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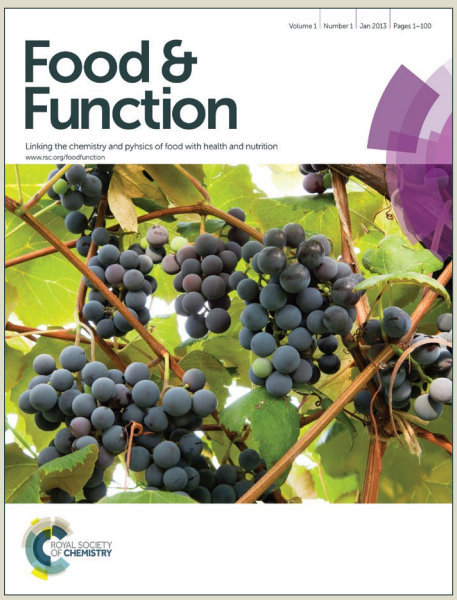


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# Potential applications of metagenomics to assess the biological effects of food structure and function

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## ABSTRACT

Metagenomics, or the collective study of genomes is an important emerging area in microbiology and related fields, and is increasingly being recognized as a tool to characterize the microbial community structure and function of diverse sample types. Metagenomics compares sequences to existing databases to enable the identification of potential microbial reservoirs and predict specific functions; yet, metagenomics has not been widely applied to understand how changes in food structure and composition affect microbial communities and their function in the human gut. Studies are needed to understand the digestion of food products, and to measure their effectiveness in preserving a healthy microbiome, as well as intestinal function. We suggest the use of metagenomics with validation techniques such as Polymerase Chain Reaction (PCR), cloning and functional assays to assess the biological effects of food structure and function.

**Keywords:** Food structure, Metagenomics, Microbiome, Milk Fat Globule

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## INTRODUCTION

Structure and functionality measurements have revolutionized the field of food science mainly because both food flavor and the potential beneficial health effects are among the consumers' interests today; yet, we are just beginning to elucidate this association. This is particularly the case for milk products, which have been extensively consumed for centuries, but formulations have been refined throughout the years to meet these criteria. The development of models capable to differentiate the digestion and potential health benefits of the different structures that can be created with modern food technology offer a fertile ground for research, and will continue to provide a forum for novel ideas in modern food processing, and a more efficient method to measure the efficiency and benefit of one structure over another. We propose that the emerging field of metagenomics, with all its variations, is of importance to understand the biological changes induced by modifications in food structure. In the present review, we have turned to the field of metagenomics with the potential of measuring the effect that the structure of different milk product components has on the function of the intestinal microbiota.

## WHAT IS METAGENOMICS?

Metagenomics, or the collective study of genomes is an important emerging area in microbiology and related fields, and is increasingly being recognized as a tool to characterize the microbial community structure and function of diverse sample types [1, 2]. One of the most important characteristics of metagenomics is the ability to characterize microbial communities

that are not able to grow under specific conditions or in pure cultures [3]. It is estimated that the great majority of microorganisms in diverse sample types are uncultivable, making metagenomics of great potential in fields such as food science, where the majority of the microbial assays still depend on those microorganisms that can be grown. In the following section, we discuss the most important approaches to study microbial communities, namely targeted amplicon sequencing (TAS) and shotgun metagenomics as a way to direct readers into the potential applications of these techniques in the field of food science.

## Sequencing approaches

### *Targeted amplicon sequencing (TAS)*

A large number of studies have characterized the microbial composition of diverse samples using TAS. TAS relies on the amplification of phylogenetic markers, which usually include the 16S (bacterial) or 18S rRNA (microbial eukaryotic) genes [4]. Variable regions from these phylogenetic markers, including the V3, V4, V5 or V6 of the 16S rRNA gene (which usually enables a reliable distinction of bacteria up to the genus level) [4], or the ITS region of the 18S rRNA gene are usually targeted [5]. Data are usually analyzed using tools such as Qiime or Mothur in order to infer phylogenetic relationships with existing reference databases such as Greengenes or Silva [6, 7]. The relative abundance of Operational Taxonomical Units (OTUs) is then used to determine taxonomy, and microbial community diversity [8]. While TAS has shown to be relatively cost-effective, some *a-priori* knowledge of the microbial composition of a sample is expected [9], and species-level resolution is often not reached [10], making techniques such as Polymerase Chain Reaction (PCR) and its variants of great utility to characterize specific

microbial communities with greater resolution. TAS data are now increasingly being coupled with shotgun metagenomics data (described below) to obtain a greater resolution of the biological structure and function of a sample. In the dairy industry, for example, TAS has been used to determine the bacterial composition of raw milk, ripened curd and mozzarella cheese. Raw milk has shown to have the greatest number of species compared to ripened curd and mozzarella cheese, possibly more than those that can be cultured [11].

***Shotgun metagenomics***

Unlike TAS, shotgun metagenomics is a non-targeted approach that provides the advantage of characterizing microbial communities (bacteria, microbial eukaryotes and archaea), and viruses (prokaryotic and eukaryotic) together [12]. While results provide insights into the taxonomy of the microorganisms in a sample, they also provide information about the enzymes and pathways that may be associated with carbohydrate, lipid and amino acid metabolism. Taxonomic composition of microbial communities from shotgun metagenomic data can be determined using tools such as the metagenomics analysis server (MG-RAST) and the Classifier for metagenomics sequences (ClaMS) [13, 14]. Taxonomic classification of shotgun metagenomic data relies on sequence annotation based on existing databases, which need to be updated and curated regularly so that sequence annotation is reliable.

