THE ESSENTIAL ROLES OF CARBOHYDRATES IN PROMOTING GUT MICROBIOTA FUNCTION THROUGH ALL STAGES OF LIFE

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The primary aim of the CSA is to mobilise the European academic and industrial community to identify generic measurement, data management and metrological challenges that must be met in order to advance and exploit carbohydrate knowledge and applications.

The potential for exploitation of carbohydrates lies in their diversity and structural complexity – subtle changes in the three-dimensional structure of a carbohydrate profoundly affects (for example) its ability to protect against or fight infectious disease. However, these subtle structural differences present a challenge for their analysis. Sophisticated measurement and metrological capabilities for analysing carbohydrates are available but are nowhere near as advanced or as routinely used in other areas such as gene sequencing.

Therefore as a first stage **CarboMet** has organised some open, Europe-wide workshops to identify key topics where our understanding needs to be advanced urgently, and where current limitations in our measurement, data management and metrological capabilities are hindering progress. The Workshops were also asked to recommend appropriate Work Programmes that should be supported by Horizon 2020 and its successor, Horizon Europe.

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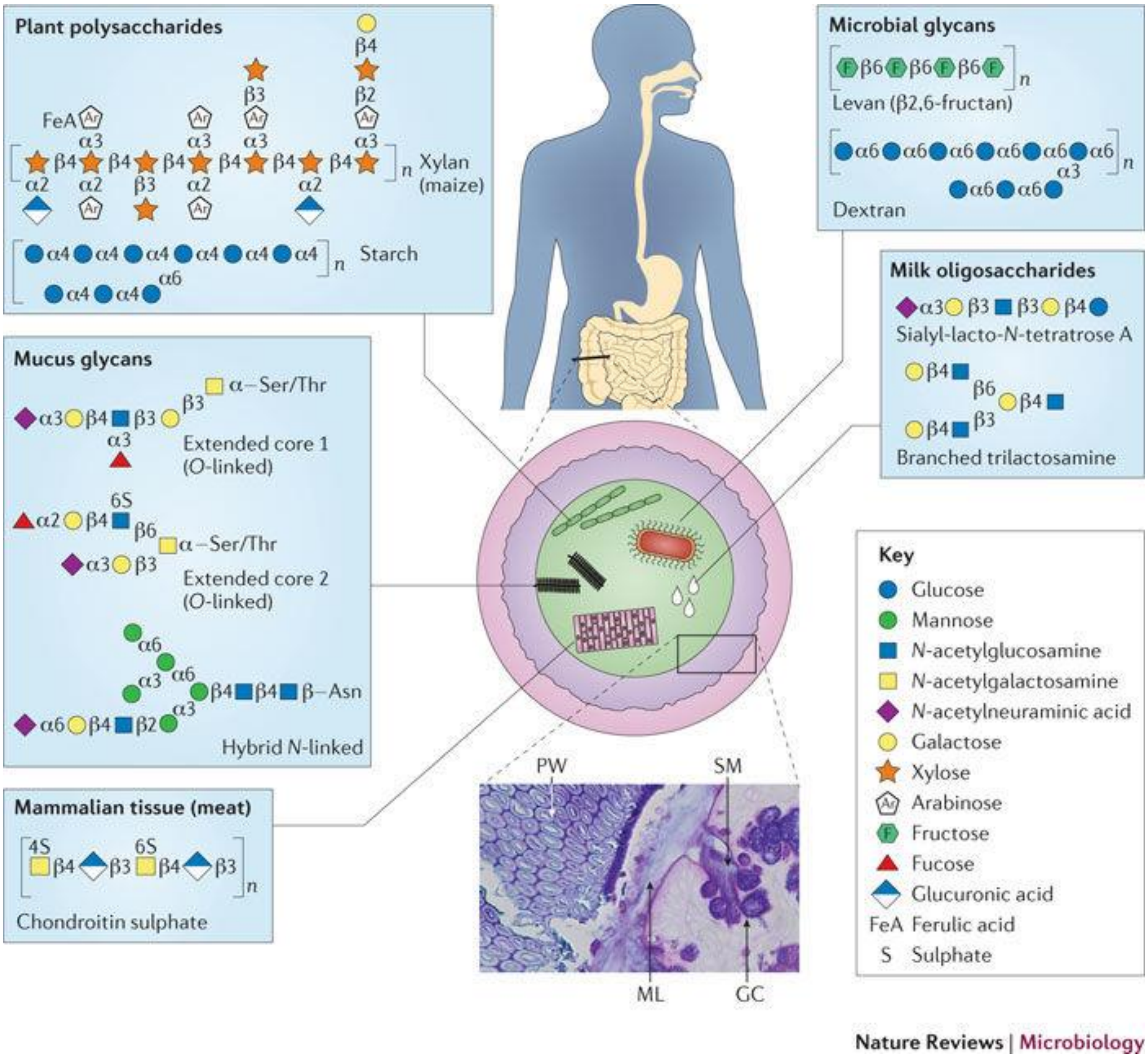

Figure 1 Carbohydrates in the Gut *(Taken from Koropatkin et. al. 'How glycan metabolism shapes the human gut microbiota[1]').*

EXECUTIVE SUMMARY

The promotion and maintenance of a healthy relationship with our gut microbiota is increasingly understood to be important for human health and well-being, through every stage of life. The **gut microbiota** fulfils a number of key biological functions[2] and imbalances in the gut microbial populations ('dysbiosis') are associated with a number of inflammation and infection conditions, such as gut disorders (e.g. Irritable Bowel Syndrome, and the Irritable Bowel Diseases, Ulcerative Colitis and Crohn's disease) and non-communicable diseases (e.g. diabetes, cardiovascular diseases and cancers).

