Chapter

# Fecal Metabolomics Insights of Agavins Intake in Overweight Mice

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## 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 β(2–1) and β(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 *at 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 tree 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 μL of the ethyl acetate phase were transferred to a vial, dried under nitrogen flux, and derivatized using 80 μL of BSFTA with 1% TMCS and 20 μL 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 μL.

### 2.4 Gas chromatography/mass spectrometry analysis

For GC/MS analysis, 1 μL 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 × 25 μm × 0.25 μm) 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 ± standard deviation. Differences between the diets were determined using one-way ANOVA followed by a Tukey post hoc test or a Dunett 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 | β-Hydroxybutyric acid | βOH-SCFA |
| 4 | 13.234 | Heptanoic acid | FA |
| 5 | 13.281 | α-Hydroxyvaleric acid | αOH-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 | *m*-Hydroxyphenylacetic acid | OH-Ar-SCFA |
| 30 | 19.228 | Cyanuric acid | Triazine |
| 31 | 19.428 | D-Arabinose | CHO |
| 32 | 19.52 | *p*-Hydroxyphenylacetic acid | OH-Ar-SCFA |
| 33 | 19.599 | *n*-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 | *cis*-9-Hexadecenoic acid | FA |
| 51 | 23.481 | *trans*-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 | *cis*-10-Heptadecenoic acid | FA |
| 56 | 24.399 | Stearic acid, methyl ester | FAME |
| 57 | 24.404 | α-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 | *trans*-11-Octadecenoic acid | FA |
| 62 | 25.315 | *cis*-11-Octadecenoic acid | FA |
| 63 | 25.481 | Stearic acid | FA |
| 64 | 26.262 | 2-O-glycerol-α-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 | α-Hydroxy sebacic acid | αOH-DiCAc |
| 83 | 28.967 | 1-O-Octadecylglycerol | Glycerol-ether |
| 84 | 29.01 | *cis*-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 | *cis*-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 | β-Tocopherol | Vitamin |
| 97 | 31.454 | Hexacosanoic acid | FA |
| 98 | 31.891 | Coprostan-3-ol | Sterol |

| ID | RT | Compound | Family |
|---|---|---|---|
| 99 | 32.551 | α-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, 3β- | Sterol |
| 106 | 34.918 | β-Sitosterol | Sterol |
| 107 | 35.064 | 24-Ethylcoprostanol | Sterol |
| 108 | 35.268 | *trans*-Dehydroandrosterone | Steroid |
| 109 | 38.518 | Urs-12-en-28-al, 3-(acetyloxy)-, (3β)- | 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; 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 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 | β-Hydroxybutyric acid | βOH-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 | α-Hydroxyvaleric acid | αOH-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 | *p*-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<br>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<br>22.483<br>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