Shotgun metagenomics data provide an advantage over TAS in that functional prediction analyses can be performed using tools such as the metagenomics server MG-RAST, Kyoto Encyclopedia of Genes and Genomes (KEGG) [15], Carbohydrate Active Enzymes (CAZy)

database [16, 17], and/or the Interactive Pathway Explorer (iPATH) [18]. These functional prediction analyses also rely on existing databases and how these have been annotated and curated. Function prediction based on shotgun metagenomics data represents one of the first steps to assign functions to specific microbial communities and identify potential reservoirs. Functional prediction from shotgun metagenomics data has provided insights into potential metabolic signatures and biomarkers of human diseases including Crohn's disease and type 1 diabetes, among many other health conditions, proving to be a reliable approach to predict the function of specific microbial reservoirs in diverse sample types.

## METAGENOMICS AND FOOD STRUCTURE

While TAS has been widely applied to understand the taxonomic composition of diverse samples, the next essential step in both microbiome research and the field of food structure and function is to identify enzymes, pathways and potential mechanisms associated with specific microbial communities. Shotgun metagenomics has the advantage over TAS in that both the taxonomy and function can be determined and predicted. Identifying the enzymes, pathways and mechanisms and how these operate under different conditions may perhaps be of greater value in the field of food structure and function than determining the taxonomy of specific samples alone. Predicted function(s) elicited by diverse food structures would have to be accompanied by the validation of metagenomics data using PCR, expression of genes of interest and function assays. We will use in great part the structure of the milk fat globule membrane (MFGM) as an example on how metagenomics can be used in studies related to food structure. MFGM are an essential component of maternal milk, and possess important functional and nutritional characteristics that are known to have beneficial health effects [19, 20].

Some of our findings using confocal microscopy, atomic force microscopy and proteomics have provided insights of the organization of the MFGM. Results have also shown that MFGM are a source of bioactive molecules including glycerophospholipids, gangliosides, cerebrosides, cholesterol and glycoproteins [21]. We have also been interested in the relationship of the MFGM structure to specific microbe interactions. We determined the effects of processing of the milk product on both the structure and composition of the MFGM by measuring the binding interactions between lactic acid bacteria and the MFGM. Table 1 shows how, by using optical tweezers, we were able to measure the difference in binding force and interactions between similar types of lactic acid bacteria and the surface of the bovine MFGM. We also devised a means to measure the amount of interaction between these bacteria and milk buttermilk powder (high in MFGM content) using a gradient centrifugation method. In this procedure, we measured the DNA recovered at the bottom of the centrifuge tubes as a result of the different binding characteristics. Cells that bound tightly to the fat globules remained at the surface along with the fat in the centrifuge tube (Figure 1A). In contrast, bacteria that did not interact with the fat globules were recovered at the bottom of the tubes (Figure 1B). These data are mainly the phenotypic effects of bacteria/MFGM interactions; therefore, a large missing part in this research area is the potential changes in gene expression resulting from bacteria/MFGM interactions. Global changes in gene expression are increasingly being explored using RNA-sequencing, or RNA-seq, a variation of shotgun metagenomics, where complementary DNA (cDNA) libraries are constructed from messenger RNA (mRNA). The identity of the genes and the level of expression can then be explored using RNA-seq. Another key question that has not been investigated in great detail is the potential physiological function(s) of MFGM to the infant gut

microbiome. TAS results have shown that breast- vs bottle-feeding can significantly alter the structure and succession of the infant gut microbiome [22, 23]. More recent studies have identified the specific structures of maternal milk that are associated with maintaining infant health during development. For instance, leptin and certain fatty acids present in the milk of obese mothers have been associated with children also developing obesity [24]; yet, how MFGM may be associated with the gut microbiome, maintaining health and preventing diseases such as obesity remain to be addressed. Also, efforts to mimic MFGM present in maternal breast milk have shown promising results, opening a new are in infant gut microbiome research [25].

Shotgun metagenomics is also increasingly being applied to identify the association of microbial communities with molecules and enzymes in specific carbohydrate, lipid and amino acid pathways. In the following section we discuss some of these findings.

**Carbohydrates**

Carbohydrates are classified as mono-, di-, oligo- and poly-saccharides, and some types are resistant to human hydrolytic enzymes. Non-digested carbohydrates provide a major energy source for the growth of certain microorganisms in the gut [26, 27]. The different assemblages of monosaccharides into higher structures results in a great diversity of carbohydrates. Complex carbohydrates in the form of fruits, vegetables and cereals constitute a good proportion of human diets [28], and their consumption is known to affect the composition and function of the gut microbiome. For instance, weight-loss diet studies, where total carbohydrate intake is decreased, have shown that there is a reduction in the detection of microbially-produced short-chain fatty

