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In Vitro Investigation into the Potential Prebiotic Activity of Honey Oligosaccharides

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The effect of honey oligosaccharides on the growth of fecal bacteria was studied using an in vitro fermentation system. Prior to treatment, glucose and fructose (31.73 and 21.41 g/100 g of product, respectively) present in honey, which would be digested in the upper gut, were removed to avoid any influence on bacterial populations in the fermentations. Nanofiltration, yeast (*Saccharomyces cerevisiae*) treatment, and adsorption onto activated charcoal were used to remove monosaccharides. Prebiotic (microbial fermentation) activities of the three honey oligosaccharide fractions and the honey sample were studied and compared with fructooligosaccharide (FOS), using 1% (w/v) fecal bacteria in an in vitro fermentation system (10 mg of carbohydrate, 1.0 mL of basal medium). A prebiotic index (PI) was calculated for each carbohydrate source. Honey oligosaccharides seem to present potential prebiotic activity (PI values between 3.38 and 4.24), increasing the populations of bifidobacteria and lactobacilli, although not to the levels of FOS (PI of 6.89).

KEYWORDS: Honeydew honey; prebiotic; oligosaccharides; nanofiltration; activated charcoal

INTRODUCTION

Honey is primarily composed of the monosaccharides glucose and fructose, which can be found in amounts of between 55 and 75%. A complex mixture of minor carbohydrates (10−25%), mainly disaccharides and trisaccharides, is also present (*1*). Moreover, the presence of four tetrasaccharides, one pentasaccharide, and one hexasaccharide has been detected in New Zealand honeydew honeys (*2*). Many attempts have been made to determine the composition of honey (*3*−*7*); however, the identity of some of the more minor carbohydrate components is still unknown.

Since antiquity, honey has been considered to be an important source of energy, being used in medical therapies and as a valuable food ingredient (*8*). Certain components of honey can provide antioxidant activities, seen as beneficial for human health (*9*−*11*), and various studies have revealed the inhibitory properties against certain pathogens (*12*, *13*). Shamala et al. (*14*) carried out in vitro and in vivo studies in the small and large intestines of rats and proposed that honey enhanced the growth of lactic acid bacteria. Moreover, honey has been shown to support lactic acid production in skim milk fermented with lactic acid bacteria in a manner similar to that of other sweeteners such as sucrose and fructose (*15*). More recently, Kajiwara et al. (*16*) showed that the growth, in pure culture, of commercial strains of bifidobacteria was enhanced by honey in a manner similar to that of other commercial prebiotic oligosaccharides [fructooligosaccharide (FOS), galactooligosaccharides (GOS), and inulin]. This study, however, used whole honey composed largely of monosaccharides, which would be metabolized in the upper human gut and would not be expected to reach the large intestine in vivo.

It is essential to realize that bacterial metabolism of carbohydrates is different in pure and mixed cultures. The end products of one species can be used as a substrate by others, and some microorganisms may grow upon substrates that they are not able to ferment (*17*). Moreover, the high amounts of glucose and fructose in honey, which are metabolized in the gastrointestinal tract, can contribute toward the growth of bacteria using in vitro systems. Therefore, to test the prebiotic properties of honey oligosaccharides such compounds must be separated.

Separation of monosaccharides from honey oligosaccharides has been mainly carried out using charcoal−Celite columns (*18*, *19*); however, this procedure is considered to be quite "lengthy and cumbersome" (*5*). Other methods have been applied in the literature for the separation of oligosaccharides from different carbohydrate sources, such as nanofiltration and yeast treatments. Nanofiltration has been previously used for the separation of monosaccharides from a mixture of galactooligosaccharides (*20*). Yields of 19% (w/w) of monosaccharides and 88% (w/w) of di- and oligosaccharides were obtained from model systems following four filtration steps (*21*). During yeast treatment,

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carbohydrates, mostly monosaccharides and disaccharides, are converted to ethanol and CO_2. *Saccharomyces cerevisiae* is known to have a high specificity for removing some common mono- and disaccharides from complex carbohydrate mixtures (*22*). Such methods have not hitherto been applied to honey samples.

In this study, the effect of honey oligosaccharides upon the growth of fecal bacteria was studied using an in vitro fermentation system. This followed monosaccharide removal by nanofiltration, yeast treatment, and adsorption onto activated charcoal.

MATERIALS AND METHODS

Honey. Artisanal honeydew honey was directly obtained from beekeepers from the central region of Spain (Riaza, Segovia).

Standard Substances. Analytical standards of carbohydrates (cellobiose, fructose, β-phenyl-glucoside, glucose, isomaltose, maltose, maltotriose, melezitose, raffinose, sucrose, and α,β-trehalose) and organic acids (acetic, butyric, lactic, and propionic acids) were obtained from Sigma Chemical Co. (Poole, U.K.). Fructooligosaccharides (FOS; Raftilose P-95) were acquired from Orafti (Tienen, Belgium).

Separation Techniques. *Nanofiltration.* A discontinuous nanofiltration process was carried out following the method of Goulas et al. (*20*). Permeate solutes were cleared from the retentate through volume reduction, followed by redilution with water and re-nanofiltration in repetitive steps (*23*). A Gyrosep 300 stirred cell (Techmate Ltd., Milton Keynes, U.K.) was fitted with a 40 cm^2 DS-5-DL membrane (Osmonics Desal, Le Mee sur Seine, France). A PTFE-coated magnetic stirrer bar was centrally positioned, gripped on a stainless steel bar, and supported on the top plate. The filtration cell was placed in a water bath, equilibrated at 50 °C, and a pressure of 10 bar was applied to the system using nitrogen as the pressure source. Conditioning was performed prior to use by filtering 300 mL of demineralized water through the membrane at a constant pressure of 10 bar. Once equilibrated, 300 mL of 5% honey solution in HPLC water was applied to the cell, and after treatment, 150 mL of permeate was collected and the process stopped. The retentate was diluted to 300 mL with HPLC grade water, and 1 mL of both retentate and permeate was collected for further analysis. The process was repeated five times, and 1 mL of sample was taken each time. Following the last step, retentate was freeze-dried for subsequent treatments.

