristaltic pumps, working semi-continuously in G and I and continuously in AC, TC and DC. The system set up, that is, the volumetric capacity, pH, anaerobiosis (O_2 and CO_2 levels), and temperature (37 °C) were controlled[16]. The pH was continuously controlled in the compartments for the stomach (following pH changes during gastric digestion, from pH 4,8 to pH 1,7) and the small intestine (pH 6.5–7), using secretions of 1 mol/L hydrochloric acid and 1 mol/L sodium bicarbonate, respectively[17]. Anaerobiosis of the system was achieved by the addition of nitrogen[16].

2.2.1. Dynamic gastrointestinal digestion

The dynamic gastrointestinal digestion consisted of two steps, a gastric digestion (G) (2 h) followed by an intestinal digestion (I) (6 h)[16]. Digestion in G was performed by adding a continuous flow of 0.03 % (w/v) pepsin (from porcine mucosa, ≥ 2500 unit/g; P7012–56, Sigma-Aldrich, Spain) to a gastric electrolytic solution. The gastric pH medium was set up according to a pH curve observed in *in-vivo* data by adding a HCl solution (1 M) (HCl, 37 % purity, VWR Chemicals, Spain). After 2 h, the gastric digested material generated was immediately transferred to I vessel, where simulated digestion was conducted via the continuous addition of an intestinal solution consisting of pancreatin (1.9 g/L) (pancreatin P1750–100 G, Sigma-Aldrich, Spain), $NaHCO_3$ (12 g/L)

(Merck, Germany), and Oxgall dehydrated fresh bile (6 g/L, bile bovine, B3883, BD, USA) in distilled water (240 mL for the whole intestinal digestion step). After that, the intestinal digested material generated was immediately transferred to the third vessel (AC) for 30 min, simulating the digestion transfer through the iliocecal valve to the AC. The transferred material was maintained in the whole colonic segment for 76 h under a continuous dynamic flow from the AC entrance to the DC exit, according to Rosès et al. (2023)[18].

2.2.2. Dynamic in-vitro colonic fermentation

Fresh feces from 4 healthy adults, non-smokers, with no history of antibiotic use in the previous three months and no background of intestinal disease were collected and transported in special anaerobic bags (BDGasPak™ systems) (Becton, Dickinson and Company, NJ, USA). The samples were diluted and regenerated in a physiological phosphate buffer with thioglycolate 20 % (w/v) (Merk, Spain). This mixture was then homogenized in a stomacher and centrifuged at 3000g for 15 min (Heraeus Multifugue x3R Centrifuga, Thermo Scientific, Spain). The supernatant was collected and inoculated in the colon vessels (Supplemental Figure S1) according to Roses et al. (2021)[19]. Hence, 50, 80 and 60 mL of the collected supernatant were placed in the AC section, the TC section, and the DC section, respectively, and filled with culture medium[20] up to a total volume of 1000, 1600 and 1200 mL, respectively, allowing simulation of the conditions of the human colon media.

2.2.3. Experimental protocol: in-vitro gastrointestinal digestion and colonic fermentation of red wine

The experimental design and set-up are depicted in Figure 1. The experiment was composed of two different phases. First, a stabilization period of 12 days (days 0 to 12), where a stable colonic microbiota was reached, followed by a wine treatment period of

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14 days (days 13 to 26), where the microbiota was fed with red wine once per day. For the microbiota stabilization period (day 0 to day 12), 200 mL of culture medium were added to G three times a day. After the stabilization period, and during the wine treatment period (days 13 to 26), the system was fed with 100 mL of red wine once a day (up to a final volume of 200 mL) and with 200 mL of cultured medium twice a day[20,21]. Samples of culture medium from the three colonic reactors (AC, TC, DC) were collected at different times during the stabilization (days 0, 5, 7 and 12) and treatment (days 13-16, 18-21, 23 and 26) periods and used for further analysis (Figure 1).

2.3. Analysis of wine flavanols and their microbial metabolites by ultra-high performance liquid chromatography with triple-quadrupole mass spectrometry (UHPLC/QqQ-MS/MS) in different digestion steps

The monitored of wine flavanol transformation during the continuous gastrointestinal digestion and colonic fermentation was conducted through UHPLC-QqQ-MS/MS. Samples (50 mL media) were collected from each section (Figure 1) at the end of the stabilization period (day 12) and during the treatment period (days 13, 14, 15, 16, 18, 20, 23 and 26) and stored at -80º C until analysis. Prior to the chromatographic analysis, the samples were filtered (PTFE syringe filters, 0.22 µm pore size, Scharlab Chemie, Sentmenat, Catalonia, Spain) and analyzed by UHPLC-QqQ-MS/MS based on the method described by Royo et al. (2021)[22]. Separation of analytes was carried out in a liquid chromatograph (Shimadzu Nexera, Shimadzu Corporation, Japan), coupled to a 3200QTRAP triple quadrupole mass spectrometer (AB Sciex, USA) equipped with an electrospray ionization source (ESI Turbo V™ Source). The (poly)phenol separation was performed in a Waters AcQuity BEH C18 column (100 mm × 2.1 mm, 1.7 µm) equipped with a VanGuardTM AcQuity BEH C18 pre-column (5 × 2.1 mm, 1.7 µm) (Milford, MA,

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USA). The electrospray (ESI) interface was used in the negative mode [M-H]⁻. Data acquisition was carried out with the Analyst® 1.6.2 software (AB Sciex, USA).

The wine flavanol metabolites were identified by comparing their spectra and retention times with those of externally injected standards. Compounds for which standards were not available were tentatively identified using MRM transitions with the mass of the parent ion (M-H) and typical MS fragmentation pattern described in the literature. Some of the compounds were quantified using the calibration curves of their corresponding commercial standards. The other compounds were tentatively quantified using the calibration curves of standards with similar chemical structures. Supplementary Table S1 shows the selected reaction monitoring (SRM) conditions, the cone voltage and collision energy and the commercial standard used for quantification. The concentration of the wine flavanols and their microbial metabolites in the gastrointestinal and colon media (AC, TC and DC) was expressed as mean of the average of two replicates.

The phenol commercial standards used for the identification and quantification were epicatechin and procyanidin B2 (Extrasynthese), procyanidin B1 (Purifa, Dongguan, China), catechin, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, 3,4-dihydroxybenzoic acid (protocatechuic acid), *trans*-coumaric acid, gallic acid and 4,4-bis(4-hydroxyphenyl)valeric acid (Sigma-Aldrich), 5-(3',4'-dihydroxyphenyl)-δ-valerolactone (TransMIT, Gießen, Germany), 3-(3,4-dihydroxyphenyl)propionic acid and 3,4-dihydroxyphenylacetic acid (Alfa Aesar, Massachusetts, USA), 3-(3-hydroxyphenyl)propionic acid (Biosynth Carbosynth, Compton, United Kingdom), hippuric acid and 3-phenylpropionic acid (Thermo Fisher Scientific, Walthman, MA, USA), catechol (TCI, Tokio, Japan), pyrogallol (Glentham Life Sciences, Corsham, United Kingdom) and gallocatechin (Target Mol, Massachusetts, USA). Stock solutions of the standard were prepared in methanol (1000 mg/L) and stored at -20º C. Methanol

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(HPLC grade), formic acid (HPLC grade), acetonitrile (HPLC grade) and HCl were purchased from VWR Chemicals BDH Prolabo (Leuven, Belgium). The water was Milli-Q quality (Millipore Corp, Bedford, MA, USA).

2.4. Microbiota analysis

The microbial population during the stabilization and wine treatment periods was checked by bacteria plate counts. Culture medium samples were collected by duplicate at different days (days 0, 5, 7, 12) of the stabilization period to monitor the maintenance of microbial populations and at different days during the wine treatment period (days 14, 19, 21 and 26) (Figure 1). Ten milliliters of media were taken from each colon reactor and serially diluted in saline solution. The plates were inoculated with 1 mL of 4 serial dilutions of the media by duplicate and incubated at 37º C under aerobic or anaerobic conditions. The following bacterial groups were quantified by the direct plating method on specific colonic culture medium (CFU/mL): *Lactobacillus* (MRS agar using the MALDI-TOF technique to verify lactobacilli colonies), *Bifidobacterium* (TOS-propionate agar enriched with MUP), *Enterobacter* (VRBD agar), *Clostridium* (TSC agar enriched with cycloserin) and total anaerobic bacteria (Schaedler agar). Results were expressed as Log CFU/mL culture medium.

2.5. Determination of short-chain fatty acids (SCFA)

Culture medium samples were collected in triplicate from each reactor (AC, TC and DC) at the end of the stabilization (day 12) and wine treatment (day 26) periods (Figure 1). Samples were pooled and filtered (0.2 μm filters) previously to chromatographic analysis. The microbial SCFA acetic, propionic and butyric acids were analyzed by gas chromatography coupled with a flame-ionization detector (GC-FID) after liquid–liquid extraction. Briefly, ethyl acetate containing capric acid as the internal standard (IS) were added to 10 mL of medium sample collected from each reactor (AC, TC and DC), mixed

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during 10 min and centrifuged. The supernatant was filtered and injected into a GC-FID (AS 800 C.U., CE Instruments, Wigan, United Kingdom) equipped with an HP-FFAP 25 m x 0.2 mm x 0.33 mm column (Agilent Technologies, Santa Clara, CA, USA). The SCFA were quantified by interpolation in the calibration curve using capric acid as IS.

