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# Fecal Metabolomics Insights of Agavins Intake in Overweight Mice

*Alicia Huazano-García, Horacio Claudio Morales-Torres,  
Juan Vázquez-Martínez and Mercedes G. López*

## Abstract

Targeted and non-targeted metabolite profiling can identify biomarkers after a dietary treatment leading to a better understanding of interactions between diet and health. This study was conducted to establish enriched or depleted metabolites in the feces of overweight mice after a diet shift plus agavins or inulins supplementation, and their possible association with beneficial effects on host health. Thirty-eight male C57BL/6 mice were fed with a high-fat diet for 5 weeks followed by a diet shift to a standard diet supplemented with agavins (HF-ST + A) or inulins (HF-ST + I) for five more weeks. Feces were collected before and after prebiotic supplementation for metabolomics analyses. HF-ST + I group increased the fecal excretion of two methyl esters: linoleic and oleic acid, while HF-ST + A mice showed a substantial augment of 2-decenal, fructose, cyclohexanol, and the acids: 10-undecenoic, 3-phenyllactic, nicotinic, 5-hydroxyvaleric, and lactic. From the metabolites identified in HF-ST + A, only lactic acid has been reported previously and associated with beneficial effects on host health. However, the identification of new metabolites, coming from the microbial fermentation of agavins, opens opportunities to transform this information into practical solutions to tackle overweight and associated metabolic syndrome.

**Keywords:** agavins, branched neo-fructans, metabolomics, postbiotics, prebiotics, overweight, fecal metabolites, biomarkers

## 1. Introduction

In the last decade, an increasing number of studies have been strongly associated with a high-fat consumption altering the gut microbiota composition and/or its functionality [1, 2]. It has also been related to overweight and obesity as well as the metabolic syndrome [3–5]. Overweight and obesity not only affects the wellbeing of an individual but also places an unwanted economic burden on society [6]. Therefore, it is necessary and urgent to find an effective way to prevent and/or treat these worldwide pathologies. In this sense, prebiotics might be a good nutritional alternative in the management of overweight and obesity and its associated metabolic syndrome, since their supplementation or consumption can modulate the

gut microbiota producing a wide range of metabolites (postbiotics), consequently generating positive effects on host health [6, 7].

Agavins are relatively new prebiotics that in pre-clinical studies have shown several beneficial effects on the health of individuals [8–10]. Agavins are neo-fructans composed of complex and highly branched molecules with  $\beta(2-1)$  and  $\beta(2-6)$  linkages as well as an internal glucose unit [11, 12].

Our research group has evidenced that agavins can decrease glucose, triglycerides, and cholesterol concentrations as well as increase the anorexigenic peptide glucagon-like peptide1 (GLP-1; appetite-suppressing peptide) secretion on mice fed with a normal diet [8, 9]. Moreover, recently, we reported that agavins intake led to the reversion of metabolic disorders in overweight mice (induced by high-fat diet consumption) and also a substantial decrement of orexigenic peptide ghrelin (appetite-stimulating) and adipokines (leptin and insulin) levels in the portal vein, in such a way that all mice showed an integral improvement on their health [10].

On the other hand, due to the structural complexity of agavins, they cannot be degraded by endogenous gastrointestinal enzymes during their passage through the stomach and the small intestine; so, they reach the colon structurally unchanged, where they are fermented by the gut microbiota present in this organ [13, 14]. Fermentation of complex carbohydrates, such as agavins, might involve the collaboration of a highly diverse selection of gut microbes, which produce a myriad of different metabolites (postbiotics) that are suggested as key links in the communication between bacterial communities of the gut and the host [15, 16]. Nonetheless, only short-chain fatty acids (SCFA) such as acetate, propionate, butyrate, lactate, and succinate are among the metabolites reported up to now, derived from the agavins fermentation in *in vitro* and/or *in vivo* studies [9, 10, 17, 18]; the generation of these acids has been associated with different beneficial effects in the context of obesity, since SCFA reduce body weight gain, through G-protein-coupled receptors (GPRs), influencing the secretion of hormones involved in appetite control [19–21]. Moreover, SCFA are used as energy sources and may contribute to several metabolic pathways, including gluconeogenesis [22] and lipogenesis [23], thus contributing to whole-body energy homeostasis.

In spite of the above, other secondary metabolic products from the microbiota such as amino acids, nucleotides, bile acids, phenolic acids, fatty acids, and sterols, to mention some, can come from the agavins-microbiota interactions that have yet to be established. In the last decades, developed metabolomic tools have allowed researchers to study and characterize a wide range of metabolites in a non-invasive manner and also on biological systems, obtaining a large set of metabolites (metabolomics) that derive from gut microbes, enriched or depleted, after a dietary intervention [24]. This area of studies has been increased on the last decade, since this opens an opportunity to propose new biomarkers with new therapeutic approaches, through selective alterations of microbial production molecules to promote host health and prevent diseases [25].

In the present work, we established general and unique metabolites in overweight mice after agavins intake. We have previously showed that agavins consumption by overweight mice led to a gut microbiota modulation (these changes differed from those originated by inulins intake [14]); then, we hypothesized that agavins structure and the changes in the composition of gut microbiota in relation to inulins could lead to changes in colonic metabolic activity. The identification of microbial metabolites derived exclusively from agavins consumption may help to propose new biomarkers with huge potential and applicability on the prevention and/or treatment of overweight and their comorbidities.