Carbohydrates play a number of crucial roles in maintaining microbial communities: (**1**) Diet has a major influence on overall gut health and dietary carbohydrates are the main modulators of the gut microbiota structure and function.[3] (**2**) Carbohydrates present on the human gut lining as part of the mucus layer are the first point of contact between the microbiota and the host. Alterations in human mucin glycosylation are associated with gut disorders[4] and involved in the selection of the mucus-associated microbiota. (**3**) All members of the microbiota are covered with cell surface carbohydrates (polysaccharides or glycoconjugates), which mediate colonisation, biofilm formation, or interaction with the host immune system.

A comprehensive understanding of the role of **carbohydrates in the gut** is essential if we are to understand the interactions between the human host and gut microbiota and learn how to maximise these relationships for the benefit of human health[5].

This report identifies a number of opportunities and remaining challenges, to fully harvest and exploit the use of carbohydrates to promote or restore gut homeostasis as an alternative or complementary to traditional medication. This is especially important for ageing populations, where strategies that can reduce healthcare costs and improve health outcomes are increasingly required. A better understanding of the role of carbohydrates in underpinning mechanisms of gut-host interactions will allow the development of **carbohydrate-based strategies**, such as prebiotics, as solutions to address Sustainable Development Goal 3 (SDG3) that seeks to promote good health and well-being for all at all stages of life, in particular targets to reduce mortality from non-communicable diseases.

European glycoscientists[6] have been leading the development of the tools and knowledge needed to probe and understand carbohydrate interactions. These are now being applied to the microbiome field to provide invaluable mechanistic insights into the function and modulation of complex gut microbial communities. The development and integration of advanced glycomics technologies is necessary to complement and enhance the knowledge gained by other '-omics' studies in order to provide a comprehensive mechanistic understanding of the biological processes involved. In addition, there is an urgent need to be able to produce carbohydrates at scale, economically and efficiently, in the quantities needed for clinical trials to allow the regulatory assessments and approvals required prior to commercialisation and for subsequent industrial production.

The key ***challenges*** that have been identified are:

1. Understanding the mechanisms underpinning and promoting healthy relationships between the microbiota and the human gut: the importance of glycometrics;
2. Developing tools for the production of carbohydrates using fermentation, enzymatic, chemical and/or automated synthesis approaches.
3. Developing new metrology and measurement tools for carbohydrates that are involved in the interactions between the gut microbiome and human host;
4. Optimising data management by integrating glycomics data with other -omics data using bioinformatic tools and databases to facilitate knowledge sharing between glycoscientists and microbiome researchers;
5. Developing carbohydrate-based strategies that target the gut microbiota to restore and/or promote good health and well-being for all at all stages of life.

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SPECIFIC CHALLENGE – Solving major health problems by understanding the influence of carbohydrates on the human gut microbiota

The promotion and maintenance of a healthy relationship with our gut microbiota is increasingly understood to be important for human health and well-being, through every stage of life. In the mutualistic relationship between the human host and the gut microbiota, the host provides shelter and nutrients whilst in return the gut microbiota carry out a number of essential roles which include:

- Metabolism of xenobiotics and drugs;
- Production of metabolites, vitamins (B, K, and, folic acid) and neurotransmitters;
- Maintaining the structural integrity of the gut barrier;
- Maturation of the host immune system;
- Protection against pathogens;
- Development of the nervous system.

Some of these roles are directly mediated by carbohydrates, others are the result of carbohydrate fermentation by the gut microbiota, as discussed further below.

It is therefore not surprising that undesirable imbalances in the gut microbial populations ('dysbiosis') are associated with a number of inflammation and infection conditions, such as gut disorders (e.g. Irritable Bowel Syndrome, Crohn's disease) and non-communicable diseases (e.g. diabetes, cardiovascular diseases and cancers).

A comprehensive understanding of the many roles that carbohydrates play in the gut is essential if we are to understand the interactions between the human host and gut microbiota and from this how the effect of diet can maximise these relationships for the benefit of human health[7].

The **gut microbiota** defines the population (types, relative quantities) of microbes that live in a particular gut and the **gut microbiome** is their collective corresponding genome. This microbial population is dynamic, developing from birth, changing over our lifetimes and influenced by external and environmental factors such as diet.

