Impossible to go Beyond Beef? A Nutriomics Comparison

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Abstract

Concerns regarding the effects of red meat on human and environmental health are prompting consumer interest in plant-based diets. As global food systems strive to meet the dietary needs of an estimated mid-century population of 10 billion, a new generation of plant-based meat alternatives—formulated to mimic the taste and nutritional composition of red meat—have attracted considerable consumer interest, research attention, and media coverage. We used untargeted metabolomics to provide an in-depth comparison of the nutrient profiles of grass-fed ground beef and a market-leading plant-based meat alternative. Metabolomics revealed a 90% difference in nutritional profiles beef and a popular plant-based meat, many of which can have important consumer health implications. This information could not be determined from their Nutrition Facts, which suggests nutritional similarity. Our findings indicate that beef and a

popular plant-based meat should not be viewed as nutritionally interchangeable, but as complementary in terms of provided nutritional entities. As society aims to increase food production with ~ 60% by 2050, the meat and the plant-based meat industries will likely coexist and have to complement each other in order this reach this goal.

Main

By 2050, global food systems will need to meet the dietary demands of almost 10 billion people. To meet these demands in a healthy and sustainable manner, it is suggested that diets would benefit from a shift towards consumption of more plant-based foods and less meat, particularly in Western countries[1]. This has raised questions of whether novel plant-based meat alternatives represent healthy and sustainable alternatives to meat[2,3].

The new generation of plant-based meats such as the Impossible™ Burger and Beyond Burger® are becoming increasingly popular with consumers. Their success has led other international food companies—including traditional meat companies—such as Purdue Farms (US), Cargill (US), Lightlife (US), Gardein Protein International (Canada), Maple Leafs (Canada), Quorn (UK), Tyson Foods (US), and Unilever (UK/The Netherlands) to invest in their own versions of these products[4]. The global plant-based meat sector is currently experiencing rapid growth and is projected to increase from $11.6 billion in 2019 to $30.9 billion by 2026[5] with a compound annual growth rate (CAGR) of 15% (Fig. 1). In contrast, the animal meat sector is "only" expecting a CAGR of 3.9% during that time (Fig. 1) and will reach a market value of $1142.9 billion by 2023[5].

The production of plant-based meats as a replacement for animal-sourced meat is nothing new. One of the earliest engineered meat alternatives was Protose™, a plant-based meat made from wheat gluten, peanuts and, soybean oil, which was designed by John Kellogg in the late nineteenth century. In 1899, Kellogg wrote the following in his patent application for Protose™:

"The objective of my invention is to furnish a vegetable substitute for meat which shall possess equal or greater nutritive value in equal or more favorable form for digestion and assimilation and which shall contain the essential nutritive elements in approximately the same

proportion as beef and mutton and which substitute has a similar flavor and is as easily digestible as the most tender meat" (U.S. Patent No 670283A).

Unlike previous products, contemporary plant-based meat alternatives have accomplished to create a taste and sensory experience that more closely resembles red meat. For example, soy leghemoglobin imitates the "bloody" appearance and taste of heme proteins in meat, while extracts from red beets, red berries, carrots, and/or other similarly colored vegetables are often embedded in plant-based products to give them a reddish 'meat-like' appearance[4]. Methyl cellulose is often used to give plant-based meat alternative a 'meat-like' texture. Modern meat alternatives also match the protein content of meat by using isolated plant proteins (*e.g.*, soy, pea, potato, mung bean, rice, mycoprotein, and/or wheat) and they are often fortified with vitamins and minerals naturally found in red meat (*e.g.*, vitamins B_{12}, zinc, and iron) to provide an even more direct nutritional replacement[6]. Indeed, a popular novel soy-based alternative closely matches the Nutrition Facts panel of beef (Fig. 2), and to consumers reading nutritional labels they appear nutritionally interchangeable[7]. Nonetheless, food sources in their natural state have considerable complexity and contain a wide variety of nutrients (e.g., phenols, anti-oxidants, peptides, amino acids, fatty acids, carboxylic acid etc.), the majority of which do not appear on nutrition labels[8], but have important health implications. Important nutritional differences are likely to exist between beef and the new generation of plant-based meat replacements; however, this has not been thoroughly assessed.

Given the scientific and commercial interest in plant-based meat alternatives, the goal of our study was to use untargeted metabolomics to provide an in-depth comparison of the nutrients in grass-fed ground beef and a popular next-gen soy-based meat alternative, both of which may be considered healthier and more environmentally friendly sources of "beef"[4,9]. Metabolomics is an analytical profiling technique that allows researchers to measure and compare large numbers of nutrients and metabolites that are present in biological samples. Metabolomics analysis enabled a look "behind the curtain" to evaluate how beef and a popular soy-based alternative differ nutritionally—beyond what their labels reveal (Fig. 2).

Untargeted Metabolomics of Plant-Based Meat and Beef

A schematic representation of the study flow is provided in Fig. 2. We purchased eighteen packages of a popular next-gen soy-based meat alternative from a local grocery store. Ground beef from eighteen grass-fed cattle was purchased from Alderspring Ranch (May, ID) and matched for fat (14 grams) and serving size (113 grams) to the soy-based alternative. To identify potential nutritional differences between beef and the soy-based meat alternative, we analyzed the relative abundance of metabolites in individually cooked samples (n=18 beef samples and n=18 soy-based meat alternative samples, respectively) using gas chromatography/electron-ionization mass spectrometry (GC/ei-MS)-based untargeted metabolomics[10]. We profiled 190 unique metabolites in the beef and soy-based meat samples, which were tested for differences between products using the Wilcoxon rank sum test with Benjamini-Hochberg adjusted P-values at 5% (False Discovery Rate; FDR < 0.05).

We found that a total of 171 out of 190 profiled metabolites (90%) were different (FDR < 0.05) between beef and the soy-based alternative (Table S1). To visualize differences and identify the top metabolites that contributed to the nutritional disparity between beef and plant-based meat, we created a ranked heatmap of the top fifty metabolites based on the Pearson distance measure and the Ward clustering algorithm, and performed unsupervised principal component analysis using software procedures from MetaboAnalyst 4.0

(http://www.metaboanalyst.ca). Both the heatmap (Fig. 3A) and unsupervised principal component analysis (Fig. 3B) revealed a distinct separation in nutritional components between the grass-fed ground beef and the soy-based meat alternative. To identify the main nutrient classes that differed between beef and the soy-based alternative, we then clustered individual metabolites into nutrient classes according to their structural similarity using Chemical Similarity Enrichment Analysis (ChemRICH) software procedures (http://chemrich.fiehnlab.ucdavis.edu/).