Yeast Treatment. Yeast treatment was based upon the method of Yoon et al. (*22*). A 20% (w/v) solution of honey in HPLC water was treated with 1% (w/v) *S. cerevisiae* (Allisons baker's yeast) at 37 °C for 48 h. Samples (1 mL) were taken at 0, 3, 6, 8, 18, 24, and 48 h, centrifuged at 7000*g* for 5 min, and filtered through 0.22 μm filters (Sartorius) to remove the yeast. The ethanol produced during fermentation was removed under nitrogen, and samples were freeze-dried for subsequent experiments.

Activated Charcoal Treatment. Oligosaccharides in honey sample were extracted using a method optimized in the laboratory (optimization data not shown). In brief, 0.5 g of honey was dissolved in 20 mL of deionized water and stirred with 3 g of activated charcoal Darco G-60, 100 mesh (Sigma Chemical Co., St. Louis, MO), in 250 mL of 10% ethanol in water for 30 min to remove mono- and disaccharides. This mixture was filtered under vacuum, and the activated charcoal was further washed with 25 mL of 10% (v/v) ethanol in water. Oligosaccharides adsorbed onto the activated charcoal were extracted by stirring for 30 min, in 250 mL of 50% (v/v) ethanol. Activated charcoal was eliminated by filtering through paper as previously described, and the ethanol was evaporated under vacuum at 30 °C. The remaining sample was filtered through a 0.22 μm filter (Millex GV) and freeze-dried.

Analytical Methods. All analyses were carried out in duplicate.

The water content of honey samples was determined following AOAC method 969.38 (*24*) using a refractometer (Abbe 60, Bellingham & Stanley Ltd., London, U.K.). A moisture content of 15.8% was obtained.

Analysis of Carbohydrates. A 0.5 g honey sample was diluted with 25 mL of 80% ethanol, and 2 mL of the solution was evaporated under vacuum.

Sample preparation for gas chromatography (GC) analysis was carried out by mixing 10 mg of carbohydrates with 1 mL of phenyl-β-D-glucoside (1 mg/mL) as internal standard and evaporation under vacuum. Sugar oximes were formed using 2.5% hydroxylamine chloride in pyridine (350 μL) at 75 °C for 30 min. Following the reaction, trimethylsilyl (TMS) derivatives of the oximes were obtained using 350 μL of hexamethyldisilazane (HMDS) and 35 μL of trifluoroacetic acid (TFA) at 45 °C for 30 min (*25*) followed by centrifugation at 7000*g* for 5 min at 5 °C (*26*).

Analysis of honey carbohydrates was carried out following the method of Sanz et al. (*1*) in an HP-5890 gas chromatograph equipped with a flame ionization detector. Separation was carried out using a 25 m × 0.25 mm i.d. × 0.25 μm film thickness fused silica column, coated with DB-1 (J&W Scientific, Folsom, CA). The injector and detector temperatures were 300 °C; the oven temperature was held at 200 °C for 20 min and then programmed to 270 °C at a heating rate of 15 °C min^{-1}, to 290 °C at 1 °C min^{-1}, and finally to 300 °C at 15 °C min^{-1} and held at that temperature for 40 min. Chromatographic peaks were measured using an HPChem acquisition system (Hewlet-Packard, Palo Alto, CA).

Peak identification was carried out by comparing their retention times with those of standard compounds and those previously published by Sanz et al. (*7*). Identity was also confirmed by mass spectrometry (MS). Quantitative values were calculated from FID peak areas. Standard solutions containing different proportions of each carbohydrate were prepared to calculate the response factor (RF) relative to phenyl-β-D-glucoside (internal standard). For di- and trisaccharide the RF was calculated as the mean value of the individual ones.

GC-MS analyses were carried out using the same capillary columns, installed in an HP-5890 gas chromatograph with an MD 5971 quadrupole mass detector (both from Hewlett-Packard) working in EI mode at 70 eV. Helium was used as the carrier gas, and injections were made in the split mode, with a split flow of 40 mL/min. Acquisition was performed using HPChem Station software (Hewlett-Packard).

Fermentation Studies. *In Vitro Fermentation Method.* A small scale in vitro fermentation method was used to study the growth of fecal bacteria in response to fermentation of the honey preparations. Ten milligrams of carbohydrates was dissolved in autoclaved nutrient basal medium to give a final concentration of 1% (w/v). This medium contained, per liter, 2 g of peptone water (Oxoid Ltd., Basingstoke, U.K.), 2 g of yeast extract (Oxoid), 0.1 g of NaCl, 0.04 g of K_2HPO_4, 0.01 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2 \cdot 6H_2O$, 2 g of $NaHCO_3$, 0.005 g of haemin (Sigma), 0.5 g of L-cysteine HCl (Sigma), 0.5 g of bile salts (Oxoid), 2 mL of Tween 80, 10 μL of vitamin K (Sigma), and 4 mL of 0.025% (w/v) resazurin solution. Samples were inoculated with 100 μL of fecal slurry, which was prepared by homogenizing fresh human feces (10%, w/v) in phosphate-buffered saline (PBS; 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, and 0.2 g/L KH_2HPO_4), pH 7.3 (Oxoid), with a manual homogenizer (Fisher, Loughborough, U.K.). Each fermentation experiment was carried out in triplicate and incubated at 37 °C. One sample was prepared, as a control, without any carbohydrate addition. All additions, inoculations, and incubations were carried out inside an anaerobic cabinet (10% H_2, 10% CO_2, 80% N_2). Samples were removed at 0 and 12 h of fermentation for enumeration of bacteria and short-chain fatty acid (SCFA) analysis.