## 2. Materials and methods

### 2.1 Animals and diets

Thirty-eight male C57BL/6 mice (12 weeks old at the beginning of the experiment) were obtained from Universidad Autonoma Metropolitana, Mexico city, Mexico) and housed in a temperature and humidity controlled room with a 12-h light–dark cycles. Mice were maintained in individual cages and subject to two experimental phases, to gain and loss weight, respectively. In the first phase, mice were fed with a high-fat diet (n = 30; 58Y1 Test Diet, St. Louis, MO, USA) for 5 weeks to induce overweight in the animals. In the second phase, overweight mice (HF) were shifted to the standard diet (5053 Lab Diet, St. Louis, MO, USA) alone (HF-ST; n = 8) or supplemented with agavins (HF-ST + A; n = 8) or inulins (HF-ST + I; n = 8) for five more weeks. Moreover, we had a healthy control group of mice (ST; n = 8), which were fed with the standard diet (5053 Lab Diet, St. Louis, MO, USA) throughout the experiment.

The high-fat diet (58Y1 Test Diet) had 20.3% calories from carbohydrates (16.15% maltodextrin, 8.85% sucrose, and 6.46% powdered cellulose), 18.1% from proteins, and 61.6% from fat (31.7% lard and 3.2% soybean oil), whereas the standard diet (5053 Lab Diet) contained 62.4% calories from carbohydrates (28.6% starch, 3.24% sucrose, 1.34% lactose, 0.24% fructose, and 0.19% glucose), 24.5% from proteins, and 13.1% from fat.

Food and water were provided *ad libitum* along the experiment. Mice experiments were conducted according to the Mexican Norm NOM-062-ZOO-1999 and approved by the Institutional Care and Use of Laboratory Animals Committee from Cinvestav-Mexico (CICUAL; protocol number 0091-14).

### 2.2 Agavins and inulins

Agavins from 4-year-old *Agave tequilana* Weber blue variety plants were extracted and purified in our laboratory and presented an average degree polymerization (DP) of 8, whereas inulins (oligofructose) was bought from Megafarma® (Mexico city, Mexico) and possess an average DP of 5. Agavins and inulins were added in the water at a concentration of 0.38 g/mouse/day [10].

### 2.3 Feces collection and preparation for untargeted and organic acids metabolic analyses

Feces were collected from each mouse at the end of the first and second experimental phases, before and after prebiotic supplementation, respectively. The feces of mice were pooled by treatment, lyophilized, triturated, and homogenized to generate fecal metabolites profiles. Untargeted metabolic analysis was carried out following a method adjusted from Eneroth et al. [26] and Gao et al. [27] as follows: 100 mg of feces were extracted three times with chloroform/methanol (2:1), 1 mL each time. After that, the extracts were combined and solvent freed. The residue was resuspended in 1 mL of chloroform/methanol (2:1) and an aliquot of 50 µL was transferred to a vial. The aliquot was solvent freed under nitrogen flux and then was derivatized using BSTFA with 1% TCMS (80 µL) and pyridine (20 µL) at 80°C for 25 min. Once the system was at room temperature, isooctane was added to a final volume of 200 µL. Heptadecanoic acid, at final concentration of 3 mg/mL, was used as internal standard.

On the other hand, extraction of organic acids was performed according to García-Villalba et al. [28]. Briefly, 100 mg of feces were suspended in 1 mL of



aqueous 0.5% phosphoric acid solution and mixed in vortex for 2 min. After that, samples were centrifuged for 10 min at 10,000 g. Then, the supernatant was transferred to a vial and was extracted with an equal volume of ethyl acetate. 2-Methyl valeric acid was used as internal standard at final concentration of 2 mM. This system was centrifuged for 10 min at 10,000 g, and then 200  $\mu$ L of the ethyl acetate phase were transferred to a vial, dried under nitrogen flux, and derivatized using 80  $\mu$ L of BSFTA with 1% TMCS and 20  $\mu$ L of pyridine. The mix was allowed to react at 80°C for 25 min. After the mix was at room temperature, isooctane was added to a final volume of 200  $\mu$ L.

## **2.4 Gas chromatography/mass spectrometry analysis**

For GC/MS analysis, 1  $\mu$ L of the organic phase was injected in the pulsed-splitless mode. Injector temperature was set to 260°C. A HP-5-MS capillary column (30 m  $\times$  25  $\mu$ m  $\times$  0.25  $\mu$ m) was used with helium as carrier gas at constant flow rate of 1 mL/min. Oven program began at 40°C (held 5 min), then increased at rate of 6°C/min until 170°C, then a second temperature ramp of 12°C/min until 290°C was applied. Transfer line temperature was set at 260°C. Mass spectrometer operated at 70 eV of electron energy, quadrupole and ion-source temperatures were 150 and 230°C, respectively. Data were obtained scanning from 40 to 550 m/z, while MassHunter Workstation software version B.0.0.6 (Agilent Technologies, Inc.) was used to collect the data. Components mass spectra and retention times were obtained using the AMDIS (automated mass spectral deconvolution and identification system, <http://www.amdis.net/>) software. Compounds identification was achieved by comparing their respective extracted mass spectrum with the mass spectra of the standards and/or with the mass spectra data of the NIST library and software (National Institute of Standards and Technology, USA).

## **2.5 Statistics and data analysis**

Results are present as mean  $\pm$  standard deviation. Differences between the diets were determined using one-way ANOVA followed by a Tukey post hoc test or a Dunnett T3 post hoc test. Differences were considered significant when  $p < 0.05$ . Statistical analyses were performed using the IBM SPSS Statistics software version 22. Principal component analysis and heatmap were conducted using a language and environment for statistical computing R and the ade4 and gplots packages.

## **3. Results and discussion**

Previous studies carried out by our research group evidenced that agavins intake led to improvement on health and wellness of the host, which has been associated with gut microbiota modulation and their metabolic products such as SCFA [10, 14]; nonetheless, other bioactive chemical compounds coming from agavins fermentation that could also contribute with the beneficial agavins consumption effects are unknown yet. In the present work, we performed a metabolomics analysis to establish and propose general and unique metabolites (postbiotics) in the feces of overweight mice after agavins (prebiotic) intake. Since it has been recommended the use of combination of methodologies to extend the metabolic coverage of microbiota [29], we performed an untargeted metabolic as well as organic acids profile analyses to carry out this task.