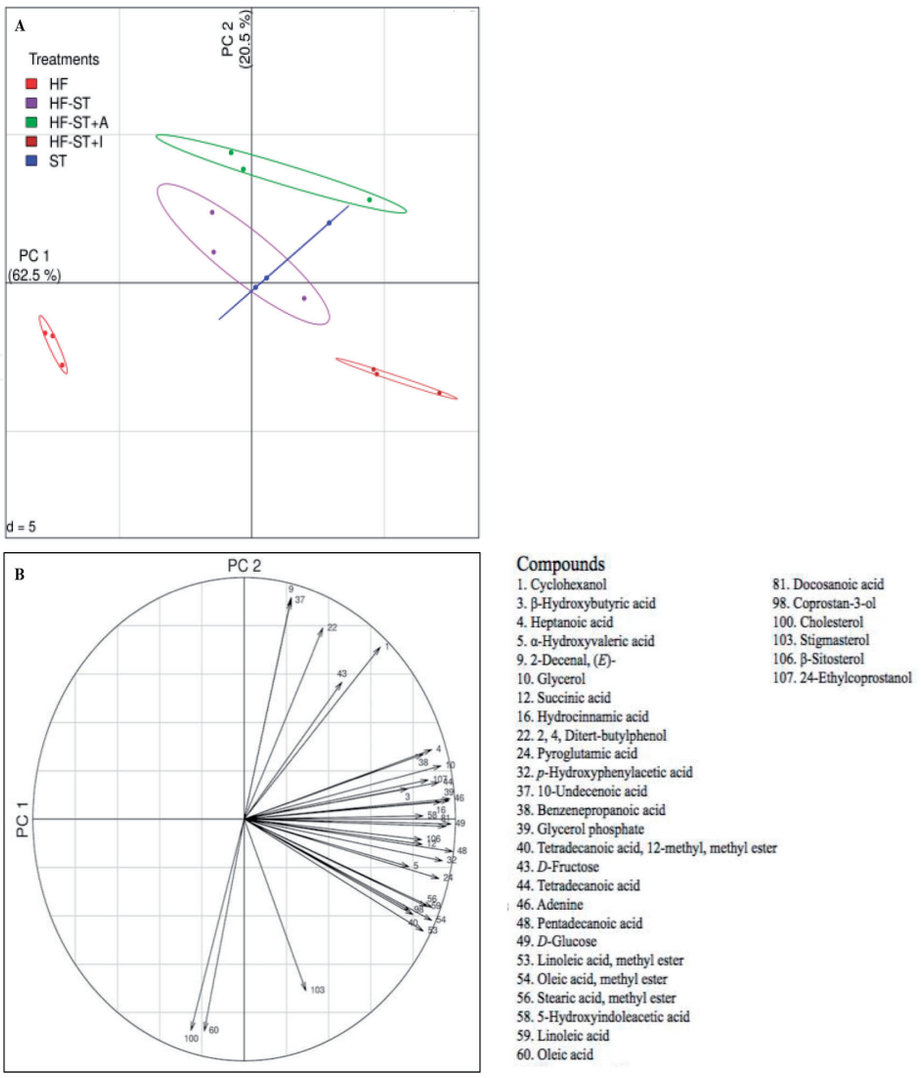


**Figure 1.**
*Metabolites enriched or depleted in the feces of overweight mice after a diet shift and prebiotic supplementation. (A) PCA and (B) loading plot of the two first PCs. Overweight mice (HF) after a diet change (HF-ST) and agavins (HF-ST + A) or inulins (HF-ST + I) supplementation. ST was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

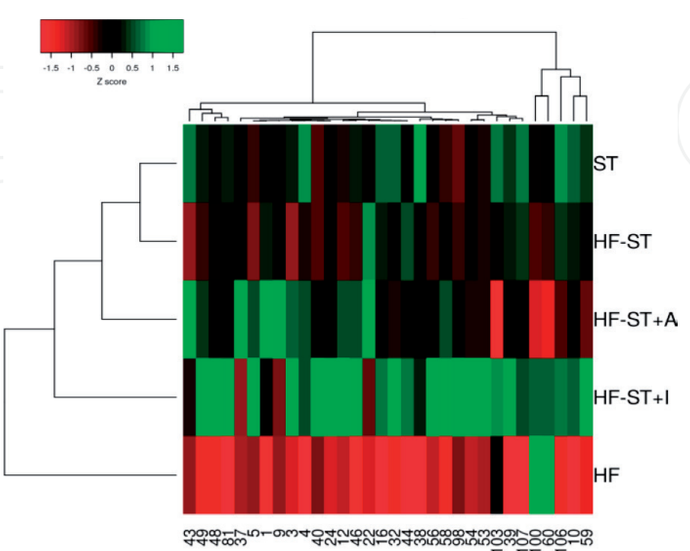


**Figure 2.**
*Heatmap of differential metabolites found in the feces of overweight mice after a diet shift and prebiotic supplementation. HF, overweight mice; HF-ST, overweight mice after a diet shift; HF-ST + A, overweight mice that were switched to standard diet plus agavins supplement; HF-ST + I, 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, α-hydroxyisovaleric, and α-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 ($HCA_2$) [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 specifics biomarkers for this prebiotic. It is known that nicotinic acid also stimulate the $HCA_2$, 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.