2.6. Cell culture assays

2.6.1. Cell cultures

Endothelial (EA.hy926) and human hepatic (Hep G2) cell lines (both from the American Type Culture Collection, Manassas, VA, USA) were used as vascular homeostasis, and cholesterol and insulin resistance models, respectively, to conduct the functional analysis of wine flavanol colonic metabolites. The cells were cultured in high glucose-DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% of fetal bovine serum (Gibco, BRL, Australia) and 1 % penicillin-streptomycin at 37º C in a humidified atmosphere of 5% CO_2. The medium was changed every 2-3 days until it reached a 90% confluence.

2.6.2. Preparation of the test samples

Medium culture samples from DC reactor at the end of the stabilization (day 12) and wine treatment (day 26) periods (Figure 1) respectively, were collected in triplicate, pooled, and filtered (0.2 µm filters). Each sample was measured, adjusted to 6-8 pH and stored at -20º C until the subsequent experiments.

2.6.3. Cell viability assay

To define the non-toxic levels of microbial wine flavanol metabolites present in the culture medium samples from DC colon reactor, cell viability was evaluated through a fluori-colorimetric assay. Briefly, 2×10^4 cells were seeded in 96-well plates and treated with different serial dilutions from the samples of the DC (days 12 and 26) at 37º C and 5 % CO_2-humidity environmental. After 24 h of treatment, the cell media was replaced

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with 10 % Alamar Blue reagent (Invitrogen, Waltham, MA, USA) in PBS for 2 h and colorimetry was measured using a spectrofluorometer (Fluoroskan, Thermo Fisher Scientific, Waltham, MA, USA) at λ excitation = 540 nm and λ emission = 590 nm. Considering the straight relation between fluorescence and cellular viability, the equation was as follows:

% Viability = (Fluorescence units in the sample / Fluorescence units in the control) x 100

2.6.4. Cell treatments

2.6.4.1. NO and END-1 production

EA.hy926 cells were seeded in 24-well plates at $1 \cdot 10^5$ cells/well. The following day, the PBS was depleted from the media and the cells were cultured for 24 h at 37 ºC. The cells were then treated for 2 h with a 1/8 dilution of the culture medium samples obtained from DC colon reactor at day 12 (control media) and at day 26 corresponding to the end of the wine treatment period (media contained microbial wine flavanols) (Figure 1). After DC media treatment, the IL-1b (100 ng/mL) was added and remained overnight. After 24 h, the cell supernatant was collected, and the NO was measured using Griess reagent (Merck, Darmstadt, Germany) and following the manufacturer's protocol. Additionally, the EA.hy926 cells were collected for RNA extraction and measurement of the END-1 gene expression.

2.6.4.2. Cholesterol metabolism

HepG2 cells were seeded in 12-well plates at $2.5 \cdot 10^5$ cells/well. After 24 h, the cells were treated with the DC culture medium samples (days 12 and 26) (Figure 1) described above and incubated for 24 h at 37 ºC. After the incubation period, the cells were collected for measurement of LDLr and HMGo-R gene expression (Thermo Fisher Scientific).

2.6.4.3. Insulin resistance

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To determine the hepatocyte glycogen storage, Hep2 cells were seeded in 12-well plates at $2.5 \cdot 10^5$ cells/well. The cells were incubated with 100 mM of insulin for 24 h at 37 ºC and then the hepatocytes were treated with the DC media samples (days 12 and 26) (Figure 1) described above for another 24 h. After this period, cells were washed three times with PBS and collected for determination of glycogen. The glycogen content in the cells was assayed by using anthrone reagent (Sigma-Aldrich), and the amount of blue compound generated by this reaction was detected on the 620 nm wavelength using a microplate reader (Thermo Fisher Scientific). In addition, the protein content of the collected HepG2 cells was quantified by the BCA method (Thermo Fisher Scientific), and the values were shown as the ratio of glycogen (mg)/protein (mg). Another 12-well plate with the same treatment was collected for measurement of the Akt gene expression (Thermo Fisher Scientific).

2.6.5. Real time quantitative RT-PCR

For determination of the gene expression, RNA extraction from different experiments was carried out automatically with the MAXWELL equipment (Promega Corporation, Madison, WI, USA). cDNA was obtained from RNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). To study the cholesterol metabolism, real-time PCR was performed using the END-1, HMGCo-R, LDLr and Akt primers (Thermo-Fisher Scientific) as biomarkers.

The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Thermo-Fisher Scientific) was used as a housekeeping gene, whose expression is constitutive in these cells. The amplification conditions in the thermocycler 7300 (Applied Biosystems, CA, USA) were universal and the quantification of gene expression was performed in a relative way, so that the magnitude of the physiological changes in the biomarker gene

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was obtained in comparison with the housekeeping gene. For calculations, the formula $2^{-\Delta\Delta Ct}$ was used.

2.7. Statistical analysis

Microbiota count and SCFA concentration, respectively, in culture medium were expressed as mean ± standard deviation (SD) of the average of two replicates. Cell line results were expressed as mean ± SD of the average of two independent studies, including 2 replicates per study (n=4). One-way analysis of variance (ANOVA), using Fisher's least significant difference (LSD) test, was used to determine significant differences ($p < 0.05$) between data from cells incubated with culture medium before and after wine treatment. All the statistical analyses were carried out using GraphPad Prism 9 version 9.4.1 for Windows (GraphPad Software, San Diego, California USA.

3. Results and discussion

The bioactivity of red wines has been associated with the presence of flavanols. In this work, two different red wines from two consecutive seasons (2020 and 2021), were submitted to a dynamic gastrointestinal digestion model, including colonic fermentation, to study the potential cardioprotective and insulin resistance effects of flavanol colonic metabolites.

3.1. Stability and kinetic of wine flavanols during the dynamic *in-vitro* gastrointestinal digestion

Absorption of dietary components occurs predominantly during gastrointestinal digestion. With only limited exceptions, the bioavailability of food phenolic compounds is low, particularly in the case of the oligomeric and polymeric forms of flavanols, like proanthocyanidins[5]. Consequently, the beneficial effects attributed to the flavanol fraction of wine appear to be primarily linked with the non-absorbed compounds that

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transit to the colon. To evaluate the stability of phenolic compounds during gastrointestinal digestion and, therefore, predict the amount and nature of flavanols reaching the colon, we performed simulated gastric and intestinal digestion. Interestingly, the digestion of the selected red wines from the two consecutive harvests (2020 and 2021) showed a similar trend (Table 2 and Supplemental Tables S2 and S5), which validates the results. In line with the data published by Tamargo et al. (2023)[23], we also observed a gradual decrease in the wine flavanol (precursors) concentration in the media throughout the dynamic *in-vitro* gastrointestinal digestion, with partial and complete disappearance after the gastric and intestinal phases, respectively (Table 2). Regarding the phenolic acids, the concentration of *p*-coumaric acid in the media increased after the intestinal digestion phase (Table 2). The concentration of protocatechuic acid was similar to that detected in the digestion media plus wine, before digestion. While gallic acid remained largely unaffected after the gastric step, it was not detected after the intestinal digestion phase. The increase in the concentration of the *p*-coumaric acids after intestinal digestion, could be related to the release on the polyphenols covalently bound to wine proteins[24].

373 **Table 2.** Phenolic composition of the media after the gastric and intestinal digestion of the 2020 and 2021 wines, respectively. Var: percentage of
374 variation in the phenol concentration after the gastric and intestinal digestion in relation to media+wine.
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Phenolic compound	2020 wine (mg/L wine)					2021 wine (mg/L wine)				
	Media+wine	Gastric digestion	Var (%)	Intestinal digestion	Var (%)	Media+wine	Gastric digestion	Var (%)	Intestinal digestion	Var (%)
p-coumaric acids	0.50	0.40	-20	1.52	204	1.04	0.91	-12	2.18	110
Protocatechuic acid	1.26	1.15	-9	0.96	-24	1.02	0.90	-12	1.00	-2
Gallic acid	10.1	9.38	-7	nd	-100	14.0	13.3	-4	0.19	-99
Catechin	4.18	3.02	-28	nd	-100	5.92	3.34	-44	0.32	-95
Epicatechin	2.13	1.50	-30	nd	-100	2.51	1.35	-46	0.09	-96
Gallocatechin	0.74	0.52	-30	nd	-100	1.30	0.78	-40	nd	-100
Epigallocatechin	0.80	0.54	-33	nd	-100	0.72	0.40	-45	nd	-100
Epigallocatechin gallate						0.04	0.07	96	nd	-100
Procyanidin B1	7.72	4.62	-40	nd	-100	9.80	4.10	-58	0.46	-95
Procyanidin B2	1.89	1.20	-37	nd	-100	2.43	1.21	-50	nd	-100
Procyanidin B3	0.52	0.42	-19	nd	-100	0.55	0.29	-47	nd	-100
Procyanidin T	0.02	0.01	-50	nd	-100	0.08	0.06	-28	nd	-100

376 Results are expressed as mean (n=2). nd: not detected.
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3.2. Flavanol kinetic metabolism in different colon segments during the dynamic colonic fermentation of wine

The kinetic of the wine flavanol colonic metabolism shows the overall progression of the precursors present in wine and the generation of their microbial metabolites during colonic fermentation (Figure 2). Consistent with observations after gastro-intestinal digestion, the behavior of flavanols from both the 2020 and 2021 wines showed similar trends. Both exhibited a similar qualitative profile of microbial metabolites, although there were some variations in their concentrations in the culture medium (Figures 3A-C and Supplemental Tables S2 to S7).