We identified 24 nutrient classes with ≥ 3 structurally similar metabolites regardless of whether these metabolites were found in beef or the plant-based meat (Table 1). We found that 23 of the nutrient classes differed significantly (FDR < 0.05) between beef and the soy-based meat alternative (Table 1). Several nutrients were found either exclusively (22 metabolites total) or in greater quantities in beef (52 metabolites total) compared with the soy-based meat alternative (Table S1). Similarly, several other nutrients were found exclusively (31 metabolites total) or in greater quantities (67 metabolites total) in the soy-based meat alternative when compared to beef.

Creatinine (product of creatine), hydroxyproline (a non-proteinogenic amino acid), anserine (a carnosine metabolite), glucosamine (a saccharide), and cysteamine (an aminothiol) are examples of nutrients only found in beef and appeared as discriminating metabolites within their respective nutrient class (Table 1). These nutrients have important physiological, anti-inflammatory, and/or immunomodulatory roles[11,12] and low intakes are associated with cardiovascular, neurocognitive, retinal, hepatic, skeletal muscle, and connective tissue dysfunction[11,12]. For example, creatine and anserine provide neurocognitive protection in older adults[13,14]. Cysteamine, a potent antioxidant, also has neuroprotective effects and is a precursor of glutathione—one of the most potent intracellular antioxidants[15]. Squalene has strong antioxidant, anti-bacterial, and anti-tumor activity[16], while dietary hydroxyproline and glucosamine stimulate collagen biosynthesis and are important for maintaining the structure and strength of connective tissue and blood vessels[11,17].

On the other hand, metabolites in nutrient classes such as phenols, tocopherols, and phytosterols (Table 1) were found exclusively or in much greater abundance in the plant-based meat when compared to beef. For instance, the plant-based meat alternative contained more tocopherols (α, γ, and δ)—a class of nutrients with vitamin E activity best known for their antioxidant effects[18]. We also found several phytosterols such as *beta*-sitosterol, campesterol, and stigmasterol in the plant-based meat, which collectively possess antioxidant, anti-inflammatory, and cancer-protective properties[19]. We also found a wider variety and greater abundance of phenolic compounds in the soy-based alternative when compared to beef (Table 1). Identified compounds include sulfurol, syringic acid, vanillic acid, and methylated/hydroxylated forms of valeric acid, which can benefit human health by dampening oxidative stress and inflammation[20].

Within the nutrient class of polyunsaturated fatty acids (PUFAs); arachidonic acid (ARA, C20:4, ω-6) and docosahexaenoic acid (DHA, C22:6, ω-3) were found exclusively (DHA) or in much greater quantities (ARA) in the grass-fed beef samples (Table 1). These essential fatty acids are major constituents of the brain phospholipid membrane and have important roles in cognition, immunomodulation, platelet function, and cell signaling[12,21]. Their deficiencies are associated with cognitive decline and increased risk of cardiovascular disease[12,21].

Important differences were also observed in saturated fatty acid and glyceride classes (Table 1). The main saturated fatty acids and glycerides (Table 1) in the plant-based meat were coconut oil-derived lauric acid, monolaurin, dilaurin, and trilaurin, which possess anti-microbial and/or anti-inflammatory properties[22]. On the other hand, we found higher levels of the dietary odd-chain saturated fatty acids (OCFAs) pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) in beef than in the soy-based alternative. These compounds are believed to exert their beneficial effects by attenuating inflammation, dyslipidemia, and cell fibrosis[23], and increased dietary intake is associated with a lower risk of metabolic disease[24,25].

For an exhaustive list of the different metabolites found in beef and the plant-based meat and their potential roles in human health, the readers are referred to Table S1. While several of these nutrients are considered non-essential or conditionally-essential based on life-stages (*e.g.*, infancy, pregnancy, or advanced age) and are often less appreciated in discussions of human nutritional requirements[8], their importance should not be ignored as low intakes can have profound impacts on human health.

Can Plant-Based Meat Alternatives Meet Human Nutritional Requirements?

A key question in the broader discussion of replacing of animal foods with plant-based substitutes is whether plant-based substitutes can adequately satisfy human nutrition requirements. The underlying dietary strategy for most of mankind now[26], and certainly throughout our evolutionary history, has been omnivory[27,28]. While overlap exists between nutritional profiles of animal and plant foods, needs for certain nutrients—including vitamins C and E (tocopherols), folate, manganese, thiamin (B_{1}), potassium, phenols, and other phytochemicals—are more readily met by consuming plant foods. However, needs for other nutrients—including heme-iron, retinol (vitamin A), vitamin B_{12}, and long-chain PUFAs, and secondary nutrients such as creatine, anserine, taurine, and cysteamine—are met more readily or exclusively from animal foods. Animal foods also facilitate uptake of several plant nutrients (*e.g*,, non-heme iron and zinc)[29,30], while plant nutrients (*e.g.*, phytochemicals and fiber) provide protective effects against potentially harmful compounds (*e.g.*, heterocyclic amines, advanced glycation end products etc.) in cooked and cured animal foods[31]. The secondary compounds in plant foods (*i.e.*, phytochemicals) also exert key antioxidant, anti-inflammatory, anticancer, and immunomodulatory roles[32]. Arguably, plant and animal foods in the human diet interact symbiotically to improve human health.

Nonetheless, those following vegan and vegetarian diets often have improved metabolic health when compared to omnivores, though differences may disappear when extensively adjusting for lifestyle and dietary factors[33,34]. For example, large-scale population based studies performed in individuals with 'healthy lifestyles' such the Oxford-EPIC Study[35] (n~64,000) and the 45-and-Up Study (n~267,000)[36] report no difference in mortality rates between omnivores and vegetarians, when omnivores also consume high amounts of fruits, vegetables, nuts, and seeds. Additionally, intra-individual differences in nutrient metabolism[37-40] may explain why some individuals can thrive on plant-based diets, while others experience health problems associated with nutrient deficiencies[41]. While discussions regarding red meat, plant-based diets, and human health have become increasingly vigorous in recent times[42,43], academics[44,45] and governing bodies[46] generally agree that population health, particularly in Western countries, would benefit from a shift towards increasing the amount of whole food plant-sources as opposed to consuming a Standard American/Western diet—rich in ultra-processed foods[47,48].

While plant-based foods are often considered to be healthy foods to consume, Hu and colleagues[2] have expressed concern in extending these notions to plant-based meat alternatives given their ultra-processed nature. Of note is a recent 8-week randomized controlled trial (RCT) that compared biomarkers of metabolic health in response to consumption of ~2.5 servings/day of a market leading plant-based alternative (Beyond Meat™) versus organic animal meats (grass-fed beef, organic chicken, and pork), both consumed as part of an omnivorous diet[49]. The authors found that serum trimethylamine-N-oxide (TMAO) concentrations were lower following 8 weeks of plant-based meat consumption when compared to animal meats, but only if the participants received the plant-based meat intervention first. Participants in the plant-based meat arm also lost weight when compared to the animal-based group, but again only if the plant-based meats were consumed first, not second. No order effect was observed for low density lipoprotein-cholesterol (LDL-C), which was lower after plant-based meat ingestion regardless of the order of intervention. No group differences were observed in other health biomarkers (high density lipoprotein-cholesterol, triglycerides, insulin, glucose and blood pressure).