acids (SCFA), which include acetate, propionate and butyrate, known to be beneficial for gut health in fecal samples [29]. In addition, there is a significant decrease in the proportion of Bifidobacteria and butyrate-producing *Lachnospiraceae*, and an increase in *Ruminococcus bromii*. Subjects with the smallest ruminococcal populations have shown to fail to fully ferment dietary resistant starch, suggesting that this bacterial group might play a key role in this process [30]. To the best of our knowledge, most metagenomics studies in the area of carbohydrate digestion focus on the effect of complex carbohydrates to the gut microbiome structure, opening the opportunity to decipher the metabolic profiles that could potentially result from the consumption of diverse forms of carbohydrates. It is also known that carbohydrate utilization and central metabolism functions seem to be more dedicated to the degradation of complex carbohydrates rather than simple sugars [31]. Metagenomic studies focusing on how simple carbohydrates affect both the human gut microbiome structure and function are still needed. This could be performed with human milk, for example, which is known to harbor a great variety of monosaccharides including D-glucose (Glc), D-galactose (Gal), N-acetylglucosamine (GlcNAc), L-fucose (Fuc), and N-acetylneuraminic acid (NeuAc) [32]. Testing the potential effect(s) of these monosaccharides using *in-vivo* and *in-vitro* models could provide insights into the metabolic response of specific microbial components that could potentially influence human gut microbiome function.

Complex carbohydrates are degraded by enzymes known as carbohydrate active enzymes (CAZymes) that can be human- or microbially-encoded [33]. Shotgun metagenomic analyses have shown to be useful in the identification of CAZYmes, where the majority are microbially-encoded and are present in diverse human surfaces including the nares, oral cavity, skin and

stool, with the latter showing the highest relative abundance [26]. The majority of the CAZYmes in the human gut aid in the degradation of plant cell wall and animal glycans, while the remaining aid in the degradation of starch, glycogen, and peptidoglycans [34]. CAZymes belonging to the glycoside hydrolases (GHs) can break the glycosidic bond between carbohydrates or between a carbohydrate and a non-carbohydrate moiety [35]. Another type of CAZyme that breaks bonds with the insertion of a water molecule are the polysaccharide lyases (PLs) [35]. GH families are more abundant than PL families, with 130 and 22 families discovered so far, respectively; thus, we will further focus on GHs.

Several human-encoded GHs including GH1, GH9, GH13, GH18, GH31, GH35 and GH37 are known to be involved in carbohydrate degradation; yet, microorganisms play the major role in carbohydrate degradation using GHs that differ from those that are human-encoded [36]. The availability of databases such as the Carbohydrate-Active enzyme (http://www.cazy.org/) makes it possible to identify GH sequence homology in a sample and identify the potential reservoirs. At the phylum level, it is known that the Firmicutes and Bacteroidetes represent the most abundant reservoirs of GHs, opening the opportunity to understand their role in carbohydrate metabolism [36]. In the field of dairy science and nutrition, oligosaccharides present in milk and their digestion by bacteria and other microorganisms in the gut is an increasing expanding area of research; however, metagenomics may perhaps represent the most comprehensive initial approach to understand the potential effect(s) of oligosaccharides to the gut microbiome, and to identify the enzymes and reservoirs involved.

## Lipids

Lipids include fats, sterols, mono-, di- and tri-glycerides, and phospholipids, and act as energy reservoirs, for signaling processes and structural components [37-39]. Lipids are highly diverse, essential food components, and their homeostasis is essential to maintain health; yet, they are also associated with health-related conditions including obesity. Obesity has been strongly linked to changes in the structure of the gut microbiome. For instance, a high-fat diet is known to affect the relative abundance of the major gut bacteria phyla. Individuals under a low-fat diet have shown to possess a lower relative abundance of Bacteroidetes and a higher relative abundance of Firmicutes. Individuals under a high-fat diet have demonstrated the opposite effect [40, 41]. Interestingly, lean, germ-free mice have been shown to gain more weight when receiving the gut contents of obese mice than recipients of the gut contents of lean mice, further supporting that the microbial components of stool have an effect on energy utilization [42]. The predicted increased capacity for dietary energy harvest by the obese mice microbiome was validated using biochemical assays. Future studies are needed to understand the specific microbial components at the species level of the human microbiome that may be directly or indirectly involved in conditions such as obesity. Identifying these components could potentially be used in the future as a tool to counteract the increased energy harvest capacity from specific microorganisms that may lead to conditions such as obesity.

The structure in which the lipids are presented to the gut is also of great relevance in the field of food science, but it remains unknown how different structures affect the human gut microbiome [43]. Identifying the specific microbial components associated with energy harvest will aid in the understanding of the microbial taxonomy structure associated with the digestion of

milkfat and related products in the human gut. Partial hydrolysis (lipolysis) of milkfat results in specific flavors of dairy products and this, in part, will depend on the degree of lipolysis. Lypolytic enzymes are involved in the degradation of milkfat and include lipases and esterases, and both are utilized to produce desire flavors in dairy products. Metagenomics has been previously utilized to search for novel lypolytic enzymes in unconventional samples such as aquatic environments, and has proven to be a successful tool in the initial screening of novel lypolytic enzymes [44]. It is not well known how the gut microbiota utilizes lypolytic enzymes to digest milkfat, opening the opportunity to utilize microbiome analyses and shotgun metagenomics to identify the specific microbial components, as well as the enzymes and pathways associated with the process. Results will provide further insights into possible changes in the gut microbiome, if any, after the digestion of milkfat and related products and/or if these are responsible for maintaining gut health. In fact, previous studies have shown potential beneficial effects of ingesting milk products in re-shaping the gut microbiota. A previous study demonstrated that consumption of fermented milk products results in changes in the gut microbiota in inflammatory bowel syndrome (IBS) patients, showing overall beneficial effects; yet, how milk products can potentially modulate the gut microbiota of healthy subjects remains to be investigated [45].

