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Antistaphylococcal activity and metabolite profiling of manuka honey (*Leptospermum scoparium* L.) after *in vitro* simulated digestion.

Luisa Mannina*,b, Anatoly P. Sobolevb, Erika Coppoc, Arianna Di Lorenzod, Seyed Mohammad Nabavie, Anna Marchesee, Maria Dagliaa

The antistaphylococcal activity against methicillin-susceptible and -resistant *Staphylococcus aureus* and the metabolite profiling of manuka honey (MH) were investigated before and after *in vitro* simulated gastric (GD), and gastroduodenal (GDD) digestion. Undigested manuka honey showed antibacterial activity against all the tested strains, the GD sample showed no activity against *S. aureus*, and the GDD honey showed an antistaphylococcal activity, which was slightly reduced in comparison with the undigested sample. To explain these results, methylglyoxal (MGO), to which most of the antibacterial activity of MH is ascribed, was submitted to *in vitro* simulated GD and GDD. After digestion, MGO showed antibacterial activity at concentrations definitively higher than those registered in digested MH samples. This result showed that the antistaphylococcal activity registered after digestion cannot be ascribed to MGO. Thus metabolite analysis, carried out using an explorative untargeted NMR-based approach and a targeted RP-HPLC-PAD-ESI-MSn analysis focused on bio-active substances, was used to highlight the chemical modifications occurring from digestion. The results showed that 1) the level of MGO decreases, 2) the content of aromatic compounds, such as leptosin and methyl syringate, markers of manuka honey, was stable under gastric and gastroduodenal conditions, whereas 3) the levels of acetic and lactic acids increase in particular after gastroduodenal digestion, being 1.5 and 2.8 times higher in GDD-MH than UND-MH, respectively. Overall, the results obtained from chemical analysis provide at least a partial explanation of the registered antibacterial activity observed after gastroduodenal digestion.

1. Introduction

The World Health Organization (WHO) reports that in 2010 there were an estimated 582 million cases of 22 different foodborne enteric diseases, with 351 000 associated deaths. Over 40% people suffering from enteric diseases caused by contaminated food were children aged under 5 years. *Staphylococcus aureus* is one of the main microorganisms involved in foodborne diseases, a commensal and opportunistic pathogen that is particularly dangerous due to a combination of toxin-mediated virulence, invasiveness and antibiotic resistance. The incidence of methicillin-resistant *S. aureus* (MRSA) has steadily increased in recent years with a rate of up to 59% among *S. aureus* clinical isolates. *S. aureus* can cause a wide range of infections including acute cutaneous infections, post-operative infections and intestinal infections. Especially, lasting colonization of *S. aureus* in the human intestinal tract may have important clinical implication ranging from enteritis and diarrhea to bacteremia, which is associated with high morbidity and mortality, when compared with bacteremia caused by other pathogens. Modern medicine pays attention to studying natural products with antimicrobial activity and their potential use in clinical application, mainly due to low cost and the absence of risk of antimicrobial resistance in comparison with conventional therapies. Manuka honey (MH), which is known for its antibacterial activity, is derived from the manuka tree (*Leptospermum scoparium* L.), native to Australia and New Zealand and belonging to the Myrtaceae family. In traditional medicine, MH is used to heal wounds, protect oral health, reduce inflammation, and treat upper gastrointestinal dyspepsia, gastroenteritis, and diarrhea. The efficacy of MH seems to be due to its antibacterial activity against a wide range of Gram positive and Gram negative bacteria including multiresistant strains, such as *Salmonella typhimurium* DT104 and methicillin-resistant *Staphylococcus aureus*. In 2008, Mavric et al. identified methylglyoxal (MGO) as the main component responsible for the nonperoxide MH antibacterial activity. In MH, MGO concentration ranges from 40 to 760 mg/kg. The high concentration of MGO can be explained both by the presence of dihydroxyacetone (DHA) as a direct precursor for MGO formation in manuka honeys, and sugar...
degradation following the caramelization reaction occurring during honey storage.\textsuperscript{10} More recently, combinational treatment of wounds with manuka honey and antibiotics has been shown to exert a synergistic enhancement of the antibacterial activity, reducing the effective dose of the antibiotic and the risk of antibiotic resistance. The same authors showed that methylglyoxal (MGO) did not act synergistically with rifampicin and is therefore not the sole factor responsible for the synergistic effect of manuka honey with rifampicin.\textsuperscript{11}