Based on the phenol composition of the media after gastro-intestinal digestion (Table 2 and Supplemental Tables S2 and S5), it can be inferred that no parent compounds of the wine flavanols enter the AC reactor with the exceptions of catechin, epicatechin and procyanidin B1 at very low concentrations following the digestion of the 2021 wine. However, during the early stage of colonic fermentation (AC), catechin, epicatechin, epigallocatechin, gallocatechin and proanthocyanidins were detected (Figure 3A and Supplemental Tables S2 and S5). The concentration of flavanols gradually decrease in TC until completely disappear in DC, with the exception of epigallocatechin, epigallocatechin gallate and procyanidin B1 in wine 2021 (Figures 3A-B). The transient disappearance of flavanols may be explained by non-specific binding interactions of some flavonoids with lipophilic carrier proteins present in the digestion media that could be disrupted by the colonic environment or gut microbiota activity[25].

The main microbial metabolites detected in the AC section were the benzoic acid related compounds: catechol, pyrogallol and gallic acid (Supplemental Tables S2 & S5). The early appearance of gallic acid may result from the breakdown of more complex molecules and/or the degalloylation of the gallic acid esters of wine flavanols. Other

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colonic metabolites in the AC were phenyl propionic acid related compounds (Figure 3C). This may indicate the premature microbial degradation of more complex phenolic compounds in the ascending colon.

In the TC reactor, a greater abundance and diversity of phenolic species were observed compared to the AC (Figures 3A-C). In the TC section, increases in valerolactones and valeric acids were observed. These are exclusively microbial metabolites of flavanols[7]. In line with that previously described[7], we identified di- and monohydroxy propan-2-ol, from which hydroxylated valerolactones are generated (Supplemental Tables S3 & S6). The subsequent microbial catabolism of valerolactones produces valeric acid derivatives with varying degrees of hydroxylation, and these were abundant in the TC. Other microbial metabolites detected in the AC, such as *p*-coumaric acid, gallic acid, pyrogallol and catechol, were also detected in the TC at lower concentrations.

In the DC section, the predominant compounds included 5-(3,4-dihydroxy phenyl) valerolactone, 5-(3,4-dihydroxy phenyl) valeric acid, 4-hydroxy-5-(4-hydroxy phenyl) valeric acid, benzoic acid related compounds and catechol (Supplemental Tables S4 & S7). The main compounds generated during the colonic fermentation in the DC section belong to the family of phenylpropionic and phenylacetic acids, especially 3-hydroxyphenyl acetic acid and 3(4-hydroxy) phenyl propionic acid (Supplemental Tables S4 & S7). This trend indicates the persistence of these compounds during colonic transit, possibly due to the continuous metabolism of wine flavanols resulting in the formation of valerolactones and valeric acids. These results are consistent with the observations made by Firman et al. (2019)[26], where the first colonic segment (AC) exhibited less diversity and abundance of phenolic species compared to the TC and DC. Additionally, our findings align with the same authors' conclusions that the TC and DC have closely related

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metabolic profiles. Similarly, a study by Cattivelli et al. (2023)[12] showed the degradation driven by the colon microbiota of cooked red-skinned onion flavonols resulted in the accumulation of three main metabolites, i.e., 3-(3'-hydroxyphenyl)propanoic acid, 3-(3'-hydroxyphenyl)acetic acid and 3-(3',4'-dihydroxyphenyl) acetic acid. This provides further evidence of significant colonic metabolism in the TC and DC sections.

Given the crucial link between the bioavailability of dietary phenolic compounds and their efficacy as bioactive molecules, numerous *in-vivo* studies have been conducted to elucidate the impact of phenolic microbial metabolites on overall bioavailability[27]. Considering that flavanols represent the principal phenolic constituents in red wine, the results presented here offer important insights into the association between health benefits, particularly cardiovascular health, and the presence of colonic microbial products derived from wine flavanols. Indeed, the relevance of valerolactones and valeric acid related compounds is evidenced as they have been proposed as intake biomarkers of food containing proanthocyanidins[27].

3.3. Impact of wine flavanols on the microbial population and SCFA production

It has been suggested that dietary polyphenols, including those from red wine, can modulate gut microbiota and/or their metabolic activity, positively impacting the reduction of CVD risk factors[28]. In this work, the plate counting technique was used to examine variations in viable gut bacteria in the media of the AC, TC and DC during the stabilization period (days 0, 5, 7 and 12) and during the wine treatment period (days 14, 19, 21 and 26) (Figure 1). During the initial phase of stabilization, the fecal bacteria introduced adapted to each reactor according to the characteristics of the media, such as pH and nutrient availability[26]. In our study (Figure 4), during the wine treatment period, (days 14, 19, 21 and 26), *Bifidobacterium* gradually increased in the three colon sections except in AC with wine 2021 treatment (Figure 4A). In contrast, the count of

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Enterobacter tended to decrease in the three reactors, particularly evident during the treatment period of with wine 2021 (Figure 4). Finally, there were no evident changes in the count of *Lactobacillus* in the AC, TC and DC during the treatment period with wine 2021, contrary, the treatment with wine 2020 tended to increase this bacteria population in the three reactors (Figure 4). In contrast to the differences observed for microbial metabolites profile of flavanols in the three reactors during colonic fermentation, no marked differences were observed in *Bifidobacterium*, *Enterobacter* and *Lactobacillus* counts between the culture mediums of AC, TC and DC (Figure 4).

SCFA are microbial products derived from the anaerobic fermentation of non-absorbed dietary compounds, especially carbohydrates and, to a lesser extent, dietary and endogenous proteins[29]. Chromatographic analysis of the culture medium from the AC, TC and DC, respectively, collected at the baseline (stabilization period, day 12) and at the end of the wine treatment period (day 26) (Figure 1) showed that wine supplementation modulates the production rate of SCFA (Figure 5). In both wines (2020 and 2021), the most marked change was the increase in the production of butyric and propionic acids in the TC and DC (Figures 5B & 5C). Regarding acetic acid, we observed that its production was stimulated during the incubation of wine 2021 in all the reactors but not with wine 2020. Since acetic and butyric acids share a common metabolic pathway, it is suggested that the presence of red wine may favor the synthesis of butyric acid at the expense of acetic acid[26,29]. This effect on the stimulation of butyric and propionic acids production may also be due to a direct interaction of phenolic metabolites with bacterial activity or a direct interaction of these compounds in the metabolism of these SCFA. These results concord with a recent study[23], in which the production of butyric acid was significantly higher when red wine was fermented alone compared to when it was combined with a lipid food model, suggesting that the non-bioavailable

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fraction resulting from wine digestion could potentiate the production of butyric acid. Conversely, Suo et al. (2021)[30] did not observe differences in the generation of various SCFA classes when fermenting isolated high-molecular-weight-polyphenolic complexes and oligomeric phenols compared with control (deionized water). This indicates that wine as a whole entity may play a role in the generation of butyric acid rather than isolated (poly)phenols. Extensive research has investigated the role of SCFA in human health, with particular emphasis on butyric acid due to its significant impact on various physiological processes in the human body[31].

3.4. Study of the potential cardioprotective activity of wine flavanol microbial metabolites in cell line models

CVD comprises a spectrum of disorders, including coronary artery disease, stroke, hypertension and heart failure. Many researchers have identified a positive association between moderate red wine intake and an improvement in cardiovascular health parameters. Over the last decade, research has emphasized the role of the microbiome in regulating cardiovascular physiology and disease progression. These findings highlight the importance of identifying whether microbial metabolites produced from wine polyphenols contribute to the observed health effects. Understanding the interplay between wine polyphenols, gut microbiota, and cardiovascular health could provide valuable insights for uncovering novel therapeutic strategies aimed at preventing and managing CVD. In order to study the cardio-protective effect of microbial metabolites, culture medium from the AC, TC and DC collected at the baseline (stabilization period day 12) and at the end of the wine treatment period (day 26) (Figure 1), containing the wine flavanol microbial metabolites, were exposed to endothelial and hepatic cell models.