Lipids can also affect the metabolic capabilities of the gut microbiome. For instance, lipid-utilizing genes are enriched in obese individuals [46]. Another study found that obese subjects have higher levels of SCFA in their stool, indicating that colonic fermentation differs in lean and obese subjects [41]. Studies utilizing mouse models suggested that the ability of the microbiome to influence energy balance is dependent on the capacity of the microbes to suppress expression of

247 angiopoietin-like protein 4, or fiaf, a gut-derived inhibitor of human lipoprotein lipase [47]. When
248 germ-free mice received the gut contents of normal mice, fiaf was suppressed, resulting in a
249 greater proportion of triglycerides being stored. While there is some conflicting evidence
250 suggesting that a high-fat diet does not always result in fluctuations of Firmicutes and
251 Bacteroidetes, the gut microbiome does respond to lipids and contribute to host energy balance,
252 probably due to the utilization of several different signaling mechanisms [46].

253

254 Metagenomic studies can provide a starting point to identify genes associated with lipid
255 metabolism and associated signaling pathways, but these would need to be coupled with other
256 techniques such as lipidomics. Lipidomics is the global study of lipids, including pathways and
257 networks in biological systems, and has been increasingly utilized during the last years due to the
258 advances in mass spectrometry (MS), computational methods and systems biology approaches.
259 In fact, lipidomic studies define the biochemical mechanisms of lipid-related diseases through
260 identifying alterations in cellular lipid metabolism, trafficking, as well as homeostasis.
261 Lipidomics may be an essential tool to understand the role that the gut microbiota plays in the
262 complex lipid-host metabolism in association with health and diverse disease phenotypes. A
263 lipidomics approach, that includes metagenomics, mass spectrometry and systems biology may
264 also be applied to understand the global changes in metabolic pathways in association with the
265 ingestion of milkfat, related products and MFGM [48].

266

267 MFGM is a conserved structure in the milk that follows a very specific function in milk
268 expression. The structure of MFGM also plays a role in digestion, a hypothesis that has some

early support [43, 49]. A previous study showed that the composition of plasma lipids can be different if milk fat is consumed as free molecules or as native fat globules (i.e. homogenized droplets). The experiment was designed so that milk samples had exactly the same composition, but in different physical states: as free fat, natural cream (which contained the basic natural structure of the MFGM), small natural fat globules in cream, and homogenized cream. Their results showed that the enrichment in plasma tricacyl glycerides (TAG) in the rats fed the different equi-caloric diets was lower with emulsified milk fat compared with anhydrous milk fat. Moreover, during digestion and absorption, fatty acids (FA) profile of plasma lipids was different for the homogenized cream than for the free fat. These data show that dairy products with the same composition, but varying fat supra-structures result in different kinetics of lipid digestion which could be a health-concern.

**Amino Acids**

Amino acids support the growth and survival of gut bacteria, and regulate energy and protein homeostasis. Gut bacteria break down proteins into peptides and amino acids, indicating that they have an important role in amino acid homeostasis. Amino acids derived from dietary protein sources may serve as substrates for bioconversion by the gut microbiome. *Clostridium* spp., *Bacillus* spp., *Lactobacillus* spp., *Streptococcus* spp., and Proteobacteria are the most abundant groups responsible for amino acid fermentation in the small intestine, while *Clostridium* spp. and Peptostreptococci appear to be the most abundant groups involved in amino acid fermentation in the large intestine. Lysine, arginine, glycine, valine, and isoleucine are among the preferred substrates for gut bacteria, and result in the generation of a complex mixture of metabolic end products including, but not limited to ammonia, branched-chain fatty acids

(BCFA), and SCFA. The production of SCFA suggests an interaction between microbial activity and host amino acid, and SCFA homeostasis. Diverse microbial enzymes may contribute to mammalian amino acid metabolism by generating bioactive metabolites in the intestine. One such class of enzymes, amino acid decarboxylases, is widely prevalent in gut microbes. When combined with amino acid transport systems, amino acid decarboxylases link dietary compounds with microbial metabolism and signaling with the gut mucosa [50, 51].

Human milk and related products could represent valuable models to understand the effect of amino acids to the gut microbiome structure and function. Human milk is known to be a source of proteins (8g/L). Approximately 70 % of (human) milk proteins are glycosylated and possess both N-linked and O-linked glycan moieties. The majority of milk glycoproteins are found in skim milk (whey and casein), but the MFGM contains a representative amount of total glycoproteins. The most abundant human milk glycoproteins are α-lactalbumin (17% of total protein), lactoferrin (Lf) (17%), and secretory IgA (sIgA) (11%), belonging to the whey fraction, and κ-casein, from the casein fraction (9%) [52].