Enumeration of Bacteria. Bacteria were counted using fluorescent in situ hybridization (FISH). Samples (100 μL) were fixed overnight at 4 °C with 4% (w/v) filtered paraformaldehyde (pH 7.2) in a ratio of 1:3 (v/v). Samples were washed twice with filtered PBS and resuspended in 200 μL of a mixture of PBS/ethanol (1:1, v/v) and then stored at −20 °C until further analysis. Hybridization of the samples was carried out as described by Rycroft et al. (*27*) using the appropriate genus-specific 16S rRNA-targeted oligonucleotide probes labeled with the fluorescent dye Cy3 (MWG Biotech) for the different bacteria or with the nucleic acid stain DAPI (4′,6-diamidino-2-phenylindole) for total cell counts. The probes used for each bacterial group, previously validated by different authors, were Bif164, specific for *Bifidobacterium* (*28*); Bac303, specific for *Bacteroides* (*29*); His150, for *Clostridium* (*histolyticum* subgroup; *30*); EREC482 for *Eubacterium* (*Clostridium coccoides-Eubacterium rectale* group; *30*); and Lab158, for *Lactoba-*

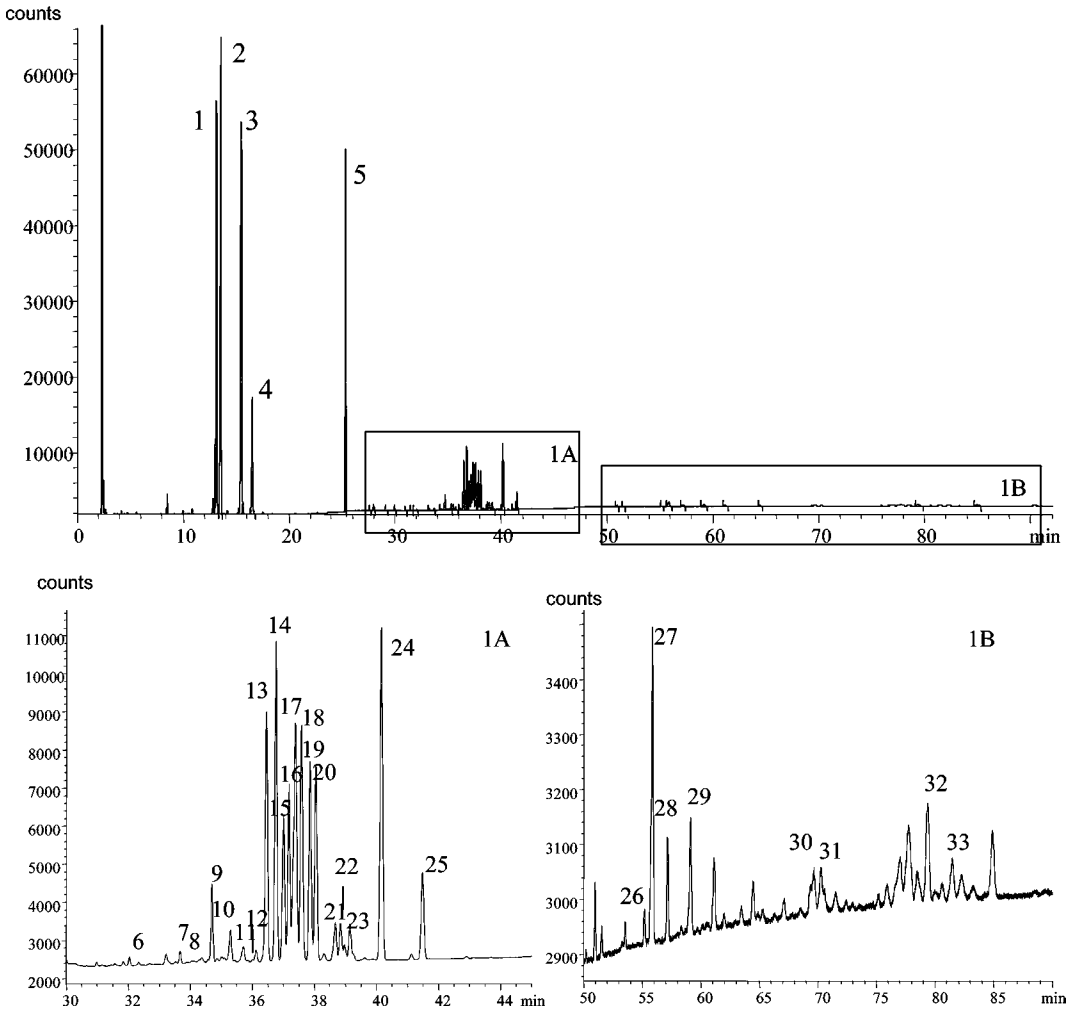

Figure 1. GC profile of TMS-oximes of carbohydrates in honeydew honey: (**A**) disaccharide fraction; (**B**) trisaccharide fraction. Peaks: (1) fructose 1; (2) fructose 2; (3) glucose (*E*, syn); (4) glucose (*Z*, anti*)*; (5) phenyl-β-D-glucoside (internal standard); (6) sucrose; (7) unknown; (8) α,α-trehalose; (9) α,β-trehalose; (10) unknown; (11) cellobiose (*E*); (12) unknown; (13) cellobiose (*Z*) + laminaribiose (*E*) + maltulose (*E*); (14) maltulose (*Z*); (15) nigerose (*E*) + leucrose 1 + unknown; (16) turanose 1 + leucrose 2; (17) laminaribiose (*Z*) + turanose 2 + maltose (*E*); (18) kojibiose (*E*); (19) maltose (*Z*) + trehalulose 1; (20) nigerose (*Z*) + trehalulose 2; (21) palatinose 1; (22) kojibiose (*Z*); (23) palatinose 2 (*Z*); (24) isomaltose (*E*); (25) isomaltose (*Z*); (26) raffinose; (27) 1-kestose; (28) erlose; (29) melezitose; (30) maltotriose (*E*); (31) maltotriose (*Z*); (32) panose (*E*); (33) panose (*Z*).

cillus/Enterococcus (*31*). The samples were then filtered onto 0.2 μm pore size filters (Millipore Corp., Watford, U.K.) and cells counted using a Nikon Eclipse E400 fluorescent microscope. A minimum of 15 random fields were counted on each slide.

