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Bioconversion of Pinot noir anthocyanins into bioactive phenolic compounds by lactic acid bacteria

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Abstract. There has been increasing interest in red wine anthocyanins in relation to changes brought about by lactic acid bacteria in the human digestive system. Anthocyanins are modified multiple times prior to being absorbed, so that intact anthocyanins are unlikely to survive the process, although the parent compounds may still act as local antioxidants within the digestive system. Metabolites such as phenolic acids are easier to absorb, and are thus more bioavailable. This study has investigated the biotransformation of the representative Pinot noir anthocyanin, malvidin-3-glucoside, into metabolites by *Lactobacillus plantarum* at pH 3.4, 4.2, 5.9 and 7.0 incubated anaerobically at 37°C for up to 24 hours. The metabolites were identified by HPLC-MS. Anthocyanin glycosides were found to be completely hydrolyzed by all selected strains at pH 5.9 after 24 h of incubation. The anthocyanins were quite stable under acidic conditions (pH 3.4) but were highly unstable at neutral pH due to both chemical and microbial degradation. The main metabolites were phenolic acids such as gallic acid and protocatechuic acid. They showed greater antioxidant activity than the parent anthocyanins, as indicated by radical scavenging assays and through an assessment of reducing strength using cyclic voltammetry.

Keywords: anthocyanins, pinot noir, polyphenol, lactic acid bacteria

1. Introduction

There is on-going research interest in the relationship between moderate red wine consumption and lowering the risk of cardiovascular heart disease, and links to the antioxidants present in wine [1, 2]. The most abundant antioxidants present in red wine are polyphenols of the flavonoid class, including flavan-3-ols and the coloured anthocyanins [3]. Pinot noir is one of the most popular red wine grapes in New Zealand, and contains a relatively simple monomeric anthocyanin profile, including malvidin-3-glucoside (75%), peonidin-3-glucoside, petunidin-3-glucoside and delphinidin-3-glucoside [4].

The health-protecting effects of anthocyanins have been suggested from epidemiological studies [5]. The *in vitro* antioxidant activity of anthocyanins is a dominant characteristic, and as a dietary source they may protect bodily tissues against free radical-induced oxidative damage thus playing a role in preventing chronic diseases and specific cancers [2]. Despite these indications of their biological activity there is only sparse information on the *in vivo* bioavailability and bioactivity of anthocyanins. Additionally, recent studies have shown that gut microflora are responsible

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for the majority of biotransformations of anthocyanins into bioavailable and bioactive metabolites [6–8]. Most previous studies have focused on the biotransformation of anthocyanins under a single pH condition, often at neutral pH. However, anthocyanins experience a gradient of progressively increasing pH when moving through the digestive system. This study aimed to investigate the bioconversion of a major Pinot Noir anthocyanin, malvidin-3-glucoside by *Lactobacillus plantarum* WCFS1 under different pH conditions, into its bioavailable and bioactive metabolites.

2. Experimental

2.1. Materials

Malvidin-3-O-glucoside was extracted and purified from a Pinot noir grape skin extract using a solid-phase extraction manifold and a semi-preparative HPLC system. Protocatechuic acid (3,4-dimethoxy-4-hydroxybenzoic acid) and malvidin-3-O-glucoside standard were purchased from Extrasynthese (Genay, France). Syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid), gallic acid (3,4,5-trihydroxybenzoic acid), and vanillic acid (4-Hydroxy-3-methoxybenzoic acid) were provided by Sigma–Aldrich. *Lactobacillus plantarum* (WCFS 1) strain was provided by Plant & Food Research, Palmerston North, NZ. Oxoid MRS Broth was purchased from Thermo Fisher Scientific NZ for bacterial growth. Strains were grown with a 2% inoculation into modified MRS fermentation broth, which did not contain glucose nor meat extract, but was supplemented with 0.5% (w/v) lactose, 0.2% (v/v) Tween 80, 0.8% (w/v) casein acid hydrolysate and 0.05% (w/v) cysteine, purchased from Sigma-Aldrich. The bacterial strain was incubated under anaerobic conditions (Gas-Pack, Oxoid Anaerogen; Thermo Fisher Scientific NZ limited) for 24 h at 37°C.