3.1 Untargeted metabolic profiles

Untargeted metabolic analysis showed a total of 300 metabolites, from which only 109 were identified completely. Those 109 compounds mainly included fatty acids and their esters, carbohydrates, sterols, alcohols, alkanes, SCFA, aldehydes, amino acids, nucleobases, bile salts, and phenylpropanoids (**Table 1**).

Of these 109 compounds (**Table 1**), 32 presented a significantly differential abundance between the different evaluated diets (Tukey’s test,  $p < 0.05$ ; **Table 2**). These 32 metabolites were grouped mostly in fatty acids and sterols and subsequently used for the principal component analysis (PCA) and heat map construction.

PCA was applied to the data to investigate metabolomics changes derived from agavins consumption. The variance explained by each principal component (PC) is displayed on the X and Y axes. PC1 and PC2 account for 62.5 and 20.5% of the variance, respectively. Very clear and separated clusters were observed among overweight mice (HF) and the other mice groups, suggesting differences on fecal metabolomics profiling (**Figure 1A**). In addition, mice that received agavins supplement are clearly separate, into a distinct cluster, from rodents fed with inulins supplementation. Interestingly, HF-ST + A group appear very close to standard diets (HF-ST and ST), evidencing a large similarity on metabolites among them compared to HF-ST + I group (**Figure 1A**).

Moreover, the loading plot illustrates the variables (metabolites) that are responsible for the discrimination (clustering of the samples) observed in the PCA plot. Then, according to the loading plot, HF and HF-ST + I groups shared the PC2 due to high content of oleic acid, cholesterol, and stigmasterol in the feces (**Figure 1B**).

ID	RT	Compound	Family
1	12.62	Cyclohexanol	Alcohol
2	13.028	Carbonic acid	Others
3	13.183	$\beta$ -Hydroxybutyric acid	$\beta$ OH-SCFA
4	13.234	Heptanoic acid	FA
5	13.281	$\alpha$ -Hydroxyvaleric acid	$\alpha$ OH-SCFA
6	14.008	Benzaldehyde, 2,5-dimethyl-	Aldehyde
7	14.049	L-Valine	Amino acid
8	14.483	Urea	Others
9	14.623	2-Decenal, (E)-	Aldehyde
10	14.94	Glycerol	Polyalcohol
11	15.074	2,5-dihydroxy hexane, 2,5-dimethyl-	Polyalcohol
12	15.406	Succinic acid	DiCAc
13	15.778	Uracil	Nucleobase
14	16.153	Butane, 1,2,4, triol	Polyalcohol
15	16.646	Thymine	Nucleobase
16	16.774	Hydrocinnamic acid	PhePr
17	17.096	Bicyclo[2.2.1]heptane-1-carboxylic acid, 7-Hydroxy, methyl ester	Ester
18	17.258	Decanoic acid	FA
19	17.55	Decane, 2,3,5,8-tetramethyl-	Alkane

ID	RT	Compound	Family
20	17.666	Dodecane, 4,6-dimethyl-	Alkane
21	17.741	Dodecane, 2,6,11-trimethyl-	Alkane
22	17.95	2,4-Ditert-butylphenol	Phenolic
23	18.011	1-Butene, 1-phenyl-3-(hydroxy)-, E	Ar
24	18.168	Pyroglutamic acid	Amino acid
25	18.422	2,6-Ditert-butylphenol	Phenolic
26	18.642	Pentadecane	Alkane
27	18.637	Undecanoic acid	FA
28	18.724	Heptadecane, 2,6,10,15-tetramethyl-	Alkane
29	19.173	<i>m</i> -Hydroxyphenylacetic acid	OH-Ar-SCFA
30	19.228	Cyanuric acid	Triazine
31	19.428	D-Arabinose	CHO
32	19.52	<i>p</i> -Hydroxyphenylacetic acid	OH-Ar-SCFA
33	19.599	<i>n</i> -Dodecanoic acid	FA
34	20.172	Hexadecane, 2,6,11,15-tetramethyl-	Alkane
35	20.204	Xylulose	CHO
36	19.153, 20.341	D-Mannose	CHO
37	20.438	10-Undecenoic acid	FA
38	20.51	Benzenepropanoic acid	PhePr
39	21.051	Glycerol phosphate	Others
40	21.159	Tetradecanoic acid, 12-methyl-, methyl ester	FAME
41	21.225	Azelaic acid	DiCAc
42	19.549, 21.389	D-Galactose	CHO
43	21.583, 21.652	D-Fructose	CHO
44	21.345	Tetradecanoic acid	FA
45	21.878	Inositol	Polyalcohol
46	22.039	Adenine	Nucleobase
47	22.352, 22.436	Methyl-tetradecanoic acid isomers (C15 fatty acid isomers)	FA
48	22.716	Pentadecanoic acid	FA
49	21.966, 22.483, 23.336	D-Glucose	CHO
50	23.428	<i>cis</i> -9-Hexadecenoic acid	FA
51	23.481	<i>trans</i> -9-Hexadecenoic acid	FA
52	23.691	Hexadecanoic acid	FA
53	24.128	Linoleic acid, methyl ester	FAME
54	24.177	Oleic acid, methyl ester	FAME
55	24.37	<i>cis</i> -10-Heptadecenoic acid	FA
56	24.399	Stearic acid, methyl ester	FAME
57	24.404	$\alpha$ -D-glucose, 2-(acetylamino)-2-deoxy	CHO
58	25.102	5-Hydroxyindoleacetic acid	IndolAc