Besides MGO, MH contains other α-dicarbonyl compounds, such as glyoxal, 2,3-butanedione, glucosone, 3-deoxyglucosone, and 3,4-dideoxyglucosone-3-ene, in higher amounts in comparison with the other commercial honeys with different botanical origins.\textsuperscript{12} The antibacterial activity of some α-dicarbonyl compounds, such as glyoxal and diacetyl compounds, against S. mutans and S. aureus has been previously demonstrated by Daglia et al.\textsuperscript{13}, recognizing these compounds as the main agents responsible for the antibacterial activity of brewed coffee.

Moreover, MH contains organic acids (such as phenyllactic acid, 4-methoxyphenyllactic acid and 2-methoxybenzoic acid) incorporated into honey via nectar and pollen\textsuperscript{14}, which have been reported to exert antibacterial activity.\textsuperscript{15,16}

Recently, Fearnley et al.\textsuperscript{17} and Kato et al.\textsuperscript{18} identified other compounds, such as leptosin and methyl syringate, which represent MH specific markers, and ascertained a correlation between leptosin and MH unique manuka factor (UMF), suggesting that leptosin has antibacterial activity or contributes to MH bactericidal activity. A recent study revealed the presence of DHA in 6 fresh manuka honeys, taken directly from the beehive, and 18 commercial manuka honey samples with a DHA concentration ranging from 600 to 2700 mg/kg and 130 to 1600 mg/kg, respectively.\textsuperscript{19} DHA exerted in vitro antifungal, antiprotozoal and antiplasmodial activities.\textsuperscript{20,21}

A recent research showed that after in vitro simulated digestion, MH MGO concentration decreases from 51.2 to 87.8% due to MGO reacting with digestive enzymes by carbonylating their free amino groups\textsuperscript{22} and -resistant \textit{Staphylococcus aureus} strains. Moreover, to evaluate the effects of the digestion process on MH metabolites and provide an explanation of the results obtained from microbiological assays, the metabolite profiling of digested and undigested MH samples was determined using an untargeted NMR-based methodology and a RP-HPLC-PAD-ESI-MSn method.

2. Materials and methods

2.1. Chemicals

HPLC water Millipore grade, pepsin from porcine gastric mucosa (2400 units/mg protein), pancreatin from porcine pancreas (8xUSP), bile salts, formic acid solution, methanol, (-)-catechin, caffeine solution (50 µg/mL), sodium bicarbonate, methylglyoxal were purchased from Sigma-Aldrich (Milan, Italy).

2.2. Manuka honey samples

Honey from the manuka tree (\textit{Leptospermum scoparium} L.) was obtained from an Italian herbalist’s shop. The content of MGO declared in the label was 550 mg/kg corresponding to a UMF of 25+.

2.3. Solid phase extraction (SPE) of manuka honey

The MH sample was subjected to SPE according to Oelschlaegel et al.\textsuperscript{14} in order to obtain two different fractions, one containing polar compounds including MGO and sugars, and one containing mainly polyphenols. Briefly, a tC18 Sep-Pak Vac 6cc cartridge (Waters, Milford, Ma) containing 1 g of stationary phase was conditioned with methanol (10 mL) and formic acid 0.1 % v/v (10 mL). Two grams of honey were dissolved in 3 mL of 0.1% formic acid containing caffeine used as internal standard (10 µg/mL). After the honey solution was loaded, the polar substances (i.e. sugars, MGO, and other dicarbonyl compounds) were recovered with 1 mL formic acid 0.1% (MH-SPE1 fraction), and the non-polar analytes (MH-SPE2 fraction) were eluted with 75% methanol (1 mL). The MH-SPE1 fraction was submitted to \textit{in vitro} simulated gastric and gastroduodenal digestion and then to HPLC-UV-PDA analysis to quantify MGO in undigested and digested samples. Moreover, the MH-SPE2 fraction was submitted to \textit{in vitro} simulated gastric and gastroduodenal digestion and both undigested and digested SPE2 fractions were analyzed by HPLC-PDA-ESI-MSn to investigate if some differences on the metabolic profiling occur after digestion processes.