## METAGENOMICS AND FOOD FUNCTION

Determining the food structure and the potential biological effects should translate to food function studies. Previous and unpublished studies in our laboratory characterizing the effect of MFGM composition and structure on the binding of lactic acid bacteria indicate that the influence of media on the binding process, and in the expression of proteins in the surface of the bacteria are of great importance in the structure of the bacterial population [53]. Figure 2 shows the

Polyacrylamide gel electrophoresis (PAGE) results of different surface proteins of seven different *Lactobacillus casei* strains when grown in different media. MRS represents the standard laboratory media for lactic acid bacteria, P represents milk permeate obtained after filtering whole milk through a 10,000 MW cut-off ultrafiltration (UF) membrane, M represents the same permeate, but with the MFGM fraction from cream, and ultra-high temperature pasteurization (UHT) represents the whole milk treated with ultra-high temperature for shelf stability. The *L. casei* strains included (NCFM, SlpA, SlpB, SlpX, MUB, FpbA and CdpA) are different in their genetic make-up on the S-layer surface proteins [54]. Microscopy results support the differences in protein composition as a result of the exposures of these bacterial strains to different substrates. This is the case for *L. casei* strain SlpA in the presence of cream (Figure 3A), buttermilk (Figure 3B) and milk (Figure 3C), where there are significant differences in binding. While understanding the phenotypic effects of lactic acid bacteria in the presence of different substrates is indeed important, it is also necessary to understand the genetic changes associated with the exposure to diverse milk products.

Application of microbiome analyses and shotgun metagenomics may provide insights into the relative abundance of genes that could potentially be associated with the exposure of diverse substrates (Figure 4). Variations of shotgun metagenomics, including RNA-seq may be used to illustrate global gene expression patterns associated with the phenotypic changes resulting from binding to different substrates. While understanding the relative abundance and global gene expression patterns associated with specific lactic acid bacterial strains is of great importance to the food science field, understanding the global changes in taxonomy, genetic

structure and gene pattern expression of the gut microbiome when exposed to variations of milk products would better capture microbial phenotypic changes, including binding properties.

Another example of bacterial binding in intestines and the effect that surface structures may play in nutrition was presented in a study of the protein-carbohydrate interaction of lactic acid bacteria and mucins found in pig guts [55]. In that study, mucins in the gut interacted with lactic acid bacteria, but the further impact in the microbial community structure and function can only be studied utilizing metagenomics tools. How these phenotypic characteristics, in association with the exposure of diverse milk products affect the function of the gut microbiome would possibly need to be investigated using *in-vitro* and *in-vivo* models. The inclusion of the effects on the human gut microbiome would be of essential value to appreciate the impact of food structure on the biology and function of the digestive system.

**Food function in association with health and disease**

Food function also has a tremendous impact in gut health and disease, possibly because there is a strong association of food function with the gut microbiome. Several of these diseases are metabolic and have been mainly studied using animal models; therefore, results would need to be replicated in humans. In the following section, we discuss recent studies that have explored changes in the gut microbiome function in association with type 2 diabetes and inflammatory bowel disease (IBD).

357 ***Type 2 Diabetes***

358 Type 2 diabetes results from an increased production of glucose and resistance to insulin.
359 It is also the combination of diverse factors including genetics, body composition, and nutrition.
360 Insulin resistance is associated with a high fat diet, which modifies the intestinal microbiota,
361 resulting in an increased intestinal permeability and susceptibility to microbial antigens [56]. More
362 recent studies have shown that type 2 diabetes is also associated with an altered gut microbiome
363 [57]. Interestingly, when obese individuals were transplanted with the fecal microbiota from lean
364 individuals it resulted in improved insulin sensitivity [58]. Clostridiales bacteria including
365 *Bifidobacterium* and *Faecalibacterium prausnitzii* are known to be in lower abundances in
366 individuals with type 2 diabetes, but it is not clear if it is associated with the development of the
367 disease or if it is a result of it. Butyrate-producing bacteria are decreased in individuals with type
368 2 diabetes, representing a risk for health as butyrate is the preferred source of energy and repair
369 in the human gut.

370

371 Administration of several different prebiotics has shown to have positive effects on the
372 onsets of type 2 diabetes [59]. For instance, an increase in *Bifidobacterium* spp. modulates
373 inflammation in obese mice and is also associated with an increase of glucagon-like peptides and
374 peptide YY, which are known to be beneficial because they decrease insulin-resistance. These
375 data open the opportunity to explore the effects of manipulating the intestinal microbiota in an
376 attempt to revert type 2 diabetes [60].

377

378 ***IBD***

IDB is a condition that includes both Crohn's disease and ulcerative colitis, and affects more than 3 million people worldwide [61]. There are several factors that affect the outcome of the condition and include age and diet, although no specific diet has shown to cause or alleviate IBD [61, 62]. More recent studies have associated IBD with alterations to the gut microbiome. Evidence suggests that *Enterobacteriaceae* are enriched in individuals suffering the condition, with *Escherichia coli* adherent-invasive strains being identified [63]. *Fusobacterium varium* has also been associated with the disease, where it can induce colonic mucosal erosion in mice [64]. Specific metabolic pathways have also shown to be different in healthy subjects and those with IBD. The metagenome of individuals with IBD has shown to have a higher representation of pathways associated with oxidative stress, type II secretion systems and bacterial virulence factors [65, 66].

