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Multi-omics profiling finds synbio milk differs nutritionally from bovine milk and contains 93 uncharacterized fungal metabolites and 236 fungal proteins.

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Abstract

Synbio milk, containing recombinant bovine β-lactoglobulin (β-LG), produced in the fungus *Trichoderma reesei,* was deficient nutritionally compared to bovine milk. It also contained both fungal proteins and fungal metabolites never verified safe for human consumption. By three independent methods synbio milk was found to contain predominantly fungal protein, not the 90% to 99% β-LG, claimed by the product developer. By (1) shotgun proteomics, (2) ELISA and classical protein analysis, and (3) simulated mixing experiments, measuring percent deviation of the amino acid composition of synbio milk from that of bovine milk, fungal protein content was found to be 75.1%, 86.5% and 90% to 95%, respectively. Additionally, high sensitivity untargeted mass spectrometry revealed substantial levels of 69 nutrients in bovine milk, of which only 7 were present in small amounts in synbio milk. This analysis also revealed 93 compounds in synbio milk, byproducts/waste products of fungal fermentation, whose chemical identities could not be established searching large mass spectral databases, suggesting they are novel compounds. Neither these nor the fungal proteins found in the synbio milk have been

tested for safety or allergenicity at exposure levels relevant for synbio milk consumption. Therefore, comprehensive toxicity and allergenicity testing are needed to assess the safety of synbio milk for human consumption.

Key words: synthetic biology, synbio, milk, β-lactoglobulin, liquid chromatography-untargeted mass spectrometry, *Trichoderma reesei*

Introduction

Synthetic biology, or synbio, is a broad and expanding technology. One of the most common uses of synbio is the development and large-scale deployment of microorganisms genetically engineered to secrete specific compounds that are not naturally produced in that organism, but that are considered valuable for industrial or commercial purposes. The organism is engineered, using either recombinant DNA or gene-editing techniques, to serve as a production system for a specific compound. Raw materials are fed into this cellular "factory" in the form of a nutrient medium containing highly processed substances including carbohydrates, nitrogenous compounds, minerals, and other nutrients, and the engineered microorganism metabolizes the feedstock, producing the target substance, other molecules, and metabolic wastes.

Synbio technology has been employed to produce a wide range of molecules for use in medicine, the food industry, energy production, and many other applications. [1,2] The range of organisms that are routinely engineered using the synbio model include, most commonly, microorganisms such as yeast, fungi, bacteria and algae, but also higher plants and animals.

Recently, this technology has been used to produce synbio milk and derivative products such as ice cream. [3] These products have now entered early-stage commercialization. [3,4] Synbio milk has been touted as having large benefits for animal welfare [5,6] and environmental protection [5–7]; some proponents suggest this technology may lead to transformative improvement in the sustainability, and the cost, of food production and may significantly strengthen food security. [8]

Open Questions and Research Gaps—However, the use of this technology requires special scrutiny, given its novelty, the rapid rate of technology development, and the known tendency of both recombinant DNA [9–11] and gene-editing [12–14] technologies to cause unintended genomic alterations, and given the ongoing controversy regarding the safety of genetically modified crops [13–17]. There are also a range of safety issues that have been noted to be directly relevant to synbio technology as well as genetic modification in general [18,19]. The introduction of novel synbio substances into the food supply raises questions similar to those related to GMO crop deployment, and additional new concerns related to food safety, allergenicity, nutrition, and health [13].

Relevant to these considerations are questions regarding the transparency with which these new products are marketed to wholesale buyers, retail shoppers, and investors. Also, of note is whether new regulations are needed to ensure these products are aligned with human and environmental health concerns.

Synbio milk raises additional questions regarding potential biohazards, environmental impacts, and social-cultural-economic implications that reach back all the way to the farmer. However, to date, little research has been published that explores these questions.

The compositional data obtained from the present investigation provides evidence useful in answering additional core questions relevant to synbio milk, questions such as the following: What do the data on composition tell us regarding the safety of the synbio products? What is the nutritional adequacy of these products relative to bovine milk? What does this data tell us about the ability of these synbio products to substitute for bovine milk? Is it justified to call these synbio products "milk" given the significant differences between the synbio product and bovine milk? In recognition that this is an open question that we intend to explore, we will, henceforth in this paper, refer to this product as synbio "milk."

Scope of applicability—Although this synbio "milk" product is in early stages of commercialization, the field has attracted significant interest from investors and entrepreneurs and is experiencing rapid growth. There are, for instance, at least six additional companies whose production systems rely on very similar fermentation methodologies for production of bovine milk proteins intended for use in milk replacement products [3]. There are substantial similarities in the production methods used by the company that produced the products studied, and the methods employed by other companies in the synbio "milk" field. Consequently, many of the findings and conclusions reported here may have relevance beyond the specific products tested and apply more broadly to the field of synbio "milk" and, in some cases, to the broader field of synbio food and beverage products.

Results

The study employed four independent analytical approaches to assess the molecular composition of a widely available milk replacement product which uses genetically modified bovine β-lactoglobulin (β-LG), produced by fermentation of recombinant *Trichoderma reesei* (*T. reesei*) fungus, also named *Hypocrea jecorina (strain QM6a)*. First, we used untargeted mass spectrometry to generate a comprehensive inventory of the low molecular weight molecules (50 to 1000 Daltons) present in both the synbio "milk" and bovine milk. Second, we used immuno-analysis and classical analytical methods to quantify the levels of β-LG and other proteins in synbio "milk" and bovine milk. Third, amino acid analysis of synbio "milk" and bovine milk was conducted to assess the nutritional value of synbio "milk" relative to bovine milk and to gain a second, independent, estimate of the levels of β-LG and fungal proteins contained in synbio "milk." Fourth, shotgun proteomics was conducted to assess the relative amounts of synbio β-LG and fungal proteins in synbio "milk" and to assess the range of fungal proteins in synbio "milk".

Inventory of low molecular weight molecules in synbio "milk"—The investigation used ultra-high performance liquid chromatography, coupled with electrospray ionization and quadrupole-time of flight mass spectrometry (UHPLC-ESI-QTOF-MS), to inventory the full range of small molecules (50 to 1000 Daltons) present in synbio "milk" and bovine milk. Figs. 1 and 2

present the results of this full spectrum analysis. Tables presenting the data of Figures 1 and 2 numerically can be found in Table S1 of Supplementary File.

Figs. 1a through 1d present semi-quantitative measurements comparing two samples of bovine milk with synbio “milk”. This analysis identified 69 nutritional compounds, present in authentic bovine milk. The nutrients identified included many lipids, vitamins, fatty acid carnitines, important intermediary metabolites, glycerol-phosphocholine, and hippuric acid. These include compounds that have a wide range of important functions, including biosynthetic, regulatory, and structural roles. It should be noted that, virtually every biological material that is analyzed using the powerful tool of untargeted metabolomics is found to contain many compounds regarding which no information can be found in the scientific literature. Typically, there are more unknown compounds in most biological materials than compounds well characterized by science. This simply points to the current limits of human knowledge and the challenges ahead for biochemists and biologists, as they explore living systems more deeply, moving toward the goal of fully understanding these systems at the molecular level.

Of the 69 compounds identified in bovine milk, only about 7 were present in synbio “milk,” all at levels much lower than those observed in bovine milk. All other bovine milk nutrients were either absent or present only in traces in synbio “milk.” One of those detected in synbio “milk” was riboflavin, also known as vitamin B2, which was intentionally added to the synbio “milk” as stated on the nutrition facts panel of the product. Three of the compounds were fatty acids, which were highly abundant in the sunflower oil, which was also a declared ingredient in the synbio “milk.” Thus, synbio “milk” lacked most of the important nutrients present in normal

bovine milk and the ones that were present were at much lower levels than found in bovine milk.

Fig. 2a through Fig. 2d present the many compounds that we found in synbio “milk.” There were 107 compounds that were abundantly present in synbio “milk.” Of these, only 14 compounds could be tentatively identified based on comparison of the mass spectra and molecular weights of all compounds to mass spectral libraries containing mass spectral and molecular weight data for tens of thousands of compounds. These 14 are presented in Fig. 2a. and include polyphenols and phenolic acids, which have antioxidant and anti-inflammatory properties, sugars, sugar phosphates, an alkaloid, and small peptides. Of the compounds identified by chemical name, only one was also present in bovine milk. Thus, again, it is apparent that synbio “milk” lacked most important nutrients present in normal bovine milk.

The 93 other compounds present in synbio milk (Figs. 2b through 2d) could not be identified by comparison with the mass spectrometric data present in mass spectral libraries. The primary reason for this lack of identification is likely because the molecules detected may not yet have been studied by any branch of science and therefore are beyond the frontiers of science, beyond the frontiers of molecular biology and nutrition that exist today. The existence of such a category of molecules is not surprising. Humankind still has much to learn regarding living systems and their molecular composition and functioning. [20–22] In some cases, a few of these molecular species may have been studied by other methods but not by mass spectrometry and therefore data regarding these compounds would not be included in mass spectral libraries. A third case may be that a given molecular species may have been studied by

mass spectrometry, but that data may not yet be consolidated into one of the mass spectral libraries that we used. Fourth, mass spectrometers differ in their sensitivity, specificity and ionization methods resulting in different fragmentation patterns leading to situations in which it is not possible to match data for a compound to the data for the same compound in a mass spectral library. Finally, sample complexity, matrix effects, and presence of isomers and structural variants can lead to mismatches between compounds detected and mass spectrometric library data.

Of the 93 other unidentified molecules found in synbio “milk”, 92 are absent from bovine milk and are apparently uncharacterized by science. Notably, there is no evidence that these compounds are safe for human consumption. Although the untargeted metabolic protocol used in this study does not provide absolute quantitation, it does indicate semi-quantitatively that a number of these unknown and untested compounds are present in substantial amounts in synbio “milk”. The peak areas of these compounds, which are roughly proportional to the abundance of the compounds, are as large or larger than the peak areas observed for the important nutrients identified in bovine milk.

It should be noted that, although synbio “milk” and bovine milk both contain many compounds that have not been identified and characterized in the scientific literature, we know that the yet-not-identified compounds in bovine milk are safe for human consumption based on hundreds of years of safe consumption of bovine milk by humans. In contrast, the compounds in synbio “milk” are not only unknown in terms of their chemistry and biology, but, in addition, they are untested for safety. It is not known if they are safe for human consumption.

Immuno-quantitation of proteins in synbio "milk"—In pursuit of understanding in more depth the nutritional composition of synbio "milk", the study next used ELISA immuno-quantitation and classical quantitative methodologies to measure the different classes of proteins present in synbio "milk."

As is shown in Table 1, ELISA analysis, employing antibodies specific for β-LG, established that recombinant bovine β-LG protein is present in synbio "milk" at 1.01±0.11 g protein/243.9 g milk. The developer defines a portion of milk as 1 cup or 236 ml or 243.9 g. The total protein content of the synbio "milk" was found to be 7.53±0.26 g/236 ml or 243.9 g. Thus β-LG accounts for 13.4% of the total protein in the synbio "milk."

The other 86.6% of the protein in synbio "milk' is fungal protein derived from the *T. reesei* secretome. According to the synbio "milk" product label (see Supplementary File, Table S2), the only source of protein in synbio "milk" is an extract of the secretome of a strain of *T. reesei* that has been engineered to produce β-lactoglobulin. If 13.4% of that protein is β-LG, the other 86.6% of the protein must be fungal in origin. Thus, according to this method, the protein in synbio "milk" consists of 13.4% recombinant bovine β-LG and 86.6% fungal protein.

β-Lactoglobulin and fungal protein in synbio "milk"	g protein/ 243.9 g milk
Total protein in synbio "milk" (by proximate analysis)	7.53
β-LG protein in synbio "milk" (by ELISA)	1.01

Fungal protein in synbio "milk" (by difference)	6.52
Total protein in bovine milk (by proximate analysis)	8.37
β-LG protein in bovine milk (by proteomic analysis)	1.20
Other proteins (casein, other wheys, etc.) in bovine milk (by difference)	7.17

***Table 1. β-lactoglobulin and fungal protein content of* synbio *"milk"*—**The dietary portion defined by the manufacturer was 1 cup, which is 236.6 ml or 243.9 g. Total Protein Values are the result of two independent analyses on separate days, n = 5 for synbio "milk" and n = 4 for bovine milk, standard deviations are 0.26 and 0.68, respectively. Synbio β-LG value is the result of independent analyses on 2 separate days, n = 14, standard deviation, 0.11. β-LG in bovine milk was measured by proteomics.