Analysis of SCFA. Samples were centrifuged at 7000*g* for 5 min, and 20 μL was injected onto an HPLC system (Hewlett-Packard HP1050 series) equipped with a UV detector and automatic injector. The column was an ion-exclusion Aminex HPX-87H (7.8 $\times$ 300 mm, Bio-Rad, Watford, U.K.) maintained at 50 °C. The eluent was 0.005 mmol L^{-1} sulfuric acid in HPLC grade water, and the flow rate was 0.6 mL min^{-1}. Detection was performed at 210 nm, and data were acquired using Chem Station for LC3D software (Agilent Technologies). Quantification of the samples was carried out using calibration curves of acetic, propionic, butyric, and lactic acids in concentrations between 0.5 and 100 mM.

Statistical Analysis. Statistical analysis was performed using SPSS for Windows version 11.5. Univariate analysis of variance (ANOVA) and LSD test were also used to determine significant differences among the bacterial populations using the different samples. The differences were considered to be significant when $P < 0.05$.

RESULTS AND DISCUSSION

Separation and Characterization of Honey Oligosaccharides. Honeydew honeys present lower values of glucose and fructose and higher levels of oligosaccharides, mainly melezitose or erlose, than nectar honeys (*32*). Therefore, a honeydew honey sample was selected to increase the yield of oligosaccharides in order to study their effect upon the growth of fecal bacteria. **Figure 1** shows the GC profile of the TMS-oximes of mono-, di-, and trisaccharides of the selected honey sample. Disaccharide and trisaccharide elution zones (panels **A** and **B**, respectively, of **Figure 1**) were very complex, and coelution of some compounds was observed. Carbohydrates having glucose as the reducing moiety presented two well-resolved peaks; the major one, which eluted first, was assigned to the syn (*E*) isomer and the minor to the anti (*Z*) isomer, as based on the results found by Funcke and Von Sonntag (*33*). Carbohydrates having fructose as the reducing moiety gave two peaks with similar abundance and lower resolution, which did not allow assignation as syn and anti isomers (*34*). Carbohydrate composition of the honeydew honey is shown in **Table 1**; 70.9% of the total quantified carbohydrates corresponded to monosaccharides, whereas 26.6% were disaccharides and only 2.5% were assigned as trisaccharides.

Figure 2 shows the GC chromatogram of the TMS-oximes of the carbohydrates obtained following five discontinuous repetitions of the nanofiltration process. Although a diminution