TMAO is a gut microbiota-dependent metabolite produced from quaternary ammonium compounds such as phosphatidylcholine, choline, betaine, and L-carnitine, which are predominantly found in animal meats, but TMAO can also be directly obtained from seafood[50]. Whether TMAO is truly an effector of metabolic disease in otherwise healthy individuals and whether increased TMAO levels in cardiovascular disease and type 2 diabetes is the result (rather than the cause) of disease-related dysbiosis is currently a focal point of discussion[50,51], and likely depends on the context in which elevated TMAO levels are observed (pathophysiological states versus dietary intakes of fish and red meat as part of an otherwise “healthy diet”)[50]. Nonetheless, this work provides preliminary evidence that a “flexitarian approach” (replacing some meat with plant-based alternatives as part of an omnivorous diet) has no negative health effects and may have slight positive benefits in terms of weight control and cardiometabolic risk profiles[49]. Future work that assesses additional health biomarkers (*e.g.,* disease-associated inflammation and oxidative stress) and is aimed at elucidating mechanistic pathways by which plant-based meat alternatives impact metabolic health are needed to confirm potential health effects of plant-based meat alternatives.

Similarities between beef and the soy-based alternative in terms of total protein content and several vitamins and minerals (Fig. 2.) suggests that a “flexitarian approach” (replacing some meat with plant-based alternatives as part of an omnivorous diet) is unlikely to negatively impact nutritional status of consumers in the long-run, but this also depends on what other foods are part of the diet and the degree to which plant-based substitutes replace animal foods (*e.g.*, the occasional replacement or full replacement of all animal foods). If a particular nutrient is obtained in sufficient quantities from other commonly consumed foods then its lack in a plant-based meat is likely of no consequence[49]. However, caution is warranted for vulnerable populations such as children, women of childbearing age, and older individuals who may be at increased risk for nutritional deficiencies with low intakes of animal foods[52,53]. Moreover, in discussions about replacing meat with plant-based substitutes on a global level, it is important that food policies do not adversely impact the estimated 2 billion people in developing countries whose basic nutritional needs and livelihoods depend on meat and livestock products[3,52].

Our work has several limitations. While the soy-based meat alternative we studied is one of the most popular products currently on the market, product formulations of next-gen plant-based meats differ slightly in terms of the type of isolated plant proteins (*e.g.*, soy, pea, potato, mung bean, rice, mycoprotein and/or wheat), fats (*e.g.*, canola, soy coconut, and/or sunflower oil), and/or other ingredients (*e.g.*, soy leghemoglobin, different vegetable extracts, and/or different flavoring agents)[6]. Nonetheless, we reasonably expect that plant-based meat alternatives are far more similar to each other than they are to red meat.

The nutritional components highlighted in our work represent only a small fraction of the currently estimated >4,000 distinct metabolites present in foods such as beef and soy (the main constituent of the studied plant-based meat alternative)[54]—many of which have known health effects, but would require extensive targeted metabolomics approaches for their systematic identification.

As the field of nutriomics (the application of metabolomics in nutrition domains) progresses, we will undoubtedly gain greater appreciation of the complexity of natural food matrices and the ability of manifold nutritional constituents to synergistically modulate human health[8]. The complexity of the natural food matrix highlights that attempting to mimic natural food sources using single constituents such as isolated proteins, vitamins, and minerals is challenging and underestimates the true nutritional complexity of food sources in their natural state.

Conclusions

Untargeted metabolomics revealed a 90% difference in nutritional profiles between beef and a market-leading soy-based meat alternative. This information could not be determined from their Nutrition Facts panels (Fig. 2.), which suggests that similar nutrients can be obtained from both products. While beef and the soy-based alternative both contain a wide range of potentially beneficial nutrients (*e.g.*, phenols, tocopherols, fatty acids, antioxidants, amino acids, and dipeptides) as well as some potentially deleterious compounds (*e.g.*, maillard reaction end-products) (Table 1 and Table S1), large differences in individual nutrients indicate that these products should not be viewed as nutritionally interchangeable (Fig. 3 and Table S1). This information does not appear to be known with consumers[7]. Thus, the new information we provide is important for making informed decisions by consumer decisions and to inform food policies and dietary advice.

As society strives to meet dietary needs of an estimated 10 billion people by 2050, the challenge is to create global food systems that are locally adapted to meet dietary needs in a sustainable, healthy, and inclusive manner[3]. Animal and plant foods—and the nutrients they provide—should arguably be viewed as complementary rather than competitive in this scenario. The observed nutritional differences between beef and a popular plant-based meat alternative further highlights this notion. As global food systems work to increase production with ~ 60% by 2050, both the meat and plant-based alternative industries will likely coexist and have to complement each other in order to meet this lofty goal[3].

Methods

Product sourcing

Eighteen different packages (340 grams or 12 oz each) of a market-leading plant-based meat alternative was bought from a local grocery store in Raleigh, NC, USA. Ground beef from eighteen grass-fed, black angus cattle (454 grams or 16 oz each) was purchased from Alderspring Ranch (May, ID) and matched for total fat content (14 grams) to the soy-based alternative, which was confirmed using proximate analysis (method AOAC 960.39; Microbac Laboratories, Warrendale, PA). Individual patties (112 grams or 4 oz each) were formed from each individual package of plant-based meat and beef, respectively. Individual patties were cooked on a non-stick skillet until the internal temperature of each patty read 71 °C as determined by a meat thermometer. One-gram microcore samples were obtained from the middle of each patty (n=18 for ground beef; n=18 for soy-based meat replacement) using a bioptome device, immediately frozen in liquid nitrogen, and stored at -80 degrees °C until metabolomics analysis.