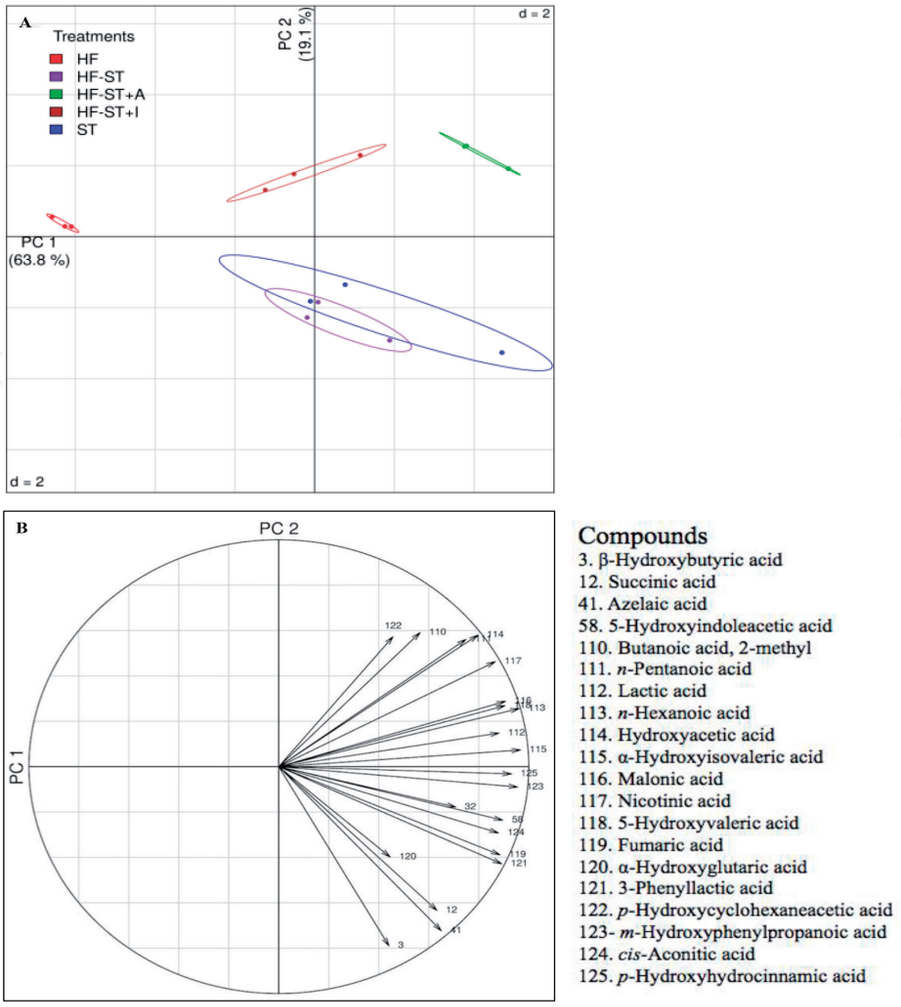


**Figure 3.**
*Organic acids detected in the feces of overweight mice after a diet shift and prebiotic supplementation. (A) PCA and (B) loading plot of the two first PCs. Overweight mice (HF) after a diet change (HF-ST) and agavins (HF-ST + A) or inulins (HF-ST + I) supplementation. ST was a healthy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

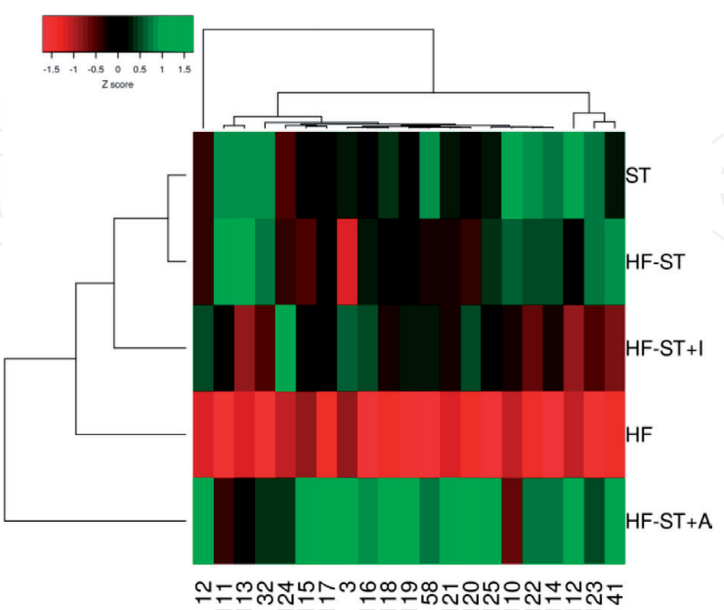


**Figure 4.**
*Hierarchically clustered heatmap of organic acids found in the feces of overweight mice after a diet shift and prebiotic supplementation. HF, overweight mice; HF-ST, overweight mice after a diet shift; HF-ST + A, overweight mice that were switched to standard diet plus agavins supplement; 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 | *n*-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 | *n*-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 | *p*-Hydroxycyclohexaneacetic acid | OH-Cy-SCFA | −0.80 | −0.15 | −0.07 | −0.53 |
| 32 | 27.406 | *p*-Hydroxyphenylacetic acid | OH-Ar-SCFA | −0.98 | −0.02 | −0.17 | −0.55 |
| 123 | 28.745 | *m*-Hydroxyphenylpropanoic acid | PhePr | −0.95 | −0.02 | −0.10 | −0.51 |
| 124 | 29.137 | *cis*-Aconitic acid | TCA | −1.00 | 0.06 | 1.62 | 3.48 |
| 125 | 29.186 | *p*-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-phenyllatic 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.

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## 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 | β-hydroxybutyric acid |
| UDA | 10-undecenoic acid |
| PCA | principal component analysis |

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