Some of the microbes present in a gut at any point in time can be **commensal,** neither causing harm nor providing benefit to the host; **mutualistic,** living in a symbiotic relationship with the host obtaining shelter and nutrients and in return for carrying out a number of roles; or **pathogenic**, disease-causing microbes that cause harm to the host. All three classes are likely to co-exist as the overall microbial population evolves within its host, either beneficially or to its detriment. (For a comprehensive review see '*Introduction to the human gut microbiota' by Thursby & Juge*[8]).

Sources of ***carbohydrates*** influencing gut microbiota composition and function include:

- ***Food-derived polysaccharides*** (also known as dietary fibre, prebiotics, or microbiota-accessible carbohydrates ['MACs']). These include plant cell wall polysaccharides (cellulose, hemicellulose, arabinoxylans, xyloglucans, mannans, pectin and beta-ß-glucan) from fruit, vegetables and whole cereals, and plant storage polysaccharides such as resistant starch, inulin type fructans and those present in bacterial/plant levan, and yeast mannan, as well as those produced by other microbes in the gut. Oligosaccharides present in milk are important for infant health and development (see also below).
- ***Host glycoconjugates***, e.g. (i) mucin glycosylated proteins in which the polypeptide backbones have attached to them large numbers of oligosaccharides via *O*-glycosidic linkages. These are a source of carbohydrates for the microbiota and a key point of interaction with the epithelial cell wall of the host. In addition, mucins protect epithelial cells from damage such as infections, dehydration, and chemical modification as well as

lubricating the passage of materials through the GI tract. The composition of the attached oligosaccharides varies along the GI tract[9] and any deficiencies or alterations are associated with gut disorders[10] and diseases, therefore presenting an opportunity as biomarkers for medical diagnosis and (in the context of precision medicine approaches) for patient stratification. (ii) Human milk oligosaccharides (HMOs) from human breast milk cover more than oligosaccharide structures made up from 5 monosaccharide building blocks, namely, glucose, galactose, fucose, N-acetylglucosamine and, N-acetylneuraminic acid. HMOs act as substrates for Bifidobacteria and help to shape the gut microbiota composition in infants. In addition, HMOs are also believed to have anti-adhesive antimicrobial properties which lower the risk of infections in infants, as well as a role in immunity thanks to their ability to modulate epithelial and immune cell responses.

- ***Microbial polysaccharide*** structures and glycoconjugates. These include lipopolysaccharides (LPS), lipo-oligosaccharides (LOS), capsular polysaccharides (CPS), extracellular polysaccharides (EPS), wall techoic acids (WTA), lipotechoic acids (LTA), peptidoglycans (PG) but also cell-surface glycoproteins which are increasingly being characterised in gut commensal bacteria. LTAs, PGNs, LPSs, and a range of CPSs, including polysaccharide A (PSA), are part of microbe-associated molecular patterns (MAMPs). Microbial glycans are important mediators of microbiota-host interactions and immunomodulation with MAMPs interacting with toll-like receptors (TLRs) and mammalian lectins.

The gut microbiota can break down or ferment these complex carbohydrates using enzymes (specifically Carbohydrate-Active Enzymes collectively known as 'CAZymes'[11]) into short chain fatty acids ('SCFAs') and other compounds. These SCFAs are vital energy sources, and have anti-inflammatory and cell regulatory roles. The repertoire of CAZymes vary with the different gut microbiota species present[12] (e.g. *Actinobacteria; Proteobacteria; Bacteriodetes, Firmicutes* and others) as encoded by their genome, collectively known as the microbiome. In contrast, the diversity of glycan structures expressed by the host or by the microbes cannot be deduced from genome analysis, highlighting the need for glycomics approaches to advance our current understand of the role of carbohydrates in the gut. For example, genome sequencing data is used to classify the microbes but strains can be, and need to be, further differentiated by their cell surface carbohydrates.

It is now acknowledged that the field of gut microbiota (which for a decade has been dominated by genome sequencing analyses) needs to move beyond association studies and tackle mechanisms. This is essential if we want to develop meaningful microbiome-targeted strategies for the benefit of human health. European glycoscientists have been leading the development of the tools and knowledge needed for probing and understanding carbohydrate interactions. These are now being applied to the microbiome field to provide invaluable mechanistic insights into the function and modulation of complex gut microbial communities. Furthermore, the development and integration of advanced glycomics technologies is necessary to complement and enhance the knowledge gained by metagenomics and metabolomics studies of the gut microbiome and provide a comprehensive mechanistic understanding of the biological processes involved.

IMPACTS ON HUMAN HEALTH & WELL-BEING

a) Human milk oligosaccharides and their effects on infant health

Around the end of the 19th century observations indicated that breast-fed infants had higher survival rates than those who were bottle-fed in cases of infection and other diseases. HMOs also lower the risk of necrotising enterocolitis, an incredibly serious condition where a portion of the bowel dies. In addition, it has been established that they provide essential nutrients for brain development and

cognition.[13] This led to a burgeoning field of research into the components of breast milk. To date more than 150 of HMO structures (out of 200 present in human milk) have been elucidated clearly and about 30 have been synthesised, which allows further study into their benefits as potential additions to formula milk.