3.4.1. Cell viability assessment

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A fluorometric assay was performed to assess the potential toxicological impact of colonic fermentation media. Based on the cell viability assay performed on EA.hy926 endothelial and HepG-2 hepatic cells treated for 24 hours with culture medium from the AC, TC and DC compartments (Figure 1), a dilution of 1/8 was selected to study the functionality of colonic metabolites as this dilution exhibited no cytotoxicity towards the cells (data not shown).

3.4.2. Effect of wine flavanol colonic metabolites on NO levels and endothelin (END-1) expression in HepG2 cells

Endothelial cells were utilized to assess the impact of flavanol microbial metabolites present in the three colonic culture medium (AC, TC and DC) on vascular tone regulation. We focused on two key vasoactive substances released from the endothelium: nitric oxide (NO) and endothelins (ETs). While NO exerts potent vasodilatory effects, ETs are among the most potent vasoconstrictors. In the present study, we investigated the release of NO and the expression level of mRNA END-1, observing significant statistical differences between cells incubated with the stabilization media at 12 days (basal) and the wine-fermented media at 26 days (wine treatment period) ($p<0.05$) (Figure 6). Specifically, the production of NO in cells incubated with the media containing the microbial metabolites (wine treatment) was significantly higher than in the stabilization media (basal) in the three reactors for the 2020 and 2021 wines. The level of mRNA of ET-1 increased significantly in all the reactors, except for the TC after the 2021 wine treatment (Figure 6B). Consistent with previous research, changes in the END-1 concentration in cells demonstrated an inverse association with the NO concentration, maintaining proper vascular tone balance and preventing endothelial cell dysfunction[32].

3.4.3. Modulation of cholesterol metabolism and insulin resistance in treated-HepG2 cells with wine flavanol colonic metabolites

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Another risk factor for cardiovascular disease is the level of lipids, particularly LDL cholesterol. Therefore, we explored the potential of the wine flavanol microbial metabolites present in the culture medium from the AC, TC and DC, before and after wine treatment, to influence the expression of the LDL receptor (LDLr) and HMG-CoA in the hepatic cell line, both of which are involved in cholesterol biosynthesis. This study revealed that the culture medium collected from the three reactors during both wines treatment (2020 and 2021) can enhance the expression level of mRNA LDLr (Figure 7A), thereby facilitating the removal of LDL cholesterol from circulation[33]. This effect can be attributed to the presence of several wine flavanol microbial metabolites in the culture medium.

The regulation in the expression of mRNA HMGCo-A showed differences between the wines from 2020 and 2021 (Figure 7B). In this instance, only cells exposed to the culture medium from the TC and DC of the 2021 wine showed a significant down-regulation in the expression of mRNA HMGCo-A, contributing with the inhibitory effect of cholesterol biosynthesis in the liver. Previous research has found an inverse relationship between the mRNA abundance of HMG-CoA reductase and LDLr mRNA[34]. These results suggest that microbial metabolites generated by colonic fermentation of wine flavanols stimulate the expression of the LDLr gene (Figure 7A), whereas transcript levels of HMG-CoA were not significantly affected by the wine treatment (Figure 7B), and these may depend on the concentration of microbial metabolites rather than the composition.

Regarding insulin resistance, a risk in CVD, the liver plays a pivotal role in regulating blood glucose levels through various processes including gluconeogenesis, glycogen synthesis, and glycogen breakdown. AKT, a key mediator in the PI3K/AKT signaling pathway, exerts influence over these metabolic processes. Reduced AKT levels

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552 can hinder glucose transportation and disrupt glycogen synthesis, potentially resulting in
553 elevated blood glucose levels and insulin resistance[35]. In the present study, the hepatic
554 cells exposed to the culture medium of the AC, TC and DC from microbial fermentation
555 of the 2020 and 2021 wines reduced the risk of insulin resistance, disrupting glycogen
556 synthesis (Figure 8A) and inhibiting hepatic glucose generation by down-regulation of
557 mRNA AKT expression (Figure 8B). These findings suggest that wine flavanol microbial
558 metabolites can modulate hepatic glucose metabolism, thereby potentially offering
559 therapeutic benefits in managing insulin resistance and glycemic control. In addition to
560 microbial metabolites, the SCFA present in the fermentation culture medium (AC, TC
561 and DC), could modulate the CVD risk parameters studied. Previous data showed that
562 butyric acid modulates insulin resistance and the accumulation of fat in the liver[11]. In
563 addition, the increment in the plasma levels of butyric acid has been associated with an
564 improvement of the endothelial function[36].

565 In conclusion, the results of the present study show that wine flavanols, submitted to
566 a dynamic *in-vitro* digestion model, reach the colon where they are transformed by the
567 colon microbiota. The colonic fermentation of wine flavanols resulted in the
568 accumulation of three main metabolites, i.e., 3-(3'-hydroxyphenyl)propanoic acid, 3-(3'-
569 hydroxyphenyl)acetic acid and 3-(3',4'-dihydroxyphenyl)acetic acid. In addition, an
570 increase in a complex mixture of valerolactone and valeric acid derivatives was observed
571 in the TC and DC sections. In parallel, a significant increase in the production of butyric
572 and propionic acids was observed respectively in the TC and DC, and an increase in the
573 count of certain bacteria, mainly *Bifidobacterium*. The functionality study shows that
574 exposing fermentation media, containing the wine flavanol microbial metabolites, to
575 endothelial and hepatic cell lines positively modulates four biomarkers associated with
576 three CVD risk factors. Specifically, an increase was observed in the vasodilator NO that

improves the blood pressure. In addition, there was an improvement in the LDL receptors and the HMGCoA enzyme, with a positive effect on the cholesterol metabolism, with the reduction of glycogen levels improving insulin resistance. The results of this study reinforce the idea that wine flavanols are intensively metabolised by colonic microbiota to generate a complex mixture of their bioactive forms that could influence host health.

Acknowledgements

This study was supported by the MCIN (Ministerio de Ciencia e Innovación) through the CDTI project IDI-20210434. Silvia Yuste has a Margarita Salas postdoctoral grant funded by the European Union-NextGenerationEU through the Ministerio de Universidades and the Universitat de Lleida. The authors are grateful to the technical staff of the Instrumental Analysis Service at the ICVV for their UHPLC-QqQ-MS/MS analytical support.

Conflicts of interest

On behalf of all the authors, the corresponding author states that there is no conflict of interest.

Contributions

Juana Mosele: Formal analysis, Investigation, Writing -original draft, Writing - review & editing; **Blanca Viadel**: Conceptualization, Project administration, Validation Data curation, Formal analysis, Investigation, Methodology, Software; **Silvia Yuste**: Formal analysis, Investigation, Methodology; **Lidia Tomás-Cobos**: Formal analysis, Investigation, Methodology; **Sandra García**: Formal analysis, Investigation, Methodology; **María-Teresa Escribano Bailón**: Conceptualization, Data curation, Formal analysis, Investigation; **Ignacio García Estévez**: Conceptualization, Data curation, Formal analysis, Investigation; **Pilar Moretón Fraile**: Conceptualization, Funding acquisition, Project administration; **Fernando Rodríguez de Rivera**:

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DOI: 10.1039/D4FO03774J

Conceptualization, Funding acquisition, Project administration; **Soledad de Domingo Casado**: Conceptualization, Funding acquisition, Project administration; **Maria-Jose Motilva**: Investigation; Methodology, Writing -original draft, and Writing - review & editing.

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Figure Captions

Figure 1. Scheme of the experimental protocol of the in-vitro gastrointestinal digestion and colonic fermentation of red wine. G: gastric digestion step, I: intestinal digestion step, AC: ascending colon section, TC: transversal colon section, DC: descending colon section, SCFA: short chain fatty acids. Bold numbers are the days of sampling media from the reactors during stabilization (0-12 days) and treatment (13-26 days) periods, respectively. The dashed circles indicate the days of sampling media to microbiota analysis during stabilization (days 0, 5, 7, 12 days) and treatment (days 14, 19, 21, 26) periods, and to SCFA and cell culture assays at the end of the stabilization (day 12) and treatment (day 26) periods. The squares indicate the days of sampling media to chromatographic analysis (UHPLC-MS/MS) of flavanoid metabolites at the end of the stabilization (day 12) and during the treatment (days 13, 14, 15, 16, 18, 20, 23 and 26) periods, respectively.

Figure 2. Kinetic disappearance of total flavanols (precursors) of the wines from 2020 (solid blue line) and 2021 (dashed blue line) harvests, and the parallel production of flavanol colonic metabolites (solid purple line and discontinued purple line for wines from 2020 and 2021, respectively) during the dynamic *in-vitro* colonic fermentation. AC: ascending colon, TC: transversal colon, DC: descending colon. Data are expressed as mean (n = 2).

Figure 3. Kinetic disappearance of flavanol monomers (A) and oligomers (B) of wines from 2020 (solid line) and 2021 (dashed line) harvests, and microbial metabolites production (C) during the dynamic *in-vitro* colonic fermentation. AC: ascending colon, TC: transversal colon, DC: descending colon. Data are expressed as mean (n = 2).