As shown in Table 1, the level of β-LG present in the synbio "milk", 1.01 g/243.9 g, is quite similar to that reported to be present in bovine milk, 1.20 g/243.9 g. The total protein content of synbio "milk", 7.40 g/243.9 g, is also roughly equivalent to that of bovine milk, 8.37 g/243.9 g.

The distinction between synbio "milk" and bovine milk is that, in addition to β-LG, the proteins in bovine milk consist of caseins, other whey proteins, and small amounts of other bovine proteins, while in synbio "milk" the proteins present, in addition to β-LG, are fungal proteins. Many of the proteins in bovine milk are described in detail in the scientific literature and all bovine milk proteins have a long history of safe use as part of the human diet [23], although a small percentage of the human population is allergic to one or more of these proteins [24]. In contrast, the proteins in synbio "milk," other than recombinant bovine β-LG, are fungal proteins derived from the *T. reesei* secretome. Although approximately 230 of these proteins have been

identified [25,26], none of them have undergone assessment for allergenicity or adverse dietary effects at the exposure levels relevant to the multi-gram per dietary dose levels relevant to synbio “milk”.

Amino acid composition of synbio “milk” and authentic bovine milk—The amino acid profile of milk serves as an important indicator of its nutritional value and plays a vital role in verifying its authenticity. We compared the amino acid composition of synbio “milk” to that of bovine milk, whey, and pure β-LG to assess both the nutritional quality of synbio “milk” and its authenticity as a replacement for bovine milk. The amino acid composition data presented in Table 2 is the basis of analyses presented in Figures 3, 4 and 5 that establish that the amino acid composition of synbio “milk” differs substantially from that of bovine milk, bovine whey, and from the exact amino acid composition of β-LG. Accredited (AOAC-International) procedures were used to determine the amino acid profiles of these proteins, except for β-LG, the exact amino acid composition of which was determined from its amino acid sequence, obtained from GenBank [27,28].

	Percent Total Amino Acid Weight

	Grams of each amino acid in 100g total amino acids							
	β-Lactoglobulin	**Synbio**	**Conventional**	**Organic**	**Biodynamic**	**Whey A**	**Whey B**	**Whey C**
Alanine	4.93	6.52	3.27	3.11	3.25	4.94	5.02	5.14
Arginine	7.88	2.58	3.00	3.33	3.25	2.34	1.88	1.81
Aspartic Acid	8.01	10.05	7.36	7.33	7.47	10.13	10.66	10.88
Cystine	1.83	2.17	0.54	0.67	0.65	1.82	1.88	1.81
Glutamic Acid	9.28	17.12	20.16	20.22	20.13	16.88	17.55	17.52
Glycine	3.27	1.90	1.91	2.00	1.95	1.82	1.57	1.51
Histidine	1.87	1.49	2.72	2.44	2.60	2.08	1.88	2.11
Isoleucine	4.78	5.30	4.90	5.11	4.87	6.23	6.58	6.65
Leucine	13.99	13.32	9.26	9.33	9.09	9.87	10.03	9.97
Lysine	2.64	10.33	8.17	8.00	8.12	8.83	9.09	9.37
Methionine	3.16	2.72	2.45	2.44	2.27	2.34	2.19	2.11
Phenylalanine	6.21	3.26	4.63	4.67	4.55	3.12	2.82	2.72
Proline	7.91	4.89	9.26	9.33	9.74	6.75	6.27	6.04
Serine	6.65	3.53	5.45	5.33	5.52	5.19	4.70	4.83
Threonine	3.95	4.62	4.36	4.22	4.55	7.01	7.21	7.25
Tryptophan	3.08	2.04	1.36	1.33	1.30	1.82	1.88	1.81
Tyrosine	7.87	3.26	4.63	4.89	4.55	2.86	2.82	2.72
Valine	2.69	4.89	6.54	6.22	6.17	5.97	5.96	5.74
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table 2. Amino acid composition of β-LG, synbio "milk", conventional, organic, and biodynamic bovine milk and bovine whey.

For each protein, Table 2 presents the grams of each amino acid present in 100 g of total amino acids. As illustrated in Fig. 3, we determined the similarity between two proteins by calculating the degree to which the level of each amino acid deviated between the two proteins, termed the "percent deviation of amino acid composition". For instance, the amount of the amino acid alanine in 100 g of synbio "milk" amino acids was 6.52 g, while the amount in β-LG was 4.93 g per 100 g total amino acids. Thus, the deviation of the level of alanine in conventional milk from the level in synbio "milk" was $|(4.93-6.52)|/4.93)\times100 = 24.4\%$. After calculating these percent deviations for all individual amino acids, these values were averaged to obtain an over-all measure of the deviation of the amino acid composition of one sample from that of the other. The results of these calculations, comparing the levels of individual amino acids in pure β-LG and in synbio "milk," are presented in the first 18 bars of Fig. 3. The final, green, bar is the average of all 18 and provides a summary of the extent to which the amino acid composition of synbio "milk" deviates from that of pure β-LG.

Fig. 3 makes it clear that the amino acid composition of synbio "milk" differs quantitatively from that of pure β-LG. Fig. 4 extends this comparison to bovine milk and to whey protein. For all milk samples examined, the percent deviation of the amino acid composition of synbio "milk" from that of conventional, organic, and biodynamic milk was between 41% and 45%, nearly as great as the percent deviation observed between synbio "milk" and pure β-LG. With whey, the percent deviation was around 20%, lower, but still substantial. In contrast when milk was compared to another milk and whey to another whey, the values were much lower, 3% to 4% for milk and 3% to 6% for whey. We conclude from the very small percent deviation in amino acid compositions between different whey samples and between different milk samples

indicates that the amino acid compositions of different whey samples are very similar and the amino acid compositions of different milk samples are very similar. In contrast, the amino acid compositions of synbio “milk” and various bovine milk samples are quite different and, similarly the amino acid composition of synbio “milk” is quite different from that of pure β-LG.

Since protein, and therefore amino acids, are a key component of milk nutrition, these observations indicate that synbio “milk” is nutritionally quite different from bovine milk. The open question following from the results presented in Figs. 3 and 4 is this: It appears that synbio “milk” is different nutritionally from bovine milk and from β-LG, but can we find a quantitative correlation between the β-LG content of a sample and its precent deviation of amino composition of the sample?

The ELISA and proximate analysis results presented in Table 1 indicate that bovine milk and β-LG are very different. According to that analysis, 13.4% of the protein in synbio “milk” is β-LG but the remaining 86.6% is fungal protein. However, the developers of synbio “milk” report in Table 1 of their GRAS Notification that synbio “milk” contained “>90% β-LG (see Supplementary File, Table S3). InTable 2 of that GRAS notification they report that three preparations contained 99.5% to 100% β-LG.

The results in Figs. 3 and 4 indicate that the percent deviation of amino acid composition of synbio “milk” from that of the β-LG gene is not consistent with a β-LG content of > 90%, the percent deviation between their amino acid compositions is much too high. If the protein contained in synbio “milk” were > 90% β-LG, then comparison of the amino acid compositions of β-LG and synbio “milk” would yield a percent deviation of amino acid composition similar to

that observed when two milk or two whey samples are compared. Similarly, the very high percent deviations of amino acid composition observed when the synbio “milk” is compared to bovine milk (Figure 4) are much higher than what would be expected if the β-LG content of synbio “milk” were > 90%. Thus, there is a correlation between the percent deviation of amino acid composition and the level of β-LG in a given sample.

The experiment presented in Fig. 5, in which we calculated the percent deviation of the amino acid composition of various simulated mixtures of fungal protein and pure β-LG, attempts to provide a quantitative answer to the question, posed earlier: Quantitatively, how different in amino acid composition are bovine milk and synbio “milk”? For the β-LG amino acid composition, we used the exact amino acid composition of β-LG, calculated from its amino acid sequence as reported in GenBank [27,28]. For the fungal amino acid composition, we used the average amino acid compositions of six major groups of fungal species, the Ascomycota, of which *T. reesei* is a member, the Basidiomycota, Microsporidia, Chytridiomycota, Mucoromycota and Zoopagomycota, as reported by Mohanta, et al. [29] Using these published amino acid compositions was necessary because the amino acid composition of the *T. reesei* secretome has not been published.

The left side of Fig. 5 presents in detail the results for mixing β-LG and Ascomycota protein. The graph shows that, increasing the proportion of fungal protein in the mixture leads to greater deviation in amino acid composition from pure β-LG with a 42% deviation observed in mixtures containing 95% fungal protein and 5% β-LG. To establish the generality of this result, the simulation was repeated using the amino acid compositions of the five additional groups of

fungal species, obtaining very similar results. As shown in the right-hand portion of Fig. 5, the percent deviation from the β-LG amino acid composition achieved values of between 41% and 45% for all five of the fungal groups when 95% fungal protein was mixed with 5% β-LG.

This simulation leads to the conclusion that a deviation from the β-LG amino acid composition of around 7% corresponds to a mixture that contains 95% β-LG and that a deviation of 40% to 45% represents a composition of about 5% to 10% β-LG. This confirms that the β-LG composition of synbio "milk" is much closer to the values that we report in Table 1 than to the values claimed in the synbio "milk" GRAS Notification. From both the amino acid analysis and the ELISA analysis, we estimate that the percent β-LG present in synbio "milk" is in the range of 7% to 13.4%.

Shotgun proteomics—The protein composition of the synbio "milk" is key to assessing the safety and nutritional value of synbio "milk". Therefore, an additional analysis, using shotgun proteomics, was carried out to independently assess the validity of the other two methods employed, as described in Tables 1 and 2 and Figures 3, 4, and 5. This methodology also provided a finer grained assessment of the relative amounts of β-LG and fungal proteins present in the synbio "milk", disclosing the identities of many of the fungal proteins that are present in the synbio "milk". The 82 most abundant fungal proteins and bovine β-LG are presented in Table 3, below, and the full shotgun proteomic analysis, including 236 fungal proteins is presented in Table S4 of Supplementary File.

The proteomic estimate of protein content was carried out by the iBAQ method [30,31], which determines the molar amounts of each protein in a sample based on TIC (Total Ion Current)

values of multiple tryptic peptides of each protein. The iBAQ values are unit-less values proportional to the molar amount of each protein in a sample. This method sums the peptide-feature intensities of all peptides derived from a particular protein and divides this by the number of theoretically observable peptides corresponding to that protein. This provides a reliable assessment of the number of molecules of that protein in the sample relative to all other proteins. To translate the iBAQ value into protein mass, the molar iBAQ value for each protein is multiplied by the molecular mass of that protein in kilodaltons. The iBAQ method has been used widely [30,32–35]. The moles-to-mass conversion was carried out for each protein in the synbio “milk” (β-LG plus fungal proteins) and then those values were summed to determine the total mass of protein per unit of synbio “milk”. The contribution of each protein to the total protein mass per unit of synbio “milk” was calculated. Finally, using the protein mass values of β-LG and the 82 most abundant fungal proteins in synbio “milk”, the percent contribution of these proteins to the total mass of protein in synbio “milk” was calculated. β-LG and the 82 fungal proteins account for 99.1 % and 99.5% of the protein mass of synbio “milk” for Lots A and B of synbio “milk”, respectively. According to this calculation, β-LG comprised 24.9% of the protein in synbio “milk” Lot A and 31.8% of the protein mass in Lot B, while fungal protein comprised 75.1% and 68.2% of Lots A and B, respectively.

The molecular masses of proteins were provided through the large learning model, Perplexity (https://www.perplexity.ai). Some of these are estimates, including the values for "predicted compounds". An alternate calculation was done excluding the predicted proteins, which took into account 88.5% to 89.2% of the protein in each sample. That calculation provided an upper

bound estimate of the grams of β-LG /gram total protein in synbio "milk". This upper-bound estimate was 32.2% for Lot A of Synbio "milk" and 39.6 % for Lot B.