However, there are major bottlenecks to overcome if HMOs are to be added to milk formulae:

Although analytical techniques have matured, there is still a lack of ISO standards ('ISO' = International Organisation for Standards) that are needed for these mass spectrometric measurement techniques[14]. Robust ISO standards are an essential prerequisite before the HMOs could safely be included in any recipe that could be put on the market.

Another major challenge, is the limited availability of HMOs available in the quantities required for study into mechanisms of action and to confirm that the observed effects are indeed safe and beneficial to the health of babies and infants. These are needed to establish claims alongside the necessary quality control procedures as defined by the proper ISO standards. New synthesis and production methods need to be developed to produce HMOs at large scale for industrial production.

There are several approaches to addressing the shortfall in availability of HMOs, each with their pros and cons[15]:

- Isolation from human breast milk is possible but only gives limited quantities. However, given a careful adherence to well-established procedures this has the advantage that the HMOs obtained are authentic;
- Isolation from dairy milk provides sufficient quantities and although not authentic they can be used as a starting point for other semi-synthetic production methods (i.e. chemoenzymatic or microbial fermentation approaches);
- Appropriate chemoenzymatic synthetic approaches will give the defined HMOs needed for structure-function studies but scale-up could be challenging;
- Bioengineered microorganisms would address scale-up issues especially in terms of costs; however each fermentation process only yields a limited set of HMOs at a time (per batch). In addition there may be GMO risks ('GMO' = "genetically modified organisms") unless GRAS procedures are used ('GRAS' = "generally recognised as safe", especially in the food ingredient context). Despite these caveats, engineered organisms open up the exciting possibility of probiotic delivery methods.

Recent efforts mean that HMO tri- and tetrasaccharides are now becoming available in the quantities required for study, which undoubtedly will boost research efforts and enable clinical studies.

Bringing HMOs to market –2'-Fucosyllactose

2'-Fucosyllactose (2'-FL) is an oligosaccharide (made up of the 3 sugar units fucose, glucose, and, galactose) and is the most abundant HMO in breast milk. It is believed to play a role in preventing infections by blocking pathogens and toxins. It is of interest as an ingredient for infant formula milk.

The infant formula ingredients market was valued at $13.99 bn in 2016 and is projected to reach around $23.79 bn by 2022, at a compound annual growth rate of 9.4% from 2017 to 2022[16].

In 2017, Jennewein, a German biotechnology company, was granted the first EU approval of a HMO as a novel food ingredient for their bacterial fermentation production of 2'-FL[17].Although 2'-FL is a natural product, production *via* chemical or biotechnology methods requires it to be assessed for safety prior to regulatory approval.

This regulatory milestone has paved the way for other companies[18] to produce 2'-FL and bring other HMOs to market, a major hurdle given commercialisation of the first HMOs (2'-FL and Lacto-N-neotetraose (LNnT)) took a similar timescale to an innovative pharmaceutical product (8-10 years)[19].

b) Gut microbiota populations are profoundly influenced by complex carbohydrates in the diet

The composition of the gut microbiota and the structure-function capability of the microbes largely depends on the **dietary carbohydrate** intake of the host. Western diets today are typically high-fat, high sugar compared to the relatively low fat, high polysaccharide diets of the past, resulting in the typically modern lower diversity of microbial composition in the gut.

This loss of diversity or unbalance, also referred to as 'dysbiosis', has been associated with a number of diseases affecting westernized or "developed" countries. These include neurological diseases (e.g. autism, Parkinson's, Alzheimer's), metabolic diseases (mainly diabetes and obesity), gut diseases (e.g. Irritable Bowel Syndrome, Inflammatory Bowel Diseases (Ulcerative Colitis and Crohn's disease), Coeliac disease, liver disease, colon cancer), food allergies or cardiovascular diseases. Many risk factors have been implicated in the perturbation of the gut microbiota such as mode of delivery, lifestyle, eating behaviours, disruption of biological clock and antibiotic over-use[20].

Increasing the complex carbohydrate content in the diet rapidly leads to an increase in microbial diversity with the associated health benefits[21]. This provides an opportunity in terms of developing carbohydrate-based strategies to promote or restore gut homeostasis.

As the field of gut microbiome develops, several carbohydrate-based functional foods have been described with the potential to modulate the gut microbiota composition and function as follows:

- "<u>Dietary fibre</u>" is a broad term for non-digestible carbohydrates; the impact on the gut very much depends on the type of fibre consumed. Some dietary fibres can be classified as '<u>prebiotics</u>', which are defined as selectively fermented ingredients that confer a health benefit[22]. Examples of prebiotic dietary carbohydrates include short-chain non-digestible carbohydrates such as inulin-type fructans, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS), which are all established prebiotics, as well as emerging prebiotics such as resistant starch, arabinoxylan oligosaccharides, xylo-oligosaccharides or HMOs.
- Recently, a new term has been proposed that specifically refers to carbohydrates that can be metabolically used by gut microbes, known as <u>Microbiota Accessible Carbohydrates</u> ('MAC')[23].
- <u>Functional carbohydrates</u> is a broad term that also includes the ability of carbohydrates to interfere directly with the host (independent of the microbiota). For example, functional carbohydrates can act as anti-adhesive agents by blocking sites where pathogens bind, e.g. HMOs[24]; or, by inducing protection through their interactions with carbohydrate-binding proteins (lectins of the immune system) e.g. β-glucans[25].