Sample preparation

Microcore samples the plant-based meat replacement and bovine skeletal muscle (*i.e.*, beef) were powdered under liquid N_2 and homogenized in 50% aqueous acetonitrile containing 0.3% formic acid (50 mg wet weight sample per ml homogenate) using a Qiagen Retsch Tissue Lyser II set to a frequency of 30 oscillations/sec for a total of 2 min with one 5 mm glass ball (GlenMills, Inc, #7200-005000TM) per tube. 100 µl of each sample homogenate was then transferred into a fresh, 1.5-ml, Reduced Surface Activity (RSA™) glass autosampler vial (catalog number 9512C-1MP-RS, MicroSolv Technology Corporation, Leland, NC). Proteins in sample homogenates were subsequently "crash" precipitated with 750 µl dry methanol spiked with C14:0-D_{27} (perdeuterated myristic acid, Sigma 366889, 6.25 mg/liter, CN167: 141; CN188: 115) and centrifuged at 13.500 x *g* rcf for 5 minutes (Vial Centrifuge™, MicroSolv, catalog C2417). The crash solvent is spiked with with C14:0-D_{27} Myristic Acid as an internal standard for retention-time locking (described below). 700 µl of the supernatant of each sample homogenate were subsequently transfered to fresh RSA™ glass vials (catalog number 9512C-1MP-RS, MicroSolv Technology Corporation, Leland, NC). Methanolic extracts were then dried in a Savant SPD111V SpeedVac Concentrator (Thermo Scientific, Asheville, NC), with the help of a final pulse of toluene (Fisher Scientific, catalog number T324-50) as an azeotropic drying agent. 25 µl methoxyamine hydrochloride (18 mg/ml in dry pyridine: Fisher Scientific, catalog number T324-50) was then added to each sample and incubated at 50 °C for 30 minutes for methoximation of certain reactive carbonyl groups. Finally, metabolites were rendered volatile by replacement of easily exchangeable protons with trimethylsilyl (TMS) groups using *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA; 75 µl per sample Cerilliant M-132, Sigma, St. Louis, MO) at 50 ºC for 30 minutes.

(GC/ei-MS) analysis

Samples were run on a 7890B GC / 5977B single-quadrupole, Inert MS (Agilent Technologies, Santa Clara, CA). This system is equipped with a MultiMode Inlet, which, in combination with a mid-column, purged ultimate union (PUU), enables hot back-flushing of the upstream half of the column at the end of each run to reduce fouling of both GC and MS with heavy contaminants ("high boilers") and carryover between injections. Briefly, the two wall-coated, open-tubular (WCOT) GC columns connected in series are both from J&W/Agilent (part 122-5512 UI), DB5-MS UI, 15 meters in length, 0.25 mm in diameter, with a 0.25-µm luminal film. This film is a nonpolar, thermally stable, phenyl-arylene polymer, similar in performance to traditional 5%-phenyl-methylpolysiloxane films. Prior to each daily run, the starting inlet pressure is empirically adjusted such that the retention time of the TMS-D27-C14:0 standard is set at ~16.727 minutes. After a quick, initial distillation within the MMI, the GC oven ramps from 60-325 ºC at a speed of 10 ºC/minute. Under these conditions, derivatized metabolites elute from the column and reach the MS detector at known times (*e.g.,* bis-TMS-lactic acid at ~6.85 minutes, and TMS-cholesterol at ~27.38 minutes). A mid-column pneumatic device (PUU) provides a means for hot back-flushing of the upstream GC column at the end of each run while the oven is held at 325 ºC for a terminal "bake-out" as an antifouling and anti-carryover measure (analogous to that devised by Chen *et al.* 2009). During this terminal "bake-out," the inlet is also held at 325 ºC while it is purged via its split-flow, waste vent with a large flow of the carrier gas, helium. Radical cations generated with conventional electron ionization via a tungsten-rhenium filament set to an energy of 70 eV are scanned broadly from 600 to 50 m/z in the detector throughout the run. Cycle time is approximately 38 minutes. We typically derivatize and run daily batches of ~28 unknowns and a processed blank ("ghost" sample). Our GC/MS methods are based on validated methods and generally follow those of Roessner *et al.* (2000)[55], Fiehn *et al.* (2008)[56], Kind *et al.* (2009)[57], McNulty *et al.* (2011)[58], Banerjee *et al.* (2015)[59], and Clinton *et al.* (2020)[60].

Data reduction

Raw data from Agilent's MassHunter software environment were imported into the freeware, Automatic Mass Spectral Deconvolution and Identification Software or AMDIS (version 2.73), developed by Drs. Steve Stein, W. Gary Mallard, and their coworkers at National Institute of Standards and Technology or NIST (Mallard and Reed 1997[61], Halket *et al.* 1999[62], Stein 1999[63]; courtesy of NIST at http://chemdata.nist.gov/mass-spc/amdis/). Deconvoluted spectra were annotated as metabolites, to the extent possible, using an orthogonal approach that incorporates both retention time (RT) from GC and the fragmentation pattern observed in EI-MS, both of which can be remarkably reproducible with contemporary instrumentation. Peak annotation was based primarily on our own RT-locked spectral library of metabolites (2059 spectra from 1174 unique compounds, and growing). Our library is built upon the Fiehn GC/MS Metabolomics RTL Library (a gift from Agilent, their part number G1676-90000; Kind *et al.* 2009[57]. Additional spectra have been gleaned from running pure reagent standards in our lab, from the Golm Metabolome Library (courtesy of Dr. Joachim Kopka and coworkers at the Max Planck Institute of Molecular Plant Physiology, Golm, Germany; Kopka *et al.* 2005[64];

http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html), and from the Wiley 10th-NIST 2014 commercial library (Agilent G1730-64000). Peak alignment and chemometrics of log-base-two-transformed areas of deconvoluted peaks were performed with our own custom macros, written in our lab in Visual Basic (version 6.0) for use in the Excel (Microsoft Office Professional Plus 2019) software environment (both from Microsoft, Redmond, WA). The full list of annotated metabolites and their retention times presented in Table S2.

Data processing

Three investigators (SVV, JRB, and MJM) subsequently performed line-by-line manual curation to fix miscalls and highlighted ambiguities inherent in certain isomeric or otherwise similar metabolites. Metabolites were retained for further analysis if detected in ≥ 80% of samples of either the plant-based meat replacement or ground beef (*i.e.,* 14 out of 18 samples per group). If Th. As can be observed from Table S1, this was the case for 53 metabolites, which were related detected in one source (e.g., beef or plant-based alternative) but not the other. A total of 31 metabolites were detected only on the plant-based meat samples but remained absent in all beef samples; while 22 metabolites were found in beef samples but remained absent in the plant-based meat. In the case of remaining missing values in other metabolites—for which a signal was detected in ≥ 14 out of 18 samples in one group (beef or plant) and ≥ 1 sample of the other group—k-nearest neighbor imputation was performed[65,66].

This decision was made after careful deliberation with colleagues at the Biostatistics and the Metabolomics Core at Duke University, and was based on the expectation that in such cases the metabolite feature was truly nonexistent (or at least below the Level of Detection) for a given group (beef or plant meat) and was not due to chromatographic non-detection. In other words, had the metabolite been present in the food source at meaningful levels, it would have registered as we detected this metabolite in ≥80% of samples in the other group (*i.e.*, 14 out of 18 samples).