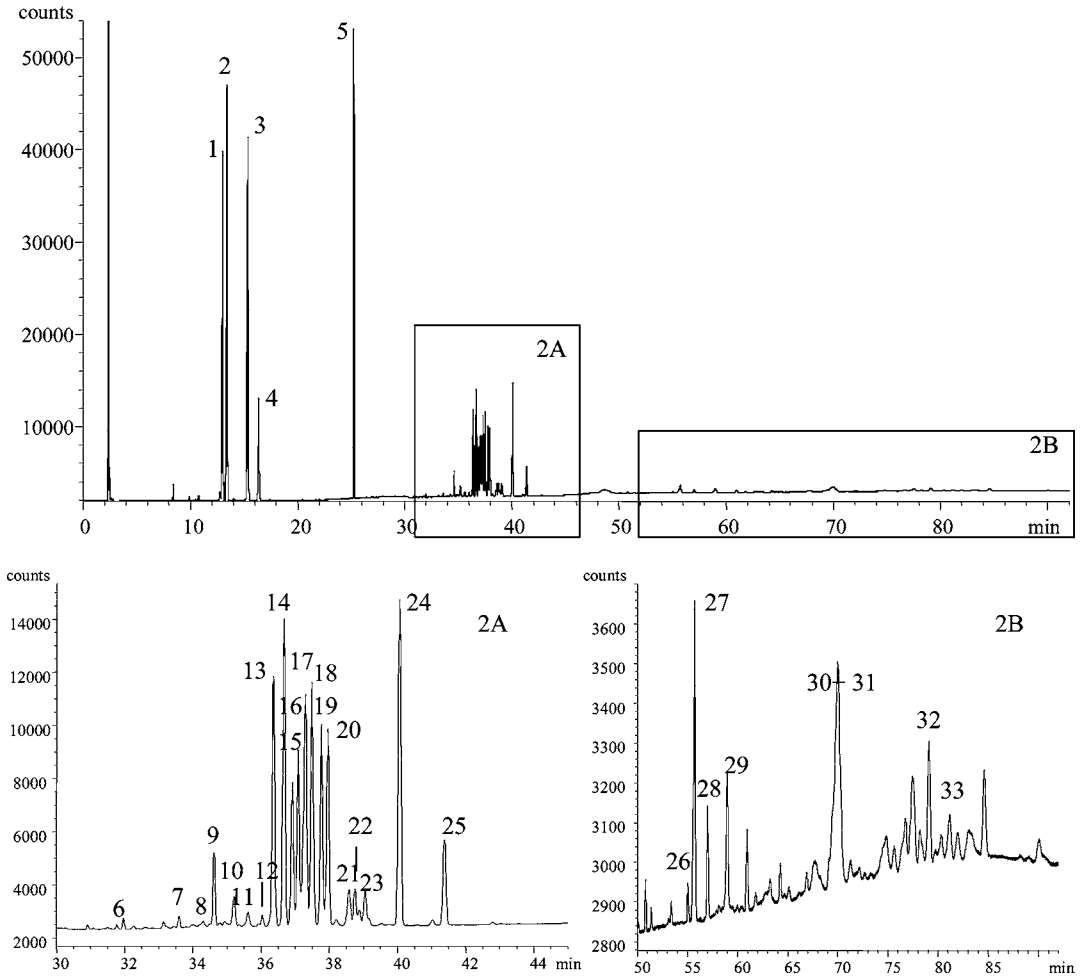

Figure 2. GC profile of TMS-oximes of carbohydrates in honeydew honey after five discontinuous repetitions of the nanofiltration process: (**A**) disaccharide fraction; (**B**) trisaccharide fraction.

Table 1. Mono-, Di-, and Trisaccharide Contents of Honeydew Honey Sample

carbohydrate	content (g/100 g of honey)
fructose	31.73 ± 1.15[a]
glucose	21.41 ± 0.65
disaccharides	19.93 ± 1.11
trisaccharides	1.90 ± 0.86

[a] Standard deviation.

of glucose and fructose peaks was observed, their presence was still detected. Decreases of 68 and 65% were observed for fructose and glucose, respectively, whereas disaccharides and trisaccharides were reduced by 30 and 14%, respectively. Goulas et al. (*20*), using a commercial galactooligosaccharide mixture and the same membrane but in a continuous system, obtained decreases of around 80% monosaccharides, 11% disaccharides, and only 2% of higher oligosaccharides. These differences can be attributed to the influence of the continuous system and also to the high concentration of monosaccharides present in honey. **Table 2** shows the concentration of carbohydrates of this honey fraction.

A previous study (*22*) showed that *S. cerevisiae* possesses a high specificity for removing some mono- and disaccharides. Therefore, the common bread yeast was used to remove glucose and fructose from the honey samples. After 18 h of treatment, almost 100% of monosaccharides was removed, and around 50% of disaccharides and 84% of trisaccharides were still remaining.

Table 2. Mono-, Di-, and Trisaccharides Contents of Oligosaccharide Fractions Obtained Following Nanofiltration Processing, Fermentation with Yeast, and Activated Charcoal Extraction

	carbohydrate content (g/100 g of product)		
	nanofiltration	yeast	charcoal
fructose	26.86 ± 1.44[a]	0.44 ± 0.01	2.51 ± 0.16
glucose	19.98 ± 1.12	0.56 ± 0.01	2.40 ± 0.14
disaccharides	37.09 ± 0.59	52.02 ± 0.45	36.83 ± 3.09
trisaccharides	4.35 ± 0.80	7.24 ± 0.86	39.83 ± 2.19

[a] Standard deviation.

Table 2 shows the final concentration of the sample. **Figure 3** shows the chromatogram of carbohydrates of a honey sample treated for 18 h with *S. cerevisiae*. The concentration of monosaccharide in the honey sample following treatment with yeast was lower than that with nanofiltration, and the concentration of di- and trisaccharides was higher. However, this treatment modifies the oligosaccharide composition of honey. As shown in **Figure 3A** some disaccharides decreased, for example, peaks 13, 14, 16, and 17, which consisted of cellobiose (*Z*) + laminaribiose (*E*) + maltulose (*E*), maltulose (*Z*), turanose 1 + leucrose 2, and laminaribiose (*Z*) + turanose 2 + maltose (*E*), respectively, whereas other compounds remained at the same levels, for example, peak 9, α,*β*-trehalose and peaks 24 and 25, isomaltose (*E*) and (*Z*). Moreover, α,α-trehalose (peak 8) increased in relation to the original honey. This carbohydrate is synthesized by *S. cerevisiae* and also can be used as a carbon