An understanding of the microbial degradation and function of carbohydrates in the gut will lead to a better design of carbohydrate-based functional foods (above) to improve health through the diet. These nutritional strategies are of great promise as a positive and less costly alternative to long term medication, especially important for ageing populations, where strategies that can both reduce healthcare costs and improve health outcomes are increasingly required. The better identification and characterization of these carbohydrates, alongside mechanistic studies into their impact on the gut microbiota structure and function, will lead to structure activity relationship know-how that should in turn allow the development of highly effective functional foods. For example, recent research has highlighted how the promotion of SCFA-producing microbes in the gut through dietary fibre intake results in improved patient blood sugar levels. This may present an effective alternative approach to managing diabetes mellitus[26].

Furthermore, carbohydrates could help alleviate the development of antimicrobial resistance (AMR), by limiting pathogen proliferation through the modulation of the gut mucosal environment or by disrupting biofilm formation on the gut mucosa. Such approaches present new opportunities to extend the utility of current antibiotics or as alternative antimicrobial therapies.

Claims of genuine health benefits of carbohydrates must be demonstrated beyond all reasonable doubt in order to obtain regulatory approval from the European Food Standards Agency ('EFSA'). So far, only one claim for the prebiotic inulin, a polysaccharide found in many plants, for the maintenance of bowel movements, has been accepted by the EFSA[27]. To establish future such claims it is important to put in place systematic metrological procedures that are internationally accepted; much has been done in these areas already but much more remains to be done.

c) Host carbohydrates on the gut surface as targets and indicators of GI diseases across life

In addition to dietary carbohydrates, **host carbohydrates** play a critical role in the maintenance of gut homeostasis. The intestinal surface is lined with mucins, which are glycosylated proteins, estimated to contain up to 80% carbohydrates. Mucin glycosylation varies along the GI tract[28]. Alterations in the mucin glycosylation pattern have been associated with intestinal diseases e.g. inflammatory bowel disease or colon cancer. As such the chemical composition of the gut lining can be indicative of GI health with these surface carbohydrates providing biomarkers of health for the appropriate precision medicine approach to be employed, either through the characterisation of mucin carbohydrates or by identifying associated changes in the microbial community residing in the mucus[29].

Experimental models have shown that a deficiency in mucin glycosylation leads to the development of colitis, accompanied with a change in gut microbiota, highlighting a direct and causal link between mucin glycosylation and GI diseases (both inflammatory and cancer)[30].

To date, most knowledge on the role of the gut microbiota in health and disease, resides on the analysis of faecal microbiota. The host carbohydrates also provide a preferential source of nutrients to the bacteria (commensals or pathogens) residing in the mucus niche, therefore in close proximity with the host immune system. A better understanding of how microbes interact with the gut surface will allow the development of preventive strategies to fight infection by major enteric pathogens such as *Clostridium difficile* or Salmonella.

Mechanistic research studies coupled with new measurement and metrology are essential to ensure that these objectives are met. Strategies would include ways to modify the composition of mucus-associated microbiota in order to improve the host immune response or competing for nutrients, a way of fighting infection or inflammation/cancer from within.

It is known that modifications in host carbohydrate structures can in turn produce signals that can modulate the cancer immune response[31]. Strategies here would include ways to modify the composition of overall gut microbiota to improve the immune response.

These approaches are particularly relevant to the design of strategies underpinning healthy ageing. As we age, changes in the GI tract and microbiota diversity lead to dysbiosis and the associated effects on health described above. The composition of the human microbiota remains relatively stable throughout most of adulthood. However, marked changes have been observed to occur between the ages 75-80 years[32]. As a result, there is an increased risk of *Clostridium difficile* infections ('CDI') and functional gastrointestinal disorders ('FGID').

Again, knowledge of these processes will allow the development of strategies to overcome these detrimental effects, and interest in this area is growing, especially since population distributions across European nations are ageing. Such alternative strategies are required to reduce significantly healthcare costs of the elderly.

d) Exploiting microbial carbohydrates for disease diagnosis and the development of highly effective medical treatments including vaccines and cancer therapies

Microbial carbohydrates present on the surface of bacteria (e.g. EPS, CPS, LPS, LOS, PG, TA – *see the 'Glossary' for definitions of these terms*) are critical mediators of the interactions between bacteria

and their environment. As such they play a major role in immunomodulation, a concept that has been thoroughly investigated in pathogens but has been less explored in the commensal bacteria of the gut microbiota. Carbohydrate epitopes decorating cell surface proteins on gut symbionts or probiotics also induce protective host immune responses.