To illustrate this with an example; anserine (β-alanyl-1-methyl-l-histidine; a methylated product of carnosine) is metabolite that is well-known to occur in beef and other animal meats, but known to be absent in plant samples[11]. Similarly, soy isoflavones such as β-sitosterol and campasterol would normally not be found in grass-fed beef, but were readily detected in all plant-based meat samples (Fig. S2.). If we used KNN imputation (or other commonly used imputation methods such as PLS, SVD, BPCA etc.) without accounting for true absence of metabolites in a given group, our data set would falsely imply that some metabolites are in the plant or beef source of which we know with certainty that they cannot be there, which we argue would be incorrect to report.

Data analysis

After data processing, individual metabolites were tested for normality using Kolmogorov-Smirnov tests ($p < 0.05$) using SAS 9.4 (Cary, North Carolina, USA). Several metabolites did not show a normal distribution after log transformation, which may be expected based on the large differences between beef and the plant-based meat alternative—53 metabolites were detected exclusively in only either the plant-based meat or beef and had log-transformed values close to 0. To test differences in individual metabolites between groups, we subsequently used the non-parametric Wilcoxon with Benjamini-Hochberg adjusted *p*-values at 5% to account for false discovery (FDR < 0.05).

Bioactivities and potential health effects of annotated metabolites were explored by entering Chemical Abstracts Service (CAS) # of individual metabolites in FooDB (https://foodb.ca/) and/or PubChem (https://pubchem.ncbi.nlm.nih.gov/) databases, while metabolic pathway identification of individual metabolites was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/). To inform the discussion of metabolomics findings, we clustered metabolites by chemical class using freely-available ChemRICH software procedures (http://chemrich.fiehnlab.ucdavis.edu/; courtesy of Dr. Oliver Fiehn and coworkers at the University of California, Davis, USA[67] (Fig. S2.). To enable cluster analysis via structural similarity and ontology mapping, InChiKeys, PubChemID and SMILES canonicals for each metabolite was retrieved by entering its respective Chemical Abstracts Service (CAS) # in the PubChem (https://pubchem.ncbi.nlm.nih.gov/) database (Table S3). After ChemRICH analysis, investigators performed line-by-line manual curation to fix any apparent miscalls or apparent misclassification of individual metabolites and to perform manual adjustment of metabolite classification when appropriate (e.g., ChemRICH classified pyrodixine as a separate “Vitamin B6” category in which case the metabolite was lumped into a larger class simply named “Vitamins”), after which analysis was re-ran. Finally, to visualize differences in individual metabolites between groups and identify the top metabolites that contributed to the nutritional differences between beef and the plant-based meat replacement, we created a ranked heatmap of the top fifty metabolites based on the Pearson distance measure and the Ward clustering algorithm and performed unsupervised principal component analysis using software procedures from MetaboAnalyst 4.0 (https://www.metaboanalyst.ca) (Fig. 3).

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Contributions

S.V.V., F.D.P., and S.L.K contributed to the conception and design of the study. S.V.V., J.R.B., and M.J.M. were responsible for the metabolomics analysis of the study. S.V.V., C.F.P., and K.M.H. performed the statistics. S.V.V and F.D.P. drafted the manuscript and all authors contributed to critical revisions of the manuscript for important intellectual content. S.V.V. had full access to the data and takes responsibility for the integrity of the data and the accuracy of the data analysis; S.V.V. affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies in the analysis have been explained.

Competing Interests

The authors declare no competing interests.

Data and materials availability.

All data that support the findings of this study are available in the main text, tables/figures, and/or the supplementary materials. The full metabolomics data set is available at Dryad: https://doi.org/10.5061/dryad.3ffbg79g3

Supplementary materials

Tables S1-S3.

Fig. S1-S2.

Figure Legends

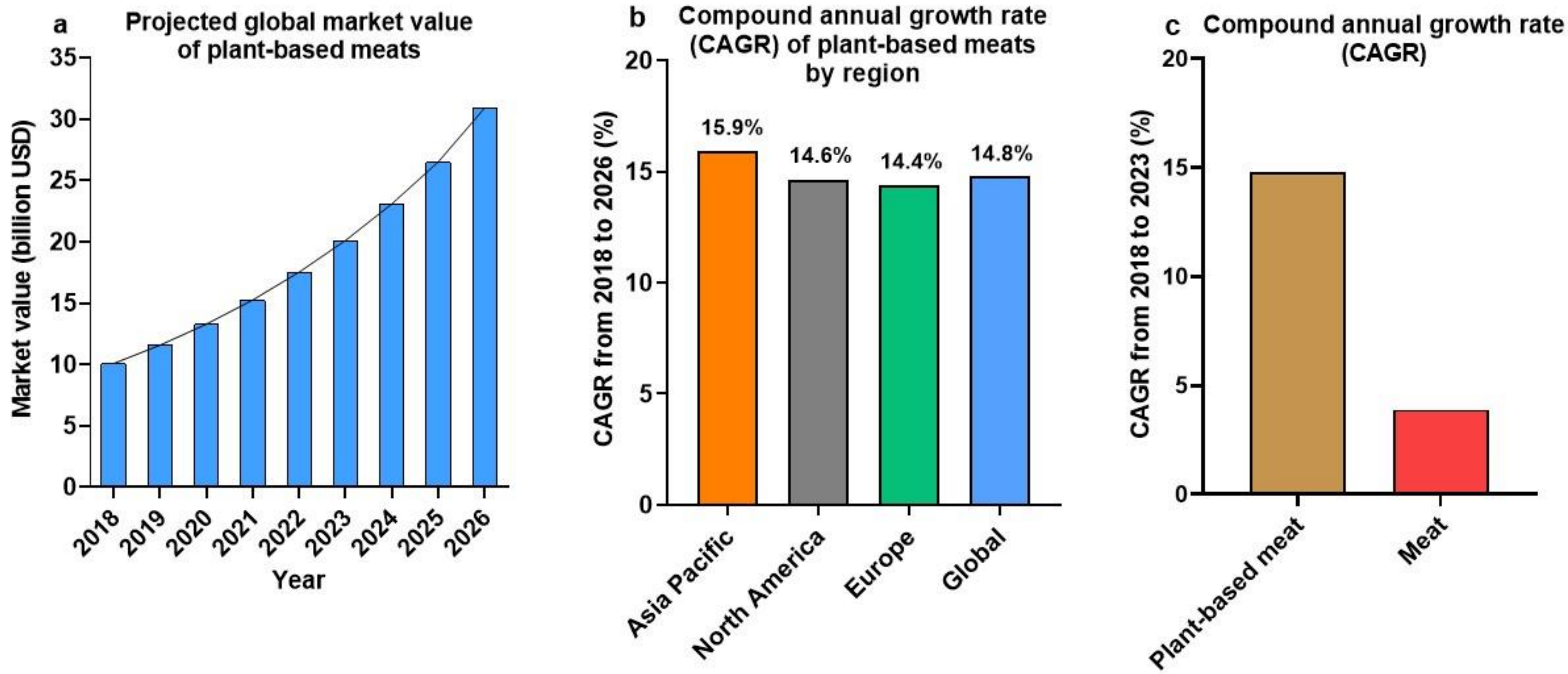


Fig. 1. The global economics of plant-based meat alternatives and meat. Market data on plant-based meat alternatives and meat were obtained from[5]. (**A**) The projected global market value of plant-based meats from 2018 to 2026 in Billion US Dollars. (**B**) The compound annual growth rate (CAGR) of the plant-based meat sector globally and by region. Amongst these regions, the largest growth is expected in the Asia Pacific. (**C**) The relative growth of the global plant-based meat sector (+14.8%) is expected to exceed the relative growth global animal meat market (+3.9%). Despite growth in absolute terms, the value share of the global animal meat

sector as a percentage of the overall food industry will remain more or less similar during 2018-2023[5]. This trend is due to a growing preference among consumers for plant-based diets, which is motivated by concerns for human and environmental health[5].