IBD relapse can be prevented using antibiotics, but these have shown to decrease the gut bacterial community diversity and possibly promote the proliferation of *E. coli*, which is known to possibly have adverse effects in IBD patients. It has recently been considered to increase gut diversity in subjects with IBD by fecal transplantation as a way to alleviate the condition, which has also shown to be promising in individuals suffering from *Clostridium difficile* infections [61]. The use of probiotics has also been evaluated in IBD patients, but these have not shown to have positive in most cases. For instance, a study found that 6 out of the 9 patients went into remission when administered a symbiotic therapy of *Bifidobacterium* and *Lactobacillus*. On the other hand, subjects with ulcerative colitis have shown more promising outcomes. A study found that treatment of ulcerative colitis with a probiotic containing 8 different bacteria including *Bifidobacterium breve*, *B. longum*, *B. infantis*, *Lactobacillus acidophilus*, *L. plantarum*, *L. casei*,

*L. bulgaricus*, *S. thermophilus* in parallel with balsalazide, an anti-inflammatory drug had a higher proportion of individuals going into remission [67]. While IBD has shown to be the result of alterations in the gut microbiota and diverse metabolic functions, further study are still needed to elucidate specific pathways, and the development of novel therapies to treat the condition.

## CONCLUSIONS

We have described how the emerging field of metagenomics and its variations can be used as tools to further our knowledge of the effects, relevance and significance of changes in food structures. Our contribution has focused on the area of dairy science and how we can measure potential changes in microbial community structure and composition. By applying the "omics" approach, molecular snapshots of biological systems can be generated, allowing the study of comprehensive molecular and metabolic profiles. We envision the field of food structure and function moving from single components towards a systems approach, and how all the components together contribute to a complex network associated with specific biological functions.

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## FIGURE LEGENDS

**Figure 1.** Frontal view of the ultracentrifuge tubes after sucrose density gradient separation from buttermilk powder (BMP). Panel A shows the BMP without bacteria, the remaining tubes (1063-

S, 23272, SD2112 and T-1) represent different *Lactobacillus ruteri* strains. Panel B shows the bottom of the tubes, with bacterial pellets that did not interact with the BMP.

**Figure 2.** Cell-surface proteins of seven lactic bacteria strains (NCFM, SlpA, SlpB, SlpX, MUB, FpbA and CdpA) grown in 4 different media as acquired from PAGE results. Media included MRS=laboratory media, P=milk permeate from 10K mw cut-off UF, M=same permeate but with added 1% w/w MFGM fraction and UHT=Ultra High Temperature treated whole milk. Surface proteins isolated from the lactic acid bacteria represent the different surface proteins, and the numbers in the color coded legend represent their respective molecular weight (MW). Stacking of the rectangles represent the bands detected for each strain under each treatment.

**Figure 3.** Confocal microscope images of the binding effects to fat globules of *L. casei* strain SlpA in cream (Panel A), buttermilk (Panel B) and milk (Panel C). Confocal laser scanning microscopy (CLSM) was used to visualize the binding of the different strains to the MFGM structure in the reconstituted BMP. The BMP was reconstituted and labeled with the phosphatidylethanolamine-lissamine rhodamine B (RH-PE) probe (Avanti, Alabaster, AL). Two-mL of the RH-PE probe was first evaporated in a microcentrifuge tube for 5 min and then suspended in 225 mL of PBS. Alternatively, cream (10% w/w in PBS, pH 7.2) isolated from raw milk by centrifugation (3200g ´ 5 min) was similarly labeled with RH-PE. The dairy product (25 mL) was added and allowed to contact with the probe for 15 min at room temperature protected from light. The bacteria suspension (A600 at 2.0 in PBS) was stained with acridine orange (AO) hydrochloride (10mg/mL in water, Sigma, St-Louis, MO) at a ratio of 1:1000. The samples were incubated for 5 min at room temperature protected from light before being washed twice with PBS. Equal volume of the labeled bacteria and the dairy products were then mixed and allowed to incubate for 15 min. The labeled samples were mixed 1:2 with agarose (0.5% w/v) before

647 being transferred to the confocal microscope slide. The samples were imaged with a CLSM
648 Fluoview FV1000 inverted microscope system (Olympus America Inc, Center Valley, PA) using
649 a Plan Apo N 60x 1.42NA immersion oil objective lens (Olympus). Laser excitation parameters
650 were set at 559 nm and 488 nm for the RH-PE and the AO probes respectively and the emission
651 spectra were recorded using a fluorescence detector (405/488/559). The images were analyzed
652 with the Fluoview FV1000 software (Olympus, Version 1.7.2.2.).

653 **Figure 4.** Flowchart of the potential applications of shotgun metagenomics to understand the
654 genetic changes resulting from the binding of *L. casei* strain SlpA to different milk products.
655 Flowchart also demonstrates the potential applications of targeted amplicon sequencing (TAS)
656 and shotgun metagenomics to understand how the consumption of milk products may potentially
657 affect microbial communities in the human gut.

658

659

**Table 1.** Binding rates and forces between different Lactobacillus strains and milk fat globules as determined using optical tweezers [44].

| Bacteria | Binding rate (%) | Binding forces (pN) |
|---|---|---|
| *Lactobacillus reuteri* 1063-S | 8 | 10-15 |
| *Lactobacillus reuteri* 23272 | 11 | 10-15 |
| *Lactobacillus reuteri* SD2112 | 33 | 15-180 |
| *Lactobacillus reuteri* T-1 | 57 | 30->200 |