In addition, bacterial cell surface carbohydrates offer a rich source of targets to help elucidate the processes that underpin antimicrobial resistance (AMR). AMR, is one of the most urgent global healthcare challenges of the 21st century. It is responsible for an estimated 33,000 deaths per year in the EU. It is also estimated that AMR costs the EU EUR 1.5 billion per year in healthcare costs and productivity losses[33]. In order to tackle the AMR challenge, we need to better understand why, when, and how, bacteria develop resistance. A key step during the development of resistance is the initially transient, adaptation of bacteria to their environment (e.g. presence of nutrients, antibiotics, host proteins, other bacteria etc.). A central, but poorly understood role for these adaptation processes is mediated by the carbohydrate molecules that are displayed on the bacterial surface.

Perhaps not surprisingly, the composition of the gut microbiota has been found to affect the effectiveness of medicinal interventions.

For example, recent work has shown that antibiotic-induced gut dysbiosis in mice caused an impaired antibody response to vaccines[34]. Knowledge of microbial cell surface carbohydrates will allow the development of targeted vaccines, as highlighted in a recent CarboMet positioning paper on carbohydrate-based vaccine development[35]. By selectively targeting the pathogenic bacteria over the commensal bacteria of the gut microbiota, these more targeted treatments have the additional advantage of reducing undesirable side effects from medical interventions.

Due consideration of the gut microbiota must be taken into account when developing treatments such as vaccines or cancer therapies. This raises procedural and regulatory issues when administering antibiotics or other drugs that may adversely affect the composition of the gut. Care is also needed in the effective design of clinical trials to avoid damage of this nature. New metrological and accompanying regulatory procedures need to be developed to analyse whether the composition of the gut microbiota in a patient will render them less amenable to the proposed medical treatment. Glycometrology will be an important part of this process.

SCOPE OF THE PROGRAMME – Glycometrology as a key part of gaining a mechanistic understanding of the human gut microbiota

Complex carbohydrates are the central components of the gut and gut microbiota function and therefore contribute crucially to human health. Analysis of the human microbiome through metagenomic studies has already delivered a huge amount of data, revealing correlations between gut microbiota and a growing number of diseases.

However, a mechanistic understanding of host-microbiota interactions is needed to design strategies that will harness the beneficial role of the gut microbiota. Mechanistic information related to both health and disease using appropriate, informed management of “big data” is needed. For an example, consider again the identification and/or invention of novel prebiotics: only with better understanding of the underpinning mechanisms can new prebiotics be identified with precision for a particular condition.

In addition to mechanistic studies, rigorous double-blinded, placebo-controlled clinical trials will be needed to prove the health benefits. This requires the synthesis and supply of multi-gram carbohydrate material coupled with targeted measurements of high sensitivity. It is here that new measurement and metrology is crucial. Then, and only then, will, society benefit, given also the

development in parallel of fast regulatory processes to allow for rapid commercialization of functional carbohydrates so that their production can be achieved at affordable cost.

In order to exploit the multifarious ways that carbohydrates can benefit human health and keep the EU its leading position in healthcare, we see that efforts in four main areas are needed to operate in a collaborative and integrated manner:

1. Understanding the mechanisms underpinning and promoting healthy relationships between the microbiota and the human gut: the importance of glycometrics

It is no exaggeration to state that the European Glycoscience community is leading the way on the International scene on providing the knowledge and technology required to understand the mechanisms behind the interactions between our gut, the microbiota and diet. Many of these tools are now ready to be brought out of specialist hands to be shared more widely with researchers in other disciplines. This will enhance and complement mechanistic research already taking place in overlapping areas of interest such as cancer research, infant health etc. where genomic approaches are proving to be insufficient to advance the field further.

To learn about the impact of host, microbial or dietary carbohydrates, it is necessary to analyse directly what carbohydrates and glycoconjugates are present. To do this, it is essential to integrate and develop glycobiology, glycosynthesis and glycometrics approaches to provide mechanistic insights to determine:

- How gut microbiota influence human health;
- Identification and validation of novel biomarkers;
- Development of novel microbiome targeted strategies.

In summary, better knowledge of carbohydrates and glycans in the gut, through glycobiology and glycometrics approaches, represent an exciting opportunity not just in terms of innovation in nutrition and food technology but also in disease prevention, diagnostic and therapies, in line with aspirations expressed throughout Horizon 2020 and currently in its successor Horizon Europe.

2. Developing tools for the production of carbohydrates using fermentation, enzymatic, chemical and/or automated synthesis approaches.