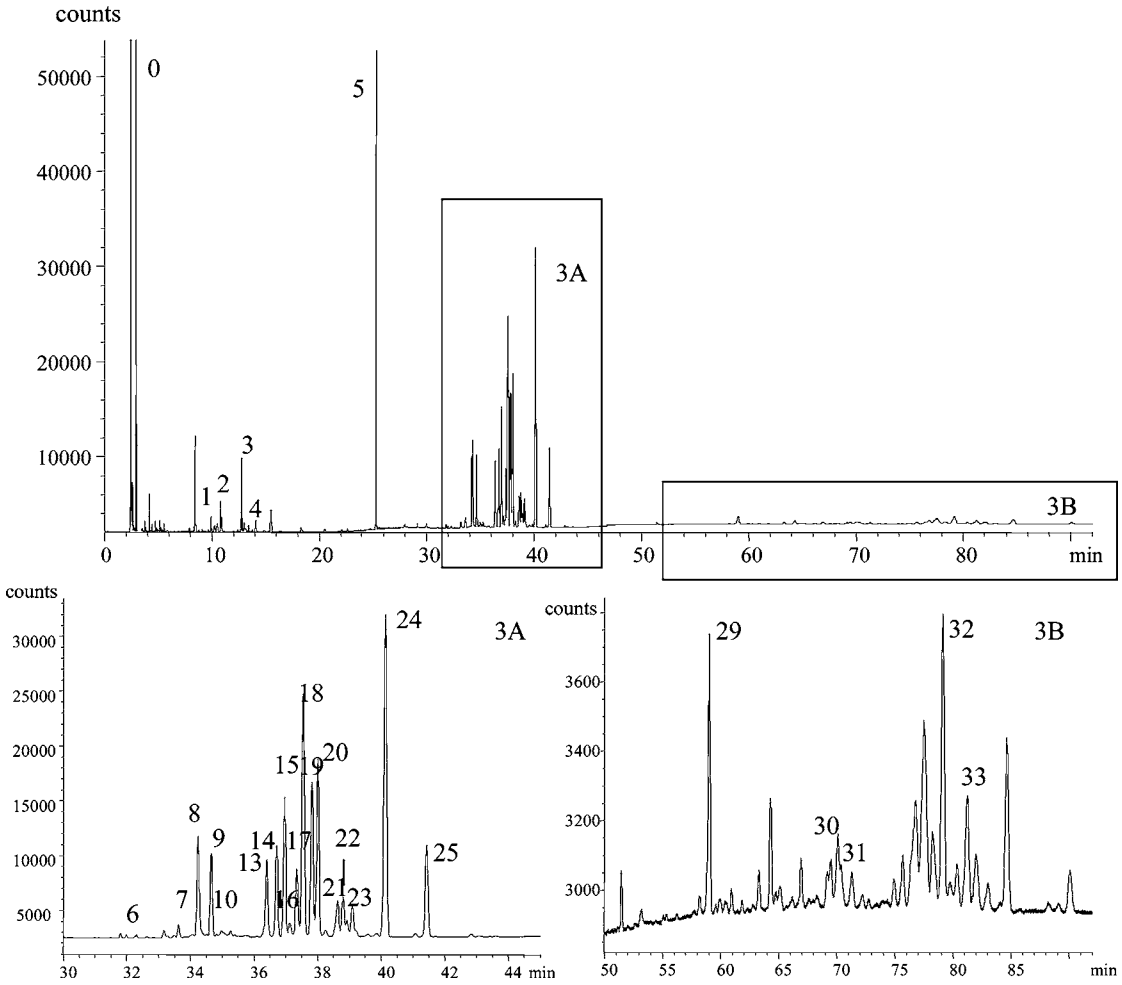

Figure 3. GC profile of TMS-oximes of carbohydrates in honeydew honey after 18 h of fermentation with yeast: (**A**) disaccharide fraction; (**B**) trisaccharide fraction.

source for growth (*35*, *36*). The high decrease of turanose and maltose during yeast treatment was previously described by Yoon et al. (*22*). Raffinose, 1-kestose, and erlose (peaks 26, 27, and 28, respectively) were completely removed, whereas melezitose (peak 29) was not fermented, as reported by Yoon et al. (*22*). According to these authors, none of the tetrasaccharides were removed by the action of the yeast. This sample also contained a large peak eluting at the beginning of the chromatogram (**Figure 3**, peak 0). This compound was identified through GC-MS as glycerol, which can be produced during the yeast fermentation process (*35*). The presence of glycerol in the carbohydrate sample may possibly have effects on the fecal microflora.

Figure 4 shows the GC chromatogram of the mono-, di-, and trisaccharides of a honey sample after treatment with activated charcoal. **Table 2** presents the concentration of these compounds in the honey fraction (grams per 100 g of product). During this treatment, most of the monosaccharides and part of the di- and trisaccharides were removed. A high concentration of trisaccharides (48.82% of total quantified carbohydrates) was detected with this method compared to nanofiltration and yeast treatment; 81.58% of the sample was shown to be carbohydrate by GC analysis. The remaining percentage (18.42%) probably corresponded to higher oligosaccharides not detected by GC. However, carbohydrate separation was also selective for some compounds. Peaks 9 and 15 (**Figure 4B**) showed higher recovery by activated charcoal than did other disaccharides. Peak 9 corresponded to α,*β*-trehalose, and peak 15 was a mixture of nigerose, leucrose, and an unknown compound. However, GC-MS analysis indicated that the remaining peak 15 corresponded to nigerose, which slightly decreased during charcoal treatment.

In Vitro Fermentation of Honey Oligosaccharides. Oligosaccharide fractions obtained from the three different separation methods, as well as the honeydew honey sample, FOS sample, and a mixture of glucose and fructose in the same proportions as found in honey, were incubated for 12 h with fecal bacteria. **Table 3** shows changes in bacterial populations during this treatment. No significant variations were detected in total bacteria and clostridia with any of the carbohydrate sources. However, generally, significant increases were detected for bifidobacteria, bacteroides, and lactobacilli with most of the carbohydrates tested.