ID	RT	Compound	Family
59	25.198	Linoleic acid	FA
60	25.25	Oleic acid	FA
61	25.298	<i>trans</i> -11-Octadecenoic acid	FA
62	25.315	<i>cis</i> -11-Octadecenoic acid	FA
63	25.481	Stearic acid	FA
64	26.262	2-O-glycerol- $\alpha$ -D-galactose	CHO
65	26.288	Nonadecanoic acid	FA
66	26.546	Arachidonic acid	FA
67	26.648	tert-Hexadecanethiol	Thiol
68	26.716	Tetratriacontane	Alkane
69	26.743	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	Alkane
70	26.774	Succinylacetone	Others
71	26.838	Hentriacontane	Alkane
72	26.9	Oleamide	Amide
73	27.006	Sebacic acid	DiCAc
74	27.095	Eicosanoic acid	FA
75	27.509	1-O-hexadecylglycerol	Glycerol-ether
76	27.584	Heneicosanoic acid	FA
77	27.618	Propyl myristate	Ester
78	27.716	2-Octadecenoic acid	FA
79	27.866	Heneicosanoic acid	FA
80	28.498	4-n-octadecylcyclohexane, 1,3,5-trimethyl-	Alkane
81	28.62	Docosanoic acid	FA
82	28.76	$\alpha$ -Hydroxy sebacic acid	$\alpha$ OH-DiCAc
83	28.967	1-O-Octadecylglycerol	Glycerol-ether
84	29.01	<i>cis</i> -4-Tetradecene, 2-methyl-	Alkene
85	29.339	Tricosanoic acid	FA
86	29.578	1-Monooleoylglycerol	Monoglyceride
87	29.709	1-Docosanol	Alcohol
88	29.895	<i>cis</i> -15-Tetracosenoic acid	FA
89	28.125, 28.661, 29.646, 29.932	Disaccharides (including sucrose)	CHO
90	30.026	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	Alkane
91	30.221	Enterolactone	Lignin
92	30.329	1-O-hexadecylglycerol	Glycerol-ether
93	30.383	Tricosanol	Alcohol
94	30.428	Cholesta-2,4-diene	Sterol
95	30.673	Cholesta-3,5-diene	Sterol
96	31.31	$\beta$ -Tocopherol	Vitamin
97	31.454	Hexacosanoic acid	FA
98	31.891	Coprostan-3-ol	Sterol



ID	RT	Compound	Family
99	32.551	$\alpha$ -Tocopherol	Vitamin
100	32.768	Cholesterol	Sterol
101	33.336	Lanosterol	Sterol
102	33.852	Campesterol	Sterol
103	34.207	Stigmasterol	Sterol
104	34.346	Chenodeoxycholic acid	Bile salt
105	34.563	Xi-Ergost-7-ene, 3 $\beta$ -	Sterol
106	34.918	$\beta$ -Sitosterol	Sterol
107	35.064	24-Ethylcoprostanol	Sterol
108	35.268	<i>trans</i> -Dehydroandrosterone	Steroid
109	38.518	Urs-12-en-28-al, 3-(acetyloxy)-, (3 $\beta$ )-	Sterol

Some metabolites have more than one retention time (RT) due to the presence of isomers. SCFA, short-chain fatty acid; FA, fatty acid; FAME, fatty acid methyl ester; CHO, carbohydrate;  $\alpha$ OH, alfa-hydroxy;  $\beta$ OH, beta-hydroxy; DiCAc, dicarboxylic acid; PyrCAc, pyridine carboxylic acid;  $\omega$ OH, omega-hydroxy; Cy, cyclic; Ar, aromatic; PhePr, phenylpropanoid; TCA, tricarboxylic acid; IndolAc, indolic acid.

**Table 1.**  
Metabolites identified in the feces of mice.

ID	RT	Compound	Family	Fold change			
				HF	HF-ST	HF-ST + A	HF-ST + I
1	12.62	Cyclohexanol	Alcohol	−1.00	0.10	0.57	−0.13
3	13.183	$\beta$ -Hydroxybutyric acid	$\beta$ OH-SCFA	−0.62	−0.48	0.15	0.34
4	13.234	Heptanoic acid	FA	−0.88	−0.39	−0.13	−0.13
5	13.281	$\alpha$ -Hydroxyvaleric acid	$\alpha$ OH-SCFA	−0.30	−0.18	0.56	1.10
9	14.623	2-Decenal, (E)-	Aldehyde	−1.00	0.18	1.79	−0.90
10	14.94	Glycerol	Polyalcohol	−0.90	−0.15	−0.16	0.01
12	15.406	Succinic acid	DiCAc	−1.00	−0.27	1.07	2.25
16	16.774	Hydrocinnamic acid	PhePr	−1.00	−0.15	−0.30	0.05
22	17.95	2,4-Ditert-butylphenol	Phenolic	−0.40	0.39	0.52	−0.16
24	18.168	Pyroglutamic acid	Amino acid	−0.78	0.00	0.17	1.29
32	19.52	<i>p</i> -Hydroxyphenylacetic acid	Ar-acid	−0.75	−0.27	−0.36	0.19
37	20.438	10-Undecenoic acid	FA	−0.75	0.01	1.03	−0.68
38	20.51	Benzenepropanoic acid	PhePr	−0.95	−0.28	−0.32	−0.24
39	21.051	Glycerol phosphate	Others	−0.98	−0.07	−0.16	0.24
40	21.159	Tetradecanoic acid, 12-methyl, methyl ester	FAME	−0.31	0.02	0.58	2.27
43	21.583 21.652	D-Fructose	CHO	−0.93	−0.88	0.36	−0.57
44	21.345	Tetradecanoic acid	FA	−0.57	0.12	0.01	0.18
46	22.039	Adenine	Nucleobase	−0.92	−0.34	0.08	0.36
48	22.716	Pentadecanoic acid	FA	−0.68	−0.12	−0.04	0.48