The carbohydrates involved in microbiota-host interactions need to be available as validated, high quality pure standards, both for allowing high-throughput analysis and for providing materials as probes for functional studies. European glycoscientists in industry and academia have made significant advances in carbohydrate synthesis and analysis and the capacity to produce a range of bespoke carbohydrates. However, there is an urgent need to be able to produce these on scale, economically and efficiently in sufficient quantities that will allow their assessment for health benefits and safety which will require multi-gram quantities (as identified in the previous section on '*Human milk oligosaccharides and their effects on infant health')*. This is in addition to eventually being able to produce beneficial carbohydrates on an industrial scale.

For the synthesis of carbohydrates, there are a number of preparation approaches that are being used:

• **Isolation of naturally occurring carbohydrates** from microbes via fermentation processes: Such biotechnological production can be from naturally producing organisms or, thanks to advances in gene technologies, optimised strains that have been engineered. There is expertise within large European companies in fermentation technologies, which are capable of being adapted for the production of particular carbohydrates and to overcome issues that are associated with scale-up, particularly for engineered systems. As shown in the example of 2'-fucosyllactose given above, regulatory breakthroughs have paved the way for fermentation approaches using engineered organisms for the production of HMOs.

• **Chemical synthesis:** Traditionally it has been very challenging to synthesise carbohydrates chemically. However, chemistry advances including the introduction of automated processes are now enabling the synthesis of larger and more complex carbohydrates. This is mostly carried out by expert academic research groups and specialist Small & Medium Enterprises (SMEs). Development of more wide-ranging synthetic methodologies that can be replicated by non-specialist organic chemists are needed and improved availability of the building blocks is also required.

• **Enzymatic synthesis:** This is being used increasingly and is often a more sustainable approach than are traditional synthetic approaches. However, a major limitation can be the availability of carbohydrate-active enzymes with the required properties and chemical activities. There is therefore an urgent need to build an arsenal of more glycoenzymes showing an expanded repertoire of chemistries.

• **Combined integrated approaches:** Using combinations of some or all of the above methods together also presents great promise (*e.g.* in sequence or in parallel chemical and/or enzymatic modification of naturally occurring carbohydrates). Such integrated approaches require cross-disciplinary strategies including chemistry, biochemistry, molecular biology and computation, and machine learning.

3. Developing new metrology and measurement tools for carbohydrates that are involved in the interactions between the gut microbiome and human host

Throughout this paper frequent references have been made to analytics, metrology, new measurement, standards, biomarkers and traceability, including the need for ISO standards. (*See the 'Glossary' for a definition and explanation of Metrology).*

This ranges from being able to classify microbial strains further based on subtle differences in their cell surface carbohydrates, the need for ISO standards for the safe development and production of bespoke carbohydrates for human intervention; through to the safe design of clinical trials and targeted drug design and delivery so as not to detrimentally affect the gut microbiota as an unintended consequence of treatments.

European glycoscientists have been leading the development of the analytical tools and knowledge needed for probing and understanding carbohydrate interactions. This requires the efficient synthesis, analysis, and also labeling of carbohydrates (e.g. 13C-labeling or *in situ* metabolic labeling of carbohydrates[36]). These tools are now being applied to the microbiome field to provide invaluable mechanistic insights into the function and modulation of complex gut microbial communities. However, these tools need to be developed further to ensure they address regulatory needs to allow the development of carbohydrate based strategies for the benefit of human health.

It is to be welcomed that the current propositions and priorities for Horizon Europe highlight metrology as an area for a major industrial partnership with the intent "to develop new tools for speed, accuracy and cost of measurement" (*sic*). This industrial partnership should extend well beyond what might be called the more recognised areas of manufacturing and process control to the very boundaries of biological and medicinal sciences, including glycobiology.

Currently, it is arguable that nowhere is this more critical than in medical technologies, disease resistance and food security, the very issues addressed here, since the future of human health, security and safety is at the core of these activities. Inevitably, as the science of glycobiology expands and demonstrates its increasing importance, relevance and utility, so will the demand for new metrological standards alongside new measurement techniques.

We therefore recommend that the European Association of National Metrology Institutes ('EUROMET') should actively include the research imperatives that are described in this paper as future priorities.

4. Optimising data management by integrating glycomics data with other -omics data using bioinformatic tools and databases to facilitate knowledge sharing between glycoscientists and microbiome researchers

Data management is an implicit part of the modern science of metrology. More and more, data management is being built in to cutting edge measurement tools, offering increasingly powerful tools that will contribute to a better understanding of the function of the gut microbiota. Given the huge quantity of datasets (glycomics, metabolomics, genomics, metatranscriptomics, metaproteomics) and the many kinetic and thermodynamic parameters involved in mechanistic studies of the microbiota, there is a critical need for accurate data analytics[37].

It has also been recognised that there is a need to organise access to glyco data which had been previously lacking in bioinformatics provision[38]. Successful efforts towards integrating glyco- and prote- omics data are already underway[39], however, further integration of glycomics with other –omics data (in particular metagenomics and metabolomics) is also needed.