Table 3--Weight-Percent of Individual Proteins in Total Protein of Synbio Milk

Organism	Protein Descriptions	Synbio "Milk" Lot A	Synbio "Milk" Lot B	Organism	Protein Descriptions	Synbio "Milk" Lot A	Synbio "Milk" Lot B
Bos taurus	Beta-lactoglobulin	24.86	31.77	T. reesei	Predicted protein	0.14	0.04
T. reesei	Alpha-glucuronidase	26.23	20.64	T. reesei	Predicted protein (Fragment)	0.14	0.07
T. reesei	Carbohydrate esterase family 5	10.27	15.81	T. reesei	Mitochondrial matrix factor	0.11	0.06
T. reesei	Predicted protein	8.19	9.37	T. reesei	Predicted protein	0.11	0.04
T. reesei	Predicted protein	7.25	4.52	T. reesei	Trehalase	0.10	0.07
T. reesei	AA9 fam. lytic polysacch. monooxygenase cel61A	5.59	5.03	T. reesei	Glycoside hydrolase family 12	0.10	0.02
T. reesei	Predicted protein (Fragment)	1.50	1.47	T. reesei	Predicted protein	0.10	0.06
T. reesei	Predicted protein	1.09	1.76	T. reesei	Glycoside hydrolase family 55	0.10	0.06
T. reesei	Cell wall protein	0.84	0.57	T. reesei	Predicted protein	0.09	0.08
T. reesei	4-O-methyl-glucuronoyl methylesterase	0.80	0.48	T. reesei	Predicted protein	0.09	0.03
T. reesei	Phospholipase C	0.74	0.52	T. reesei	FAD-binding PCMH-type dom.	0.09	0.03
T. reesei	Carbohydrate esterase family 5	0.65	1.06	T. reesei	Amidase	0.08	0.05
T. reesei	alpha-galactosidase	0.62	0.54	T. reesei	Predicted protein	0.07	0.03
T. reesei	Beta-hexosaminidase	0.62	0.32	T. reesei	Predicted protein	0.07	0.09
T. reesei	Predicted protein	0.57	0.22	T. reesei	Predicted protein	0.07	0.04
T. reesei	alpha,alpha-trehalase	0.55	0.28	T. reesei	Predicted protein	0.07	0.03
T. reesei	Carbohydrate-binding module family 1	0.54	0.29	T. reesei	Predicted protein	0.06	0.04
T. reesei	non-reducing end alpha-L-arabinofuranosidase	0.49	0.52	T. reesei	Predicted protein	0.06	0.30
T. reesei	Predicted protein	0.44	0.20	T. reesei	Predicted protein	0.06	0.02
T. reesei	Ubiquitin fusion protein (Fragment)	0.41	0.14	T. reesei	1,3-beta-glucanosyltransferas	0.06	0.04
T. reesei	Glucanase	0.37	0.28	T. reesei	Predicted protein	0.06	0.03
T. reesei	Acid phosphatase-like protein	0.33	0.21	T. reesei	Predicted protein	0.06	0.03
T. reesei	Glycoside hydrolase family GH30	0.33	0.22	T. reesei	EEF1-gamma domain-contain	0.06	0.01
T. reesei	Predicted protein	0.32	0.21	T. reesei	cellulase	0.06	0.01
T. reesei	Beta-mannosidase A	0.31	0.14	T. reesei	Predicted protein	0.05	0.02
T. reesei	Glycoside hydrolase family 92	0.28	0.15	T. reesei	Predicted protein	0.05	0.04
T. reesei	Predicted protein	0.26	0.07	T. reesei	Predicted protein	0.05	0.01
T. reesei	Glycoside hydrolase family 31	0.25	0.18	T. reesei	xylan 1,4-beta-xylosidase (Fra	0.05	0.02
T. reesei	Xyloglucanase	0.22	0.12	T. reesei	Aldose-1-epimerase	0.04	0.03
T. reesei	glucan endo-1,3-beta-D-glucosidase (Frag.)	0.22	0.14	T. reesei	Predicted protein	0.04	0.01
T. reesei	Predicted protein	0.22	0.11	T. reesei	Predicted protein	0.04	0.02
T. reesei	1,3-beta-glucanosyltransferase	0.21	0.08	T. reesei	Glycosidase	0.04	0.04
T. reesei	Glucoamylase	0.21	0.08	T. reesei	Predicted protein	0.04	0.02
T. reesei	Phosphatidyl-glycerol/-inositol transfer prot.	0.21	0.10	T. reesei	Endo-1,4-beta-xylanase 3	0.04	0.00
T. reesei	Predicted protein	0.20	0.04	T. reesei	Glycoside hydrolase family 79	0.04	0.03
T. reesei	Predicted protein	0.18	0.15	T. reesei	Predicted protein	0.04	0.03
T. reesei	Transaldolase	0.18	0.08	T. reesei	Glycoside hydrolase family 28	0.03	0.01
T. reesei	Carboxylic ester hydrolase	0.18	0.18	T. reesei	Peptide hydrolase	0.03	0.02
T. reesei	Alpha-galactosidase	0.16	0.11	T. reesei	alpha-galactosidase (Fragmen	0.03	0.01
T. reesei	Glycoside hydrolase family 71	0.15	0.05	T. reesei	Lysophospholipase	0.02	0.03
T. reesei	Predicted protein	0.15	0.08	T. reesei	Endo-1,4-beta-xylanase 2	0.02	0.00
T. reesei	DNase1 protein	0.14	0.05				

Table 3. Proteomic Analysis of Synbio Milk.

Two lots of synbio milk were subjected to shotgun proteomic analysis, as described in Methods. A single bovine protein was detected, β-LG, and 236 *T. reesei* proteins were detected. The most abundant 87 of these are presented in Table 3. A full listing of all detected *T. reesei* proteins is presented in Table S4 of Supplementary File.

While ELISA and proximate analysis estimated that β-LG accounted for 13.4% of the protein in synbio "milk", and amino acid analysis estimated that the β-LG content of synbio "milk" was around 7%, shotgun proteomic analysis, found that about 24.9% of the protein in synbio "milk" was attributable to β-LG, while 75.1% was attributable to 236 fungal proteins. As shown in Table 3, the ten most abundant fungal proteins individually contribute from 26.23 % to 0.74 %, totaling 62.5% of the protein content of synbio "milk. The remaining 12.6%, is distributed among the remaining 236 fungal proteins (Listed in Supplementary File, Table S4).

Together, the results of these three methods for estimating the β-LG content of synbio "milk" clearly confirm that fungal protein comprises the bulk of the protein present in synbio "milk", from 75.1 % to 95%. The shotgun proteomic analysis was conducted later than the other work reported in this paper. At that time, we were able to obtain a second lot of the unsweetened flavor of the synbio "milk". These results are presented in Table 3, Lot B. In these preparations, the weight percent of β-LG was 31.8% of the total. This suggests that there is significant lot-to-lot variation in the protein composition of synbio "milk" but confirms again that synbio "milk" contains predominantly fungal proteins, and that, at minimum, the fungal protein content of the synbio "milk" is equal to what was measured in Lot B, 68.2%.

Discussion

This research compared the nutritional properties of synbio "milk," and bovine milk. Both contain similar levels of β-lactoglobulin, and total protein, but the inventory of small molecules, the amino acid profiles, and the protein compositions, of these two beverages were found to be very different. These differences have nutritional consequences and may also have impacts on consumer safety.

Nutrition—UHPLC-ESI-QTOF-mass spectrometric analysis revealed that synbio "milk" lacks all but a handful of the low molecular weight (<1000 Daltons) nutrients found in cow's milk, such as lipids, vitamins, and amino acids. We also found quite significant differences in the amino acid composition of synbio "milk", compared to bovine milk, including substantially lower levels of essential amino acids, most notably histidine, phenylalanine and valine. Thus, synbio "milk" and bovine milk are not nutritionally equivalent. At the same time, untargeted mass spectrometry revealed that the synbio "milk" contains 93 uncharacterized low-molecular weight molecules , likely fermentation byproducts. These are novel compounds apparently not previously studied. Some of these compounds are quite abundant. Based on mass spectrometric signal intensity (TIC), several of these compounds are as abundant as the most abundant nutritional compounds in bovine milk. They remain chemically and biologically undefined, and their food safety and nutritional properties have not been assessed.

Protein composition of synbio "milk" vs bovine milk—We found that the protein composition of synbio "milk" differed significantly from that of bovine milk. While both contain similar levels of β-lactoglobulin (β-LG), the other proteins present in synbio "milk" are very different from

those in bovine milk. Three lines of evidence support this conclusion. First, total protein quantification, and ELISA quantitation of β-LG, indicate that synbio "milk" contains about 13.4% β-LG and that the remaining 86.6% of the protein in synbio "milk" is fungal protein. Second, the amino acid profile of synbio "milk" diverges by about 40% from that of bovine milk. According to the simulation presented in Figure 5, this degree of deviation in amino acid composition indicates that the synbio product contains 5% to 10% β-LG. Third, shotgun proteomics indicates that β-LG represents a minor proportion of the protein content of synbio "milk", while about 237 fungal proteins are present in the product.

Based on these findings, we conclude that synbio "milk" contains substantially more fungal protein than it contains β-LG. This raises questions regarding what is known about these fungal proteins and how they compare with the proteins present in bovine milk.

The bovine milk and *T. reesei* proteomes––Fortunately, information is already available regarding the composition of both the bovine milk proteome and the proteome of the *T. reesei* secretome, as a result of in-depth shotgun proteomic analysis of these materials [25,26,36–39] and assessments of the safety for human consumption of certain *T. reesei* enzymes [40–42].

Shotgun proteomics has catalogued a large number of proteins present in the bovine milk proteome [36–38]. These proteins fall into several categories, and are known to be safe for most people, based on an extensive history of safe use. The exception is the limited segment of the human population that is allergic to a few milk proteins, primarily β-lactoglobulin and α-lactalbumin. Other people are sensitive to other milk constituents, particularly lactose, resulting in non-allergenic milk sensitivities.

Shotgun proteomics has also elucidated hundreds of proteins present in the *T. reesei* secretome. Some of these have been characterized structurally and functionally, defining their chemical identities and biochemical functions. An extensive but non-exhaustive list of the most abundant proteins present in the *T. reesei* secretome, has been reported [25,26,39] and the list of T. reesei proteins that we detected is to be found in the Supplementary File, Table S4 of this paper.

When we compared the proteomes of bovine milk and the T. reesei secretome, we found that these are strikingly different in function as well as composition.

Functions of bovine milk proteins vs *T. reesei* proteins—Most of the proteins in milk are well-characterized. From their functions, it is clear that they evolved to support mammalian nutrition, development and health. Many, like the caseins and the whey proteins, including β-lactoglobulin and α-lactoglobulin, are nutritional, providing a wide range of amino acids. Caseins, lactoferrin and others are abundant and, therefore, nutritional but they also function to transport minerals. Proteins, such as β-lactoglobulin and bovine serum albumin, transport fatty acids, vitamins and other hydrophobic compounds. Other proteins contribute to immune defense, and many other low-abundance bioactive proteins have regulatory and signaling functions.

In contrast, the *T. reesei* proteome evolved primarily to support biomass degradation, breakdown of complex carbohydrates, proteins and lipids for fungal metabolism, as well as cell wall maintenance. Based both on published data [25,39,43] and on our shotgun proteomic results, the proteins of the *T. reesei* secretome are predominantly cellulases, hemicellulases,

proteases, lipases and metabolic enzymes. Also identified are oxidoreductases that facilitate redox reactions and modify lignins. Structural proteins, such as hydrophobins are also common.

These enzymes have functions very different from the nutritional, developmental and health-protective functions of milk proteins. Based on this information and the compositional differences identified, we infer that there are likely important functions of bovine milk proteins that synbio "milk" proteins may not be equipped to perform.

Safety of *T. reesei* proteins—Based on the shotgun proteomic data, it is concluded that, although the proteome of the *T. reesei* secretome is quite different from that of bovine milk, there is no scientific evidence at this time that any specific protein identified in the *T. reesei* secretome is harmful to the consumer.