2.4. \textit{In vitro} simulated gastric and gastroduodenal digestion process

Two aliquots of MH, MH-SPE1, and MH-SPE2 fractions, obtained as reported in the previous section, and MGO solution were submitted to \textit{in vitro} simulated gastric and gastroduodenal digestion process as reported by Daglia et al. with some modifications.\textsuperscript{2}

In brief, two MH aliquots (20 g) dissolved in 10 mL of Millipore grade water (or the same volume of Millipore grade water, used as a control), were added to 5 mL of freshly prepared pepsin (1.6 g in 10 mL of 0.1 M HCl), the pH was adjusted to 2.00 ± 0.02 using 4 M HCl, and the mixture was incubated at 37 °C for 2 h in a shaking water bath. After gastric digestion, the second aliquot was adjusted to pH 7.00 ± 0.02 with 0.1 M NaHCO\textsubscript{3} and added to 1.25 mL of freshly prepared pancreatin bile mixture (0.04 g of pancreatin and 0.25 g of bile salts in 5 mL of 0.1 M NaHCO\textsubscript{3}), before incubating for 1 h at 37 °C. At the end of the digestion process, gastric digested (GD) and gastroduodenal digested (GDD) MH samples were freeze-dried, reconstituted to 20 mL (1 g/mL) and submitted both to microbiological assays and NMR analysis.

Two aliquots of 1 mL of MH-SPE1 and MH-SPE2 fractions were digested as reported above for the MH sample, adjusting the volume of enzymes solutions used (i.e. 0.5 mL of pepsine
solution and 0.125 mL of freshly prepared pancreatin bile mixture). At the end of the digestive processes, both MH-SPE1 and MH-SPE2 samples were brought up to 2 mL, filtered with non-sterile disposable syringe filters 0.22 μm (Millex-LG) and submitted to RP-HPLC-DAD analysis for the quantification of MGO and RP-HPLC-PAD-ESI-MSn analysis for the determination of the metabolite profiling.

Finally, two aliquots of 23.9 μL of standard MGO solution, corresponding to the MGO concentration reported in the label of MH sample (550 mg/kg or 7.5 mM), added to 10 mL of MilliPore grade water (or the same volume of MilliPore grade water, used as a control) were digested as reported above for MH sample digestion. At the end of digestion both samples and controls were brought up to 2 mL (10x corresponding to 75 mM) and submitted to microbiological assays and to RP-HPLC-DAD analysis.

2.5. Bacterial strains and growth conditions

Ten recent clinical S. aureus strains (5 methicillin-resistant and 5 methicillin-susceptible) stored at the Institute of Microbiology, University of Genoa, Italy were studied. Among the five methicillin-resistant isolates, three strains were multi-resistant (resistant to at least three classes of antibiotics). S. aureus strains were identified to the species level by using API STAPHi system (bio Mérieux, Marcy l’Etoile, France). The antibiotic was determined using the disk diffusion test suggested by the Clinical and Laboratory Standards Institute guidelines.25 Strains were cultured in Mueller-Hinton broth and Mueller-Hinton agar (Biolife, Milan, Italy) at 37°C.

2.6. Susceptibility tests

Minimum inhibitory concentrations (MICs) of MH and MGO, before and after simulated gastric and gastroduodenal digestion, were determined by the broth microdilution method suggested by the Clinical and Laboratory Standards Institute guidelines.25 In brief, exponentially growing bacteria (5X105 cells per mL, final inoculum) were added to the various concentrations of UND, GD, and GDD MH and MGO, 2-fold serially diluted in 96-well microtitre plates Mueller-Hinton broth. After 18-24 h of incubation at 37°C the MH and MGO concentrations which prevented a visible bacterial growth were recorded as the MIC. All tests were performed in triplicate and were repeated three times.