754 **Figure 4.** Bacteria abundance in culture medium (A) AC: ascending colon, (B) TC:
755 transversal colon, (C) DC: descending colon, sampled at different days of the stabilization
756 period (days 0, 5, 7 and 12 in lighter colour) and wine treatment period (days 14, 19, 21
757 and 26). Wines from 2020 (solid bars) and wines from 2021 (grid fill). Data are expressed
758 as mean ± SD (n = 2).
759
760 **Figure 5.** Amount of short chain fatty acids quantified in (A) AC: ascending colon, (B)
761 TC: transversal colon and (C) DC: descending colon at the beginning (basal, day12) and
762 at the end of the treatment (day 26) period after the supplementation with wines from
763 2020 (solid bars) and 2021(grid bars). Data are expressed as mean ± SD (n = 2).
764
765 **Figure 6**. Evaluation of endothelial function parameters through (A) nitric oxide (NO)
766 production and (B) expression levels of nitric oxide synthase (ENDT) mRNA in
767 EA.hy926 cells exposed to media obtained from different colonic reactors representing
768 the ascending colon (AC), transversal colon (TC) and descending colon (DC) sections,
769 before and after wine 2020 (purple solid bars) and 2021 (purple grid bars)
770 supplementation. Data are expressed as mean ± SD (n = 4). *p < 0.05; ***p < 0.001;
771 ****p < 0.0001 respect to control.
772
773 **Figure 7**. Cholesterol metabolism evaluated by expression levels of (A) LDL receptor
774 mRNA and (B) HMGCo-R mRNA in HepG2 cells exposed to media obtained from
775 different colonic reactors representing the ascending colon (AC), transversal colon (TC)
776 and descending colon (DC) before and after wine 2020 (purple solid bars) and 2021
777 (purple grid bars) supplementation. Data are expressed as mean ± SD (n = 4). *p < 0.05;
778 **p < 0,005; ****p < 0,0001 respect to control.

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779 **Figure 8.** Carbohydrate metabolism evaluation by (A) synthesis of glycogen and (B)
780 expression levels of Akt mRNA in HepG2 cells exposed to media obtained from different
781 colonic reactors representing the ascending colon (AC), transversal colon (TC) and
782 descending colon (DC) before and after wine 2020 (purple solid bars) and 2021 (purple
783 grid bars) supplementation. Data are expressed as mean ± SD (n = 4). *p < 0.05; ****p
784 < 0.0001 respect to control.

Data Availability Statement

View Article Online
DOI: 10.1039/D4FO03774J

The data supporting this article have been included as part of the Supplemental Information (Figure S1 and Tables S1-S7)