However, there are four caveats to this statement. First, where safety assessments of *T. reesei* proteins have been done, they have assessed only very low levels of these proteins. This is because the safety testing has been done to verify that *T. reesei* enzymes were safe to be used in food processing. Only levels of these enzymes in the μg/kg-food range are required in food and beverage processing. Consequently, the published safety assessments are not relevant to exposure of consumers to the multiple-gram quantities of fungal proteins that result from consumption of synbio "milk". Safety assessments of *T. reesei* proteins must be conducted at much higher exposure levels to verify the safety of synbio "milk". Thus, the safety assessments conducted to date are in general not relevant to the safety of synbio "milk". This is discussed in the context of the GRAS status of this product, below.

The second caveat is that the fungal forms of two enzymes, common to virtually all fungal species, but not yet identified in *T. reesei*, are commonly allergenic to humans. These are enolase[44] and aldehyde dehydrogenase[45], although the evidence for aldehyde dehydrogenase is controversial [46]. The open question is whether the levels of these two enzymes in synbio "milk" are high enough to elicit allergic reactions. With a product that contains nearly seven grams of fungal protein per dietary portion, allergenic reactions would not be surprising.

The third caveat, relevant to the *T. reesei* proteins identified by shotgun proteomics, is that, in cases where allergenicity has been assessed, it has been done in silico, by comparing the amino acid sequence of the protein to a database of sequences of proteins that are known to be allergenic. For instance, this approach has been used to assess the allergenicity of certain transgenic proteins present in the secretome of genetically modified *T. reesei* lines [40–42]. Although this approach can be helpful, it fails to comprehensively assess the risk of allergenicity because it only assesses the presence of amino acid sequence motifs known to be allergenic in other proteins. It fails to assess the significant possibility that novel allergenic motifs could be present in the protein in question. This is of relevance, since *T. reesei* is still novel to the human diet.

A fourth caveat relates to the current technical limitations of shotgun proteomics. This method is not capable of exhaustively characterizing the proteins of the *T. reesei* secretome. The most advanced shotgun metabolomic methods cover 80% to 88% of the human proteome and the typical proteomic analysis will cover 30% to 50% of a proteome[47,48], depending on the

methods used and materials analyzed. Even the most up-to-date and technically elaborate shotgun proteomic methodologies are unable to deliver complete proteomic information [43,47–50]. Thus, shotgun proteomics is not a viable approach to comprehensively establishing the identities of the full range of proteins present in the *T. reesei* secretome.

Furthermore, this method only identifies these proteins. It does not assess toxicity, allergenicity or nutritional value. Based on the protein identities that this method provides, shotgun proteomics can indirectly lead to information in the scientific literature regarding the toxicity, allergenicity and nutritional value of proteins, but these properties have been assessed for only a small number of *T. reesei* proteins, based on search of the scientific literature.

Concerns in this area are amplified by the fact that shotgun proteomics analyses of the *T. reesei* secretome, have revealed a substantial number of proteins that remain unidentified in terms of function and safety. Shotgun proteomic studies of the *T. reesei* secretome have identified between 138 and 230 proteins, but of these, one paper[25] reports 10 unidentified proteins or 4.3% of the proteins detected in the secretome, while the other, a more recent paper [51] reports 48 proteins or 34.8% of the total secretome as being of unknown identity and function. Our analysis identified about 100 proteins encoded in the T. reesei genome that were not yet characterized as to function or safety.

Protein identification aside, the more urgent scientific question is not, what additional fungal proteins might be present in synbio “milk”, but what biological effects the entourage of proteins present in the T. reesei secretome may have when this complex mixture of proteins is ingested in several-gram amounts, as occurs when consuming synbio “milk”. Systematic, *in vivo*

toxicological and immunological assessments of synbio “milk” as a complete product are needed to answer this question. Not only will this resolve questions regarding the fungal proteins, but this will also resolve questions regarding the safety of the 93 unidentified and untested low-molecular weight compounds (<1000 Daltons) that we have detected in synbio “milk”.

Generally recognized as safe—The synbio “milk” product line has entered the US food market based on a FDA-reviewed GRAS notification submitted by the company that manufactures the synbio ingredient present in these “milk“ products (see Supplementary Information Table S3).

The key argument in this GRAS notification is that several long-standing GRAS notifications demonstrate the safety of synbio food processing enzymes produced by fermentation in recombinant *T. reesei*. For example, one of the GRAS notifications cited is GRN32 [52], which provides evidence that recombinant pectin lyase, produced by genetically modified *T. reesei*, is safe. The synbio “milk” GRAS notification argues that the same fungal constituents present in the pectin lyase are also present in the synbio “milk” and since the pectin lyase has been demonstrated to be safe in GRN32, these same fungal constituents, present in the synbio “milk” must also be safe.

However, the synbio “milk” GRAS notification neglects to mention one critical fact, namely, that use of these two products results in very different levels of exposure of the consumer to fungal proteins and to the small-molecule fungal waste products generated by *T. reesei* fermentation, which are residual in the synbio “milk”. In foods treated with a recombinant enzyme preparation, produced in *T. reesei*, the contaminating proteins and small molecules are present

in vanishingly small amounts because the amount of the enzyme that must be added to the food for food processing purposes is very small, in the range of μg/kg-food to mg/kg-food.

In contrast, synbio β-LG is a major ingredient in the synbio "milk", and therefore, fungal (*T. reesei*) proteins are also abundant major ingredients in the synbio "milk". Based on the data presented here, fungal proteins are present in multi-gram quantities in each dietary portion of synbio "milk". If β-LG represents 24.9% of the protein in synbio "milk", the fungal protein is about 3 times as abundant as β-LG, in synbio "milk". Fungal protein is the dominant protein ingredient of the product, 75.1% of synbio "milk" protein, or 6.0 ± 0.6 g per serving.

This is illustrated in Table 4, below, which shows that the level of exposure of the consumer to fungal proteins is orders of magnitude lower with foods processed using recombinant pectin lyase, produced in T. reesei, compared to the level of exposure resulting from consumption of a single serving of synbio "milk".

Table 4	**Minimum**	**Maximum**
Fungal protein in one serving of food processed with recombinant pectin lyase produced in *T.reesei*	0.0068 mg	0.3402 mg
Fungal protein in one serving of synbio "milk" produced with *T. reesei*	5400 mg	6600 mg
Exposure to fungal protein from synbio "milk" consumption compared to exposure from consumption of food processed with recombinant pectin lyase	19,400-fold higher	794,118-fold higher

Table 4. Dietary exposure to T. reesei proteins from foods processed using recombinant enzymes produced in T. reesei compared to dietary exposure to T. reesei proteins in synbio "milk".

The safety assessment of pectin lyase was used as an example of an enzyme produced in *T. reesei* [23]. Total protein in synbio "milk" was determined by proximate analysis. Total β−LG and total fungal protein were determined by shotgun proteomic analysis. The calculations supporting the analysis presented in Table 4 can be found in Table S5 of Supplementary File.

Table 4 shows that a single portion of food produced using recombinant enzymes produced in *T. reesei* would deliver a dose of fungal proteins of less than 0.0068 mg to 0.3402 mg for a typical portion of 113.4 g (4 oz.). This calculation is based on the information provided in the European Food Safety Authority's safety assessment [53] of the enzyme declared generally recognized as safe in GRAS statement GRN32 [52]. The dose, 0.0068 mg to 0.3402 mg/113.4 g, translates into 0.06 to 3.00 ppm, which is well below the levels recognized to be of concern for most allergens. For instance, the US Food and Drug Administration allows any product containing less than 20 ppm gluten per portion to be labeled "gluten free"[54]. In contrast, synbio "milk" delivers a dose of fungal protein of between 5.40 g and 6.60 g/243.9 g portion, or 22,140 ppm to 27,060 ppm in a single dietary portion, resulting in 19,400-fold to 794,118-fold higher levels of exposure to the consumer than occurs with foods processed with enzymes produced in *T. reesei*.

From this analysis, it is concluded that the earlier GRAS notifications for genetically engineered enzymes produced in *T. reesei* are not a valid basis for establishing the safety of the *T. reesei*,

preparations used in synbio “milk”. Instead, the fungal preparations derived from *T. reesei*, engineered to produce bovine β-LG, should be tested directly for safety at the concentrations of *T. reesei* proteins and small molecules that are present in synbio “milk”. These opinions are based on current available data and do not make blanket statements about FDA compliance failures.

Limitations of the research—We have conducted analytical research, characterizing in depth, the molecular composition of synbio “milk”. This analysis provides detailed quantitative and qualitative information regarding the molecular composition of the product. Comparative analysis of synbio “milk” and bovine milk was conducted to identify compositional similarities and differences, demonstrating directly that synbio “milk” exhibits a molecular profile, a nutritional profile, quite distinct from that of bovine milk.

The impact of this strikingly different nutritional composition upon the health of consumers of this product is an important question. However, direct assessment of the impacts of this product on health of consumers is beyond the scope of the current study, but we highlight this as a key question that should be answered before this product is offered to the public. Similarly, direct assessment of the safety of synbio milk is beyond the scope of the current study. However, the extent to which this product has been verified as safe for human

consumption is documented in the GRAS notification for this product, which is in the public domain. This data, in conjunction with our results regarding the molecular composition of synbio “milk”, provide an evidential basis for concluding that additional toxicity and allergenicity testing is required to assure the safety of synbio “milk” for human consumption.

Overall, the results of this study indicate that synbio “milk” is a distinct beverage with substantial compositional differences from bovine milk, raising questions about whether it can serve as a nutritional replacement for bovine milk. The fungal proteins and the unidentified, but abundant, low molecular weight (<1000 Daltons) fungal compounds present in synbio “milk” have not been evaluated for toxicity, allergenicity, or nutritional effects at exposure levels comparable to those resulting from consumption of synbio “milk”. Further research is essential to answer these questions.

Conclusions

The following conclusions derive from the findings reported here:

Safety Testing and Regulatory Oversight—Comprehensive, fit-for-purpose testing is essential to verify the safety of novel food products, as genetic engineering can introduce alterations that can cause unintended harm [15,16]. It is also recognized that synbio foods raise new safety and biosecurity issues [18,19]. While regulations in Europe [55], Canada [56], and several other countries [57,58] mandate strict safety testing for novel foods, such as synbio foods, U.S. FDA safety assessment relies on a voluntary “Generally Recognized as Safe” (GRAS) protocol, which

essentially leaves pre-market safety assessments to the discretion of product developers. In previous years, there was strong pressure on developers to participate in a voluntary FDA safety assessment process. In recent years products have begun to enter the market without participating in the FDA safety assessment process.[59–61] Developers continue to be legally responsible for ensuring the safety of their food products, but they are not required by regulation to have pre-market safety assessment of products reviewed by the FDA unless the new product is classified as a food additive.

Around the world, GMO regulations vary widely. At one end of the spectrum is the US, which focuses on enabling innovation and commercial development. At the other end of the spectrum is the EU, which takes a precautionary, consumer-protective approach in which strict pre-market safety assessment is combined with strong traceability and mandatory labeling for GMO-containing products. [62,63] Regulations in other countries represent a spectrum of rigor regarding safety assessment and support of innovation that range between the EU and US poles. Superimposed on this is the evolution in technological development that drives further regulatory change. Currently, the emergence of gene-editing is catalyzing debate within the EU system, as well as within the regulatory systems of other countries.

The U.S. Coordinated Framework for the Regulation of Biotechnology was updated in 2017, which improved regulatory efficiency, leading to accelerated innovation and commercialization and increased international competitiveness. However, regulatory gaps and ambiguities remained after the update. Among the remaining vulnerabilities is the continued voluntary status of premarket FDA safety assessment and the continued use of the GRAS protocol. The

GRAS protocol has two options. The self-affirmed option allows the developer to conduct a private safety assessment that is retained internally and is neither reviewed by the FDA nor disclosed to the public. The second option of the GRAS protocol is for the developer to submit safety data for FDA review, successful completion of which leads to issuance of a "no questions" letter. Thus, with the GRAS process, the product goes to market either without independent safety verification or with less than consistently robust safety verification. The core vulnerability regarding the US safety assessment process is that without consistent regulatory assessment of safety, products may go to market before they are ready. Additional concerns have also been raised regarding the updated U.S. Coordinated Framework for the Regulation of Biotechnology including identification of other regulatory gaps and ambiguities, lack of transparency in rulemaking and safety assessments, over-reliance on industry data , and lack of attention to long-term safety and ecological risks. [60,64–71]

Transparency and Factual Marketing––Transparency about synbio ingredients is crucial for building consumer trust, enabling informed choices, and ensuring accountability and safety. Transparency and openness regarding the composition of products is strongly desired by consumers [72–75], a large portion of whom distrust genetically modified products [72–76].