2.2. Chemical stability and bacterial activity on anthocyanin glycosides

Malvidin-3-O-glucoside was prepared as a 1000 mg/L stock solution in acidified Milli-Q water (0.5% (v/v) formic acid) and stored at –80°C between analyses. Chemical degradation or bacterial activity towards anthocyanins was determined in triplicate by mixing 1% of stock solution with 99% of a potassium phosphate-citric acid buffer, at pH 3.4, 4.2, 5.9 and 7.0 and in control samples. For the bacterial activity, 9% of bacterial culture ($A_{600\,nm}$ ~1 corresponds to $1–2 \times 10^9$ CFU/mL) was also added to 90% buffer solution and 1% of malvidin-3-glucoside stock solution. 1 mL samples were incubated at 37°C for up to 24 h in the dark, and samples were taken out after 8 h, 12 h, 16 h, 20 h and 24 h of incubation. The reactions were stopped by adding 200 μL of methanol containing 0.5% formic acid on ice, and the remaining cells were removed by centrifugation (10,000× *g*, 10 min, 4°C). The samples were stored at –20°C until chromatographic analysis.

2.3. HPLC Analysis and identification of the anthocyanins and their metabolites

Analysis of phenolic compounds was carried out using a Hewlett–Packard Agilent 1200 Series liquid chromatography with a quaternary pump and a photodiode array detector. Samples were filtered through a 0.45 μm filter and injected onto a Phenomenex C-18 column (Synergi 4 μm; Fusion-RP 80 Å; 4.6 × 150 mm). The solvents used were aqueous 5% formic acid (B), Milli-Q water (A), and HPLC-grade acetonitrile (C), at a constant flow rate of 1.4 mL/min. The gradient started at 3% C from 0 to 15 min, then 23 min (15% C), 30 min (20% C), 35 min (45% C), 40 min (70% C), 45 min (100% C) and finally from 45 to 51 min 3% C. While anthocyanins were detected at 520 nm, most phenolic acids and other polyphenols were detected at 280 nm, and their peak areas were referred to calibration curves obtained with the commercial phenolic standards.

2.4. Isolation and confirmation of the anthocyanin metabolites

Isolation of each compound was performed using semi-preparative HPLC system, with an automatic fraction collector and a Phenomenex C-18 Semi-column (Synergi 4 μm; Fusion-RP 80 Å; 10 × 150 mm). The solvent system and gradient were the same as above but the flow rate was 6.62 mL/min. The isolated compounds were then concentrated using N_2 and a vacuum concentrator (Speedvac). Further identification of these compounds was carried out using UV-Vis and HPLC-MS (micrOTOF-QII, Bruker Daltonics) to confirm their identities.

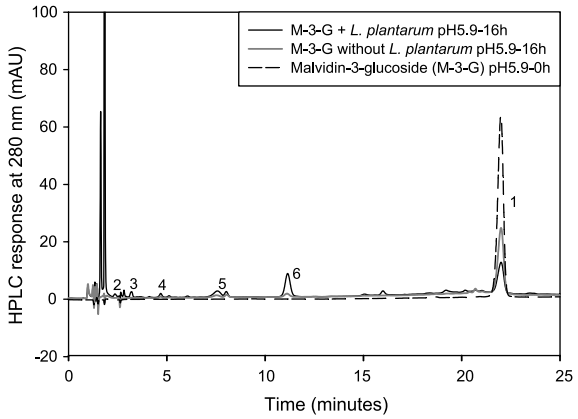

Fig. 1. HPLC chromatogram at 280 nm for malvidin-3-glucoside at time zero hour (black dashed line), incubated with *L. plantarum* WCFS 1 at pH 5.9 for 16 h (black solid line), and without a bacterial strain (grey line).

3. Results and discussion

3.1. Identification of anthocyanin metabolites

Figure 1 shows an HPLC chromatogram of malvidin-3-glucoside incubated for 24 h at pH 5.9 with *L. plantarum* (WCFS 1) recorded at 280 nm. The major metabolites that were identified were gallic acid, homogenistic acid, protocatechuic acid, vanillic acid and syringic acid (Table 1). In addition, there were further minor peaks that were tentatively assigned as protocatechualdehyde (at 6.1 min) and *p*-coumaric acid (15.5 min).