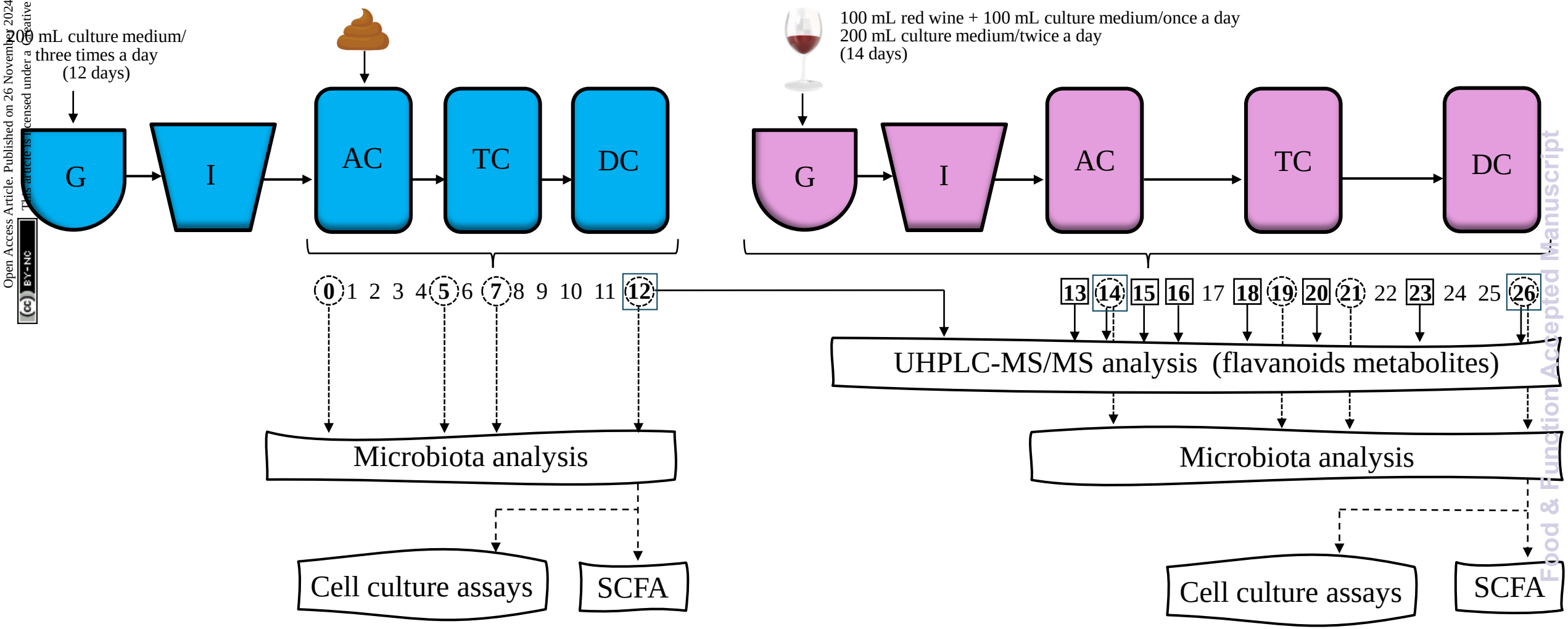


Figure 1

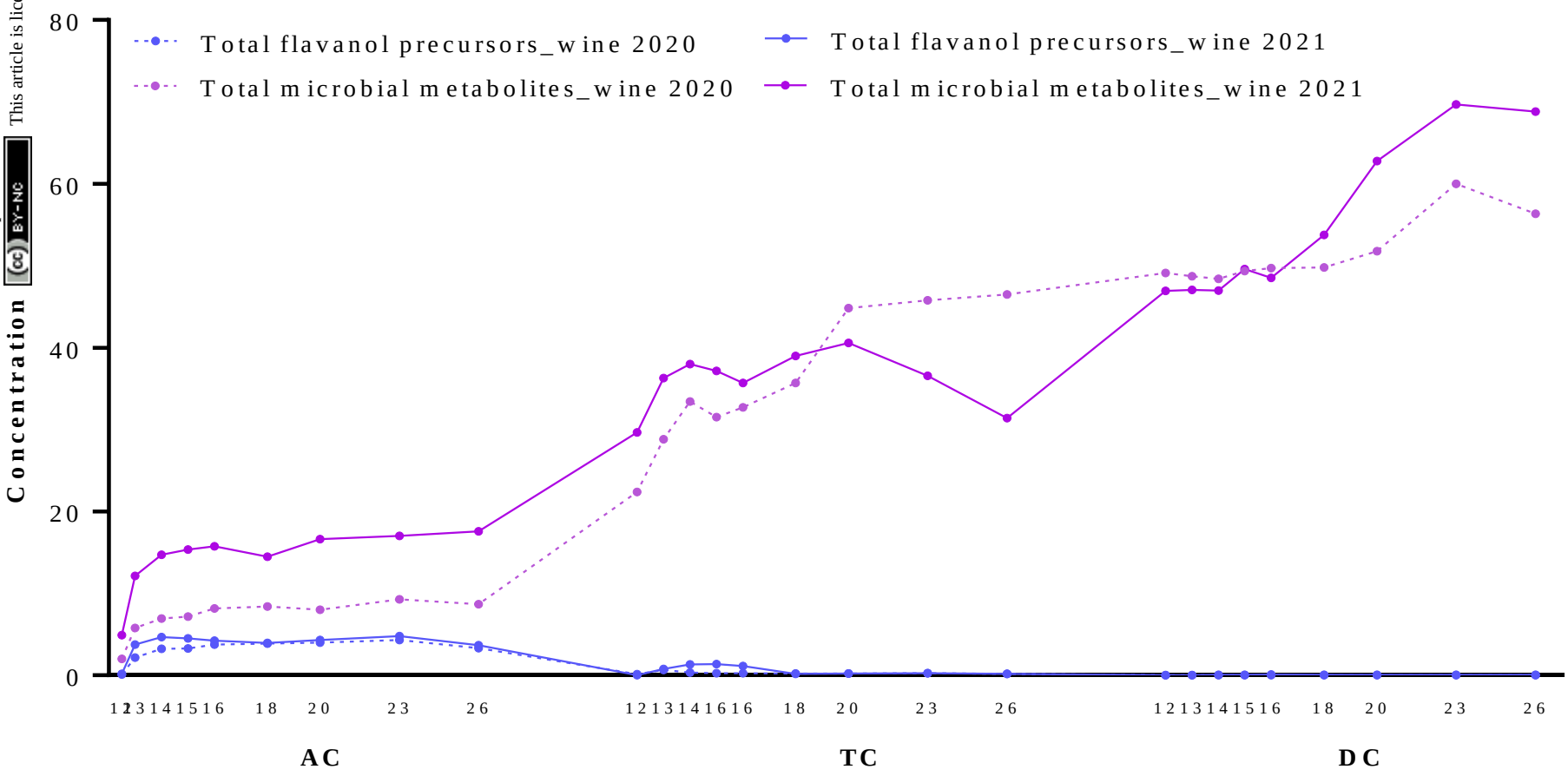
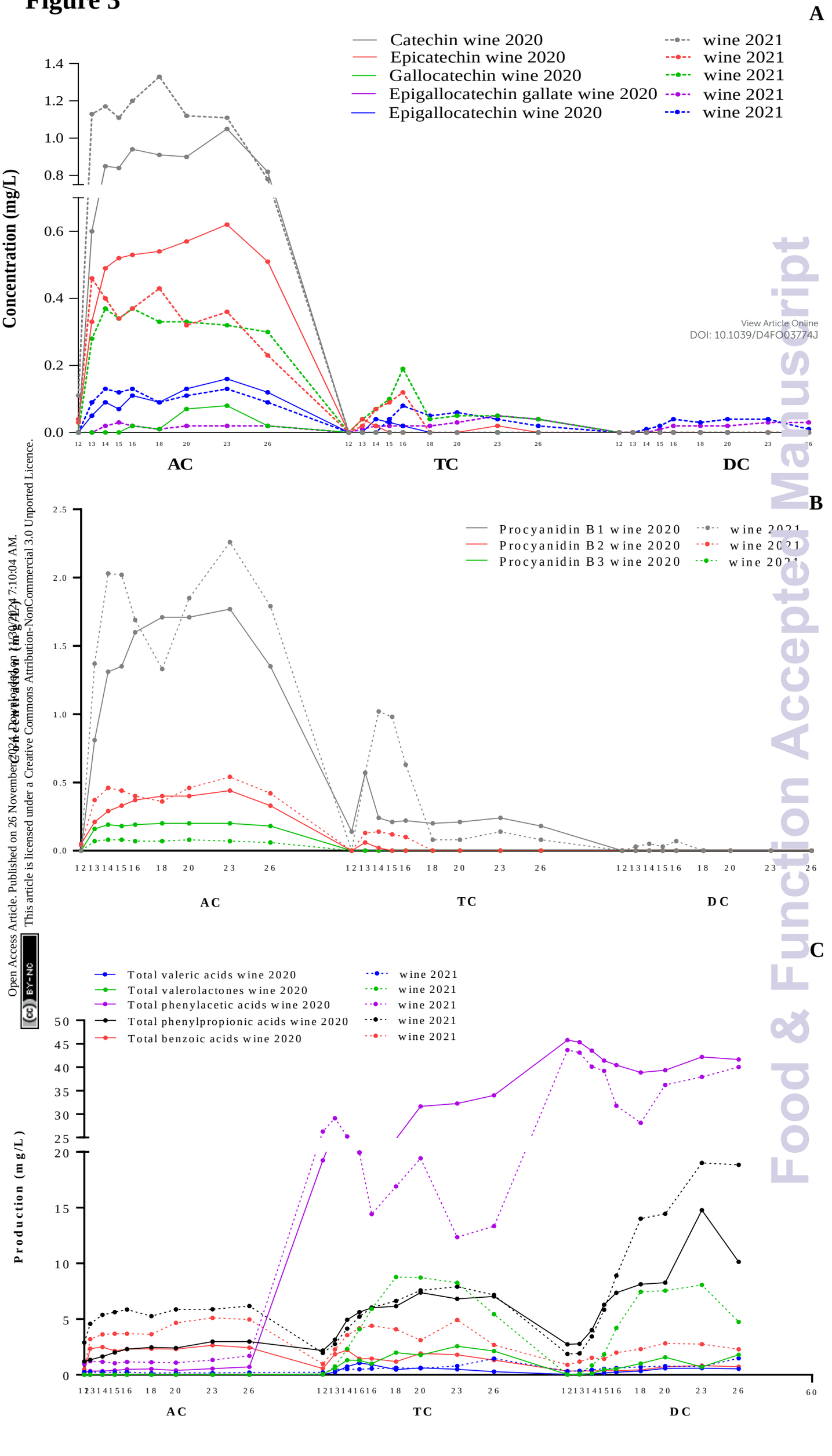


Figure 2

Figure 3



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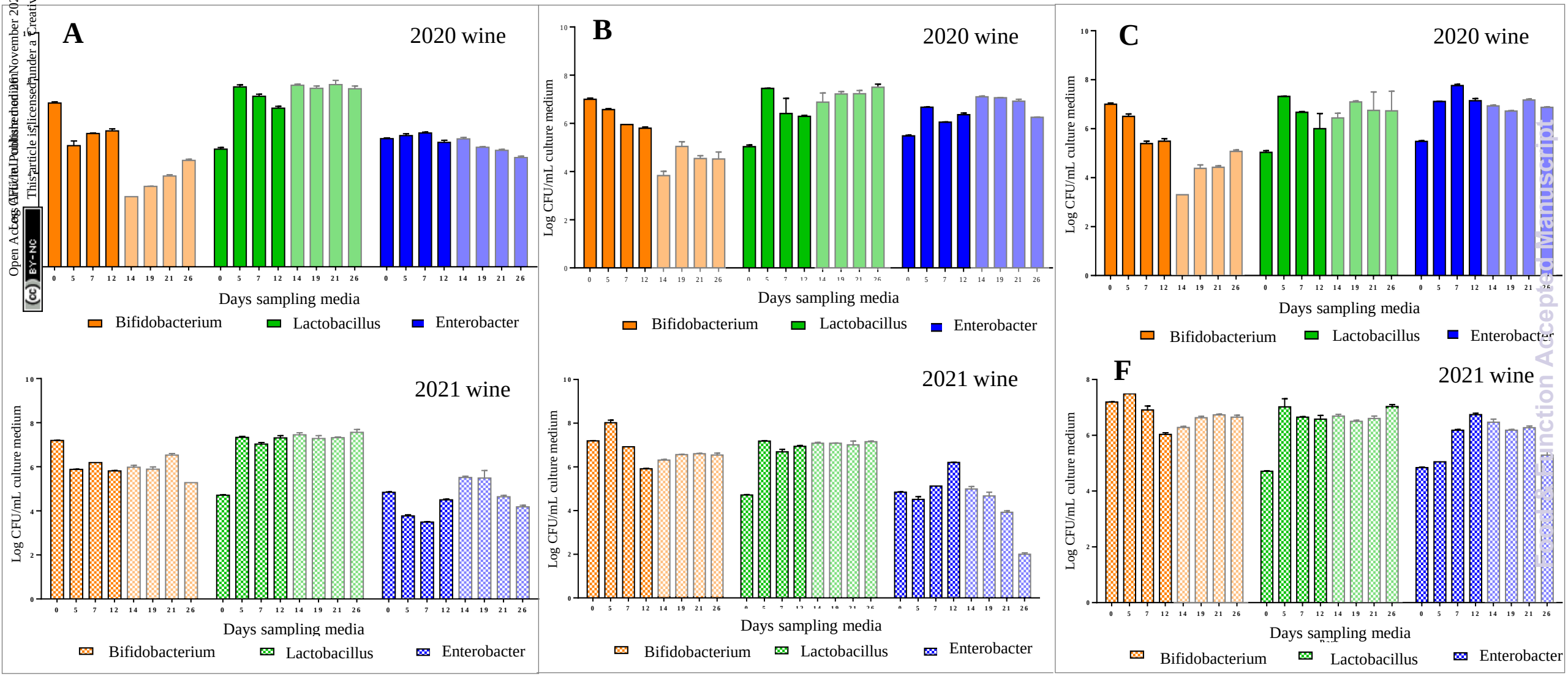