Claims made by synbio product developers, such as "identical to traditional milk" and "milk-identical protein," are not transparent, but are misleading, as are their safety claims. For instance, the synbio "milk" developer implies that the FDA has determined their product is safe. Yet, the FDA's letter of response to the developer's GRAS declaration states, "This letter is not an affirmation that beta-lactoglobulin is GRAS under 21 CFR 170.35[77]." Such communication

practices, not only undermine transparency and consumer trust, but could lead to nutritional insufficiencies or unintended exposure to hazardous compounds. Accurate, fact-based marketing is essential to support consumer confidence and to enable consumers to weigh benefits and risks [72–75].

Standards for Product Purity—Developers should implement practices that remove extraneous fermentation by-products, such as the 93 unidentified compounds in the synbio “milk”, which the study examined. Economical purification methods exist and could prevent harmful effects, safeguard company reputations, and increase consumer confidence.

Environmental Impact—While synbio products are marketed as environmentally friendly, current analyses often overlook the full impact of their supply chains, particularly the industrial agriculture involved in producing fermentation feedstocks. This gap raises questions about the true environmental benefits of these products. Additionally, the use of recombinant DNA, gene-editing, and genetically modified crops, along with the introduction of novel synbio substances into the food supply, presents further environmental concerns. Moreover, products like synbio vanillin, because it is significantly cheaper than natural vanilla [78], risk disrupting livelihoods in developing regions where hundreds of thousands of farmers are dependent for their livelihood on natural vanilla production, leading to broader socio-economic and environmental impacts [79]. The potential impact of synbio “milk” on the economic welfare of farmers and the economic stability of the agricultural sector has not been considered by proponents of this new technology.

Materials and Methods

Sample acquisition—Samples of synbio "milk" were purchased online at the brand's website and at an online vendor site. Organic milk and biodynamic milk were obtained from the dairies where they were produced, Radiance Dairy (Fairfield, IA) and Churchtown Dairy (Hudson, NY), respectively. Organic milk is milk that has been produced in a system compliant with the standard set by the USDA National Organic Program. Biodynamic milk is raw milk that was produced in a system that is not only compliant with the standard set by the USDA National Organic Program, but that is also compliant with the Biodynamic standard, administered by Demeter USA. The conventional milk used in this study was purchased at the local grocery store. It was produced by the Anderson and Ericson Dairy company (Des Moines, IA) under conditions that comply with USDA standards for milk production. The term "bovine milk" is used throughout the paper to refer to one or more of the three forms of bovine milk described above. We found that there were only small differences between conventional, organic and biodynamic milk for the parameters we were measuring. For instance, only small differences were observed between the three bovine milk types in protein levels (Table 1 less than 9% standard deviation for averages of organic and conventional milks) and in amino acid composition (Table 2, amino acid composition of the three milk varieties). For the untargeted mass spectrometric analysis of low molecular weight compounds (Figures 1 and 2) we used only two milk types for economic reasons. Fresh bovine milk samples were used for each experiment. Only one lot of synbio "milk" was available for most of the period when we were conducting this research. We purchased the first lot in April 2023 and then purchased a second sample in August, 2023, expecting to receive a different lot, but that sample was from the same

lot as the first. Sealed containers of the synbio “milk” were quite stable at 4 degrees centigrade and provided consistent results. Finally, when we carried out the shotgun proteomics analysis in spring 2025, a second lot of product was available and we incorporated it into the analytical plan.

Sample preparation for mass spectrometric analysis—Each sample was extracted in triplicate with four volumes of acetonitrile/water//1/1 and centrifuged 15 minutes at 14,800 rpm. The supernatant was then diluted with an equal volume of 0.1% formic acid and centrifuged again at a relative centrifugal force of 21,000 for 15 minutes. The supernatant (300 µL) was then added to a liquid chromatography (LC) vial and 10 µl of internal standard (diclofenac-chloramphenicol mixture, each 50ng/mL) was added to the LC vial and samples were subjected to untargeted UHPLC-QTOF-MS analysis.

Untargeted mass spectrometric analysis using ultra-high performance liquid chromatography, coupled with electrospray ionization and quadrupole-time of flight mass spectrometry (UHPLC-ESI-QTOF-MS)— To study the qualitative composition and the constituents of milk, we employed reverse-phase ultra-high-performance-liquid-chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS/MS). UHPLC-Q-TOF-MS/MS is a specialized, high-resolution, and very sensitive analytical technique for the detection of metabolites, especially small molecules (50-1000 Da).

Two independent UHPLC-MS/MS experiments were performed using electro spray ionization to ensure the detection of both positively and negatively charged metabolite ions, [M + H]+ and [M-H]−, respectively, where H denotes a single proton. Our goal was to achieve broad coverage

of a diversity of metabolites. Several research groups across the scientific community have utilized similar techniques to profile metabolites in many biological materials. Diverse metabolites were reported to have a wide range of abundances, signal intensities. Studies have employed this technique widely to investigate the differences in milk nutritional composition [80,81].

Semiquantitative full spectrum (untargeted) analysis was performed in triplicate for each extracted milk sample. In brief, the extracted bovine milk and synbio “milk” samples were analyzed using ultra-high performance liquid chromatography, coupled with electrospray ionization and quadrupole-time-of-flight mass spectrometry (UHPLC-ESI-QTOF-MS). We have employed this system previously for analysis of bioactive compounds in a diversity of biological materials. [82,83] The analysis was carried out by reverse-phase UHPLC using a Shimadzu Nexera UHPLC system (Shimadzu, Kyoto, Japan), which was directly connected to a Sciex 5600 Quadrupole Time-of-Flight mass spectrometer (AB Sciex, Concord, CAN) in direct injection mode. The auto sampler (Shimadzu SIL30AC, Kyoto, Japan) was operated in full injection mode filling a 50 µl loop with 10 µl analyte for optimal sample delivery reproducibility. Briefly, after injection, sample mixtures were transferred onto the analytical C18 HPLC column (Polar C-18 Luna Omega, 2.1mm I.D. x 10 cm, 1.6 µm particle size, 100 Å pore size, Phenomenex, CA, USA) and eluted at a flow rate of 200 µL/min. Pumps (Shimadzu LC30AD, Kyoto, Japan) were operated using the following multi-step linear gradient, consisting of two components, Mobile Phase A (0.1% acetic acid in mass-spec grade water) and Mobile Phase B (0.1% acetic acid in acetonitrile). Each step of the gradient consisted of different proportions of Mobile Phases A and B: 0 min, 1% B; 11 min, 95% B; 17 min, 95% B; 17.1 min, 1% B; 20 min, 1% B, with a total

runtime of 20 min including mobile phase equilibration. Column oven (Shimadzu CTO30A, Kyoto, Japan) was set to 40°C.

Here we used the strategy of untargeted metabolomic analysis, in which UHPLC-MS/MS was performed in the data-independent acquisition (DIA) mode. The analysis algorithm was based on SWATH (sequential window acquisition of all theoretical fragment ion spectra mass spectrometry) technology.

Data-Independent Acquisition (DIA), MS/MSALL with the Sequential Window Acquisition of All Theoretical Fragment-ion Spectra—SWATH Acquisition and feature annotation—Mass spectra and tandem mass spectral data were recorded using electrospray ionization (ESI) in 'high-sensitivity' mode with a resolution of ~35000 full-width half-maximum in both 'positive-ion' and 'negative-ion' modes on the Sciex 5600 UHPLC-QTOF-MS. The ion spray needle voltages were 5500/-4500 V respectively with drying gas temperature, 600 °C, ion source Gas 1 (nebulizer) and Gas 2 (heater) values were 50 psi each, curtain gas was 35 psi. The collision-energy value for TOF MS was 5 eV and for MS/MS, 25eV with a spread of 15eV. For collision-induced dissociation tandem mass spectrometry, the mass window for precursor ion selection of the quadrupole mass analyzer was set to ± 1 m/z. The precursor ions were fragmented in a collision cell using nitrogen as the collision gas. In the SWATH-MS2 acquisition, variable SWATH windows were set to cover the mass range of m/z 50-1000 in 16 segments (15 x 48.5 msec), yielding a cycle time of 0.7 sec, which includes one 50 msec MS1 scan. SWATH-MS2 produces complex MS/MS spectra, which are a composite of all the analytes within each selected Q1 m/z window. During the execution of the HPLC method, the mass spectrometer was externally

calibrated using a known mixture of masses from Sciex (P/N 4460134, AB Sciex, Concord, CAN). The mixture was injected at the beginning of each run, and all the spectra were calibrated prior to compound identification.

Compounds were annotated based on their accurate mass (m/z) and molecular (m/z) ion fragmentation pattern using Sciex OSoftware (ver.3.0.0.339, AB Sciex, Concord, CAN), with the SCIEX All-in-one HR-MS/MS Spectral Library (version 2.1) databases and with NIST 2017 (P/N 5084470, AB Sciex, Concord, CAN).

Quantitation of β-lactoglobulin, total protein and amino acids—β-lactoglobulin was quantitated using a β-lactoglobulin-specific ELISA kit (#MBS2019635, MyBioSource). Total protein was determined by proximate analysis (Ward Laboratories, Kearney, NE, USA), and total amino acid profile of samples was determined by Medallion Laboratories (Minneapolis, MN, USA), all, using accredited methods.

Shotgun proteomic sample preparation—Protein samples were subjected to clean-up / reduction / alkylation / tryptic proteolysis by using suspension-trap (ProtiFi) devices. S-Trap is a powerful Filter-Aided Sample Preparation (FASP) method that consists in trapping acid aggregated proteins in a quartz filter prior enzymatic proteolysis. Here, proteins were resuspended in 50 µL SDS solubilization buffer, reduced with dithiothreitol and alkylated with iodoacetamide. Proteins were digested with trypsin 1:100 enzyme: protein (wt/wt) overnight. The eluted tryptic peptides were dried in a vacuum centrifuge and re-constituted in water with 2% acetonitrile (ACN).

Proteomic LC-MS Analysis—Proteomic mass spectrometry data and search results are available from the Massive data repository (massive.ucsd.edu) and Proteome exchange (www.proteomexchange.org) using the repository numbers MSV000099870 and PXD070657 respectively. For questions, contact ccms@proteomics.ucsd.edu.

For each sample, 500ng total peptide was loaded onto a disposable Evotip C18 trap column (Evosep Biosytems, Denmark) as per the manufacturer's instructions. Briefly, Evotips were wetted with 2-propanol, equilibrated with 0.1% formic acid, and then loaded using centrifugal force at 1200*g*. Evotips were subsequently washed with 0.1% formic acid, and then 200 µL of 0.1% formic acid was added to each tip to prevent drying. The tipped samples were subjected to nanoLC on a Evosep One instrument (Evosep Biosystems). Tips were eluted directly onto a PepSep analytical column, dimensions: 150umx25cm C18 column (PepSep, Denmark) with 1.5 µm particle size (100 Å pores) (Bruker Daltonics), and a ZDV spray emitter (Bruker Daltonics). Mobile phases A and B were water with 0.1% formic acid (v/v) and 80/20/0.1% ACN/water/formic acid (v/v/vol), respectively. The standard Evosep pre-set method of 60 samples-per-day was used. Peptides were directly eluted into a hybrid trapped ion mobility spectrometry-quadrupole time of flight mass spectrometer (*timsTOF HT*, (Bruker Daltonics, Bremen, Germany) with a modified nano-electrospray ion source (CaptiveSpray, Bruker Daltonics). In the experiments described here, the mass spectrometer was operated in PASEF mode. Desolvated ions entered the vacuum region through the glass capillary and deflected into the TIMS tunnel which is electrically separated into two parts (dual TIMS). Here, the first region is operated as an ion accumulation trap that primarily stores all ions entering the mass spectrometer, while the second part performs trapped ion mobility analysis.