Current Horizon Europe thinking has identified as one of its key Industry Partnerships "Key digital technologies, including novel technologies such as 'artificial intelligence' and linking to downstream sectors". In studying the role of carbohydrates in the gut microbiota, this interface between key digital technologies on one hand, and glycoscience and glycometrics developments on the other, is crucial if we are to exploit the full benefit of the gut microbiota. While improving correlations between gut microbiota and health, full integration of glycomics data with other –omics data will also provide key mechanistic insights into the function and adaptation of microbial communities within specific niches in the gut. Data integration has the potential to fully support computational modelling approaches that will reduce or perhaps in time, completely remove the need for animal experiments. By integrating and aligning microbiome studies with this wealth of -omics data compilation of profiles will be possible which has exciting implications for patient stratification and precision medicine approaches to healthcare.

5. Developing carbohydrate-based strategies that target the gut microbiota to restore and/or promote good health and well-being for all at all stages of life

The **above 4 topics** will all contribute to the development of strategies that target the gut microbiota to restore and/or promote good health and well-being for all at all stages of life. Double-blinded, placebo controlled clinical trials will need to be carried out, exploiting the mechanistic knowledge and capacity building from 1-4, to provide evidence for the health benefits in humans. There will also need to be a strong engagement with consumers to ensure not only that the development of new functional food products is palatable but that consumers are informed on their health benefits and that the consumers' needs and expectations are taken into account.

TOPIC RELEVANCE - Within the evolving Horizon Europe programme

At the time of publication of this document (December 2018), the debate on the nature, priorities and size of the successor programme to Horizon 2020 (called currently "Horizon Europe") is entering its final stages. While emphases might change before the process is finalised, it is of interest to note that some of the current major priorities identified to date have a direct bearing on the main thrusts of this paper, which, whilst its prime concern is on the importance of carbohydrates to understand gut microbiota function, aims to highlight generally the key importance of the glycosciences to the future health and prosperity of Europe.

Five "Research Missions" have been shortlisted as early priorities for the EU's 2021-2027 research programme[40]. These are 'Digitisation', 'Health', 'Food', 'Agriculture' and a 'Clean Europe'; the first three of these relate directly to the discussions here and the fourth is also of indirect significance. In

addition, Member States have been requested to consider the merits of ten shortlisted options for "Industry Partnerships"; **glycosciences and glycometrology** are directly relevant to four of these, namely:

1. "Health innovation, for the rapid development, deployment and safe use of medical treatments, devices and technologies enhanced by digital technologies;
2. "Global health, including links to national health research systems and philanthropic funding;
3. "Key digital technologies, including novel technologies such as 'artificial intelligence' and linking to downstream sectors;
4. "Metrology, to develop new tools for speed, accuracy and cost of measurement."

We draw attention to the first, second and last of these four proposed Industry Partnerships: this is <u>precisely</u> in line with what are advocating in this on behalf of glycoscience and glycometrics, aspects of which were discussed in this paper.

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GLOSSARY OF TERMS

Antigen – any substance or entity (e.g., a virus or micro-organism) which is foreign to the host's body and evokes a defensive immune response.

Bioinformatics – the use of mathematics and computer science to collect, classify, store, retrieve and analyse biochemical and biological data.

Carbohydrates - also known as **sugars**, mono- oligo- and poly-**saccharides** and **glycans.**

CPS – Capsular Polysaccharide.

EPS – Extracellular Polysaccharides.

Epitope – specific molecular region on the surface of an antigen which can elicit an immune response from the host organism.

GI – Gastro-Intestinal.

Glycoconjugates - carbohydrates linked covalently to other biomolecules for example glycoproteins or glycolipids.

Glycosylation reactions are catalysed by **glycosyltransferase** enzymes that add glycosyl radicals to other molecules (usually proteins) in a site-specific manner. **Glycomics** – study of the structure and function of carbohydrates in biological systems.

GMO – Genetically Modified Organism.

GRAS – Generally Recognised As Safe.

HMO – Human Milk Oligosaccharide.

ISO-standards - "requirements, specifications, guidelines and characteristics that should be used to ensure that molecules, products, processes and services are fit for purpose" [https://www.iso.org/standards.html (ISO = International Organization for Standardization)].

LOS – Lipo-oligosaccharide.

LPS – Lipopolysaccharide.

LTA – Lipotechoic Acid.

Metrology – is the science of measurement. (See https://www.bipm.org/en/worldwide-metrology/). It is an umbrella term covering new measurement, standards, biomarkers and traceability. It allows the reliable determination of conformity with product specifications and/or technical requirements as well as standards developments. ISO standards are built on sound metrological procedures. It is a scientific discipline which tends to be taken for granted, yet it underpins and above all provides confidence for all aspects of modern technology. Safety, sustainability, product quality, efficacy, reliability in medical treatments, forensic science, security, manufacture, food technology and agriculture, to name but a few sectors, all depend on accurate, traceable metrology.

Pathogen - any disease-producing agent, applies especially to viruses, bacteria and other micro-organisms such as fungi.

Serotype - group of organisms, micro-organisms or cells that are distinguished by their shared specific antigenic properties, as determined by serological testing.

WTA – Wall Techoic Acid.