A comparison of the effects of FOS, honey, and the mixture of glucose and fructose on bacterial changes was carried out. Similar values of lactobacilli were found using the three carbohydrate sources. No statistically significant differences were detected in the numbers of bacteroides, although the mixture of glucose and fructose resulted in the greatest value (8.94 log). However, significant variations were detected in the number of bifidobacteria among the three samples. The highest value corresponded to FOS, followed by the honey sample, whereas no growth of these bacteria was detected using the mixture of glucose and fructose. Although an increase in bifidobacterial populations with the honey sample was detected, these levels did not achieve the values seen in FOS fermentation, as indicated in previous studies with pure cultures (*16*). The

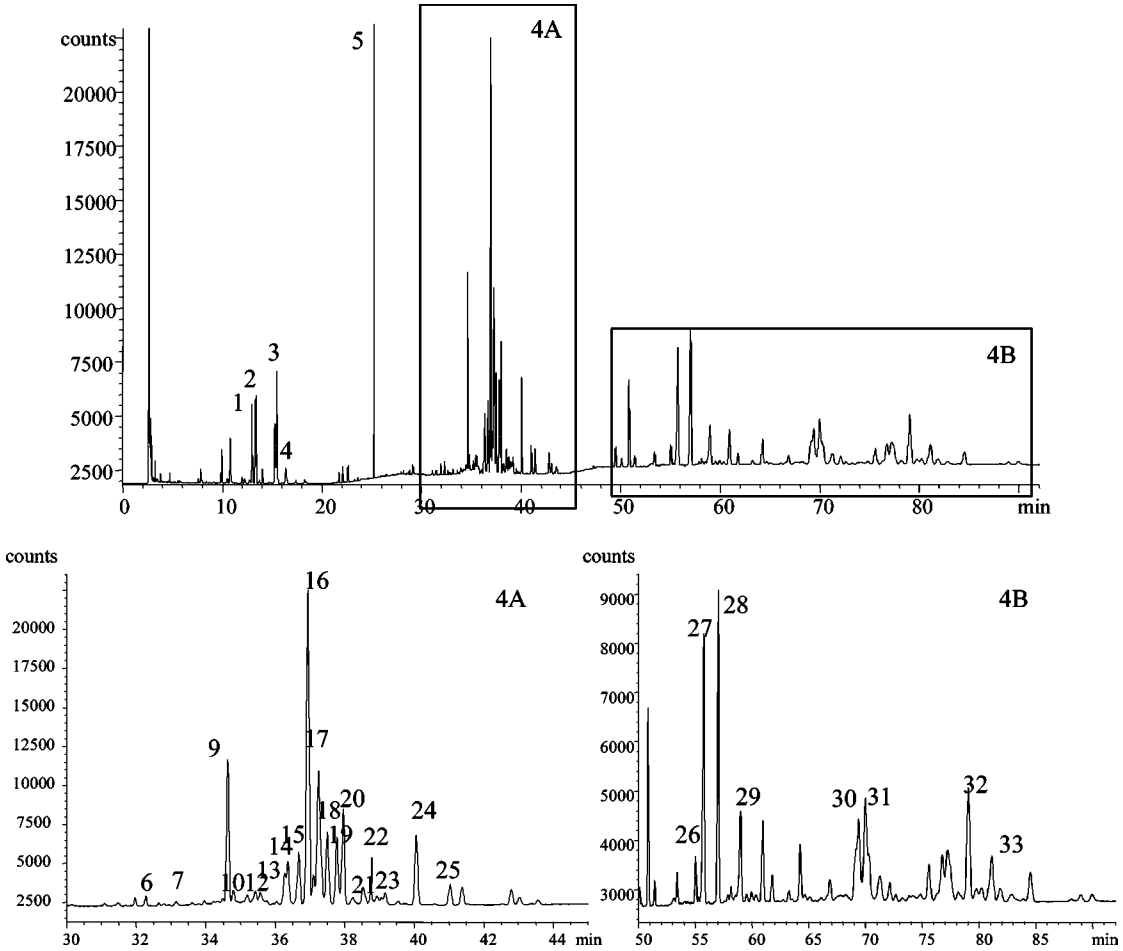

Figure 4. GC profile of TMS-oximes of carbohydrates in honeydew honey after fractionation on activated charcoal: (**A**) disaccharide fraction; (**B**) trisaccharide fraction.

Table 3. Changes in Bacterial Populations (log 10 Cells per Milliliter) in Batch Cultures after 12 h of Incubation in the Presence of Different Substrates and Prebiotic Index (PI) Scores for These Samples

sample	total bacteria	*Bifidobacterium*	*Bacteroides*	*Clostridium*	*Eubacterium*	*Lactobacillus*	PI
0 h	9.49 ± 0.12a[a,b]	8.19 ± 0.08ab	8.29 ± 0.13a	7.13 ± 0.30a	8.63 ± 0.04a	7.73 ± 0.17a	
control[c]	9.45 ± 0.13a	8.02 ± 0.15a	8.47 ± 0.14ac	7.28 ± 0.07a	8.68 ± 0.06a	7.47 ± 0.05a	–0.47
FOS	9.50 ± 0.04a	8.65 ± 0.13c	8.57 ± 0.25ab	7.13 ± 0.05a	8.36 ± 0.11ab	8.55 ± 0.08bc	6.89
glucose + fructose	9.47 ± 0.09a	8.20 ± 0.14ab	8.94 ± 0.13b	7.43 ± 0.38a	8.27 ± 0.21b	8.50 ± 0.09bc	0.67
honeydew honey	9.47 ± 0.07a	8.56 ± 0.15d	8.60 ± 0.22ab	7.19 ± 0.19a	8.43 ± 0.21ab	8.43 ± 0.25bc	5.62
fraction obtained by nanofiltration	9.53 ± 0.08a	8.43 ± 0.13bd	8.76 ± 0.28bc	7.32 ± 0.09a	8.30 ± 0.02ab	8.55 ± 0.19c	3.99
fraction obtained by yeast treatment	9.52 ± 0.17a	8.62 ± 0.09cd	8.52 ± 0.32ac	7.03 ± 0.34a	8.25 ± 0.41b	8.21 ± 0.25b	3.38
fraction obtained by charcoal extraction	9.62 ± 0.23a	8.49 ± 0.09d	8.77 ± 0.11bc	7.26 ± 0.06a	8.48 ± 0.12ab	8.61 ± 0.13c	4.24