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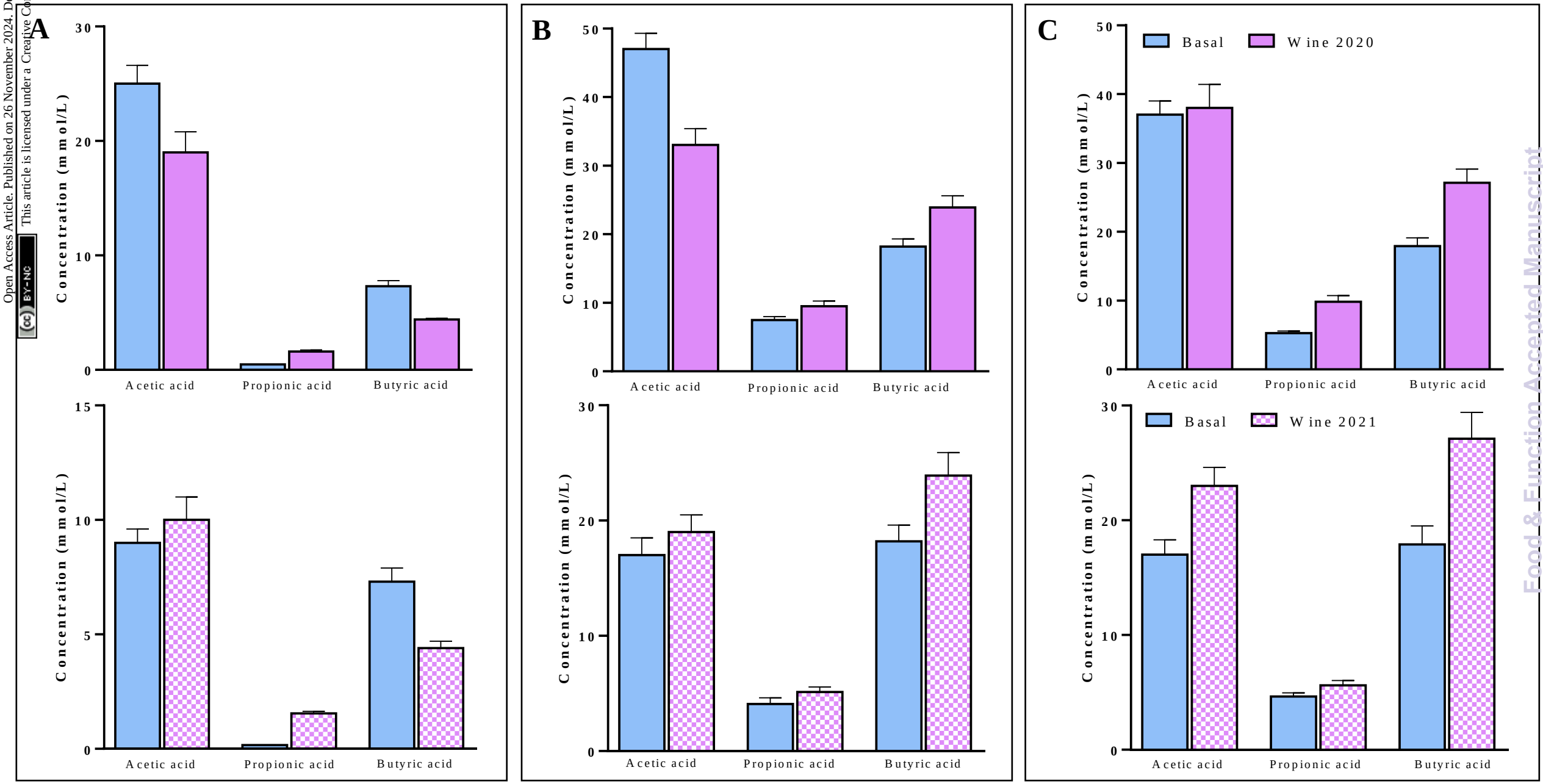

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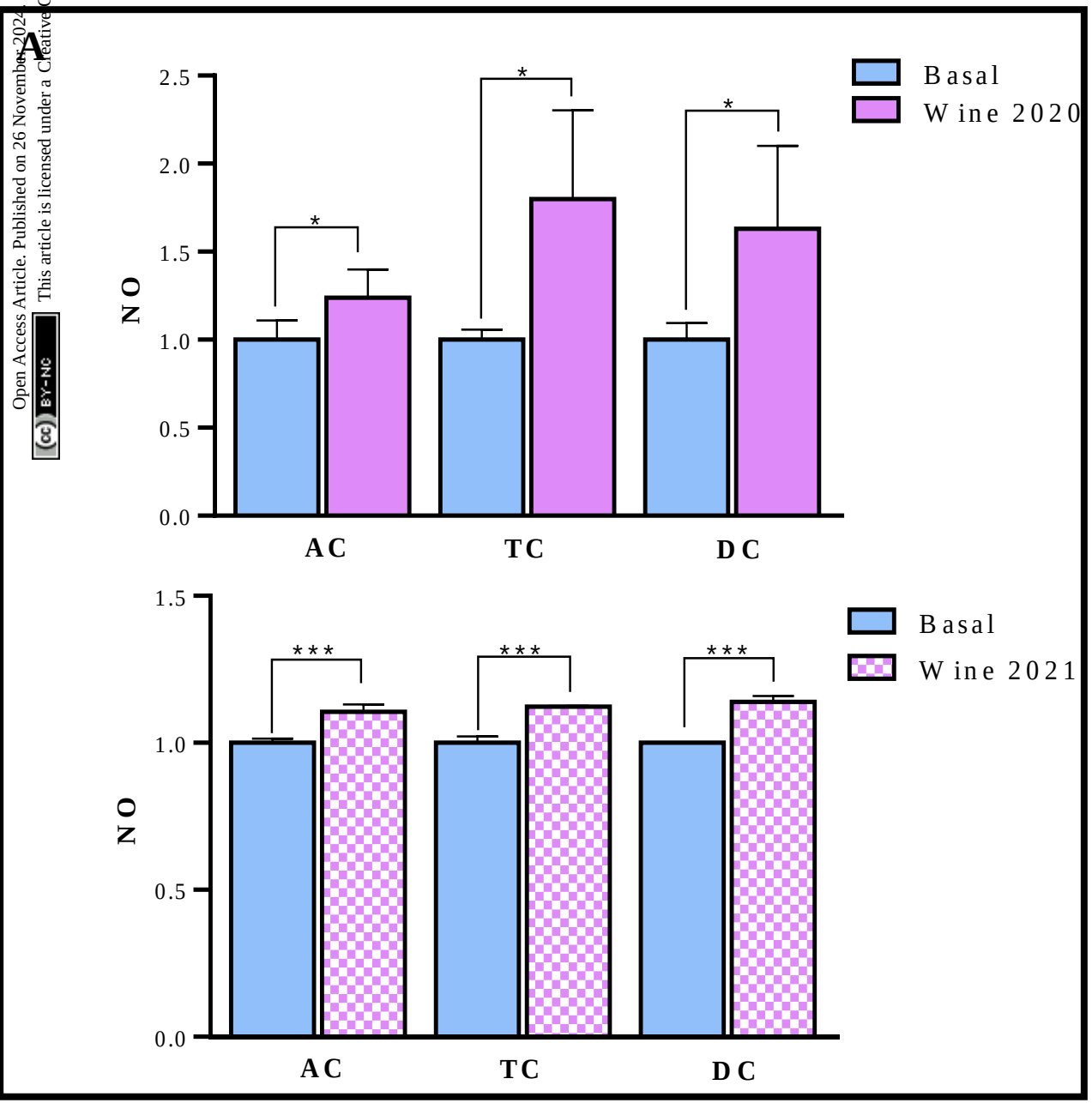