2.7. NMR analysis

UND, GD, and GDD MH samples were analyzed in aqueous solution. The NMR spectra were recorded at 27 °C on a Bruker AVANCE 600 spectrometer operating at the proton frequency of 600.13 MHz and equipped with a Bruker multinuclear z-gradient inverse probe head capable of producing gradients in the z-direction with a strength of 55.4 G cm−1. Samples (10 mg) were dissolved in 0.7 mL of D2O containing 2 mM of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) as an internal standard. The mixture was agitated for several minutes. The limpid solution (0.7 mL) was transferred to a standard 5 mm NMR tube. The 1H spectra of the aqueous extracts were acquired by co-adding 64 transients with a relaxation delay of 12 s and using a 90° pulse of 9 μs, 32 K data points. The water signal was suppressed using solvent presaturation with a soft pulse during the last two seconds of relaxation delay just before the application of the hard pulse. In order to assign 1H NMR spectrum 2D NMR experiments, namely, 1H–1H TOCSY, 1H–13C HSQC26 were performed. The experimental conditions for all 2D experiments were previously reported.26 The mixing time for the 1H-1H TOCSY was 80 ms.

2.8. RP-HPLC-DAD and RP-HPLC-PAD-ESI-MSn analyses.

The quantification of MGO in UND, GD and GDD MH-SPE1 fractions and of UND, GD and GDD MAGO solutions was performed by means of a RP-HPLC-DAD method, developed and validated by Daglia et al.27 The evaluation of the MH-SPE2 fraction metabolic profiling was performed using a Thermo Finnigan Surveyor Plus HPLC apparatus equipped with a quaternary pump, a Surveyor UV–Vis PDA detector, and a LCQ Advantage Max ion trap mass spectrometer (all from Thermo Fisher Scientific, Waltham, MA) through an ESI source. Separation was achieved on AERIS PEPTIDE 3.6 5μm XB-C18 (250 mm × 4.6 mm i.d., 3.6 μm) with a Ultra Cartridge C18-Peptide Security Guard column (for 4.6 mm i.d.) both from Phenomenex, Torrance, CA. The mobile phase consisted of 0.1% formic acid in water (elucent A) and methanol (elucent B) at a flow rate of 0.3 mL/min. The injection volume was 10 μL. With regards to the MH samples, gradient elution was carried out using the following timeframe: from 1% B to 40% B in 30 min, then to 70% B in 18 min, to 90% B in 7 min, and to 100% B in 5 min. An isotropic elution with 100% B was then carried out for 10 more minutes. Then to 1% B in 15 min. The sample tray was set at 4 °C and the column oven temperature was set at 25 °C. The chromatogram was recorded at λ 210, 254, 280 nm. Spectral data were acquired in the range of 200 - 800 nm for all peaks. The ion trap operated in data-dependent, full scan (100 2000 m/z), zoom scan, and MSn mode to obtain fragment ion m/z with a collision energy of 35%. When greater discrimination was required, additional targeted MS2 and experiments were performed on selected pseudomolecular ions to a ionization voltage of 3.5 kV, a capillary temperature of 200 °C, a sheath gas flow rate of 45 arbitrary units, and an auxiliary gas flow rate of 20 arbitrary units. Electrospray ionization was applied either in the negative or positive mode. The negative-ion mode ESI source parameters had previously been optimized by flow injection analysis using (−)-catechin (10 ppm in 0.1% formic acid–methanol solution, 50:50, v/v) and the positive-ion mode ESI source using caffeine (50 μg/mL). The Thermo Fisher Scientific Excalibur 2.0 software was used for data acquisition and processing. Three independent assays were performed to analyze each UND, GD, and GDD MH-SPE2 fractions (filtered through a cellulose acetate/cellulose nitrate mixed esters membrane - 0.22 μm; Millipore Corporation, Billerica, MA) by RP-HPLC-PDA-ESI/MSn; no relevant variations attributable to the nature of the detected fragments or their relative intensities were observed.