The dual TIMS analyzer was operated at a fixed duty cycle close to 100% using equal accumulation and ramp times of 85ms each. Data-independent analysis (DIA) scheme consisted of one MS scan followed by MSMS scans taken with 36 precursor windows at width of 25 Th at 1.09 sec cycle, over the mass range 300-1200 Dalton. The TIMS scans layer the doubly and triply charged peptides over an ion mobility -$1/k_0$- range of 0.7-1.3 V*sec/cm^2. The collision energy was ramped linearly as a function of the mobility from 59 eV at $1/k_0$ = 1.4 to 20 eV at $1/k_0$ = 0.6.

Proteomics Data Analysis—LCMS files were processed with Spectronaut version 19.8 (Biognosys, Zurich, Switzerland) using DirectDIA analysis mode. Mass tolerance/accuracy for precursor and fragment identification was set to default settings. The reviewed FASTA for *Bos taurus*, UP00009136, was downloaded from Uniprot (on 28/09/2024). A database of 112 common laboratory contaminants (https://www.thegpm.org/crap/) was used, as well as FASTA for *T. reesei*/*Hypocrea jecorina (strain QM6a)*, UP000008984.

A maximum of two missing cleavages were allowed, the required minimum peptide sequence length was 7 amino acids, and the peptide mass was limited to a maximum of 4600 Da. Carbamidomethylation of cysteine residues was set as a fixed modification, and methionine oxidation and acetylation of protein N termini as variable modifications. A decoy false discovery rate (FDR) at less than 1% for peptide spectrum matches and protein group identifications was used for spectra filtering (Spectronaut default). Decoy database hits, proteins identified as potential contaminants, and proteins identified exclusively by one site modification were excluded from further analysis.

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Contributions

All authors contributed to design of the research. R.V. and T.Y. carried out analytical procedures. R.V. conducted UPLC-QTOF data analysis and compound identification. All authors analyzed results. J.F. wrote the first draft, except for Methods, which were written by R.V. All authors reviewed and revised the manuscript. J.F. conceived of, planned and supervised the research.

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Data Availability

All data used in this research are freely available and can be obtained on reasonable request from the corresponding author. Proteomic mass spectrometry data and search results are available from the Massive data repository (massive.ucsd.edu) and Proteome exchange (www.proteomexchange.org) using the repository numbers MSV000099870 and PXD070657 respectively. For questions, contact ccms@proteomics.ucsd.edu.

Ethics Declarations

Competing Interests

T.Y. declares no competing interests. J.F. is Chief Science Officer and CEO, and K.V. is Laboratory Director at the Health Research Institute, which provides authenticity, nutritional and residue testing to companies within the food, agriculture and nutritional supplements sectors, including organic food companies. None of the authors hold patents or have financial investments related to the content of this manuscript. JF has collaborated with Rodale Institute on research regarding organic agriculture. Maharishi International University has a department that specializes in regenerative organic agriculture. The funders of this project did not have input into the design, execution or interpretation of the research, nor did they have input into the writing and publication of the article.

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Figure Legends

Figures 1a,1b,1c and 1d—Nutrients and other compounds present in bovine milk Samples of synbio "milk" (blue bars), biodynamic milk (orange bars), and organic milk (green bars) were subjected to untargeted mass spectrometric analysis. The milk samples were described in detail, in Materials and Methods. For each compound the analysis generates a molecular weight accurate to 4 or 5 decimal places and a mass spectrum. Specialized software and mass spectral libraries were used to tentatively assign chemical names to mass spectral features. The height of bars is roughly proportional to the abundance of each compound. Each sample was analyzed in triplicate. The error bars indicate the standard deviation of the triplicates. Compounds labeled with numbers such as NCGC00169781-02 have been produced as part of initiatives that combine combinatorial chemistry with high throughput screening to identify new drug candidates. Little is known about their functions/activities in natural products such as milk, but their structures are known. Two of these were present in synbio "milk" and others were present in authentic bovine milk.

Figure 2a Chemically identified compounds in synbio "milk"—Samples of synbio "milk" (blue bars), biodynamic milk (orange bars), and organic milk (green bars) were subjected to untargeted mass spectrometric analysis. The milk samples are described in detail in the Materials and Methods section. For each compound the analysis generates a molecular weight accurate to 4 or 5 decimal places and a mass spectrum. Specialized software and mass spectral libraries were used to tentatively assign chemical names to mass spectral features. The height

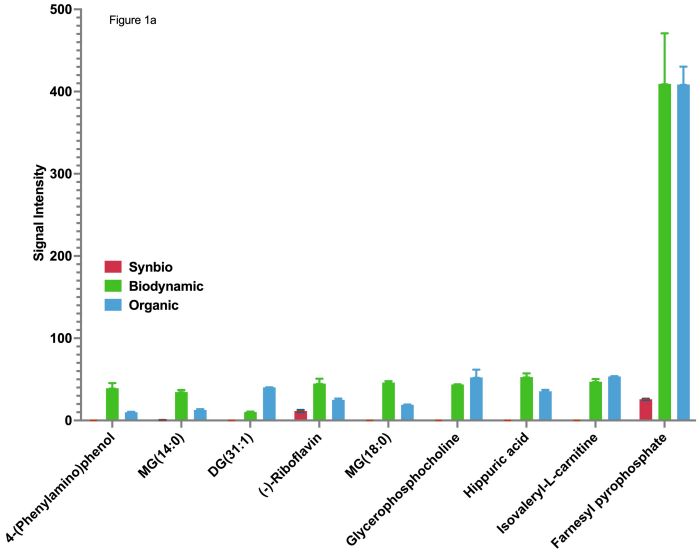
of bars is roughly proportional to the abundance of each compound. Each sample was analyzed in triplicate. The error bars indicate the standard deviation of the triplicates. Compounds labeled with numbers such as NCGC00169781-02 have been produced as part of initiatives that combine combinatorial chemistry with high throughput screening to identify new drug candidates. Little is known about their functions and activities in natural materials like milk; however, their structures are known. Of the identified compounds, only one, 6-alpha-mannobiose was present in both bovine milk and synbio "milk".

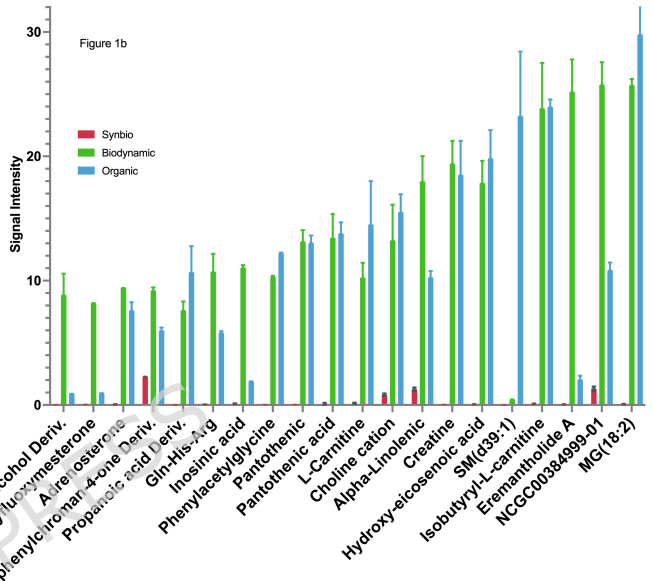
Figure 2b, 2c and 2d Compounds that are not identified chemically and are present almost exclusively in synbio "milk," not bovine milk—Samples of synbio "milk" (blue bars), biodynamic milk (orange bars), and organic milk (green bars) were subjected to untargeted mass spectrometric analysis, as described in Materials and Methods. The height of bars is roughly proportional to the abundance of each compound. Each sample was analyzed in triplicate. The error bars indicate the standard deviation of the triplicates. For each compound the analysis generates a molecular weight accurate to 4 or 5 decimal places and a mass spectrum. The specialized software and mass spectral libraries that were used to tentatively assign chemical names to mass spectral features were unable to find matches in the mass spectral libraries for all the compounds in Figures 2b, 2c, and 2d. Thus, these compounds are not likely to have been researched chemically or biologically and there is no evidence that they have been assessed for safety of consumption by humans. Most of these compounds were not detected in bovine milk.

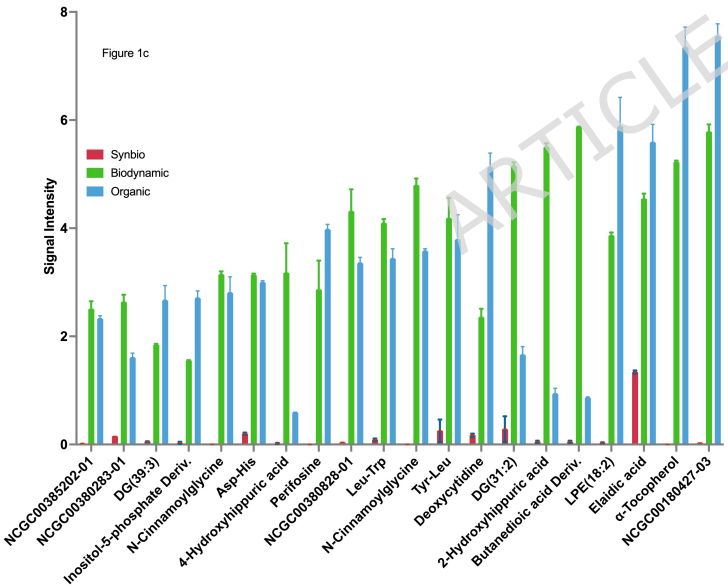
Figure 3 Percent deviation of the amino acid composition of synbio milk from the amino acid composition of pure b-lactoglobulin. This figure presents the percent deviation of the amino acid composition of synbio milk from the amino acid composition of β-lactoglobulin.

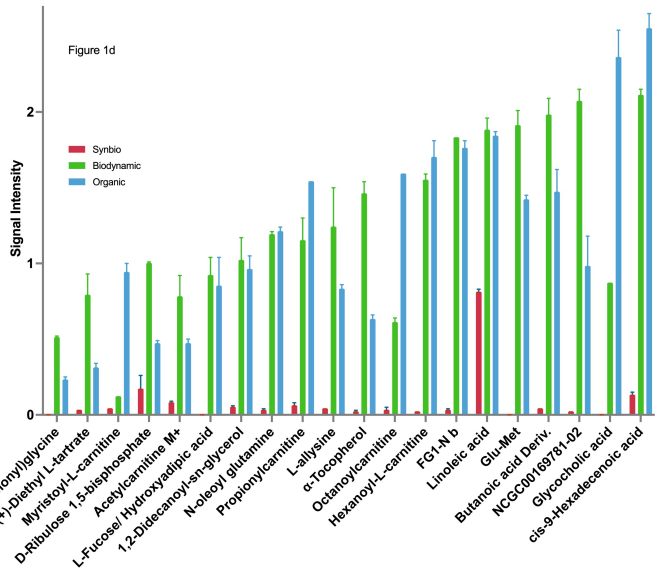
Figure 4—Percent deviation of amino acid composition This figure presents the percent deviation of the amino acid composition of synbio milk from the amino acid composition of β-lactoglobulin, conventional milk, organic milk, biodynamic milk, bovine whey, and percent deviation of amino acid composition among three bovine whey preparations and three bovine milk samples**.**

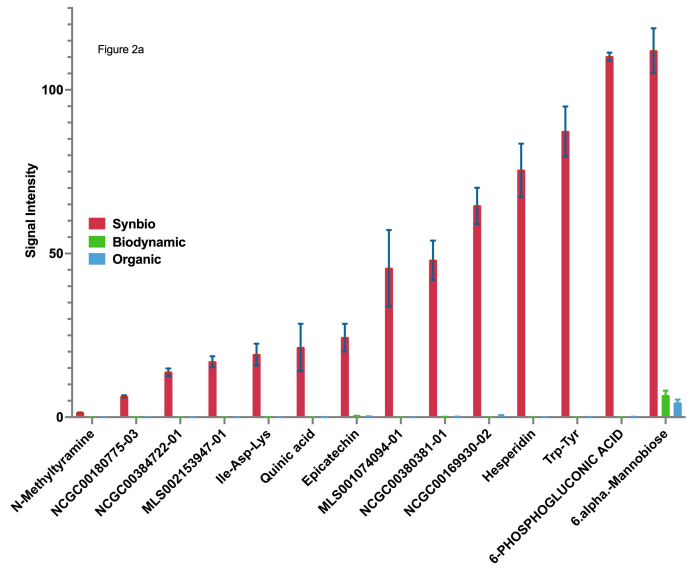
Figure 5 Percent Deviation of the Amino Acid Composition of Simulated Mixtures of β-Lactoglobulin and Fungal Protein from the Amino Acid Composition of β-Lactoglobulin as the Percent of Fungal Protein in the Mixtures Increases—*T. reesei* is a member of the Ascomycota phylum. The amino acid composition of *T. reesei* is not reported in the literature. Therefore, we used the average amino acid composition reported for the Ascomycota [29] group of fungal species. For comparison, we also did this calculation for the other groups of fungi, the Basidiomycota, Microsporidia, Chytridiomycota, Mucoromycota and Zoopagomycota groups [29].