ID	RT	Compound	Family	Fold change			
				HF	HF-ST	HF-ST + A	HF-ST + I
49	21.966 22.483 23.336	D-Glucose	CHO	−0.98	−0.40	0.02	0.47
53	24.128	Linoleic acid, methyl ester	FAME	−1.00	0.02	0.01	2.94
54	24.177	Oleic acid, methyl ester	FAME	−1.00	−0.07	−0.15	1.90
56	24.399	Stearic acid, methyl ester	FAME	−0.60	−0.15	0.02	1.15
58	25.102	5-Hydroxyindoleacetic acid	IndolAc	−1.00	0.57	0.86	1.38
59	25.198	Linoleic acid	FA	−0.71	−0.12	−0.41	0.34
60	25.25	Oleic acid	FA	0.34	−0.09	−0.35	0.18
81	28.62	Docosanoic acid	FA	−0.55	−0.01	−0.03	0.25
98	31.891	Coprostan-3-ol	Sterol	−0.08	0.21	0.54	1.53
100	32.768	Cholesterol	Sterol	0.52	−0.09	−0.35	0.27
103	34.207	Stigmasterol	Sterol	−0.13	−0.17	−0.47	0.04
106	34.918	β-Sitosterol	Sterol	−0.75	−0.14	−0.44	−0.04
107	35.064	24-Ethylcoprostanol	Sterol	−0.86	−0.12	−0.29	−0.09

Fold change value was calculated by comparison with the healthy mice fed with a standard diet (ST). HF, overweight mice; HF-ST, overweight mice that were switched to a standard diet; HF-ST + A, overweight mice changed to standard diet plus agavins; HF-ST + I, overweight mice changed to standard diet plus inulins. All the metabolites listed here have significant difference at least in one treatment  $p < 0.5$ . ID numbers correspond with those of **Table 1**. SCFA, short-chain fatty acid; FA, fatty acid; FAME, fatty acid methyl ester; CHO, carbohydrate; αOH, alfa-hydroxy; βOH, beta-hydroxy; DiCAc, dicarboxylic acid; Ar, aromatic; PhePr, phenylpropanoid; IndolAc, indolic acid.

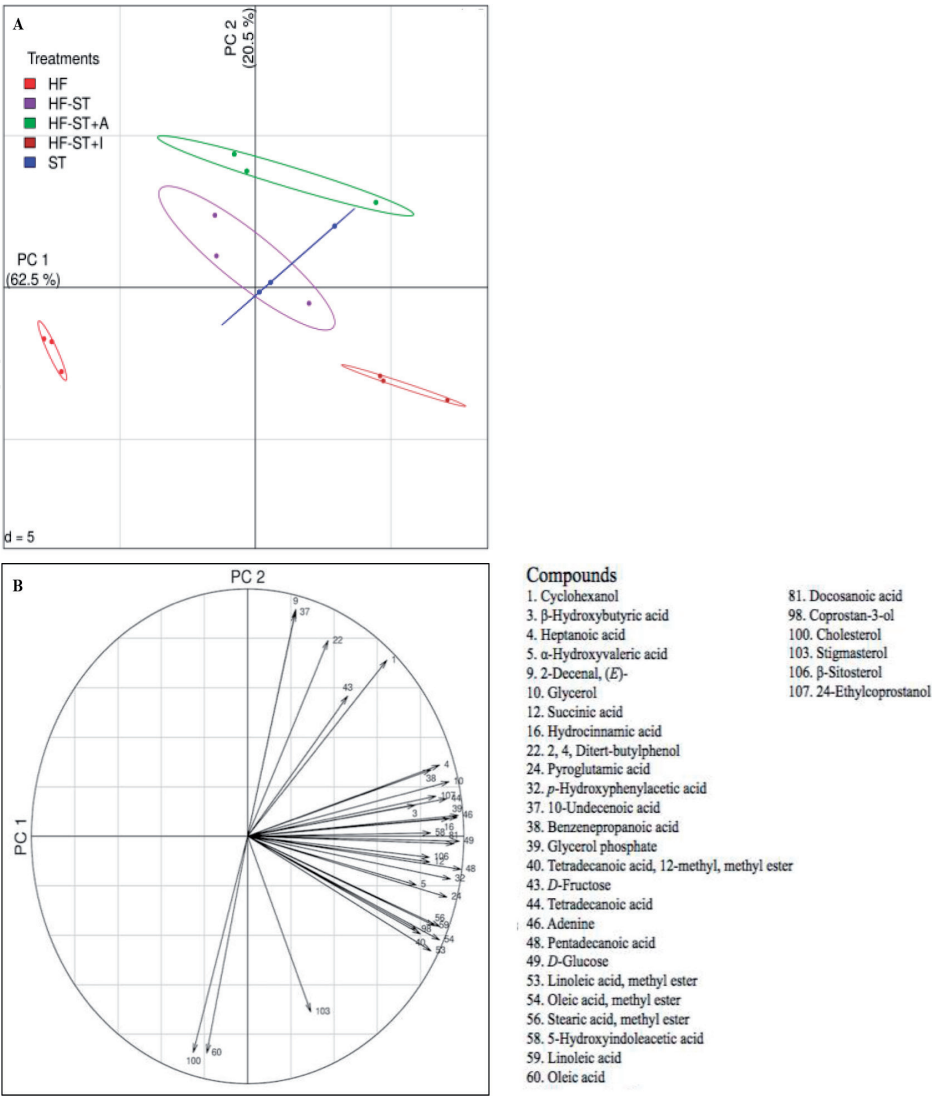
**Table 2.**  
Fold-change of differential metabolites detected in the feces of overweight mice after a diet switch and prebiotic supplementation.

Besides, hierarchical clustering analysis (**Figure 2**) revealed that HF group had a very low content of most identified metabolites, with exception of cholesterol and oleic acid. In addition, the bile salt chenodeoxycholic acid was detected exclusively in the HF treatment, and although it was not included in the hierarchical analysis since it was not detected in any other treatment, this metabolite could be used a biomarker for HF diet.