3. Results and discussion

The antistaphylococcal activity of UND, GD, GDD MH samples were determined against 10 strains of methicillin-susceptible and -resistant S. aureus (Table 1). The UND MH showed antistaphylococcal activity, according to literature data.2,3 Interestingly, although methicillin-resistant S. aureus strains
usually display resistance to several drugs, no relevant differences were observed between methicillin-susceptible and -resistant strains. As far as digestion was concerned, the GD MH sample showed no activity against *S. aureus* (MIC >1mg/mL), whereas GDD MH showed lower antistaphylococcal activity than UNDMH (MIC range 0.06 – 1.00 mg/mL). Water submitted to gastric and gastroduodenal digestion processes as a control, and thus containing the same concentration of digestive enzymes used for preparing MH samples, did not show any antibacterial activity. These results demonstrated that gastric digestion causes the loss of antibacterial activity against the tested bacteria in the applied experimental conditions. Conversely, gastroduodenal digestion induces only a slight decrease of MH antibacterial activity against *S. aureus*.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Staphylococcus aureus - MIC (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>range</td>
</tr>
<tr>
<td>UNDMH</td>
<td>0.06 – 0.50</td>
</tr>
<tr>
<td>GD-MH</td>
<td>&gt;1.00</td>
</tr>
<tr>
<td>GDD-MH</td>
<td>0.06 – 1.00</td>
</tr>
</tbody>
</table>

Table 1 In vitro Antibacterial Activity of Undigested (UND) and Gastric (GD) and Gastroduodenal Digested (GDD) MH against 10 *S. aureus* (5 Methicillin-Resistant and 5 Methicillin-Susceptible) strains.

As reported above, MGO has been identified as one of the major contributors to MH antibacterial activity. Our previous results showed that MGO concentrations of MH decrease after *in vitro* gastric and gastroduodenal digestion. These results were confirmed by Degen et al. who found that after *in vitro* digestion only 5-20% of the initial MH MGO was recovered. Therefore, we wanted to assess whether the decrease in MH antibacterial activity after digestion could be ascribed to a decrease in MGO concentration. Moreover, our aim was to ascertain if the carbonylation products, produced after the reaction between MGO and digestive enzymes, which are known to show cytotoxic activity, are active as antibacterial agents. Consequently, the *in vitro* antimicrobial activity of UND, GD and GDD MGO solutions (at 7.5 mM concentration, which is the same as that of the tested MH sample) was assayed. Moreover, MGO in UND, GD and GDD MH-SPE1 fractions was quantified to confirm the effect of digestion on MGO and to determine the actual concentration of MGO in MH. The results showed that the UND MGO sample possesses antistaphylococcal activity, according to literature data. GD and GDD MGO samples, whose MGO concentration decreased by about 20% and 50%, respectively, showed antibacterial activity at MIC values definitely higher than MGO concentrations found in MH. In fact, MIC values of GD and GDD MGO ranged from 15.2 to 31.0 mM (Table 2). These results in part justified the loss of GD and the decrease in GDD MH antibacterial activity.

The RP-HPLC-DAD analyses of UND, GD and GDD MH-SPE1 fractions revealed that, for the UND sample, MGO concentration is about 16% higher than that declared on the label, likely due to the long-term storage of the tested MH sample, though it remains well within the literature-based concentrations limits. In GD and GDD MH-SPE1 samples, the concentration of free, and therefore detectable, MGO decreased by 20% and 52%, respectively, confirming our previous data on the effect of digestion on MH MGO concentration (Table 3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Staphylococcus aureus - MIC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>range</td>
</tr>
<tr>
<td>UND-MGO</td>
<td>0.11 – 0.23</td>
</tr>
<tr>
<td>GD-MGO</td>
<td>15.20 – 31.00</td>
</tr>
<tr>
<td>GDD-MGO</td>
<td>15.20 – 30.40</td>
</tr>
</tbody>
</table>

Table 2 *In vitro* Antibacterial Activity of Undigested (UND) and Gastric (GD) and Gastroduodenal Digested (GDD) MGO against 10 *S. aureus* (5 Methicillin-Resistant and 5 Methicillin-Susceptible) strains.