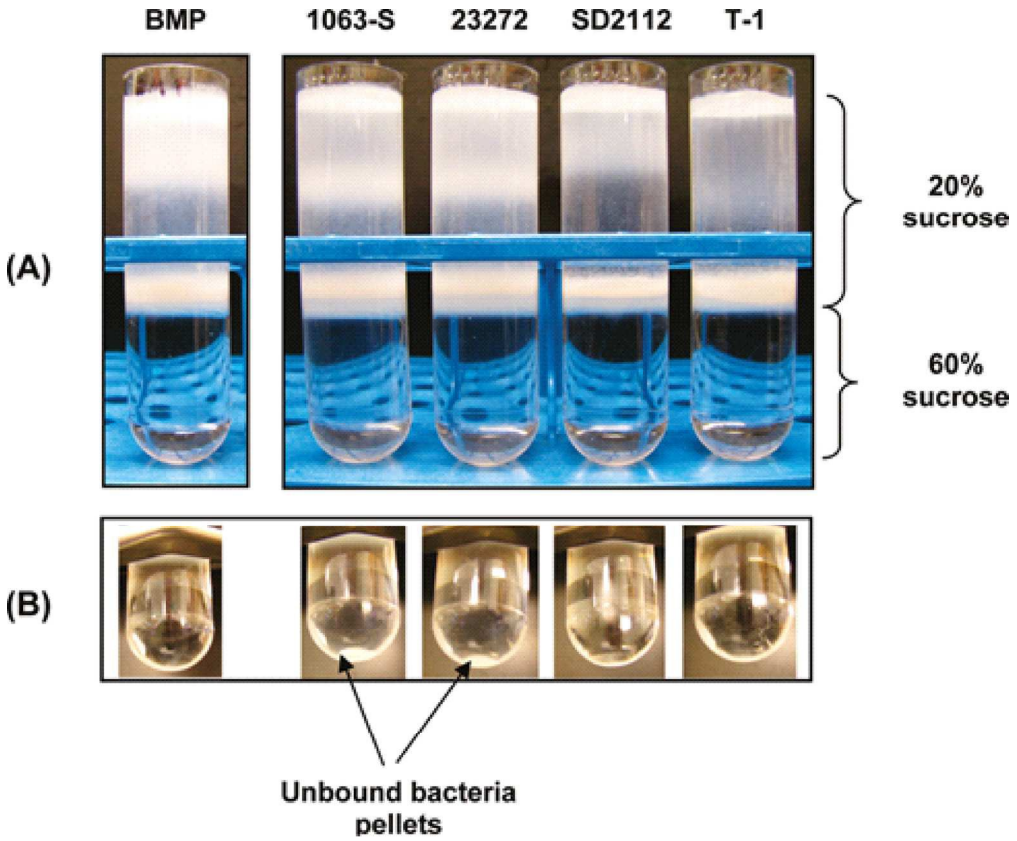


Figure 1. Frontal view of the ultracentrifuge tubes after sucrose density gradient separation from buttermilk powder (BMP). Panel A shows the BMP without bacteria, the remaining tubes (1063-S, 23272, SD2112 and T-1) represent different Lactobacillus ruteri strains. Panel B shows the bottom of the tubes, with bacterial pellets that did not interact with the BMP.
167x138mm (300 x 300 DPI)

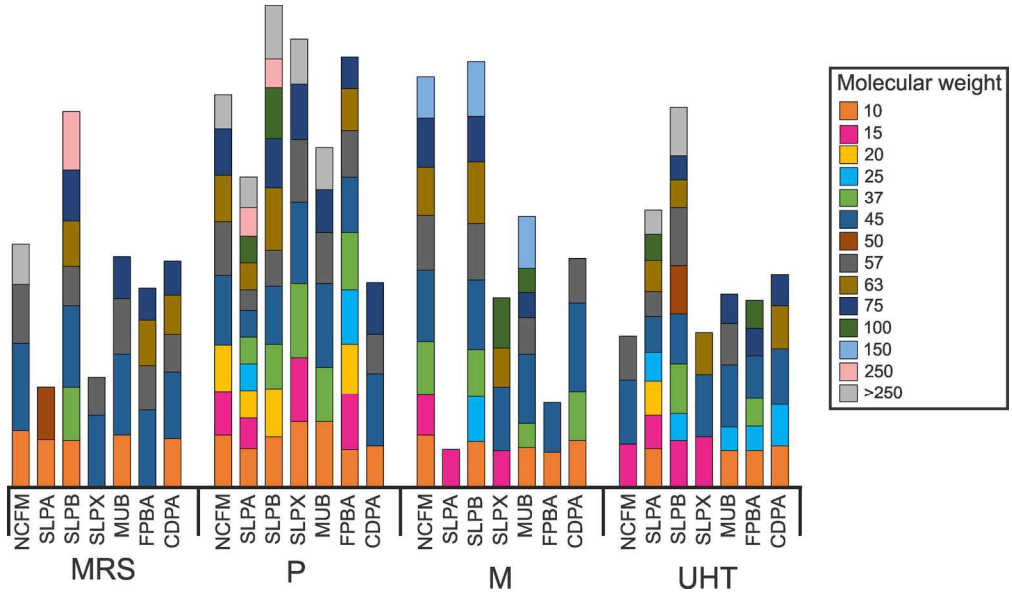


Figure 2. Cell-surface proteins of seven lactic bacteria strains (NCFM, SlpA, SlpB, SlpX, MUB, FpbA and CdpA) grown in 4 different media as acquired from PAGE results. Media included MRS=laboratory media, P=milk permeate from 10K mw cut-off UF, M=same permeate but with added 1% w/w MFGM fraction and UHT=Ultra High Temperature treated whole milk. Surface proteins isolated from the lactic acid bacteria represent the different surface proteins, and the numbers in the color coded legend represent their respective molecular weight (MW). Stacking of the rectangles represent the bands detected for each strain under each treatment.