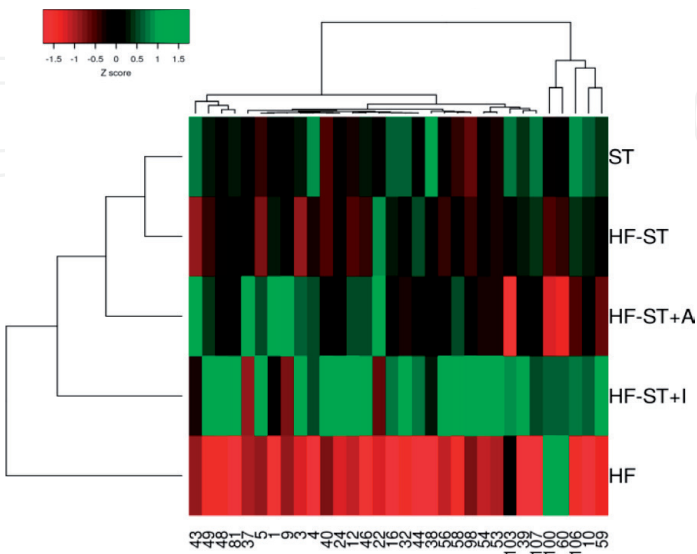
Interestingly, only HF-ST + I mice presented a high content of cholesterol and oleic acid in its feces; therefore, in the heatmap, this group appears very close to HF (**Figure 2**). Moreover, HF-ST treatment is closely linked to ST group due to similar content of all evaluated compounds. While, HF-ST + A group is located as the link between HF-ST + I and the cluster of standard diets (HF-ST and ST **Figure 2**).

In contrast to HF-ST group, HF-ST + A and HF-ST + I exhibited an enrichment of the following acids: succinic, β-hydroxybutyric (BHB), α-hydroxyvaleric, and pyroglutamic; as well as 12-methyl-tetradecanoic acid methyl ester and adenine, which could be used as biomarkers for mice groups with prebiotics.

On the other hand, HF-ST + A mice showed the highest content of 2-decenal, 10-undecenoic acid (UDA), cyclohexanol, and fructose as well as the lowest levels of oleic acid and cholesterol compared to HF, HF-ST, and HF-ST + I groups; hence, these metabolites might be used as biomarkers for agavins prebiotic. While, HF-ST + I mice had an increment of three methyl esters: linoleic, oleic, and stearic



**Figure 1.** Metabolites enriched or depleted in the feces of overweight mice after a diet shift and prebiotic supplementation. (A) PCA and (B) loading plot of the two first PCs. Overweight mice (HF) after a diet change (HF-ST) and agavins (HF-ST + A) or inulins (HF-ST + I) supplementation. ST was a healthy mice group.



**Figure 2.** Heatmap of differential metabolites found in the feces of overweight mice after a diet shift and prebiotic supplementation. HF, overweight mice; HF-ST, overweight mice after a diet shift; HF-ST + A, overweight mice that were switched to standard diet plus agavins supplement; HF-ST + I, overweight mice that were switched to standard diet plus inulins supplement. ST was a healthy mice group.

acid in relation to the other mice groups. These results evidence a clear difference in the fecal metabolites profiles between agavins and inulins, which was associated to their structural differences, such as the presence of fructose observed exclusively in HF-ST + A group. Agavins structure presents at least four terminal fructose units, so that some gut bacteria might start breaking down this prebiotic releasing fructose and agavins of smaller degree of polymerization [30]. In addition, 2-decenal and UDA were the metabolites mostly enriched with agavins intake. 2-decenal has a broad antimicrobial spectrum against pathogenic bacteria [31], while UDA is a neuroprotectant compound [32, 33] used as a nutritional supplement for maintaining a healthy balance of gut microbiota [34]. Besides, UDA also might be acting through GPR84 (a newly described medium chain fatty acid receptor) associated to immunological responses [35], and this mechanism could also contribute to improve the host health. Whereas in comparison to agavins, inulins led to a significant increase of methyl esters and sterols, coinciding with previous studies which proposed this event as a mechanism to improve the lipid metabolism of host [36, 37].