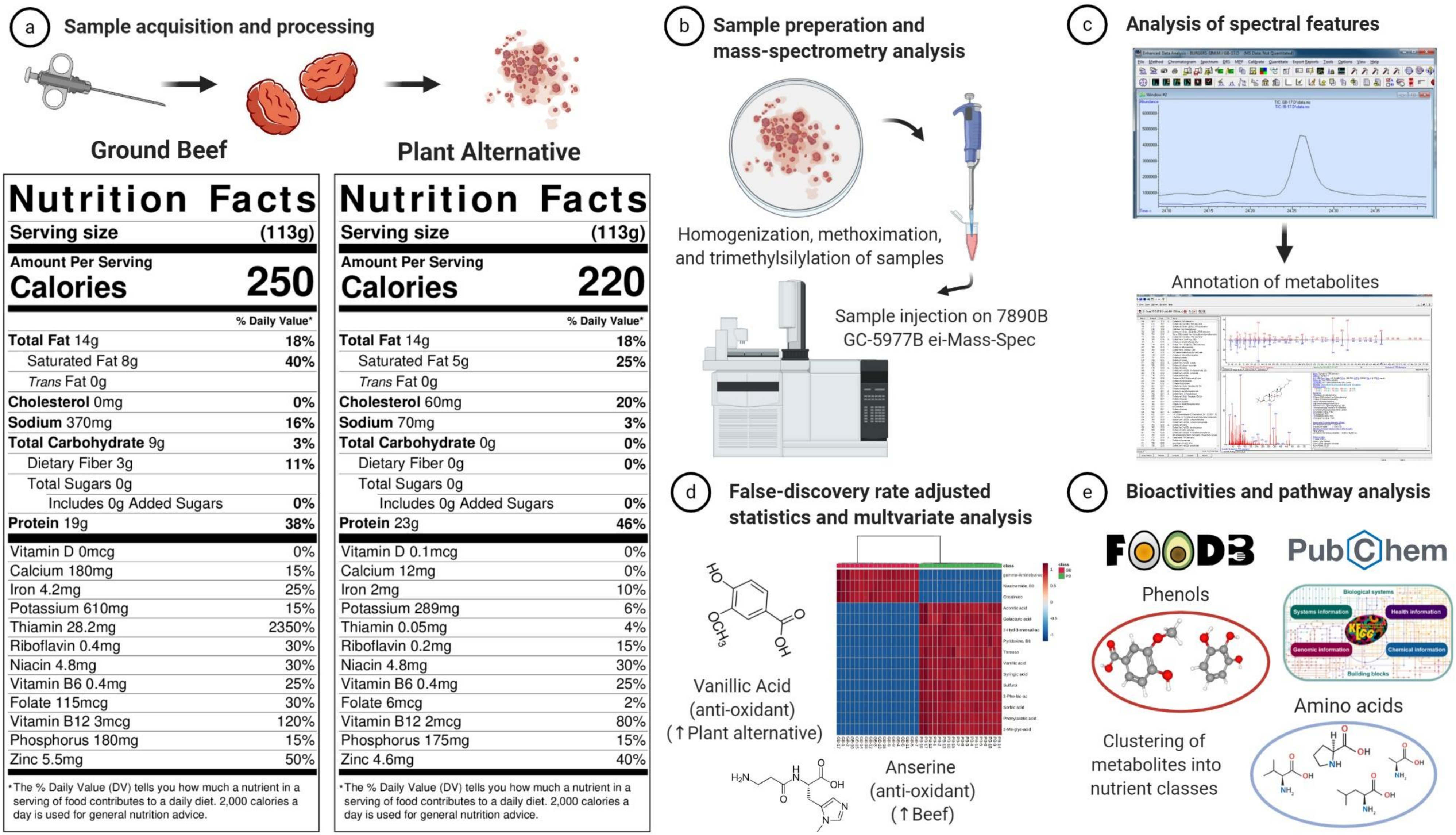
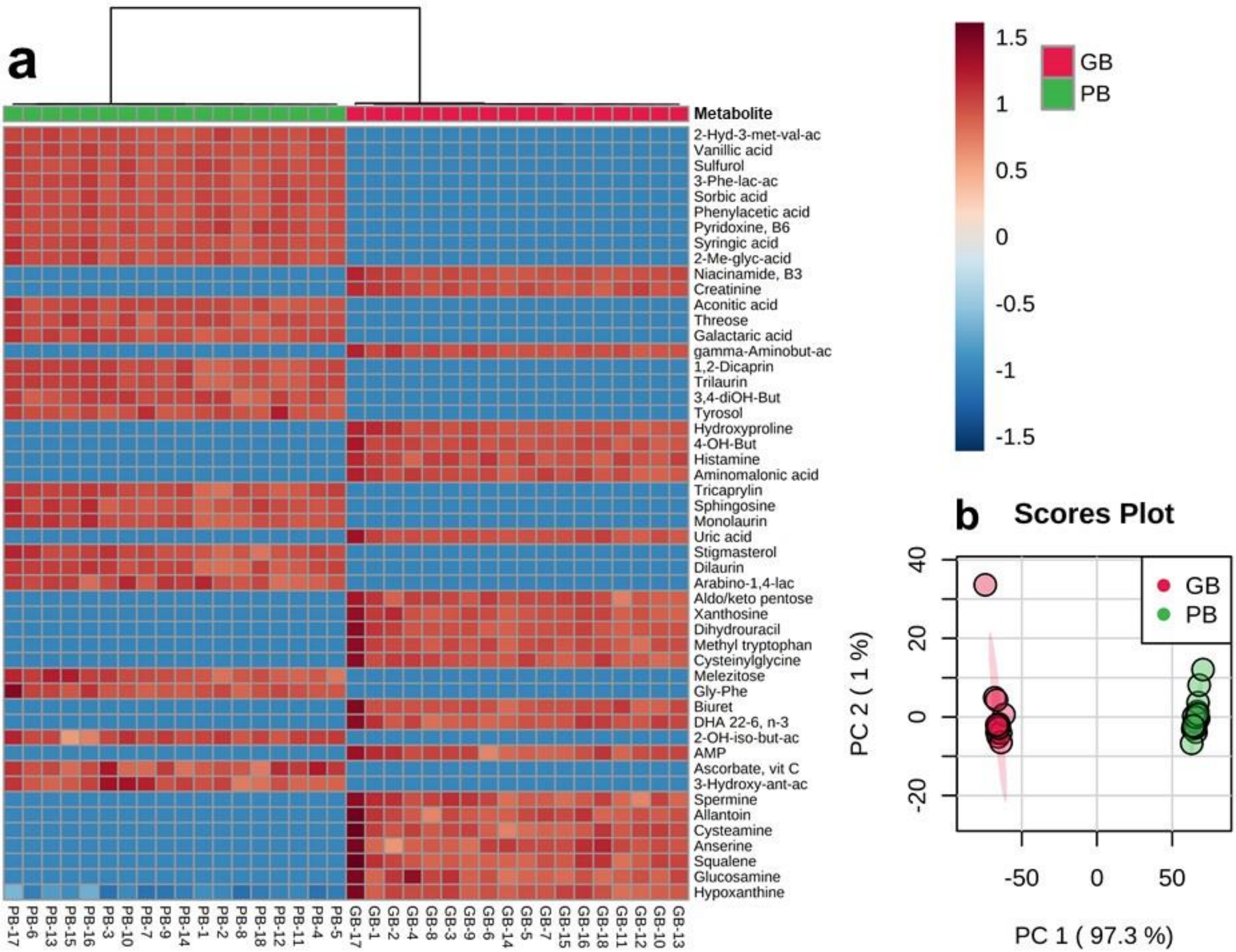