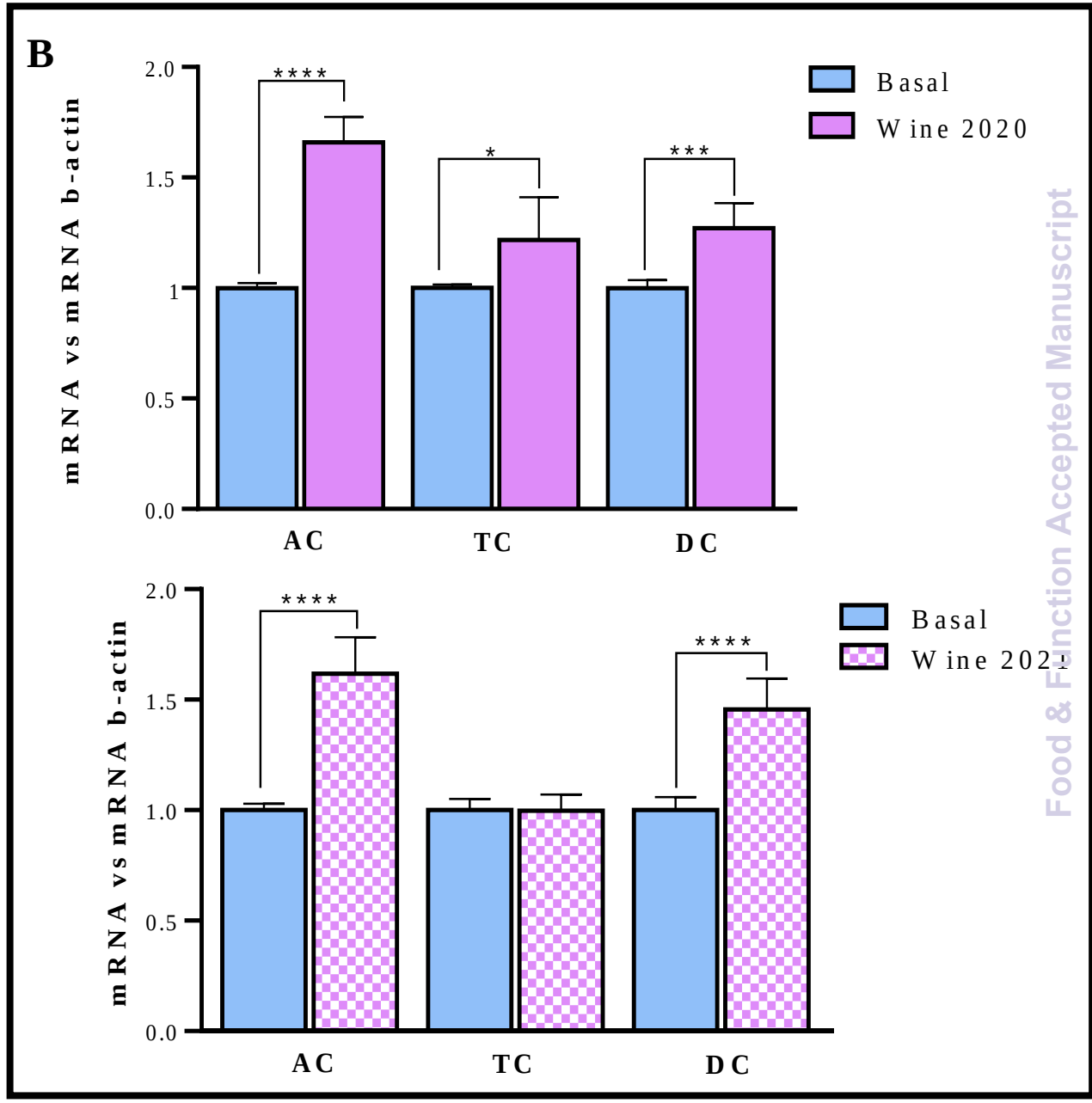

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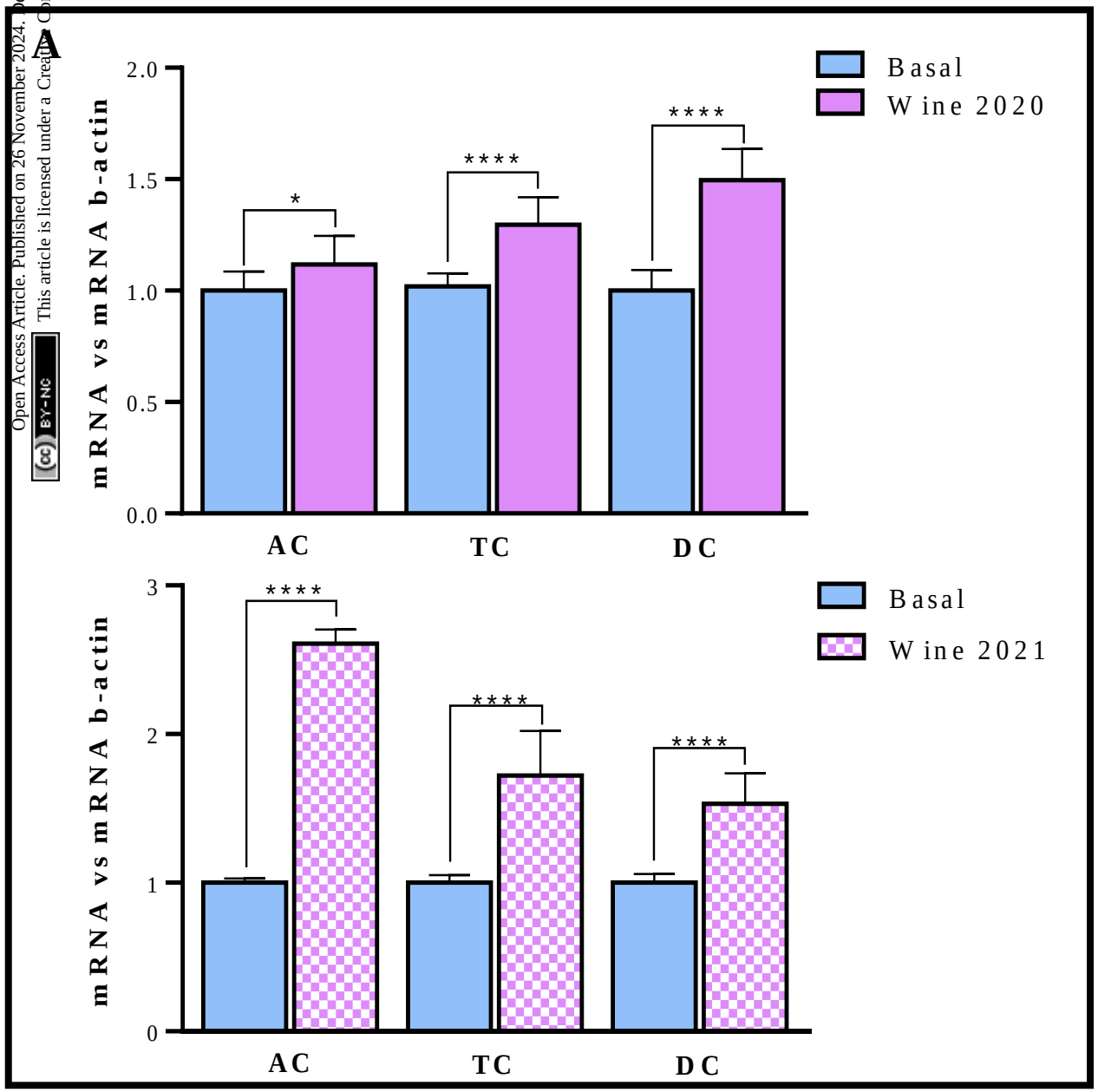

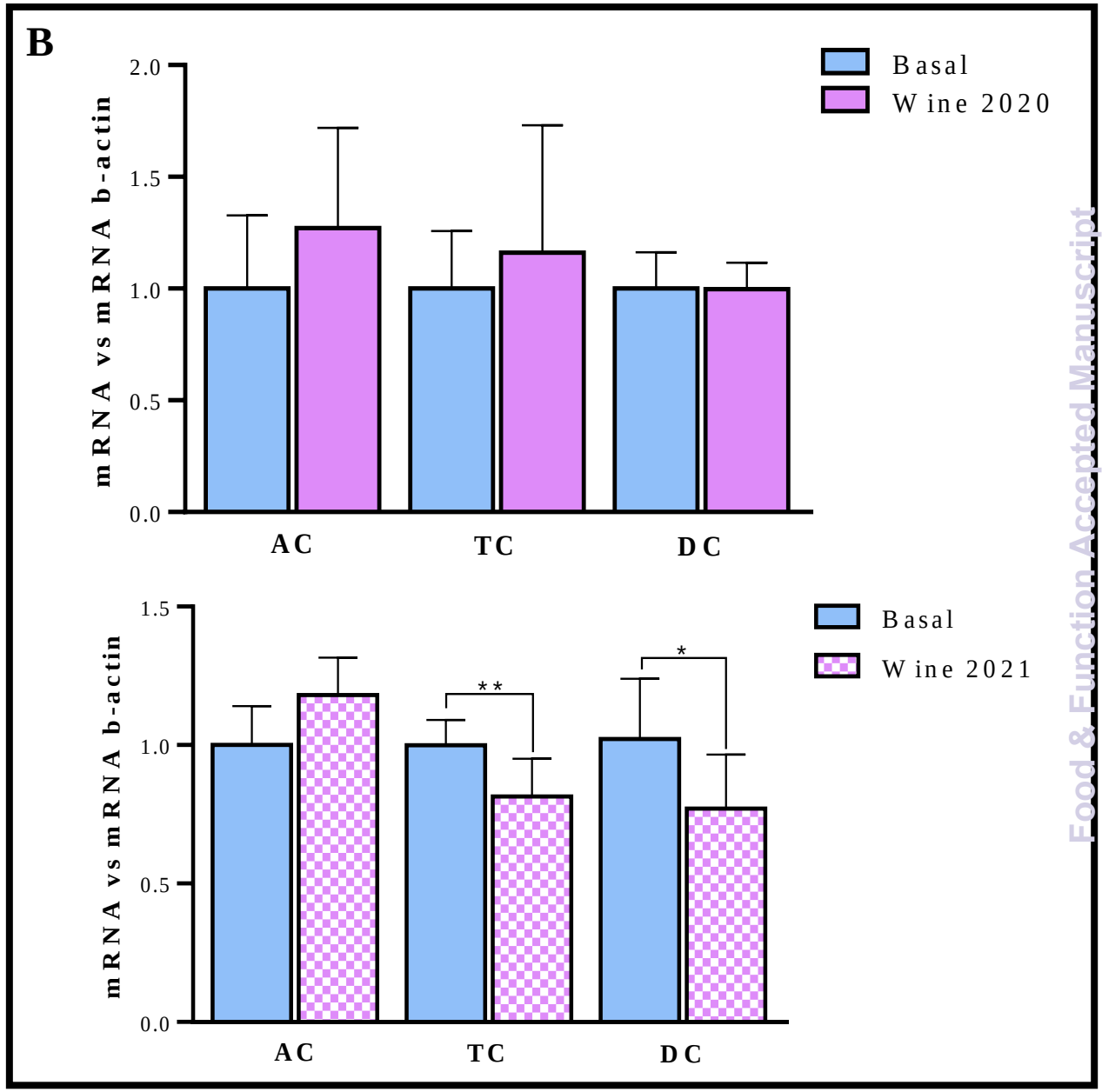

Figure 8