168x98mm (300 x 300 DPI)

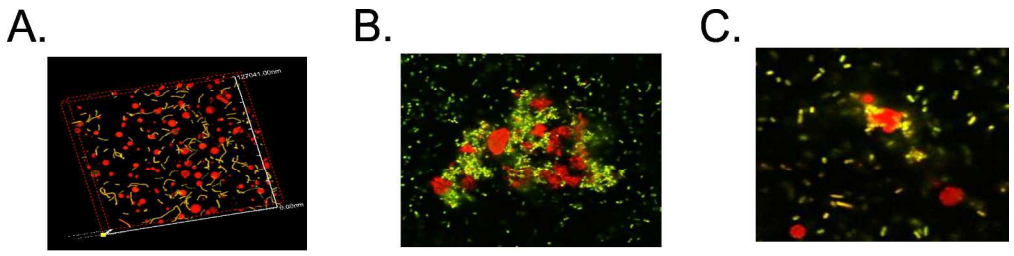


Figure 3. Confocal microscope images of the binding effects to fat globules of L. casei strain SlpA in cream (Panel A), buttermilk (Panel B) and milk (Panel C).

293x70mm (300 x 300 DPI)

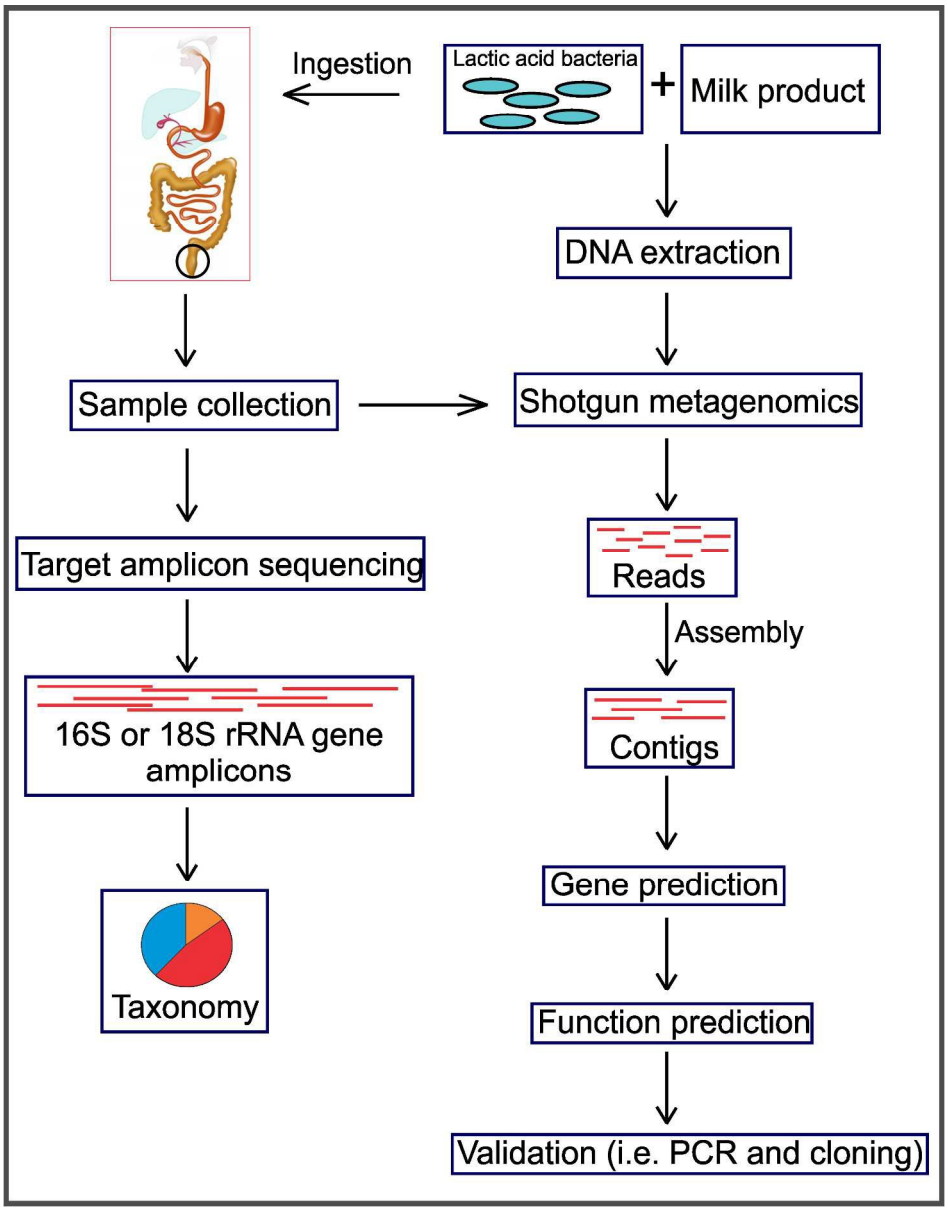


Figure 4. Flowchart of the potential applications of shotgun metagenomics to understand the genetic changes resulting from the binding of L. casei strain SlpA to different milk products. Flowchart also demonstrates the potential applications of targeted amplicon sequencing (TAS) and shotgun metagenomics to understand how the consumption of milk products may potentially affect microbial communities in the human gut.

246x313mm (300 x 300 DPI)