3.2. Effect of pH on chemical and biological degradation of malvidin-3-glucoside

Reactions without incubation with bacterial strains showed that sample pH greatly affected the chemical degradation rate. During the initial 10 mg/L solution preparation, some difference in the initial concentrations was evident, with between 8–25% losses observed, and more so at the higher solution pH (Fig. 2). During the 37°C incubation, the chemical degradation process was again faster at neutral pH, and by the end of the 24 h period only 4.69 ± 0.07 mg/L (47%) of the malvidin-3-glucoside remained. By contrast, at pH 3.4, 4.2 and 5.9, the recovery was 79%, 73% and 75%, respectively, after 24 h. Likewise, only traces (<0.2 mg/L) of metabolites such as syringic acid, gallic acid and protocatechuic acid were detected after incubation at pH 5.9 and 7.0 for 24 h (data not shown). This is consistent with earlier findings by Avila et al. (2009), who found that the methoxylated malvidin-3-glucoside was more stable than other anthocyanins such as the non-methoxylated delphinidin-3-glucoside.

Table 1
Major malvidin-3-O-glucoside metabolites produced by *L. plantarum* WCFS 1

Peak	Compounds	Retention time (minutes)	λ_{max} (nm)	Molecular ions *m/z* (amu)
1	Malvidin-3-O-glucoside	22.4	528	493
2	Gallic acid	2.4	220, 271	171
3	Homogentisic acid	3.2	292	169
4	Protocatechuic acid	4.7	260, 294	239
	Protocatechualdehyde	6.1	280, 320	
5	Vanillic acid	7.5	292	168
6	Syringic acid	11.1	274	199
	p-Coumaric acid	15.5	296, 310	164

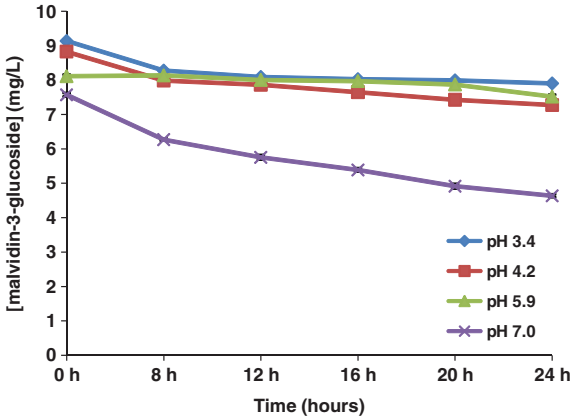

Fig. 2. Chemical degradation of malvidin-3-glucoside at different pH values.

Figure 3 shows the micro-biological induced degradation of malvidin-3-glucoside by *L. plantarum* WCFS1 under different pH conditions. Even after 8 h, the losses were 48%, 51%, 61% and 91% for incubation at pH 3.4, 4.2, 5.9 and 7.0, respectively. The rate of degradation at pH 3.4 and 4.2 were statistically the same after 12 h and 16 h of incubation, but by 24 h a greater loss was apparent for the pH 3.4 sample. A clear loss trend was observed at pH 5.9 where the decreasing concentration was 85%, 94%, 97% and 100% after incubation for 12, 16, 20 and 24 hours, respectively. However, the fastest rate of degradation occurred at

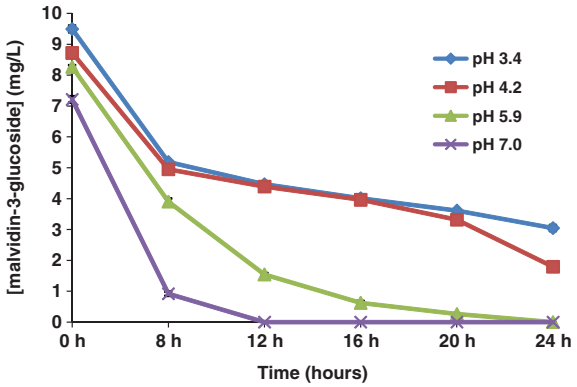

Fig. 3. Malvidin-3-glucoside degradation by *L. plantarum* WCFS1 under different pH conditions.

neutral pH, where all of the malvidin-3-glucoside was degraded after only 12 h of incubation.