a
Sample acquisition and processing
Ground Beef
Plant Alternative
Nutrition Facts
Serving size (113g)
Amount Per Serving
Calories 250
% Daily Value*
Total Fat 14g 18%
Saturated Fat 8g 40%
Trans Fat 0g
Cholesterol 0mg 0%
Sodium 370mg 16%
Total Carbohydrate 9g 3%
Dietary Fiber 3g 11%
Total Sugars 0g
Includes 0g Added Sugars 0%
Protein 19g 38%
Vitamin D 0mcg 0%
Calcium 180mg 15%
Iron 4.2mg 25%
Potassium 610mg 15%
Thiamin 28.2mg 2350%
Riboflavin 0.4mg 30%
Niacin 4.8mg 30%
Vitamin B6 0.4mg 25%
Folate 115mcg 30%
Vitamin B12 3mcg 120%
Phosphorus 180mg 15%
Zinc 5.5mg 50%
*The % Daily Value (DV) tells you how much a nutrient in a serving of food contributes to a daily diet. 2,000 calories a day is used for general nutrition advice.
Nutrition Facts
Serving size (113g)
Amount Per Serving
Calories 220
% Daily Value*
Total Fat 14g 18%
Saturated Fat 5g 25%
Trans Fat 0g
Cholesterol 60mg 20%
Sodium 70mg 3%
Total Carbohydrate 0g 0%
Dietary Fiber 0g 0%
Total Sugars 0g
Includes 0g Added Sugars 0%
Protein 23g 46%
Vitamin D 0.1mcg 0%
Calcium 12mg 0%
Iron 2mg 10%
Potassium 289mg 6%
Thiamin 0.05mg 4%
Riboflavin 0.2mg 15%
Niacin 4.8mg 30%
Vitamin B6 0.4mg 25%
Folate 6mcg 2%
Vitamin B12 2mcg 80%
Phosphorus 175mg 15%
Zinc 4.6mg 40%
*The % Daily Value (DV) tells you how much a nutrient in a serving of food contributes to a daily diet. 2,000 calories a day is used for general nutrition advice.
b
Sample preperation and mass-spectrometry analysis
Homogenization, methoximation, and trimethylsilylation of samples
Sample injection on 7890B GC-5977B ei-Mass-Spec
c
Analysis of spectral features
Annotation of metabolites
d
False-discovery rate adjusted statistics and multvariate analysis
Vanillic Acid (anti-oxidant) (↑Plant alternative)
Anserine (anti-oxidant) (↑Beef)
e
Bioactivities and pathway analysis
FOODB
PubChem
Phenols
Clustering of metabolites into nutrient classes
Amino acids
Biological systems
Systems information
Health information
Genomic information
Chemical information
Building blocks

Fig 2. Schematic description of sample preparation and metabolomics analysis. (a) Nutrition Facts panels of grass-fed ground beef and a market-leading plant-based meat alternative. Protein and fat content of the grass-fed ground beef was determined by proximate analysis (Microbac Laboratories, Warrendale, PA), while the content of other nutrients in grass-fed beef were adapted from US Department of Agriculture databases[69]. Nutrient composition of the plant-based meat alternative was determined from its Nutrition Facts panel. Eighteen burger patties of each product were cooked until an internal temperature of 71 °C, sampled using a bioptome, and immediately frozen in liquid nitrogen (LN_2) prior to further analysis. **(b)** Frozen **s**amples were homogenized in 50% aqueous acetonitrile containing 0.3% formic acid. Dried extracts were methoximated and trimethylsilylated, and untargeted metabolomic analysis was conducted via gas chromatography/electron-ionization mass spectrometry (GC/ei-MS) on a 7890B GC-5977B ei-MS (Agilent Technologies, Santa Clara, CA) in the Metabolomics Laboratory of the Duke Molecular Physiology Institute. **(c)** Raw spectral data from Agilent's MassHunter software environment were imported into the freeware—Automatic Mass Spectral Deconvolution and Identification Software or AMDIS. Peak annotation of metabolites was based primarily on our own RT-locked spectral library of metabolites (2059 spectra from 1174 unique compounds). **(d)** To determine differences in abundance of metabolites between beef and soy-based meat alternative, log-transformed metabolites were tested using the Wilcoxon rank sum test with Benjamini-Hochberg adjusted *P*-values at 5% (False Discovery Rate; FDR < 0.05). **(e)** Bioactivities and potential health effects of annotated metabolites were explored by entering metabolites in FooDB (https://foodb.ca/) and/or PubChem (https://pubchem.ncbi.nlm.nih.gov/) databases, while metabolic pathway identification of individual metabolites was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/). To further inform discussions of metabolomics findings, metabolites were clustered according to structural similarly ChemRICH software procedures (http://chemrich.fiehnlab.ucdavis.edu/). For further detail on these analyses see Methods section.