<table>
<thead>
<tr>
<th>Sample</th>
<th>mg/kg</th>
<th>% concentration loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>UND-MH-SPE1</td>
<td>635 ± 10</td>
<td>--</td>
</tr>
<tr>
<td>GD-MH-SPE1</td>
<td>505.5 ± 14</td>
<td>20.4%</td>
</tr>
<tr>
<td>GDD-MH-SPE1</td>
<td>301.6 ± 8.1</td>
<td>52.5%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>mM</th>
<th>% concentration loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>UND-MGO</td>
<td>7.5 ± 0.1</td>
<td>--</td>
</tr>
<tr>
<td>GD-MGO</td>
<td>2.43 ± 0.25</td>
<td>67.6%</td>
</tr>
<tr>
<td>GDD-MGO</td>
<td>0.81 ± 0.14</td>
<td>89.2%</td>
</tr>
</tbody>
</table>

Table 3 Quantification of MGO (expressed as mg/kg of edible food) in MH-SPE1 fraction, and of MGO standard solutions (mM) before digestion (UND) and after gastric (GD) and gastroduodenal digestion (GDD). Data are expressed as the mean of three independent measurement ± SD.

To study the effect of gastric and gastroduodenal digestion on the chemical composition of MH, the metabolite profiling of UND, GD, and GDD MH samples was investigated using NMR and RP-HPLC-ESI-MSn methodologies. The choice of NMR as an untargeted methodology was driven by the non-specific high-throughput character of the NMR approach well suited to the requirements of metabolite profiling with the advantage of detecting signals from many different classes of compounds simultaneously in the same experiment. NMR has allowed significant information on the geographical and the botanical origin and adulteration of different honeys to be obtained. Moreover the 1H DOSY NMR methodology has been applied to characterize different types of honey including MH. Figure 1 shows the 1H NMR spectra of UND, GD, and GDD MH samples in aqueous solution. The spectral region between 3.2-5.7
ppm due to sugars did not show any difference after gastric and gastroduodenal digestion, except for additional small signals from lactose (a component of digestive enzyme mixture) and from dihydroxyacetone (DHA) a precursor of MGO. The $^1$H signal of DHA is a singlet at 4.41 ppm. The spectral region between 1.3 and 2.5 ppm of the UND MH sample showed the signals of methylglyoxal at 2.37 ppm, methylglyoxal monohydrate at 2.31 ppm, acetaldehyde at 2.15 ppm, methylglyoxal dihydrate at 1.38 ppm, lactic acid at 1.33 ppm and acetic acid at 1.93 ppm.  

With regards to the effect of digestion on MH metabolite profiling the levels of acetic acid and lactic acid, well known for their bacteriostatic activity$^{29,34}$, increased after gastric (1.2 and 2.1 times, respectively) and especially after gastroduodenal digestion (1.5 and 2.8 times respectively), whereas acetaldehyde level decreased in both types of digests. The signal intensity of methylglyoxal, methylglyoxal monohydrate and methylglyoxal dihydrate decreased after both gastric and gastroduodenal digestions, reaching the lowest levels after gastroduodenal digestion. The results relative to MGO agreed with literature data.$^{2,23}$ The intensity of the DHA signal was about 7 times lower after gastric digestion, whereas it was partially restored after gastroduodenal digestion: to twice that recorded after gastric digestion. In the aromatic spectral region, the $^1$H NMR spectrum of the UND MH sample showed the signals of 3,4,5–trimethoxybenzoic acid, phenyllactic acid, methyl syringate and 4-methoxyphenyllactic acid. The signal intensity of these compounds after gastric and gastroduodenal digestions remained unchanged (Fig. 1).

![Fig. 1](Image)

The chemical stability of the aromatic compounds, which recent studies have identified as specific to MH and related to its antibacterial activity, prompted us to use a targeted RP-HPLC-PAD-ESI-MSn method for their analysis. The RP-HPLC-PAD-ESI-MSn analysis of the UND sample (Fig. 2A) showed the presence of 4-methoxyphenyllactic acid, leptosin, methyl syringate, and phenyllactic acid, according to literature data. Compound identification was based on chromatographic behavior, UV-Visible and mass spectra, and comparisons with the literature (Table 4). As far as leptosin is concerned, its molecular ion at m/z 581 corresponded to the formic acid adduct. After gastric and gastroduodenal digestion all the identified MH compounds (Fig. 2B and 2C) were still present in both the digested sample.