[a] Different letters indicate significant differences ($P < 0.05$) for each bacterial genus. [b] Standard deviation. [c] Without carbohydrate source.

Table 4. Short-Chain Fatty Acid (SCFA) Concentrations (Millimolar) Produced by Honey Oligosacharide Fermentations

	lactic	acetic	propionic	butyric
0 h	0.00a[a]	1.19 ± 0.06[b]a	0.00a	0.00a
control[c]	0.00a	15.83 ± 0.24b	0.00a	2.44 ± 0.06b
FOS	13.77 ± 0.58b	28.51 ± 0.25c	22.49 ± 0.83b	5.35 ± 0.21c
glucose + fructose	4.16 ± 0.36c	18.94 ± 1.13d	19.40 ± 2.03c	7.72 ± 0.56d
honeydew honey	15.55 ± 0.18d	31.00 ± 0.31e	22.48 ± 0.22b	5.69 ± 0.17c
fraction obtained by nanofiltration	12.57 ± 0.54e	28.50 ± 0.35c	20.78 ± 0.49c	5.86 ± 1.02c
fraction obtained by yeast treatment	9.57 ± 0.71f	31.53 ± 0.28e	15.34 ± 0.31d	14.61 ± 0.42e
fraction obtained by charcoal extraction	13.21 ± 0.47be	35.51 ± 0.78f	17.62 ± 0.14e	9.94 ± 0.02f

[a] Different letters indicate significant differences ($P < 0.05$) for each acid. [b] Standard deviation. [c] Without carbohydrate source.

mixture of glucose and fructose might have been expected to give a similar effect to honey; however, significant differences were detected, probably due to the oligosaccharide composition of the honey. Ustunol and Gandhi (*37*) compared the effect of honey on the growth of pure bifidobacterial cultures to glucose, fructose, and sucrose as the carbohydrate source. They proposed honey as the preferred sweetener for use in skim milk products, increasing numbers of bifidobacteria, although the contents of

fructose and glucose could also contribute to it, suggesting a synergistic effect between the carbohydrates of honey. However, the effect of the mixture of glucose and fructose on the growth of bifidobacteria in this study was not significant.

With regard to the oligosaccharide fractions, similar population changes of lactobacilli were found with samples obtained by nanofiltration and charcoal treatment (**Table 3**). Significant differences were seen in the number of lactobacilli with the fraction obtained by yeast treatment, which presented the lowest value. However, this sample resulted in the highest bifidobacterial population corresponding to 8.62 log, which was a similar level to FOS (8.65 log). Nevertheless, no significant differences in numbers of bifidobacteria were detected among the honey fractions. Differences in lactobacilli resulting from the yeast-treated honey could be due to the different carbohydrate compositions. Some of the oligosaccharides present in the other honey fractions could influence the growth of lactobacilli and could have been hydrolyzed during yeast treatment.

To obtain a relative quantitative measure of all these changes in bacterial population, a prebiotic index (PI) was calculated (*38*, *39*). This measure gives a comparative relationship between the growth of beneficial fecal bacteria (for example, bifidobacteria, lactobacilli, and eubacteria) and the less desirable ones (for example, clostridia and bacteroides), related to the changes of the total number of bacteria (**Table 3**). FOS produced the greatest PI values, followed by the honeydew honey sample. The three oligosaccharide fractions resulted in similar PI values, the highest being for the charcoal fraction, which contained the greatest oligosaccharide content.

Table 4 shows the values obtained for SCFA in the cultures. Lactic and acetic acids are fermentation end-products of bifidobacteria (*40*). The mixture of glucose and fructose presented the lowest concentration of these acids, and also they were the carbohydrates that resulted in the lowest numbers of bifidobacteria. Honey, FOS, and the charcoal fraction showed the higher lactic acid values, and also this last sample presented the highest concentration of acetic acid. Butyric acid is not a major end-product of bifidobacteria or lactobacilli. It is generated mostly by clostridia and eubacteria (*41*). Butyrate is considered to be a desirable metabolite of gut bacterial function, and some in vitro studies have demonstrated that butyrate can induce apoptosis in colonic tumor cell lines (*42*). Highest butyrate production was detected with the yeast-treated honey, followed by the charcoal-derived oligosaccharide fraction and the glucose and fructose mixture, whereas honey, FOS, and the nanofiltration sample presented lower and similar levels.

On the basis of these data obtained through in vitro studies, oligosaccharides from honey seem to present potential prebiotic activity, increasing the populations of bifidobacteria and lactobacilli, albeit not to the levels seen with FOS. Recently, some honey oligosaccharides (1-kestose, neokestose, nystose, 6-kestose, raffinose, stachyose, isomelezitose, and fructosylisomelezitose) have been proven to be resistant to enzymes of an in vitro digestive system (stomach, pancreatic gland, and small intestine; *43*). However, more studies on the digestibility of honey oligosaccharides need to be performed.

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