a
Metabolite
2-Hyd-3-met-val-ac
Vanillic acid
Sulfurol
3-Phe-lac-ac
Sorbic acid
Phenylacetic acid
Pyridoxine, B6
Syringic acid
2-Me-glyc-acid
Niacinamide, B3
Creatinine
Aconitic acid
Threose
Galactaric acid
gamma-Aminobut-ac
1,2-Dicaprin
Trilaurin
3,4-diOH-But
Tyrosol
Hydroxyproline
4-OH-But
Histamine
Aminomalonic acid
Tricaprylin
Sphingosine
Monolaurin
Uric acid
Stigmasterol
Dilaurin
Arabino-1,4-lac
Aldo/keto pentose
Xanthosine
Dihydrouracil
Methyl tryptophan
Cysteinylglycine
Melezitose
Gly-Phe
Biuret
DHA 22-6, n-3
2-OH-iso-but-ac
AMP
Ascorbate, vit C
3-Hydroxy-ant-ac
Spermine
Allantoin
Cysteamine
Anserine
Squalene
Glucosamine
Hypoxanthine
PB-17 PB-6 PB-13 PB-15 PB-16 PB-3 PB-10 PB-7 PB-9 PB-14 PB-1 PB-2 PB-8 PB-18 PB-12 PB-11 PB-4 PB-5 GB-17 GB-1 GB-2 GB-4 GB-8 GB-3 GB-9 GB-6 GB-14 GB-5 GB-7 GB-15 GB-16 GB-18 GB-11 GB-12 GB-10 GB-13
1.5
1
0.5
0
-0.5
-1
-1.5
GB
PB
b
Scores Plot
GB
PB
PC 2 (1 %)
40
20
0
-20
-50
0
50
PC 1 (97.3 %)

Fig. 3. Metabolomics revealed distinct differences in nutritional profiles between grass-fed ground beef (GB) and the plant-based meat alternative (PB). (a) Heatmap of the top 50 metabolites, ranked by False Discovery Rate (FDR) adjusted *P*-values (lowest to highest), that were significantly different (FDR < 0.05) between beef and the plant-based meat alternative. Red (intensity ranges from 0 to 1.5) means higher abundance of the corresponding metabolite, whereas blue means lower abundance (intensity ranges from −0 to −1.5). The numbers below the heatmap represent individual samples (GB-1 to 18 and PB-1 to 18 respectively; n = 18 for each group). Metabolites in beef and the plant-based meat were compared by the Wilcoxon rank sum test with Benjamini-Hochberg adjusted *P*-values at 5% (FDR < 0.05). (**b**) Principal Component Analysis (PCA) analysis of beef and plant-based meat revealed a distinct difference in nutritional composition between the grass-fed ground beef and the plant-based meat, with 97.3% of the variance explained within the first principal component (PC1)—which illustrates the large nutritional differences that exist between beef and the plant-based meat. The 95% confidence interval of the groups is depicted in each color. Red and green colors above the heatmap (**a**) and the PCA plot (**b**) represent the ground beef and the plant-based meat, respectively. A full list of potential bioactivities and health effects of each individual metabolite is reported in Table S1.

Table 1. Metabolites clustered into nutrient classes according to structural similarity using ChemRICH software procedures. Arrow (↑) indicates higher abundance for a particular nutrient class or nutrient.

Nutrient Class	Class size	No. different plant vs beef	↑ Plant based	↑ Beef	FDR	Key Compound	Metabolic pathway, bioactivities/potential health effects
Amino acids	19	18	12	6	<.001	Glutamine (↑Plant)	Protein metabolism, neurotransmitter, anti-sickling, anti-ulcer
Non-protein amino acids	14	10	5	6	<.001	Creatinine (↑Beef)	Energy metabolism, antioxidant, neuroprotective, ergogenic
Saccharides	13	12	8	4	<.001	Keto pentose-5-phos (↑Beef)	Energy metabolism, flavor
Saturated fatty acids	11	9	3	6	<.001	Pentadecanoic acid (↑Beef)	Odd-chain fatty acid biosynthesis, anti-bacterial, anti-oxidant,
Dicarboxylic acids	10	10	3	7	<.001	Aminomalonic acid (↑Beef)	Glycine metabolism, unknown
Phenols	10	10	7	3	<.001	Vanillic acid (↑Plant)	Plant/microbial metabolism, anti-bacterial, anti-inflammatory
Dipeptides	8	6	2	4	<.001	Anserine (↑Beef)	Carnosine metabolism, antioxidant
Purines	7	7	3	4	<.001	Uric acid (↑Beef)	Microbrial/purine metabolism, unknown
Sugar alcohols	7	6	4	2	<.001	Myoinositol (↑Beef)	Biosynthesis, cholesterolytic, liver-protective, neuro-protective
Hydroxybuyrates	6	6	4	2	<.001	4-Hydroxybutyric acid (↑Beef)	Biosynthesis, neurotransmitter, neuroprotective
Vitamins	5	5	3	2	<.001	Vitamin C (↑Plant)	Biosynthesis, anti-oxidant, liver-protective, kidney-protective
Glycerides	5	4	4	0	<.001	Monolaurin (↑Plant)	Lipid metabolism, anti-microbial, anti-inflammatory
Pentoses	4	4	2	2	<.001	Arabinose/aldopentose (↑Beef)	Energy metabolism, antioxidant, flavor
Sugar acids	4	4	3	1	<.001	Glyceric acid (↑Beef)	Biosynthesis, cholesterolytic, diuretic, kidney-protective
Unsaturated fatty acids	4	4	2	2	<.001	Sorbic Acid (↑Plant)	Fatty acid biosynthesis, preservative
Amino alcohols	4	4	3	1	<.001	Phosphoethanolamine (↑Beef)	Sphingolipid metabolism, neurotransmitter
Pyrimidines	4	3	1	2	.001	Dihydrouracil (↑Beef)	Pyrimidine metabolism, neuro-protective
Amines	4	3	0	3	.001	Cysteamine (↑Beef)	Taurine metabolism, antioxidant, neuroprotective
Phytosterols	3	3	3	0	.003	Stigmasterol (↑Plant)	Biosynthesis, anti-inflammatory, antioxidant, cancer-protective
Tocopherols	3	3	3	0	.003	γ-Tocopherol (↑Plant)	Biosynthesis, antioxidant, cardio-protective, cancer-protective
Biogenic polyamines	3	3	2	1	.003	Spermidine (↑Plant)	Glutathione metabolism, antioxidant
Polyunsaturated fatty acids	3	2	0	2	.008	DHA, 22-6, ω-3 (↑Beef)	Essential fatty acid, neuroprotective, cardio-protective
Pyridines	3	2	0	2	.017	3-Hydroxypyridine (↑Beef)	Maillard reaction end-product, flavor
Fatty acid esters	3	1	1	0	1.00	1,2-Dicaprin (↑Plant)	Energy metabolism, biosynthesis

DHA, docosahexaenoic acid; phos, phosphate.