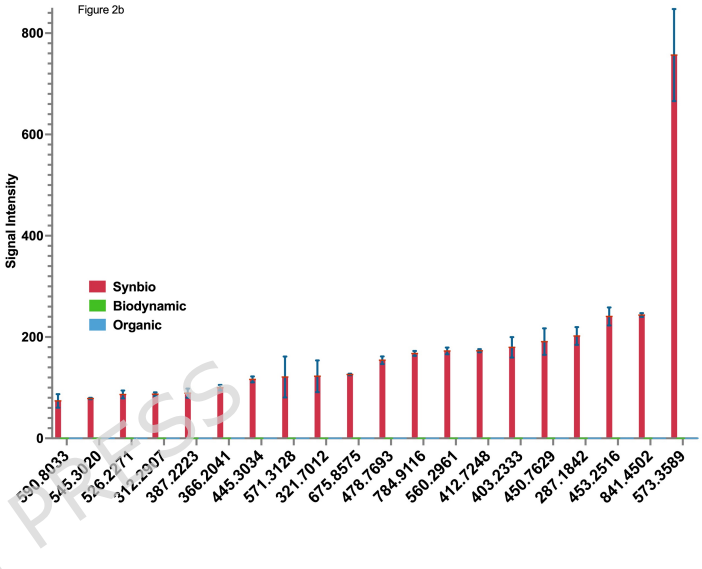


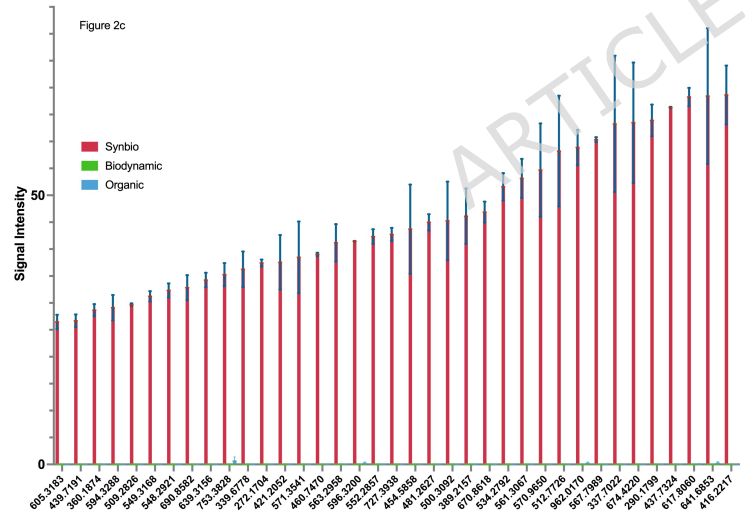


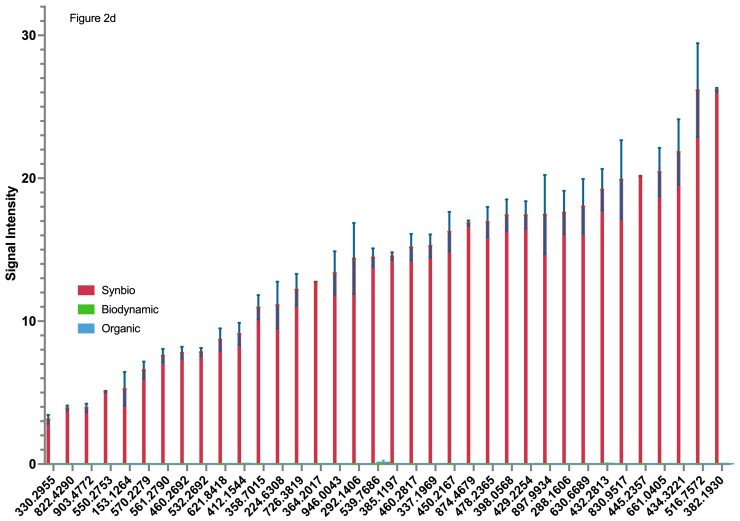


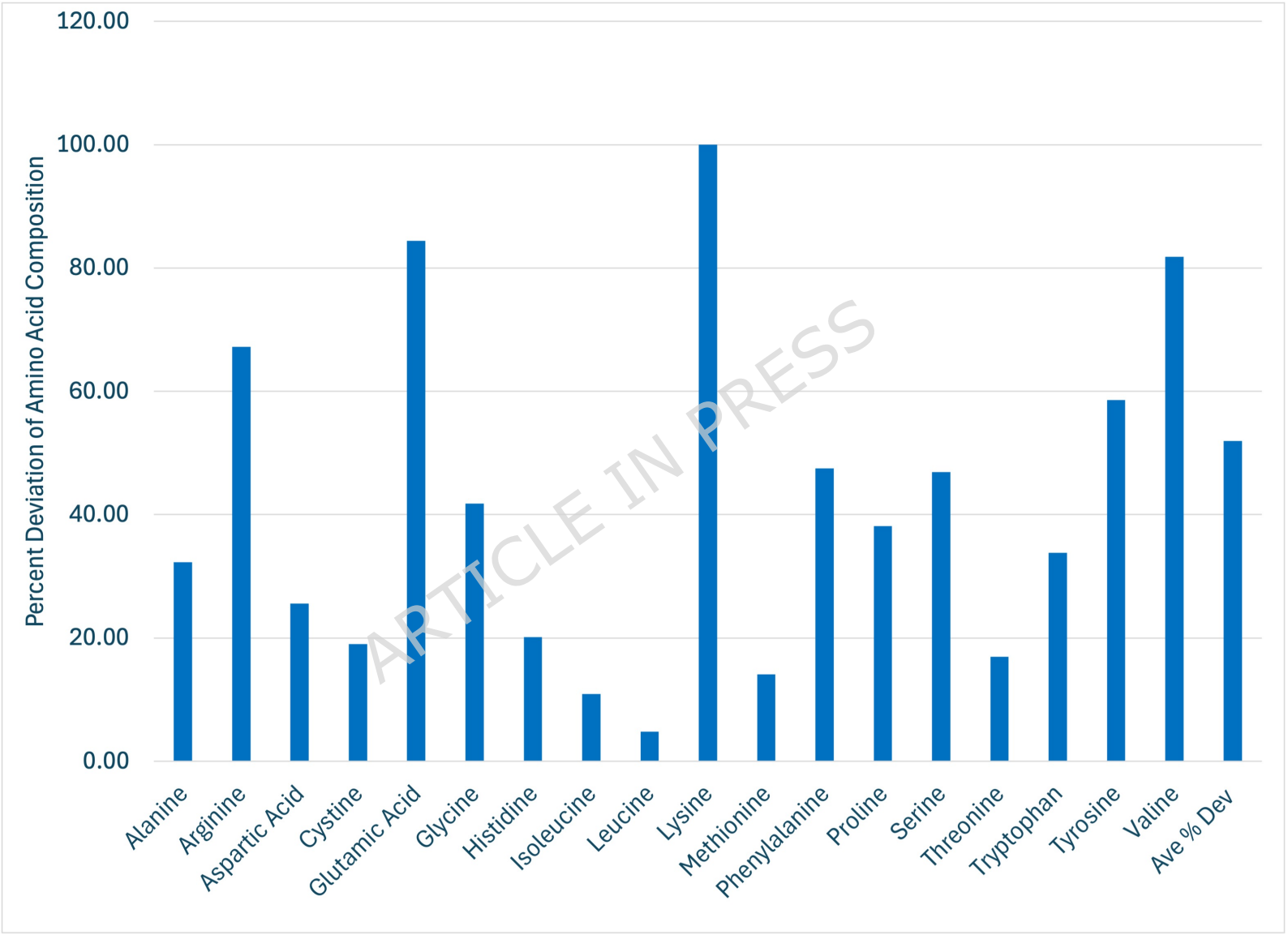


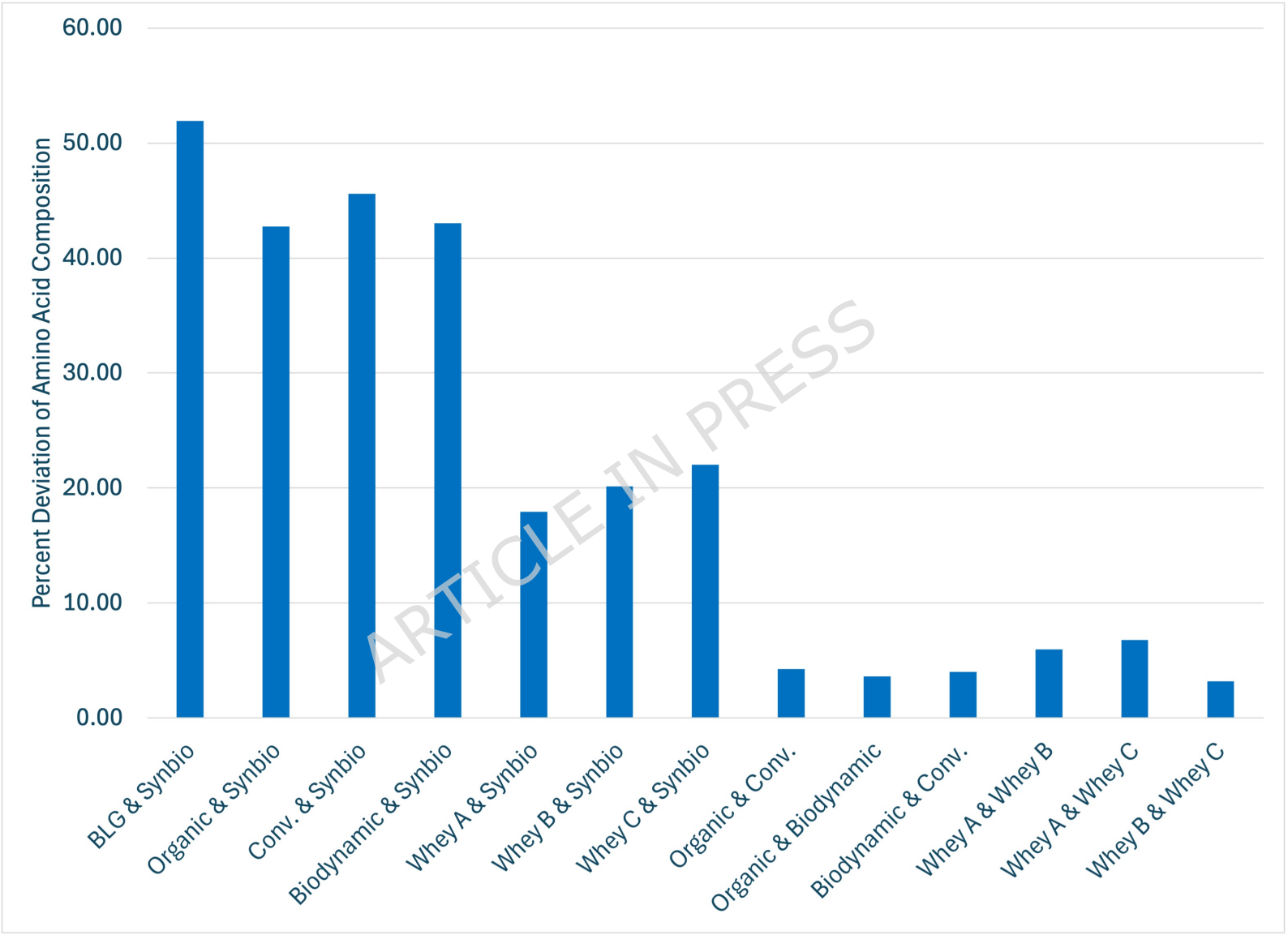












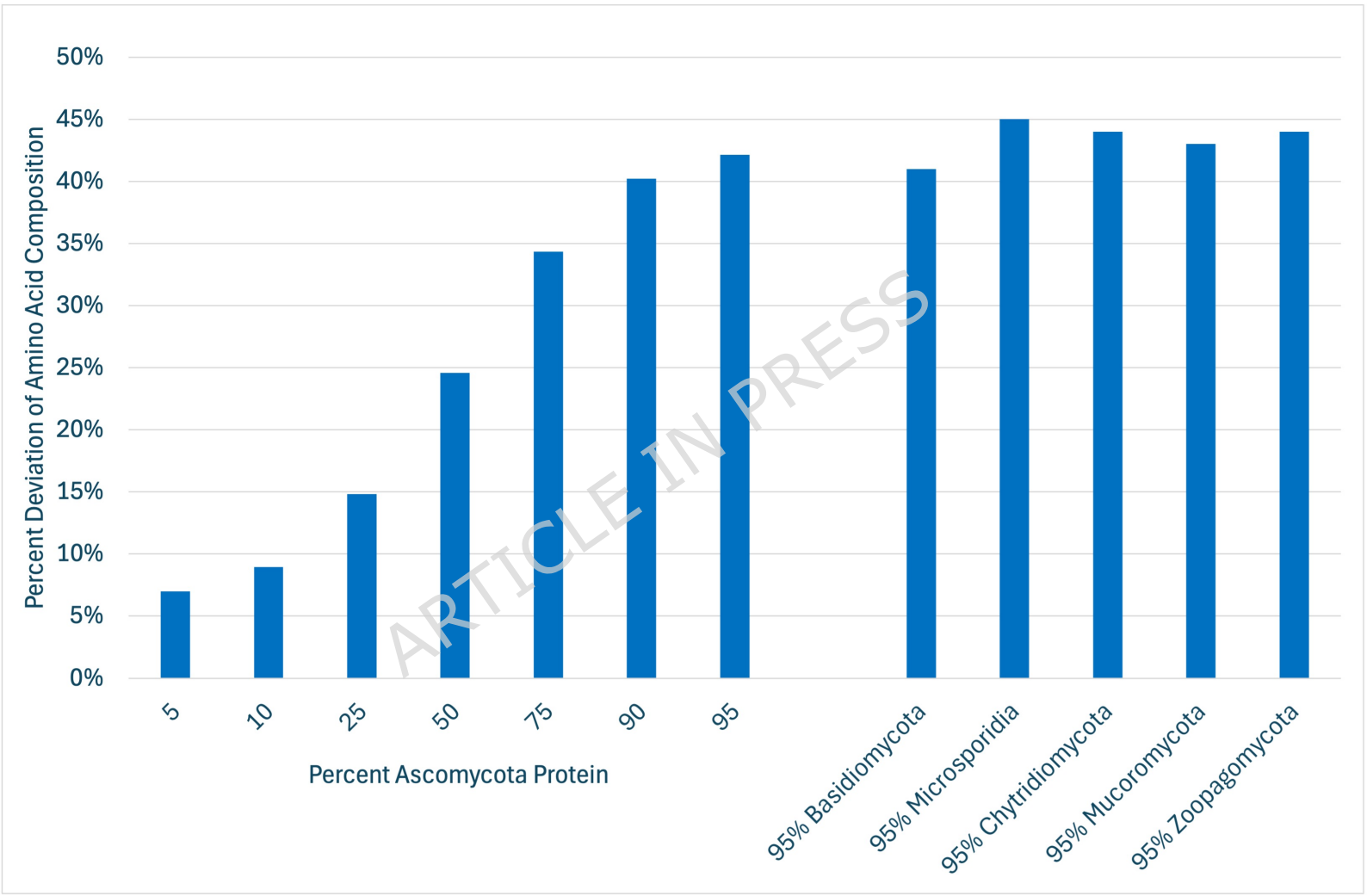


Table 1 b-Lactoglobulin and Fungal Protein in Synbio "Milk"

Total protein in synbio "milk" (by proximate analysis)
b-LG protein in synbio "milk" (by ELISA)
Fungal protein in synbio "milk" (by difference)
Total protein in bovine milk (by proximate analysis)
b-LG protein in bovine milk (by proteomic analysis)
Other proteins (casein, other wheys, etc.) in bovine milk (by d

g protein/243.9 g milk
7.53
1.01
6.52
8.37
1.2
7.17

	Table 2 Percent Total Amino Ac				
	Grams of each amino acid in 100g tot				
	b-Lactoglobulin	**Synbio "Milk"**	**Conventional Milk**	**Organic Milk**	**Biodynamic Milk**
Alanine	4.93	6.52	3.27	3.11	3.25
Arginine	7.88	2.58	3	3.33	3.25
Aspartic Acid	8.01	10.05	7.36	7.33	7.47
Cystine	1.83	2.17	0.54	0.67	0.65
Glutamic Acid	9.28	17.12	20.16	20.22	20.13
Glycine	3.27	1.9	1.91	2	1.95
Histidine	1.87	1.49	2.72	2.44	2.6
Isoleucine	4.78	5.3	4.9	5.11	4.87
Leucine	13.99	13.32	9.26	9.33	9.09
Lysine	2.64	10.33	8.17	8	8.12
Methionine	3.16	2.72	2.45	2.44	2.27
Phenylalanin	6.21	3.26	4.63	4.67	4.55
Proline	7.91	4.89	9.26	9.33	9.74
Serine	6.65	3.53	5.45	5.33	5.52
Threonine	3.95	4.62	4.36	4.22	4.55
Tryptophan	3.08	2.04	1.36	1.33	1.3
Tyrosine	7.87	3.26	4.63	4.89	4.55
Valine	2.69	4.89	6.54	6.22	6.17
Total	100	100	100	100	100

id Weight		
tal amino acids		
Whey A	**Whey B**	**Whey C**
4.94	5.02	5.14
2.34	1.88	1.81
10.13	10.66	10.88
1.82	1.88	1.81
16.88	17.55	17.52
1.82	1.57	1.51
2.08	1.88	2.11
6.23	6.58	6.65
9.87	10.03	9.97
8.83	9.09	9.37
2.34	2.19	2.11
3.12	2.82	2.72
6.75	6.27	6.04
5.19	4.7	4.83
7.01	7.21	7.25
1.82	1.88	1.81
2.86	2.82	2.72
5.97	5.96	5.74
100	100	100

Table 3--Weight-Percent of Individual Proteins in T

Organism	Protein Descriptions	Symbio "Milk" Lot A	Symbio "Milk" Lot B	Organism
Bos taur	Beta-lactoglobulin	24.86	31.77	T. reesei
T. reesei	Alpha-glucuronidase	26.23	20.64	T. reesei
T. reesei	Carbohydrate esterase family 5	10.27	15.81	T. reesei
T. reesei	Predicted protein	8.19	9.37	T. reesei
T. reesei	Predicted protein	7.25	4.52	T. reesei
T. reesei	AA9 fam. lytic polysacch. monooxygenase c	5.59	5.03	T. reesei
T. reesei	Predicted protein (Fragment)	1.50	1.47	T. reesei
T. reesei	Predicted protein	1.09	1.76	T. reesei
T. reesei	Cell wall protein	0.84	0.57	T. reesei
T. reesei	4-O-methyl-glucuronoyl methylesterase	0.80	0.48	T. reesei
T. reesei	Phospholipase C	0.74	0.52	T. reesei
T. reesei	Carbohydrate esterase family 5	0.65	1.06	T. reesei
T. reesei	alpha-galactosidase	0.62	0.54	T. reesei
T. reesei	Beta-hexosaminidase	0.62	0.32	T. reesei
T. reesei	Predicted protein	0.57	0.22	T. reesei
T. reesei	alpha,alpha-trehalase	0.55	0.28	T. reesei
T. reesei	Carbohydrate-binding module family 1	0.54	0.29	T. reesei
T. reesei	non-reducing end alpha-L-arabinofuranosid	0.49	0.52	T. reesei