### Table 4: Chromatographic, UV-Vis and MS Data of MH-SPE2 fractions potential antistaphylococcal compounds identified in undigested (UND) sample.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention time (min)</th>
<th>m/z [M-H]$^{-}$</th>
<th>m/z Fragment ions (% of base peak)</th>
<th>$\lambda_{max}$ (nm)</th>
<th>Proposed structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>52.09</td>
<td>165</td>
<td>147 (100), 101 (5), 119 (5)</td>
<td>258</td>
<td>phenyllactic acid</td>
</tr>
<tr>
<td>2</td>
<td>53.39</td>
<td>195</td>
<td>177 (100), 149 (15)</td>
<td>274</td>
<td>4-methoxy phenyllactic acid</td>
</tr>
<tr>
<td>3</td>
<td>43.43</td>
<td>581</td>
<td>535 (100), 211 (5)</td>
<td>263</td>
<td>leptosin</td>
</tr>
<tr>
<td>4</td>
<td>56.52</td>
<td>213</td>
<td>181 (70), 154 (15)</td>
<td>275</td>
<td>methyl syringate</td>
</tr>
</tbody>
</table>

### 4. Conclusions

In conclusion, the results obtained from microbiological assays show that MH does not exert its antibacterial activity against the tested 10 strains of methicillin-susceptible and -resistant *S. aureus* after gastric digestion. However, MH does retain its antibacterial activity against the tested *S. aureus* strains after gastroduodenal digestion. Moreover, MGO, which showed antibacterial activity against Gram-positive bacteria at the concentrations found in MH, showed much lower antibacterial activity after in vitro simulated gastric and gastroduodenal digestion, with higher MIC values than those registered for undigested MGO sample. Metabolite analysis, carried out using the untargeted NMR-based approach and targeted HPLC-PAD-ESI-MSn method, confirms our previous results suggesting that MGO levels decrease under gastric and gastroduodenal conditions. Moreover, the results of the chemical analysis show that, unlike MGO, the aromatic compounds, and especially leptosin, a marker of manuka honey and considered correlated to MH antibacterial activity, are stable.$^{35}$ Finally, an increase of acetic acid and lactic acid is registered after gastric and above all gastroduodenal digestion. These results provide a potential explanation of the data obtained from the microbiological assay. In fact, the degradation of MGO after gastric and gastroduodenal digestion, is likely the main cause for the decrease of MH antibacterial activity. Furthermore, comparing these results with those of microbiological tests, leptosin did not result as playing a role in MH antibacterial activity.
activity after digestion. On the contrary, the increase of the concentration of the organic acids (i.e. acetic acid and lactic acid), after gastroduodenal digestion (1.5 and 2.8 times higher in GDD than UND samples, respectively) at least in part, may justify the antistaphylococcal activity of GDD MH sample against *S. aureus*. In fact, we could assume that the organic acids reach the concentration required to inhibit bacterial growth only after gastroduodenal digestion. These compounds have a long history of being used as food additives and preservatives for preventing microbial contamination and dissemination in foods and have been reported as effective agents in inhibiting the growth of pathogenic bacteria. This can be considered as just a partial explanation and the actual cause of the restoration of the activity after the gastroduodenal digestion remains to be deeply investigated. Our results demonstrate that MH maintains its antibacterial activity against laboratory and clinical strains of *S. aureus* including MRSA strains and support the potential use Manuka honey in support of the treatment of *S. aureus*-related intestinal infection. These encouraging results prompt to propose a controlled clinical study, needed to define the *in vivo* efficacy of MH.

Fig. 2 RP-HPLC-PAD chromatograms of UND (A), GD (B), GDD MH-SPE2 fractions (C). Assignments: Phenyllactic acid (1); 4-Methoxyphenyllactic acid (2); leptosin (3); Methyl syringate (4).
5. References