### 3.2 Organic acid profiles

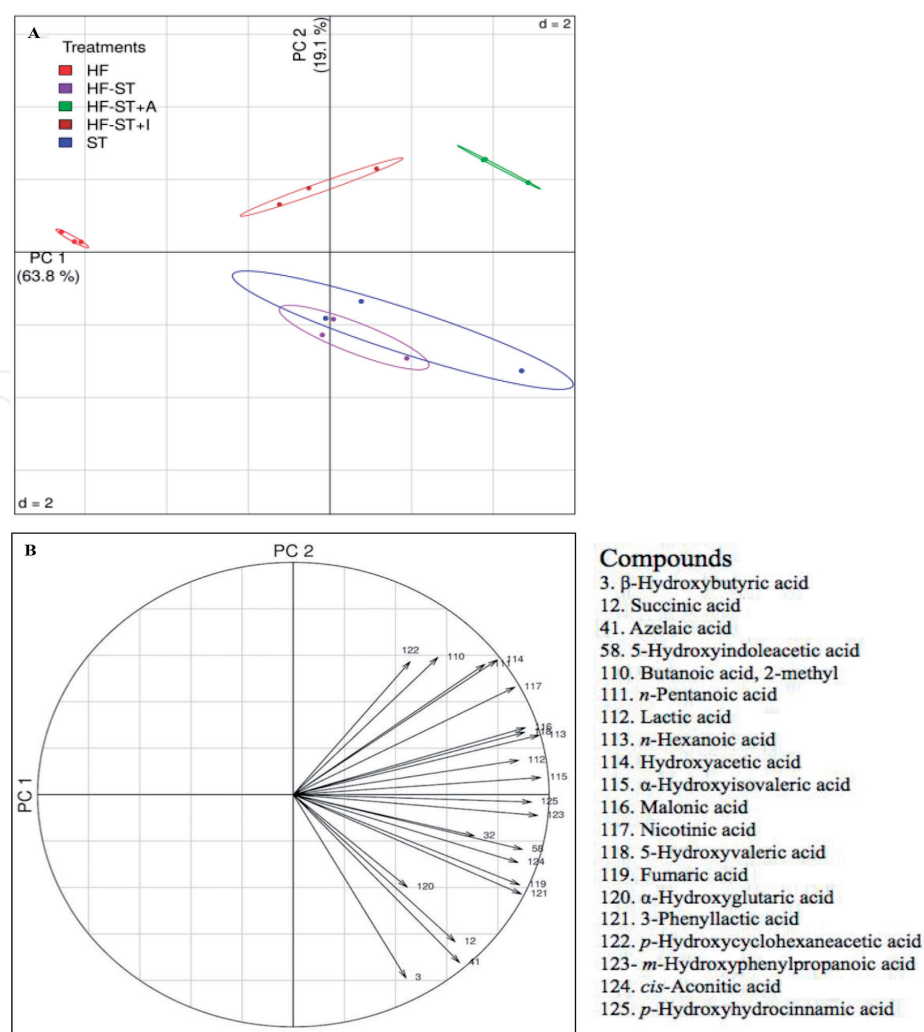
A total of 21 organic acids were identified, including SCFA, hydroxy-SCFA, dicarboxylic acids, and aromatic carboxylic acids (**Table 3**). PCA analysis of organic acids showed the HF group in a separate cluster, while standard diets (HF-ST and ST) displayed an overlap due to presence of similar metabolites in both groups (**Figure 3A**). In addition, PCA and the loading plot evidenced that HF-ST + A and HF-ST + I groups had a more similar organic acids profiles among them in relation to the other diets (**Figure 3B**).

Moreover, hierarchical clustering analysis evidenced that HF group had the lowest content of all organic acids, while HF-ST + A mice exhibited the highest content of the majority of them; therefore, this group is located at one end of the heatmap (**Figure 4**). Noticeably, HF-ST + I group showed various metabolites with a similar content as HF group; for instance the following acids: 2 methyl butanoic, lactic, and hexanoic (**Table 3**); hence, HF-ST + I appears very close to HF group in the heatmap (**Figure 4**). Once again, HF-ST and ST are the closes groups because they presented similar levels of most analyzed organic acids.

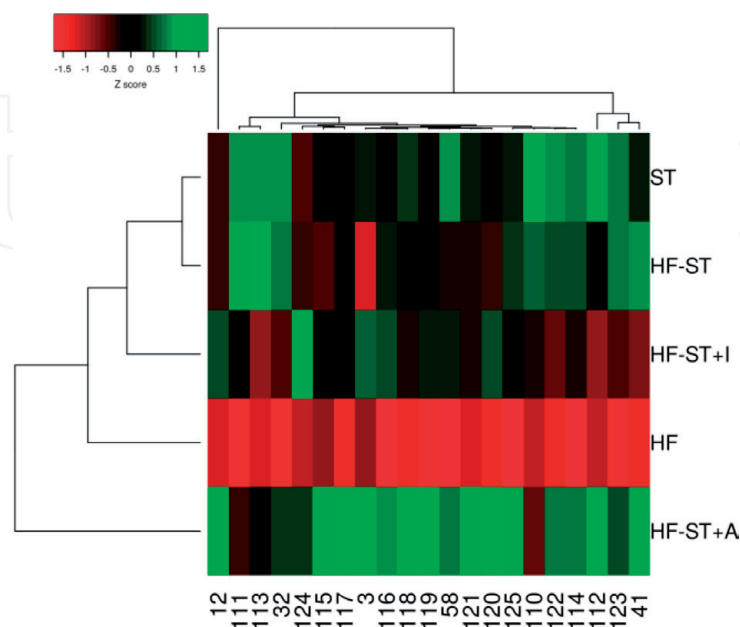
Interestingly, and despite that the hierarchical analyses locate fructans diets very distant from each other (in addition to the great structural differences between agavins and inulins), HF-ST + A and HF-ST + I groups showed an increment of the following acids: succinic, BHB,  $\alpha$ -hydroxyisovaleric, and  $\alpha$ -hydroxyglutaric. Stunningly, succinic acid is involved in glucose homeostasis [38], while BHB has a neuroprotective effect in mice [39] as well as may inhibit the release of fatty acids from adipose tissue by the hydroxy-carboxylic acid receptor 2 (HCA<sub>2</sub>) [40]. Then, these metabolites might be employed in general as biomarkers of prebiotic supplementation.

On the other hand, only agavins supplementation led to a notably enrichment of four acids: nicotinic, 3-phenyllactic, 5-hydroxyvaleric, and lactic; therefore, these metabolites could be used as specific biomarkers for this prebiotic. It is known that nicotinic acid also stimulate the HCA<sub>2</sub>, thereby decreasing plasma lipids and protecting against atherosclerotic disorders [41], and this event could be contributing to improve lipid metabolism of overweight mice that we previously reported [10]. Whereas 3-phenyllactic (the metabolite with the highest increment after agavins consumption) is synthesized by *Lactobacillus* strains and exerts direct antipathogenic activities against bacteria, viruses, and fungi [42].

Surprisingly, we did not find any differential abundance of any organic acid with inulins intake.



**Figure 3.** Organic acids detected in the feces of overweight mice after a diet shift and prebiotic supplementation. (A) PCA and (B) loading plot of the two first PCs. Overweight mice (HF) after a diet change (HF-ST) and agavins (HF-ST + A) or inulins (HF-ST + I) supplementation. ST was a healthy mice group.