T. reesei	Predicted protein	0.44	0.20	T. reesei
T. reesei	Ubiquitin fusion protein (Fragment)	0.41	0.14	T. reesei
T. reesei	Glucanase	0.37	0.28	T. reesei
T. reesei	Acid phosphatase-like protein	0.33	0.21	T. reesei
T. reesei	Glycoside hydrolase family GH30	0.33	0.22	T. reesei
T. reesei	Predicted protein	0.32	0.21	T. reesei
T. reesei	Beta-mannosidase A	0.31	0.14	T. reesei
T. reesei	Glycoside hydrolase family 92	0.28	0.15	T. reesei
T. reesei	Predicted protein	0.26	0.07	T. reesei
T. reesei	Glycoside hydrolase family 31	0.25	0.18	T. reesei
T. reesei	Xyloglucanase	0.22	0.12	T. reesei
T. reesei	glucan endo-1,3-beta-D-glucosidase (Frag.)	0.22	0.14	T. reesei
T. reesei	Predicted protein	0.22	0.11	T. reesei
T. reesei	1,3-beta-glucanosyltransferase	0.21	0.08	T. reesei
T. reesei	Glucoamylase	0.21	0.08	T. reesei
T. reesei	Phosphatidyl-glycerol/-inositol transfer pro	0.21	0.10	T. reesei
T. reesei	Predicted protein	0.20	0.04	T. reesei
T. reesei	Predicted protein	0.18	0.15	T. reesei
T. reesei	Transaldolase	0.18	0.08	T. reesei
T. reesei	Carboxylic ester hydrolase	0.18	0.18	T. reesei
T. reesei	Alpha-galactosidase	0.16	0.11	T. reesei
T. reesei	Glycoside hydrolase family 71	0.15	0.05	T. reesei
T. reesei	Predicted protein	0.15	0.08	T. reesei
T. reesei	DNase1 protein	0.14	0.05	

Total Protein of Synbio Milk

Protein Descriptions	Synbio "Milk" Lot A	Synbio "Milk" Lot B
Predicted protein	0.14	0.04
Predicted protein (Fragm	0.14	0.07
Mitochondrial matrix fac	0.11	0.06
Predicted protein	0.11	0.04
Trehalase	0.10	0.07
Glycoside hydrolase fami	0.10	0.02
Predicted protein	0.10	0.06
Glycoside hydrolase fami	0.10	0.06
Predicted protein	0.09	0.08
Predicted protein	0.09	0.03
FAD-binding PCMH-type	0.09	0.03
Amidase	0.08	0.05
Predicted protein	0.07	0.03
Predicted protein	0.07	0.09
Predicted protein	0.07	0.04
Predicted protein	0.07	0.03
Predicted protein	0.06	0.04
Predicted protein	0.06	0.30
Predicted protein	0.06	0.02
1,3-beta-glucanosyltrans	0.06	0.04
Predicted protein	0.06	0.03
Predicted protein	0.06	0.03
EEF1-gamma domain-co	0.06	0.01
cellulase	0.06	0.01
Predicted protein	0.05	0.02
Predicted protein	0.05	0.04
Predicted protein	0.05	0.01
xylan 1,4-beta-xylosidase	0.05	0.02
Aldose-1-epimerase	0.04	0.03
Predicted protein	0.04	0.01
Predicted protein	0.04	0.02
Glycosidase	0.04	0.04
Predicted protein	0.04	0.02
Endo-1,4-beta-xylanase 3	0.04	0.00
Glycoside hydrolase fami	0.04	0.03
Predicted protein	0.04	0.03
Glycoside hydrolase fami	0.03	0.01
Peptide hydrolase	0.03	0.02
alpha-galactosidase (Fra	0.03	0.01
Lysophospholipase	0.02	0.03
Endo-1,4-beta-xylanase 2	0.02	0.00

Table 4	Minimum	Maximum
Fungal protein in one serving of food processed with recombinant pectin lyase produced in *T. reesei*	0.0068 mg	0.3402 mg
Fungal protein in one serving of synbio "milk" produced with *T. reesei*	5400 mg	6600 mg
Exposure to fungal protein from synbio "milk" consumption compared to exposure from consumption of food processed with recombinant pectin lyase	19,400-fold higher	94,118-fold higher