3.3. *Conversion of malvidin-3-glucoside to phenolic acids under different pH conditions*

Figure 4A-C shows the formation of phenolic acid metabolites after incubation of malvidin-3-glucoside with *L. plantarum* WCSF1 for up to 24 h. An increasing release of metabolites corresponded to a decreasing concentration of malvidin-3-glucoside, and considerably more than seen in the absence of the bacteria. It was suggested by Avila et al. [6] that the biotransformation process starts from bacterial enzymatic deglycosylation of the malvidin-3-glucoside to an aglycon, which is further degraded to phenolic acids.

Only at pH 3.4 was only minimal production of gallic acid detected (data not shown), and the malvidin-3-glucoside was quite stable. This pH is similar to that found in both red wines and the stomach, where a very slow formation of metabolites can be expected. By contrast, more metabolites were produced gradually at pH 5.9 (matching the pH of the duodenum), and the more rapid formation of metabolites occurred at pH 7.0 (comparable to the large intestine). While not all of the malvidin-3-glucoside breakdown products were accounted for, the contribution of solution pH is evident.

Among the phenolic acids identified, gallic acid and protocatechuic acid have been shown to have high radical scavenging activities, compared to a lower activity for maldivin-3-glucoside [9, 10]. This relative antioxidant power was confirmed by tests using cyclic voltammetry, where the peak for the oxidation of gallic

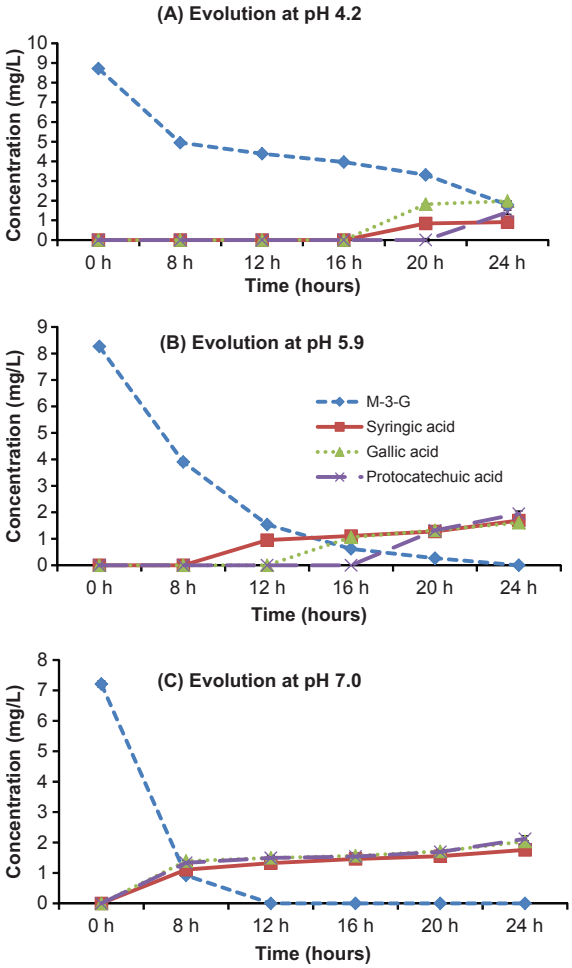

Fig. 4. Evolution of phenolic acids under different pH conditions, alongside the loss of malvidin-3-glucoside (M-3-G).

acid and protocatechuic acid occurred at a less positive potential than that for malvidin-3-glucoside, indicating that these metabolites were stronger reducing agents.

In addition to the information provided about anthocyanin degradation within the digestive system, these results suggest that *L. plantarum* WCSF1 strain might be applied as a functional culture to produce a range of more bioavailable and bioactive phenolic acids from anthocyanin-rich sources. This would offer a potential basis for new food products and dietary supplements.

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