**Figure 4.** Hierarchically clustered heatmap of organic acids found in the feces of overweight mice after a diet shift and prebiotic supplementation. HF, overweight mice; HF-ST, overweight mice after a diet shift; HF-ST + A, overweight mice that were switched to standard diet plus agavins supplement; HF-ST + I, overweight mice that were switched to standard diet plus inulins supplement. ST was a healthy mice group.



ID	RT	Compound	Family	tFold change			
				HF	HF-ST	HF-ST + A	HF-ST + I
110	11.015	Butanoic acid, 2-methyl	SCFA	−0.46	−0.15	−0.38	−0.32
111	12.382	<i>n</i> -Pentanoic acid	SCFA	−0.71	0.07	−0.37	−0.23
112	14.666	Lactic acid	αOH-SCFA	−1.00	−0.41	0.05	−0.89
113	14.826	<i>n</i> -Hexanoic acid	SCFA	−0.66	0.12	−0.27	−0.54
114	15.028	Hydroxyacetic acid	αOH-SCFA	−0.89	−0.08	0.00	−0.39
3	17.177	β-Hydroxybutyric acid	βOH-SCFA	−0.74	−0.98	0.62	0.23
115	17.312	α-Hydroxyisovaleric acid	αOH-SCFA	−0.86	−0.44	2.26	0.11
116	18.248	Malonic acid	DiCAc	−0.53	0.08	0.29	0.18
117	20.133	Nicotinic acid	PyrCAc	−0.80	−0.18	0.56	−0.06
12	20.773	Succinic acid	DiCAc	−0.88	0.01	2.01	0.96
118	21.186	5-Hydroxyvaleric acid	ωOH-SCFA	−1.00	−0.18	0.51	−0.40
119	21.483	Fumaric acid	DiCAc	−1.00	0.29	1.05	0.38
120	26.404	α-Hydroxyglutaric acid	αOH-SCFA	−1.00	−0.24	0.88	0.39
121	26.483	3-Phenyllactic acid	αOH-Ar-SCFA	−0.80	−0.32	0.66	−0.32
122	26.993	<i>p</i> -Hydroxycyclohexanecetic acid	OH-Cy-SCFA	−0.80	−0.15	−0.07	−0.53
32	27.406	<i>p</i> -Hydroxyphenylacetic acid	OH-Ar-SCFA	−0.98	−0.02	−0.17	−0.55
123	28.745	<i>m</i> -Hydroxyphenylpropanoic acid	PhePr	−0.95	−0.02	−0.10	−0.51
124	29.137	<i>cis</i> -Aconitic acid	TCA	−1.00	0.06	1.62	3.48
125	29.186	<i>p</i> -Hydroxyhydrocinnamic acid	PhePr	−0.62	0.04	0.18	−0.09
41	29.634	Azelaic acid	DiCAc	−0.69	0.20	0.25	−0.41
58	29.2929	5-Hydroxyindoleacetic acid	IndolAc	−0.90	−0.40	−0.03	−0.22

Fold change value was calculated by comparison with the healthy mice fed with a standard diet (ST). HF, overweight mice; HF-ST, overweight mice that were switched to a standard diet; HF-ST + A, overweight mice changed to standard diet plus agavins; HF-ST + I, overweight mice changed to standard diet plus inulins. All the metabolites listed here have significant difference at least in one treatment  $p < 0.5$ . ID numbers correspond with those of **Table 1**. SCFA, short-chain fatty acid; αOH, alfa-hydroxy; βOH, beta-hydroxy; DiCAc, dicarboxylic acid; PyrCAc, pyridine carboxylic acid; ωOH, omega-hydroxy; Cy, cyclic; Ar, aromatic; PhePr, phenylpropanoid; TCA, tricarboxylic acid; IndolAc, indolic acid.

**Table 3.**  
Fold-change of differential organic acids detected in the feces of overweight mice after a diet switch and prebiotic supplementation.

4. Conclusions

Microbial metabolites found in agavins group exhibited greater similarity to healthy mice, plus enrichment of specific metabolites (biomarkers) such as 2-decenal, UDA, cyclohexanol, fructose as well as some organic acids that undoubtedly are

playing a very important role on overweight mice health. For instance, 2-decenal possess antimicrobial properties; UDA is a neuroprotectant compound; nicotinic acid can decrease plasma lipids levels; while 3-phenyllactic acid shown antipathogenic activities versus bacteria, viruses and fungi. Nevertheless, further studies are needed to clarify the underlying mechanisms by which metabolites derived from agavins fermentation induce a beneficial effect on health of host. Finally, these findings open new and exciting opportunities to explore new biomarkers with applicability on prevention, therapy, or treatment of overweight people.

**Acknowledgements**

We deeply appreciate Inulina y Miel de Agave, S.A. de C.V. for its constant support.

**Conflict of interest**

All authors report no financial interests or potential conflicts of interest.

**Nomenclature**

HF	overweight mice
HF-ST	overweight mice that were switched to a standard diet
HF-ST + A	overweight mice changed to standard diet plus agavins
HF-ST + I	overweight mice changed to standard diet plus inulins
ST	healthy mice
SCFA	short-chain fatty acids
GC/MS	gas chromatography/mass spectrometry
BHB	$\beta$ -hydroxybutyric acid
UDA	10-undecenoic acid
PCA	principal component analysis

**Author details**

Alicia Huazano-García, Horacio Claudio Morales-Torres, Juan Vázquez-Martínez and Mercedes G. López\*  
Department of Biotechnology and Biochemistry, Center of Research and Advanced Studies of the National Polytechnic Institute, Irapuato, Mexico

\*Address all correspondence to: mercedes.lopez@cinvestav.mx

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